Environmental Contamination Remediation and Management

Mirta L. Menone
Chris D. Metcalfe *Editors*

The Ecotoxicology of Aquatic Macrophytes

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There are many global environmental issues that are directly related to varying levels of contamination from both inorganic and organic contaminants. These affect the quality of drinking water, food, soil, aquatic ecosystems, urban systems, agricultural systems and natural habitats. This has led to the development of assessment methods and remediation strategies to identify, reduce, remove or contain contaminant loadings from these systems using various natural or engineered technologies. In most cases, these strategies utilize interdisciplinary approaches that rely on chemistry, ecology, toxicology, hydrology, modeling and engineering.

This book series provides an outlet to summarize environmental contamination related topics that provide a path forward in understanding the current state and mitigation, both regionally and globally.

Topic areas may include, but are not limited to, Environmental Fate and Effects, Environmental Effects Monitoring, Water Re-use, Waste Management, Food Safety, Ecological Restoration, Remediation of Contaminated Sites, Analytical Methodology, and Climate Change.

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To the memory of Julia E. Aizpún de Moreno (July), one of the first women specialist in pollution studies in Argentina.

Foreword

Aquatic macrophytes are extremely important for the functioning and maintenance of aquatic ecosystems, as they are a source of organic matter, influence the cycling of nutrients and also provide food, shelter and a breeding place for fauna. In addition to these ecological benefits, they are of great interest since many macrophyte species are used for the treatment of domestic and industrial wastewater. My experience with macrophytes is related to studies of phytoremediation as an emerging alternative for water decontamination. The use of plants to metabolize, stabilize and/or accumulate contaminants and pollutants in their biomass has shown promising results for the removal of pharmaceuticals from water, for example. More specifically, pteridophyte species have been identified as effective phytoremediator agents. Among them, aquatic macrophytes have shown prominence in water decontamination, mainly due to their rapid growth and ability to reclaim contaminants.

Therefore, this book is a timely and important review and discussion of new approaches for the use of macrophytes. In addition to phytoremediation, the application of bioindicators for pollutants are being considered in the ecological risk assessment (ERA) for chemicals and other contaminants that could affect their services. There are some challenges in fullfilling this toxicity assessment which include: which macrophytes should be used, including submerged and emergent species, local species or varieties, and consideration of the diversity of climates, the complexity of stressor exposures and ecological contexts. The chapters in this book discuss some microcosm and mesocosm studies and modeling approaches, providing a global perspective on the use of macrophytes for ERA.

The book also addresses both traditional and innovative approaches for monitoring responses to contaminant exposure, addressing current issues at hand, and providing a research guide for studying the effects of pollution on aquatic macrophytes in the field. An integrated model for monitoring multiple biomarkers from bench-scale studies of biomarker responses to metals, pesticides, pharmaceuticals and per- and poly-fluoroalkyl substances is described.

This book is a comprehensive overview of the current knowledge on aquatic macrophyte ecotoxicology and was written for specialist scientists from different

countries. It was a pleasure to review this book and I congratulate the co-editors for this scientific contribution. In the chapters, detailed reviews are introduced, as well as important concepts and future developments for macrophyte applications.

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Abbreviations

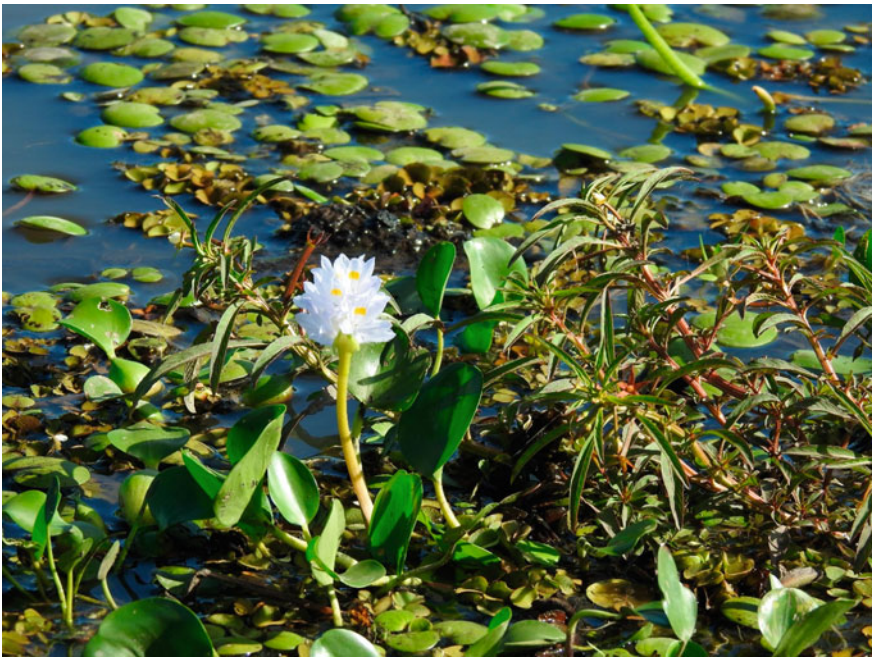
ALS	Acetolactate Synthase
AM	Abnormal Metaphases
AMRAP	Aquatic Macrophyte Risk Assessment for Pesticides
Antho	Anthocyanins
APX	Ascorbate Peroxidase
ASC	Ascorbic Acid
ASTM	American Society for Testing and Materials
BOD	Biochemical Oxygen Demand
CAAT	Chromosomal Aberration in Anaphase-Telophase
Caro	Carotenoids
CAT	Catalase
CEC	Contaminants of Emerging Concern
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Chl F	Chlorophyll a Fluorescence
Cm	C-mitosis
CNSC	Canadian Nuclear Safety Commission
COD	Chemical Oxygen Demand
CUPs	Current-Used Pesticides
CYP450	Cytochrome-P450
DNA	Deoxyribonucleic Acid
EC	Environment Canada
ECx	Effect Concentration for x% of the Test Population
ER	Ecosystem Respiration
ERA	Ecological Risk Assessment
ErC50	Growth Rate
ETR	Electron Transport Rate
F_0	Initial Fluorescence
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FOCUS	FOrum for the Co-ordination of Pesticide Fate Models and their USe
F_m	Maximum Fluorescence

F_v: F_m/F_0	Maximum Quantum Yield of Photosystem II
G6PDH	Glucose-6-Phosphate Deshidrogenase
GAC	Granulated Activated Carbon
Gene-Exp	Gene Expression
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione-S-Transferase
GPP	Gross Primary Productivity
GPx	Glutathione Peroxidase
HC5	Hazard Concentration for 5% of the Species
H_2O_2	Hydrogen Peroxide
ISO	International Organization for Standardization
LCx	lethal Concentration for x% of the Test Population
LOC	Level Of Concern
LOEC	Lowest Observed Effect Concentration
LPO	Lipid Peroxidation
MDA	Malondialdehyde
METs	Metals, Metalloids and Organometals
Mn-SOD	Manganese Superoxide Dismutase
MTs	Metallothioneins
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
CNSC	Canadian Nuclear Safety Commission
NOEC	No Observed Effect Concentration
NPQ	Non-Photochemical Quenching
OECD	Organisation for Economic Co-operation and Development
PC synthase	Phytochelatin Synthase
PCs	Phytochelatins
PECs	Predicted Environmental Concentrations
PFASs	Per- and Polyfluoroalkyl Substances
PFOA	Perfluorooctanoic Acid
Phae	Phaeophytins
Phae a	Phaeophytin a
Phae b	Phaeophytin b
P-O_2	Photosynthetic Oxygen Production
POD	Guaiacol Peroxidase
PPCPs	Pharmaceutical and Personal Care Products
PQ	Plastoquinone
PR	Photosynthesis Rate
PRIMET	Pesticides RIsks in the tropics to Man, Environment and Trade
PSI	Photosystem I
PSII	Photosystem II
QA/QC	Quality Control and Quality Assurance
qP	Photochemical Quenching
RAC	Regulatory Acceptable Concentration

Ratio a/b	Ratio Chlorophyll a/ Chlorophyll b
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RQ	Risk Quotient
SOD	Superoxide Dismutase
SRP	Soluble Reactive Phosphorus
SSD	Species Sensitivity Distribution
TAN	Total Ammonia Nitrogen
TChl	Total Chlorophyll
tGSH	Total Glutathione
TKTD	Toxicokinetic–Toxicodynamic
UbQ	Ubiquinone
UbQoxi	Oxidized Ubiquinone
UbQred	Reduced Ubiquinone
USDA-ARS	Agricultural Research Service of the United States Department of Agriculture
US EPA	United States Environmental Protection Agency
VADDs	Agricultural Drainage Ditches
VTS	Vegetated Treatment System

Symbols

$^1O_2$	Singlet Oxygen
8-OHdG	8-Hydroxy-Guanidine
ΦPSII	Quantum Efficiency of PSII
$OH^{\bullet}$	Hydroxyl Radical
$O_2^{\bullet -}$	Superoxide Radical Anion



Chapter 1
The Ecotoxicology of Aquatic Macrophytes: An Overview

Mirta L. Menone, Braedon W. Humeniuk, and Chris D. Metcalfe

Abstract Aquatic macrophytes are a morphologically and physiologically diverse group of vascular plants that are distributed all over the world in a variety of aquatic habitats. They provide a range of ecological services, as well as habitat for aquatic vertebrates and invertebrates, and are important primary producers that support both herbivores and detritivores. Aquatic macrophytes are exposed to a range of contaminants of both geogenic and anthropogenic origin. In order to protect aquatic ecosystems from the impacts of these contaminants, toxicity studies with species of aquatic macrophytes should be essential components of ecological risk assessments. This chapter provides an overview of the challenges and the opportunities for ecotoxicology studies using aquatic macrophytes and provides an introduction to the more detailed reviews and reports in subsequent chapters of the book.

1.1 Introduction

Aquatic macrophytes constitute an assemblage of taxonomically diverse macroscopic plants that are characterized by a life cycle that takes place completely or partially in the aquatic environment. Macrophytes have evolved mechanisms that allow them to adapt to environmental heterogeneity (e.g., changing water levels) and to inhabit various types of aquatic habitats, including lakes, rivers, streams, wetlands, swamps, seasonally flooded areas, as well as brackish and marine environments (Lesiv et al. 2020). Vascular plants represent the largest group among macrophytes, including aquatic ferns (*Azolla* spp., *Salvinia* spp.) but mostly Angiosperms;

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both monocots and dicots (Rejmánková 2011). These vascular aquatic macrophytes (hereafter referred to as aquatic macrophytes) are represented by 33 orders and 88 families, with about 2,614 species distributed worldwide. Overall, the diversity is highest in the Neotropics (984 species), intermediate in the Indomalayan, Nearctic and Afrotropics (664, 644 and 614 species, respectively), lower in the Palearctic and Australasia (497 and 439 species, respectively), and in the Oceanian (108 species), while only a very few vascular macrophyte species have been found in the Antarctica bioregion (Chambers et al. 2008).

The most common classification for aquatic macrophytes is by their growth form or the basis of attachment to the substratum, which includes four groups: (1) emergent macrophytes that are rooted in sediments or soils that are periodically inundated, but with aerial leaves; (2) floating leaved macrophytes rooted to the bottom substrate in streams and lakes with leaves that float on the surface of the water; (3) free-floating macrophytes that typically float on or under the water surface but are not attached to the bottom; and (4) submerged macrophytes that grow completely submerged under the water, with roots attached to, or closely associated with the substrate (Wetzel 1975; Chambers et al. 2008; Srivastava et al. 2008; Hanson 2013). Examples of the types of macrophytes are illustrated in Fig. 1.1.

This introductory chapter describes the importance of aquatic macrophytes for the functioning of aquatic ecosystems and for the well-being of humans, and also provides an overview of approaches for using aquatic macrophytes in ecotoxicology studies and for risk assessments. In subsequent chapters in this book, experts in the field of the ecotoxicology of aquatic macrophytes provide in-depth descriptions of the use of these plants for assessing the impacts of environmental pollution through biomonitoring and biomarkers, evaluating recoveries from contamination and for conducting risk assessments, as well as the potential for using macrophytes for bioremediation.

1.2 The Importance of Macrophytes in Aquatic Ecosystems

Aquatic macrophytes are primary producers at the base of both herbivorous and detritivorous food chains. They also provide physical structure to aquatic ecosystems, increase habitat complexity and heterogeneity, affect oxygen and nutrient concentrations, provide refuge from predation and release dissolved organic carbon which can be used by microbial complexes in periphyton or plankton (Bakker et al. 2016). Thus, aquatic macrophytes play an important role in the structure and the functioning of aquatic ecosystems. Photosynthesis driving primary production by macrophytes provides energy flow to the food webs of a range of aquatic ecosystems. In addition to the role of carbon derived from microalgae to higher trophic levels, there is evidence that carbon from the detritus generated by macrophytes may be an important carbon source for invertebrates and fish. In addition to providing organic matter for detritivores, macrophytes also provide food resources to aquatic and terrestrial herbivores (Thomaz 2021).

Plant biodiversity is also the foundation of food security for humans and in some cases, the basis for identifying new medicines. Aquatic macrophyte communities

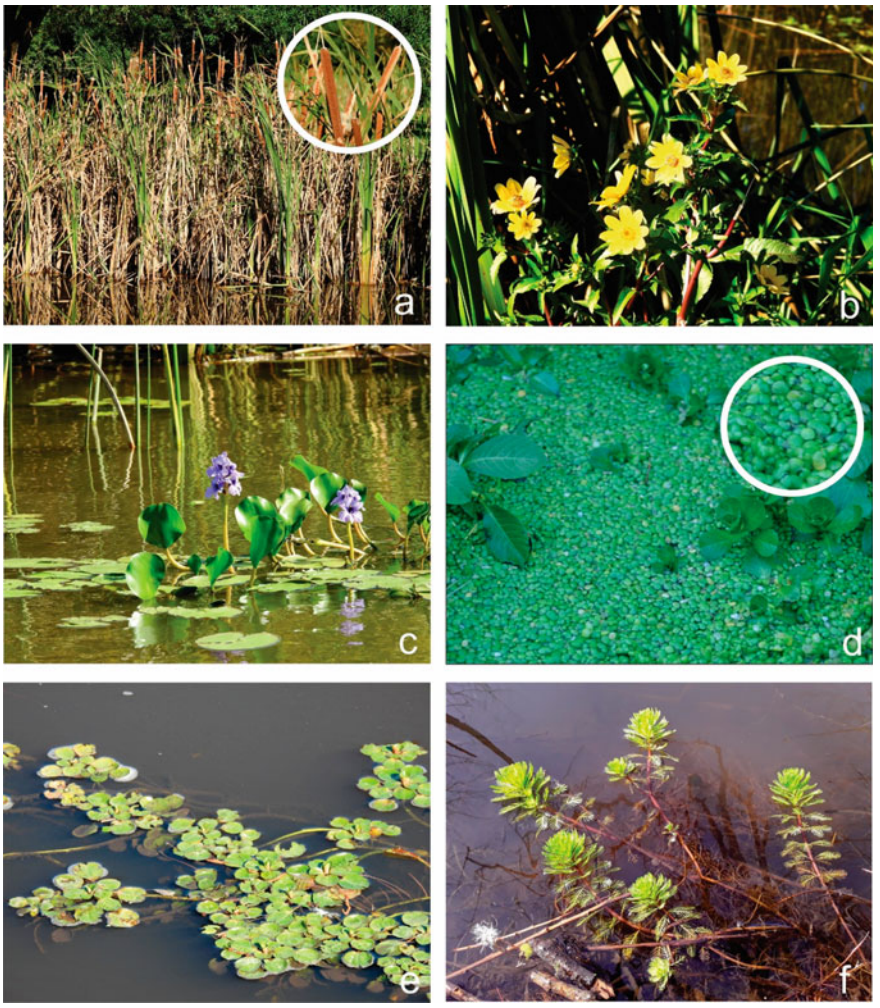

Fig. 1.1 Selected freshwater macrophytes that have been used in ecotoxicology studies: (a) cattail (*Typha* spp.), (b) beggartick (*Bidens* spp.), (c) water hyacinth (*Eichhornia* spp.), (d) duckweed (*Lemna* spp.), (e) primrose-willow (*Ludwigia* spp.), (f) water milfoil (*Myriophyllum* spp.). Photographs a, b, c and e were provided by Silvina Bachmann, photograph d by Nicolás Chiaradía and photograph f by Débora Pérez

offer multiple other benefits to humankind in terms of ecosystem functions, as well as resilience to climate change and other perturbations (Ebert and Engels 2020). Thomaz (2021) recognized these benefits within the paradigm of "ecosystem services" and identified more than 26 types of ecosystem services provided by aquatic macrophytes. These services were classified into supporting (e.g., photosynthesis and production of oxygen), provisioning (e.g., food and fiber provided by plant biomass), regulating (e.g., water purification through retention of nutrients and pollutants) and cultural

(e.g., local knowledge systems of communities which depend on ecosystems with macrophytes for survival).

1.3 Ecotoxicology Studies with Aquatic Macrophytes

Ecological stressors such as climate change, eutrophication, acidification or introduced species have been recognized as drivers of reduced macrophyte diversity in aquatic ecosystems (Chambers et al. 2008). In addition, natural ecosystems are subject to contamination by a number of elements of geogenic or anthropogenic origin, as well as xenobiotics. Anthropogenic activities such as discharges of industrial and municipal wastewater, and wastes originating from households, industry and agriculture are the main sources of the contaminants transported into aquatic ecosystems (Piwowarska and Kiedrzynska 2022). Aquatic macrophytes have been used as bioindicators of water quality in lentic and lotic systems in studies that focus on changes in plant communities (Thiebaut and Muller 1999; Ceschin et al. 2010), as well as studies of effects at the organismal level (Menone et al. 2000; Bonanno et al. 2017; Pérez et al. 2017). Despite the crucial role of macrophytic plants in aquatic ecosystems, these organisms have been underemployed for evaluating the impacts of anthropogenic activities, if compared to the number of comparable studies conducted with animals. Even so, the majority of ecotoxicology studies with aquatic macrophytes have focused on a narrow range of plant species, including *Lemna* spp., *Myriophyllum* spp. and *Hydrilla* spp. (Ceschin et al. 2021). These and other macrophytes species that have been used in ecotoxicology studies conducted in the laboratory and in the field are listed in Table 1.1.

Ecological risk assessments typically involve two main experimental or predictive approaches, as illustrated in Fig. 1.2. "Exposure Assessments" consist of measurements of the concentration of a toxicant of interest in a relevant environmental matrix (e.g., water, sediment, soil, air) or alternatively, calculations to predict what the concentration is expected to be. These data are used to determine a Predicted Exposure Concentration (PEC). "Effects Assessments" consist of measurements of the acute or chronic toxicity of the toxicant of interest to a range of organisms and these data are used to determine a Predicted No Effect Concentration (PNEC). The "Risk Characterization" step involves comparing the PEC to the PNEC to determine if exposure concentrations are likely to exceed the thresholds for toxicity (Fig. 1.2). Risk Management steps may be needed if there is a clear risk of impacts to aquatic or terrestrial species. For Effects Assessments that focus on threats to aquatic ecosystems where there are macrophytes (e.g., wetlands), there is no specific species or taxonomic group that is consistently more sensitive to the toxic effects of contaminants, including the standard duckweed (*Lemna* spp.) test organisms (Fairchild et al. 1998; Arts et al. 2008; Giddings et al. 2013). This highlights the need to incorporate toxicity studies with a suite of macrophytic test species into risk assessments (Lemly et al. 1999; Hanson and Arts 2007; Repetto 2013).

Duckweed species offer several advantages as a model test organism, as they have a wide geographic range (i.e., environmentally relevant), are exceptionally easy to

Table 1.1 Aquatic macrophytes that have been used in ecotoxicological studies, classified as emergent, floating leaved, free floating and submerged

Emergent	Free floating	Floating leaved	Submerged
Bidens laevis (FW)	*Eichhornia crassipes* (FW)	*Ludwigia peploides* (FW)	*Elodea canadensis* (FW) *E. nutalli*
Bruguiera gymnorrhiza (MG) *Kandelia candel* (MG) *Rhizophora mucronate* (MG)	*Ceratophyllum demersum* (FW)	*Potamogeton natans* (FW)	*Hydrilla verticillate* (FW)
Glyceria maxima (FW)	*Lemna minor* (FW) *L. gibba*		*Myriophyllum aquatium* (FW) *M. alterniflorum*, *M. quitense*, *M. spicatum*
Oryza sativa (FW)	*Spirodela polyrhiza* (FW)		*Posidonia oceánica* (M)
Phragmites australis (FW)			*Vallisneria neotropicalis* (FW) *V. natans*
Spartina densiflora (SM) *S. alterniflora*			*Zostera marina* (M)
Typha latifolia (FW) *T. domingensis*			

FW: freshwater (lakes, streams, wetlands); SM: saltmarsh; MG: mangrove; M: marine

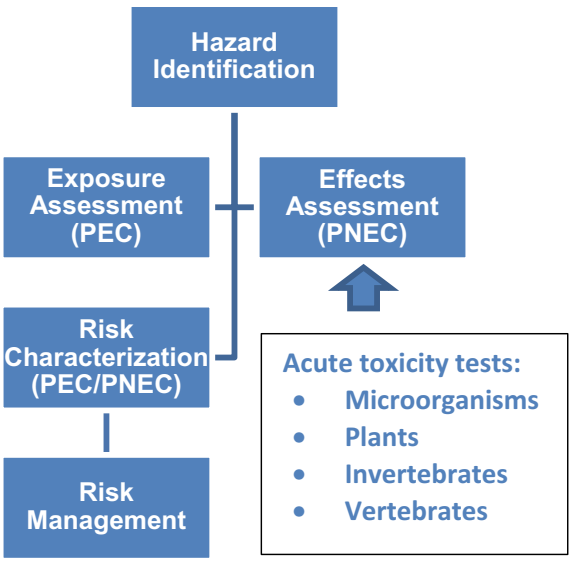

Fig. 1.2 The elements of an ecological risk assessment. PEC = Predicted Exposure Concentration; PNEC = Predicted No Effect Concentration; PEC/PNEC = Hazard Quotient

culture, bioassays are relatively inexpensive and simple to conduct, and it is possible to measure toxicity over a relatively short period of time (Rand et al. 1995; Brain and Solomon 2007; Hanson 2013). Duckweed species have been used to evaluate the cytotoxic and mutagenic effects of several classes of contaminants, including pesticides, pharmaceuticals, polycyclic aromatic hydrocarbons, metals, metalloids, organometal compounds and radionuclides (Mkandawire et al., 2013). However, duckweed species lack stems, true leaves and a sediment-interacting root system, and therefore, concerns have been raised about the suitability of *Lemna* spp. as a surrogate for all macrophytes, especially when testing compounds with herbicidal activities or assessing the risks to wetland ecosystems (Maltby et al. 2009; Arts et al. 2010; Hanson 2013). There are also limitations when evaluating responses under controlled field assessments (e.g., microcosm or mesocosm studies), as these systems are not typically eutrophic. This can mean that growth responses of duckweed are reduced under conditions of nutrient deficiency, especially in comparison to rooted submerged and emergent macrophytes, which are able to access nutrients available from both the sediments and the water column (Maltby et al. 2009; Hanson 2013). Additionally, effects of stressors that may impair light availability within the water column (e.g., turbidity) are not effectively captured by duckweed, as they typically float at the surface with ample access to light (Brain et al. 2005).

Because of the limited predictive capabilities of duckweed for evaluating the effects of sediment-bound contaminants, there are also standardized test methods for the rooted, submerged eudicot, *Myriophyllum* spp. Toxicity tests with *M. sibiricum* or *M. spicatum* have been applied when assessing the risks from exposure to herbicides that partition into sediments or for studies of eudicot targeted herbicides such as chlorophenoxy compounds (Arts et al. 2010; OECD 2014). Although there have been numerous other macrophytes used in toxicity tests (Table 1.1), the widespread adoption of additional test species for risk assessments has been limited, due in part to the lack of standardization and validation of testing procedures (Hanson 2013). However, there is ample evidence that macrophytes should be an essential component of effects assessments for a range of aquatic ecosystems (Hanson and Arts 2007; Arts et al. 2010; Giddings et al. 2013; Hanson 2013).

Because of the diversity of growth forms or the basis of attachment to substrata, macrophytes can be exposed to contaminants through several pathways, such as in sediments, in the water column, or through aerial exposure (Vonk and Kraak 2020). It is imperative for risk assessments to address the different pathways of exposure that apply to a particular ecosystem or to a specific toxicant of interest. Single-species toxicity testing introduces high levels of uncertainty for an effects assessment, especially when used as a sole line of evidence rather than in a weight-of-evidence approach (Maltby et al. 2009; Taylor and Scroggins 2013). To reduce uncertainty when characterizing the risk to non-target organisms, studies with macrophytes with different morphologies and exposure pathways must be included in the standard regulatory risk assessment process. In Chap. 5, wild rice (*Zizania* spp.) is presented as a candidate species for assessing risks to wetland ecosystems, as this rooted and emergent plant can be exposed to contaminants in sediment, water and air. Chapter 4 provides a global perspective on the use of macrophytes for risk assessments.

As was pointed out decades ago, simply determining the contaminant loads of organisms does not necessarily provide information on the toxicological significance of the body burden, or on the many factors which can influence the accumulation of contaminants. An alternative and potentially more useful approach is to evaluate indexes of sublethal stress, or "biomarkers" (Padinha et al. 2000). There are several studies in the literature on biomonitoring with macrophytes that include data on stress biomarkers, which are mostly biochemical responses (Lytle and Lytle 1998; Nimptsch et al. 2005; Turull et al. 2017; Bertrand et al. 2019). Chapter 3 in this book, provides a review of studies of bioaccumulation and biomarker responses with an emergent freshwater macrophyte, *Potamogeton pusillus,* and with mangrove species exposed in the laboratory and in the field to metals and metalloids. In this book, Chap. 2 provides a review of physiological, biochemical and genotoxicity biomarkers that have been measured in aquatic macrophytes in response to exposures to different classes of contaminants, including metals and metalloids, current use pesticides and emerging contaminants such as pharmaceuticals and personal care products (PPCPs) and per- and polyfluoroalkyl substances (PFASs). In addition, Chap. 6 includes a discussion of the potential for recovery by aquatic macrophytes from the effects of exposure to herbicides.

Due to the detrimental effects of toxic elements and xenobiotics on living organisms, there is a pressing need to develop strategies for eliminating or mitigating exposures to the contaminants that are discharged into the aquatic environment (Piwowarska and Kiedrzynska, 2021). On this subject, Chap. 7 describes best practices using drainage ditches vegetated with macrophytes as a management strategy to reduce the levels of contaminants (primarily pesticides and nutrients) entering surface waters in runoff from agricultural lands. Similarly, Chap. 8 describes "Green Liver" systems applied at laboratory and field scales, as low-impact, low-energy and low-cost systems for the remediation of pollutants in water.

1.4 Conclusions

Overall, this book provides a valuable addition to the literature on the use of macrophytes to assess the impacts of contaminants in aquatic ecosystems, and also, the potential for using macrophyte communities to reduce pollutant loading to the environment. Clearly, there is a need to develop standardized methods for toxicity testing using alternative test species, in addition to the standard operating procedures that have been developed with *Lemna* spp. and *Myriophyllum* spp. Continued work is needed to identify stress responses that can be used as biomarkers of exposure to toxicants, including employing -omics approaches. Finally, communities of macrophytes offer promise as "Nature-based Solutions" for mitigating the effects of substances that enter the aquatic environment from geogenic and anthropogenic sources.

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Chapter 2
Biomarkers in Aquatic Macrophytes: Traditional and Novel Approaches for Monitoring Responses to Exposure to Pollutants

Mirta L. Menone and Débora J. Pérez

Abstract The present work is a compilation and a discussion of articles published from 2008 to date, focusing on physiological, biochemical and genotoxicity biomarkers in aquatic macrophytes, including those most frequently used methods as well as novel approaches. This review indicates that batteries of biomarkers mainly related to dysfunction of the photosynthetic process and to oxidative stress/damage have been applied for testing responses of aquatic macrophytes to a range of pollutants. These include metals, metalloids and organometals (METs), current use pesticides (CUPs), polynuclear aromatic hydrocarbons (PAHs) and contaminants of emerging concern, which includes pharmaceuticals and personal care products (PPCPs) and per- and polyfluoroalkyl substances (PFASs), among others. Some research gaps emerged from the analysis of the literature, such as few ecotoxicological bioassays that evaluate the effects of environmental conditions (abiotic factors) on toxicity to macrophytes at realistic environmental concentrations. There is also a lack of studies focusing on marine species versus freshwater macrophytes. Traditional biomarkers like markers of oxidative stress and the content of photosynthetic pigments are still the most widely used methods. Other biomarkers related to the function of the mitochondrial electron transport chain and chlorophyll fluorescence (Chl F) are also recommended. Novel molecular biomarkers (e.g., gene expression) have shown promising results. For most of the types of pollutants studied, batteries of biomarkers in bench-scale tests have demonstrated modes of action (MoAs) but there is still a lack of validation of these methods under natural exposure scenarios

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since in situ studies are scarce. To guide researchers studying pollution effects on aquatic macrophytes in the field, we developed integrated models for monitoring of multiple biomarkers from bench-scale studies of biomarker responses to copper, atrazine, ciprofloxacin and PFASs.

2.1 Biomarker Definition and Classification

The term biomarker has been defined as "biological responses to an environmental chemical at the individual level or below; demonstrating a departure from the normal status" (Walker et al. 2001). The principal biomarkers tested have been 'measurable responses' that occur in photosynthetic activity, enzymatic processes of nutrition, secondary metabolite synthesis, oxidative stress and/or detoxification mechanisms (Ferrat et al. 2003). However, the development and availability of sophisticated techniques has made it possible to study biological pathways in greater depth, and to include other biological responses as biomarkers. In this sense, novel measured responses which involve mitochondrial respiration, DNA damage, gene expression, metabolomics, epigenetic changes, among others, are now considered as "novel biomarkers."

In this chapter, there is a discussion of the biological implications of changes in biomarkers in aquatic plants exposed to a range of contaminants, such as metals, metalloids and organometals (METs), polynuclear aromatic hydrocarbons (PAHs), current use pesticides (CUPs), contaminants of emerging concern, which include pharmaceuticals and personal care products (PPCPs) and per- and polyfluoroalkyl substances (PFASs), among other xenobiotics, such as phthalates and other plasticizers and surfactants. The cited literature is a compilation of original articles published from 2008 to date, taking as a starting point the seminal work of Brain and Cedergreen (2008). In that work, the authors concluded that all assessed biomarkers provided valuable information on the physiological effects of specific stressors, and that they were valuable tools in identifying modes of action (MoAs). In addition, these authors predicted that studies of aquatic plant biomarkers would be confined primarily to laboratory studies until further knowledge is gained regarding the time, dose and growth-factor dependence of biomarkers, in different species. The present work confirms this prediction since most of the research conducted subsequent to 2008 involves bench-scale bioassays, while examples of the application of biomarkers as tools for in situ biomonitoring are scarce. The studies reviewed in the present work show the sensitivity and versatility of biomarkers in aquatic plants for identifying MoAs, but these techniques have been applied to a much lesser extent in field studies. Studies have been classified according to major classes of biomarkers such as physiological, biochemical, genotoxicity and molecular.

- **Physiological biomarkers**: including cellular responses involved mainly in the processes of photosynthesis and mitochondrial respiration.

- **Biochemical biomarkers**: including the activity of detoxification enzymes, antioxidant enzymes, intermediate metabolism enzymes and biosynthesis and degradation enzymes; levels of scavengers or antioxidant molecules like glutathione (GSH), anthocyanins (Antho), free proline, ascorbate acid (ASC, vitamin C), α-Tocopherol (vitamin E), β-carotene (vitamin A); levels of metallothioneins (MTs) and phytochelatins (PCs); metabolites from phase I and II detoxification and products of oxidative damage of biomolecules, such as malondialdehyde (MDA).
- **Genotoxicity biomarkers**: including DNA damage such as chromosomal abnormalities and DNA fragmentation, both observed by microscopy.
- **Molecular biomarkers**: including sub-lethal responses which involve direct and indirect effects on DNA biomolecule, gene expression (e.g., expression of antioxidant enzymes), transcript levels as well as epigenetic changes (e.g., DNA bases methylation).

2.2 Physiological Biomarkers

The electron transport for photosynthesis in chloroplasts and the electron flux that drives respiration in mitochondria are key physiological processes in plants. Due to the universality and sensitivity of both processes in plants, biomarker responses related to these MoAs have been widely used to study the effects of environmental pollutants to aquatic macrophytes. The misdirection of electron flux, or the inability to transfer them correctly, will result in the break-down of plant homeostasis (Gill and Tuteja 2010), that can be monitored through various physiological biomarkers.

In particular, changes in the content of photosynthetic pigments, which can be measured as total chlorophyll (TChl), chlorophyll a (Chl a), chlorophyll b (Chl b), the ratio of Chl a/b, phaeophytins (Pheo), carotenoids (Caro) and re-emission of light measured as chlorophyll a fluorescence (Chl F) are the main physiological biomarkers used in aquatic plants to evaluate the effects of stressors on photosynthesis (Table 2.1). Other biomarkers, such as photosynthetic oxygen production ($P–O_2$), levels of plastoquinone (PQ) and the electron transport rate (ETR) in chloroplasts have also been utilized as biomarkers of the photosynthetic process (Vervliet-Scheebaum et al. 2008; Gomes et al. 2017; Pietrini et al. 2019). The biomarkers used to study effects on mitochondrial respiration in aquatic plants include the ETR in mitochondria, activity of complex I, II, III, IV of the electron transport chain, and the total levels of ubiquinone (UbQ), and their redox status as the ratio of the oxidized (UbQoxi) and reduced (UbQred) forms of this coenzyme (Table 2.1). These parameters include some of the more novel physiological biomarkers (Gomes et al. 2017; 2020).

By far the greatest number of biomarker studies have focused on the effects on photosynthesis. To understand the MoAs for biomarker responses related to plant photosynthesis, it is necessary to describe in more detail the photosynthetic process. In vascular plants, the absorption of photons is carried out mainly by Chl a (primary pigment) and Chl b (accessory pigment) located in the chloroplasts. Photons absorbed

Table 2.1 Physiological biomarkers used to evaluate effects in aquatic macrophytes

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Metals, metalloids and organometals (METs)					
Chl a, Chl b, Phae a and b	*Potamogeton pusillus*	Cu	5, 20, 40, 100 µg/L - 1, 3, 7 d	↓ Pigment contents	Monferrán et al. (2009)
Chl a, Chl b, Caro	*Bruguiera gymnorrhiza, Kandelia candel*	Cd Pb Hg	T1: 100 µg/L Cd + 1000 µg/L Pb + 100 µg/L Hg; T2: 5 × T1, T3: 10 × T1, T4: 15 × T1- -3 d	↓ Chl contents in both species T2, T3, T4, T5	Huang and Wang (2010)
Chl a, Chl b, Caro	*Vallisneria neotropicalis*	As Hg	As: 1.04–2.77 µg/g DW, Hg: 3.76–15.18 ng/g DW - Field	No effects	Lafabrie et al. (2011)
Chl a, Chl b, Caro	*Eichhornia crassipes*	Cd	5, 10, 15, 20 mg/L - 21 d	↓ Pigment contents	Das et al. (2016)
TChl, Antho	*Elodea nuttallii*	Hg	77 ng/L, 77 µg/L - 1 d	↓ Chl and Antho	Rieger et al. (2016)
Chl a, Chl b, Caro	*Hydrilla verticillata*	Cu Cd	0.01, 0.05, 0.1 mg/L - 5 d	↓ Chl a, Ratio a/b	Shi et al. (2017)
Caro	*Myriophyllum alterniflorum*	Cd	6.69 µg/L - 27 d	↓ Caro contents	Decou et al. (2018)
Chl a, Chl b	*Vallisneria natans*	Mixture As(III)/As(V)	As(III) 100 µg/L + As (V) 200 µg/L - 3, 7, 14 d	↓ Pigment contents	Li et al. (2018)
Chl F	*Zostera marina*	Cu Cd	0.8, 2.4 mM Cu - 6 d 0.89, 8.9 mM Cd - 6 d	↓ Fv/Fm	Greco et al. (2019)

(continued)

Table 2.1 (continued)

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Chl a, Chl b, Caro	*Limonium brasiliense*	Pb	45 and 90 μM - 30 d	↓ Pigment contents	Idaszkin et al. (2019)
Chl a, Chl b, Caro	*Myriophyllum alterniflorum*	As(V)	100 μg/L and 200–500 μg/g DW - 21 d	↓ Pigment contents	Krayem et al. (2019)
Polycyclic aromatic hydrocarbons (PAHs)					
Chl a, Chl b, TChl	*Ceratophyllum demersum*	Phenanthrene	0.02, 0.04, 0.06, 0.08, 0.1 mg/L - 10 d	↓ Pigment contents	Yin et al. (2010)
Chl F	*Lemna gibba*	Anthracene	5, 10 μg/mL - 6 h	↓ F_v/F_m	Mallakin et al. (2002)
Current use pesticides (CUPs)					
Chl F, P–O_2	*Elodea canadiensis*	Benzalkonium chloride (BAC) Atrazine (ATZ)	BAC: 0.5, 1, 2.5, 5, 7.5, 10, 25 mg/L - 4 h ATZ: 10, 50, 100, 500 μg/L - 4 h	↓ Chl F, P–O_2	Vervliet-Scheebaum et al. (2008)
ΦPSII	*Elodea canadensis* *Myriophyllum spicatum* *Potamogeton lucens*	Atrazine (ATZ) Isoproturon (ISO) Diuron (DIU)	ATZ: 70 μg/L - 34 d ISO: 14 μg/L - 34 d DIU: 5 μg/L - 34 d Mixture: 23.3 μg/L ATZ + 4.7 μg/L ISO + 1.7 μg/L DIU- 34 d	↓ ΦPSII in *M. quitense* with ATZ ↓ ΦPSII in *E. canadensis* and *M. quitense* with ISO ↑ ΦPSII in *P. luces* with ISO at 12 days ↑ ΦPSII in *E.* canadensis, *M. quitense* and *P. luces* with DIU and mixture	Knauert et al. (2010)

(continued)

Table 2.1 (continued)

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
TChl	*Hydrilla verticillata*	Flurprimidol (FLUR) Imazamox (IMA) Bensulfuron-methyl (BEN-M)	150, 300 μg/L - 4, 8, 12 wk 50, 100 μg/L - 4, 8, 12 wk 5 μg/L - 4, 8, 12 wk	↓ TChl with FLUR ↑ TChl with IMA and BEN-M	Theel et al. (2012)
Chl a, Chl b, Ratio a/b, ΦPSII	*Iris pseudacorus*	Atrazine	1, 2, 4, 8, 16, 32 mg/L - 1, 5, 10, 20, 35 d	↓ Pigment contents, ΦPSII	Wang et al. (2014)
Chl a, Ratio a/b	*Acorus calamus* *Lythrum salicaria* *Scirpus tabernaemontani*	Atrazine	1, 2, 4, 8 mg/L - 15, 30, 45, 60 d	↓ Chl a	Wang et al. (2015)
PR, ETR, TChl, Chl a, Phae a, Ratio Phae a/ Chl a, Complex I, II, III, IV, ETR	*Lemna minor*	Glyphosate	5, 10, 100, 500 mg/L - 45 min	↓ PR, ETR, TChl, ETR, Complex I, II, III, IV ↑ Ratio Phae a/ Chl a	Gomes and Juneau (2016)
TChl and ΦPSII	*Myriophyllum aquaticum* *Ruppia maritime*	Glyphosate	0.9, 1.8, 3.6, 7.2, and 36 g/L - 5 d	↓ TChl and PSII	Kittle and McDermid (2016)
Chl a, Chl b, Phae a, Phae b,	*Potamogeton pusillus*	Chlorpyrifos	0.0035; 0.0105, 0.0315, 0.0945 μg/L - 4 d	↓ Pigment contents	Bertrand et al. (2017)
TChl, Chl a, Chl b, Ratio a/b	*Bidens laevis*	Imidacloprid	0, 1, 10, 100, 1000 μg/L - 1 d	↑ Pigment contents	Lukaszewicz et al. (2019)
TChl, Chl a, Chl b, Ratio a/b	*Bidens laevis*	Tebuconazole	0, 1, 10, 100 μg/L - 2, 14 d	No effects	Moreyra et al. (2019)
TChl, Chl a, Chl b, Ratio a/b, Chl F, TChl, SPAD	*Bidens laevis*	Azoxystrobin	0, 0.1, 1, 10, 50, 100 μg/L - 2 d	↓ Chl b ↑ Ratio a/b	Pérez et al. (2019)

(continued)

Table 2.1 (continued)

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Chl a	*Oriza sativa*	Fenclorim (FEN) Pretilachlor (PRE)	10 μM FEN 10 μM PRE 10 μM FEN + 10 μM PRE - 3 d	↓ Chl a	Hu et al. (2020)
Pharmaeuticals and personal care products (PPCPs)					
Chl a, Chl b, TChl, Caro	*Typha* spp.	Ibuprofen	0.5, 1, 2 mg/L - 7, 14, 21 d	↓ TChl, Chl a	Dordio et al. (2011a)
Chl a, Chl b, TChl, Ratio a/b	*Phragmites australis*	Ciprofloxacin Oxytetracycline Sulfamethazine	Mixture: 0.1, 1, 10, 100, 1000 μg/L - 14, 28, 42, 56 d	↑ Chl ↓ Chl	Liu et al. (2013)
Chl a, Chl b, Ratio a/b, TChl, Caro	*Lemna gibba*	Ibuprofen	1 mg/L - 8 d	No effects	Pietrini et al. (2015)
ΦPSII	*Lemna minor*	Sucralose	0.6, 6, 60, 600, 6000 μg/L - 1, 7, 14, 21 d	↓ ΦPSII	Amy-Sagers et al. (2017)
Chl a,Chl b, Caro, Antho, Photo Gas Exchange, ΦPSII	*Lemna gibba*	Ibuprofen	20, 200, 1000 μg/L - 8 d	↓ TChl, Caro ↑ F_0, F_m	Di Baccio et al. (2017)
TChl, Caro, Chl F, qP, UbQred, UbQoxi, UbQ, ETR, Complex I, II, III, IV	*Lemna minor*	Ciprofloxacin	750, 1050, 2250, 3050 μg/L - 5 d	↓ ΦPSII (F_v and F_v/F_m), NPQ, UbQred, Complex I–IV activities and ETR ↑ F_0, qP and UbQoxi	Gomes et al. (2017)

(continued)

Table 2.1 (continued)

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Chl a, Chla b, TChl, Caro, Antho, Chl F	*Lemna minor* *Lemna gibba*	Chlorpromazine (CPZ) Paracetamol (PCM) Diclofenac (DCF)	CPZ: 0.032, 0.16, 0.8, 4, 20 μg/L PCM: 0.2, 1, 5, 25, 125 μg/L DCF: 0.16, 0.8, 4, 20, 100 μg/L - 4 d	*L. minor*: ↑ Chl b with CPZ, PCM and DCF ↑ TChl with DCF *L. gibba*: ↑ Chl b with CPZ ↑ TChl with DCF ↑ Caro with CPZ and DCF ↓ Antho with CPZ, PCM and DCF	Alkimin et al. (2019a, b)
Chl a, Chl b, TChl, Caro	*Lemna minor*	Diclofenac (DCF) Salicylic acid (SA)	4, 20, 100 μg/L of each PPCPs, 6, 10, 14 d	↓ Pigment contents with DCF	Alkimin et al. (2019a, b)
Chl a, Chl b, Caro, ΦPSII	*Lemna minor* *Lemna gibba*	Ciprofloxacin	5, 13, 31, 78, 195 μg/L - 5 d	*L. gibba*: ↓ Caro	Nunes et al. (2019)
Chl a, Chl b, Ratio a/b, TChl, Caro	*Spirodela polyrhiza*	Ofloxacin	10, 50, 100, 500, 1000 μg/L - 7 d	↑ TChl; ↓ Caro	Singh et al. (2019)
Chl a, Chl b, Caro	*Lemna minor*	Ketoprofen	0.24, 1.2, 6, 30 μg/L - 4 d	No effects	Alkimin et al. (2020a, b)
Chl a, Chl b, TChl, Caro	*Lemna minor* *Lemna gibba*	Clotrimazol	0.008, 0.04, 0.2, 1, 5 μg/L - 4 d	*L. gibba*: ↑ Chl b, TChl	Alkimin et al. (2020a, b)
Chl F, ΦPSII, Complex I, II, III, IV,	*Lemna minor*	Amoxicillin (AMX) Enrofloxacin (ENR) Oxytetracycline (OXY)	AMX: 2 μg/L ENR: 2 μg/L OXY: 1 μg/L AMX + ENR AMX + OXY ENR + OXY AMX + ENR + OXY - 14 d	↓ F_v/F_m (ΦPSII) with OXY and mixtures ↓ ETR in all treatments ↓ Complex II, III and IV with ENR and OXY and some combinations	Gomes et al. (2020)

(continued)

Table 2.1 (continued)

Physiological biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Chl a, Chl b, Caro	*Oriza sativa*	Cyprofloxacin	0.5, 1, 2, 4, 8 mg/L - 21 d	↓ Pigment contents	Hu et al. (2021)
Other organic xenobiotics					
Chl a, Chl b	*Lemna minor*	Polyethylene	10, 50, 100 mg/L - 7 d	No effects	Kalcikova et al. (2017)
Chl a, Chl b, Caro	*Eichhornia crassipes* *Cyperus alternifolius*	PFOS	1, 100, 10,000 μg/L - 42 d	↑ Pigment contents at 100 μg/L; ↓ Pigment contents at 1000 μg/L in *C. alternifolius*	Li et al. (2020)
ΦPSII, F_v/F_m, NPQ, ERT, TChl	*Lemna minor*	PFOA	2, 20, 200, 200 μg/L - 7 d	Transient effects	Pietrini et al. (2019)
Chl a, Chl b, TChl, Caro, Antho	*Spirodela polyrhiza*	Fluoride (FLU) Diethyl phthalate (DEP) Diallyl phthalate (DAP)	FLU: 50 mg/L DEP and DAP: 75, 150, 300 mg/L individually and their single and binary combination - 1, 3, 5, 7 d	↓ TChl with single and binary treatments of DEP, FLU and DAP ↓ Caro with FLU, DEP and DAP ↑ Antho with FLU, DEP and DAP	Sharma et al. (2019)
Chl a, Chl b, TChl, Caro	*Salvinia cucullata*	Glyphosate (GLY) Fluorescent polystyrene microplastics (PS-MPs)	GLY: 5, 25, 50 mg/L - 7 d PS-MPs: 3, 15, 75 mg/L PS-MPs + GLY (mg/L): 3 + 5; 15 + 25; 75 + 50	GLY: ↓ Chl, Caro	Yu et al. (2021)

Chl a: Chlorophyll a; Chl b: Chlorophyll b; Phae a: phaeophytin a, a; Phae b: phaeophytin b; Caro: carotenoids; TChl: Total Chlorophylls; Antho: anthocyanins; Chl F: chlorophyll a fluorescence; d: days; h: hours; m: min; P–O_2: photosynthetic oxygen production; ΦPSII: quantum efficiency of photosystem II; Ratio a/b: Ratio chlorophyll a/ chlorophyll b; qP: photochemical quenching; PR: photosynthetic rate; PFOS Perfluorooctane sulfonate; PFOA Perfluorooctanoic acid; ETR: electron transport rate; UbQred: reduced ubiquinone; UbQoxi: oxidized ubiquinone; UbQ: ubiquinone; Fm: maximum fluorescence; Fv: ratio between initial fluorescence and Fm; F_v/F_m: maximum quantum yield of photosystem II; NPQ: non-photochemical quenching; wk: weeks

can undergo one of three fates: (a) the photosynthesis process (photochemistry); (b) dissipation of excess energy as heat; (c) re-emission as light (referred to as Chl F) (Maxwell and Johnson 2000). Chlorophylls are assembled with membrane polypeptides, constituting the photosystem complex, named photosystem I (PSI) and photosystem II (PSII). When Chl a absorbs a photon, the molecule acquired an excited unstable state (Chl a*). The excited state of Chl a* can be converted to energy by the transfer of electrons down the electron transport chain to the PQ in the binding site QA and later to QB (reaction center 'closed'). At that point, the Chl a* will return to the stable state (Chl) by releasing the absorbed energy, by re-emitting a photon of light as fluorescence, most commonly referred to as Chl F. When the rate of reaction center 'closed' is high due to light-induction, the Chl F increases, and such quenching is referred as 'photochemical quenching' (qP), which represents the proportion of 'reaction center open' PSII. Concomitantly, a proportion of Chl a* causes dissipation of excess energy by conversion to heat, referred to as 'non-photochemical quenching' (NPQ), as described by Maxwell and Johnson (2000). The NQP reflects the dissipation of excess excitation energy in the form of harmless heat from the PSII, thus protecting the plant from the potential damage from the formation of reactive oxygen species (ROS), as described by Pietrini et al. (2019).

Under natural conditions, plants exhibit a characteristic Chl F kinetics, as the initial fluorescence (F_0) represents the Chl F before that energy has migrated to the reaction center, and maximum fluorescence (F_m) is reached when the PSII Chl a is fully reduced. The variation between F_m and F_0 is referred as F_v, and reflects the reduction of PSII electron acceptors. The relation between F_v and F_m represent the maximum quantum yield of PSII (F_v/F_m), the quantum efficiency of PSII (ΦPSII) represent the relation between F_m and F_t (i.e., the level of Chl F immediately before the saturating flash), as described by Maxwell and Johnson (2000). Moreover, the electron transport flux through PSII, measured as ETR can be monitored (Pietrini et al. 2019). However, under conditions of exposure to pollutants, the electron transfer chain can become uncoupled, and the effects on the photochemical and non-photochemical Chl F parameter can be monitored quickly, efficiently and non-destructively to evaluate the effects on photosynthesis. For example, in *Lemna minor* exposed to 2 μg/L of perfluorooctanoic acid (PFOA), transient effects were observed on F_v/F_m, ΦPSII, NPQ and ETR (Pietrini et al. 2019). Moreover, Gomes et al. (2017) observed decreases in F_v, F_v/F_m, NPQ, UbQred, complex I–IV activities and ETR, and increases in F_0, qP and UbQoxi in *L. minor* exposed to the antibiotic ciprofloxacin (Table 2.1). When the electron transport chain is uncoupled, the Chl a* cannot return to its ground state, because the blocked electron acceptor is not able to accept the electron and the Chl F decreases (de Albuquerque et al. 2020). Moreover, the Chl* induces production of ROS, resulting in oxidative damage, enzyme activation, inhibition or degradation, reductions in CO_2 assimilation rate, among other effects. In this way, a direct adverse consequence is the damage, oxidation and degradation of the main photosynthetic pigments (Chl a, Chl b) and the accessory pigments (Caro, Phae and Antho).

The catalytic activity of some enzymes involved in the metabolic pathways for synthesis and degradation of pigments can be targeted by different xenobiotics,

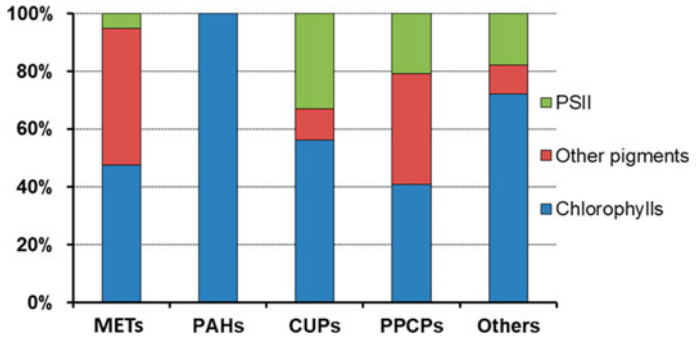

Fig. 2.1 Percentage of the different types of biomarkers used to evaluate effects on plant photosynthesis used for each group of contaminants: METs, PAHs, CUPs, PPCPs and other xenobiotics (e.g., PFASs, solvents and plasticizers). PSII: parameters related to the function of Photosystem II: Chlorophyll a fluorescence, F_m/F_v, ΦPSII; Other pigments: Caro, Phae, Antho

mainly herbicides. For example, the enzyme protoporphyrinogen IX oxidase that catalyzes the conversion to protoporphyrinogen IX from protoporphyrin IX, is a target for the diphenyl ether class of herbicides (Tanaka and Tanaka 2006). As a result of this damage to the photosynthetic pathways, chlorosis, necrosis and early senescence can occur in the plant. The photosynthetic gas exchange rates, referred to as the CO_2 uptake and O_2 production (Di Baccio et al. 2017), are used as indicators of photo-inhibition and photosynthetic capacity, but these parameters have been poorly explored in ecotoxicology studies.

In conclusion, among the physiological biomarkers, the photosynthetic pigment contents constitute the main biomarkers that are used to monitor responses to a range of contaminants, while the PSII parameters are more restricted to identify effects caused by exposure to CUPs and PPCPs (Table 2.1). Figure 2.1 illustrates the percentage of the various types of biomarkers that have been utilized to monitor effects on photosynthesis from exposure to various classes of contaminants, as indicated from the data compiled in Table 2.1.

2.3 Biochemical Biomarkers

2.3.1 Oxidative Stress

Oxidative stress is described as the imbalance between the generation and the neutralization of ROS by antioxidant mechanisms within an organism (Davies 1995), or in other words, intracellular levels of ROS increase to such a level that cellular antioxidant defenses are insufficient to maintain these harmful molecules at a level below a toxic threshold (Cnubben et al. 2001). The production of low levels of oxygen free radicals and non-radical reactive species, collectively called ROS, is normal in several

processes like the mitochondrial electron transport involved in the reduction of O_2 to water (Rand 1995). In addition, several other sources of endogenous ROS production have been identified such as the electron transport chains of chloroplasts and the activities of a number of metabolic cytochromes, such as cytochrome P450 (CYP450), as described by Valavanidis et al. (2006) and Sharma et al. (2012). If during these metabolic processes, a small proportion (2–3%) of ROS escapes from the various antioxidant mechanisms, oxidative damage to cellular macromolecules (e.g., lipids, proteins, DNA) can take place. However, enzymatic and non-enzymatic antioxidant mechanisms have evolved in both plants and animals to protect cellular components from damage by maintaining ROS below a critical level (Valavanidis et al. 2006). Plants particularly use ROS as messengers in signal transduction cascades in processes as diverse as mitosis, tropisms and cell death, and their accumulation is crucial to plant development, as well as defense (Foyer and Noctor 2005). Therefore, the concept of oxidative stress in plants has been proposed to be called 'oxidative signalling', that is, a critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology (Foyer and Noctor 2005).

ROS includes singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). The generation of toxic ROS occurs in various cellular sites, such as in the mitochondria, chloroplasts, peroxisomes and apoplasts. Particularly, the chloroplast is one of the leading ROS production sites in plants where ROS generation, directly and indirectly, depends on the interaction of Chl and light (Hasanuzzaman et al. 2020). As was explained previously, chloroplasts convert light energy to the energy required for creating chemical bonds. Light absorption by the Chl molecules of PSI and PSII triggers a sequence of redox reactions along the thylakoid membrane of chloroplasts that result in the oxidation of water, reduction of $NADP^+$ to NADPH and formation of a proton gradient across the membrane. The reduction in O_2 by reduced forms of electron carriers in the photosynthetic electron transfer chain can produce $O_2^{\bullet -}$ and H_2O_2 (Khorobrykh et al. 2020). For example, in PSI, $O_2^{\bullet -}$ is produced by Mehler reaction and then the metalloprotein manganese superoxide dismutase (Mn-SOD) converts this molecule into H_2O_2 (Hasanuzzaman et al. 2020). On the other hand, in plant organs that do not contain chlorophyll, particularly in the root, mitochondria are the major sites of ROS generation (Bose et al. 2014).

Plants have evolved several enzymatic and non-enzymatic ROS-scavenging and quenching mechanisms, and ROS-mediated signaling and ROS detoxification are coupled (Khorobrykh et al. 2020). The antioxidant defense system consists of several non-enzymatic antioxidants that are low molecular weight biomolecules, including ascorbic acid (ASC), carotenoids, phenolic compounds, α-tocopherol and some alkaloids. Antioxidant enzymes include different isoforms of superoxide dismutases (SOD), catalases (CAT), guaiacol peroxidase (POD) and ascorbate peroxidases (APx). Like in animals, the reactions involved in the cycling of the tripeptide glutathione between its oxidized form (GSSG) and reduced form (GSH) are catalyzed by glutathione reductase (GR) and glutathione peroxidase (GPx) and these redox reactions neutralize ROS (Fig. 2.2). Glutathione and these enzymes that catalyze

the redox reactions that neutralize ROS are mainly associated with the cytosol and endoplasmic reticulum, but are also found in chloroplasts, mitochondria and other organelles (Hasanuzzaman et al. 2012). However, plant GPx probably make a very small contribution to overall peroxide metabolism, compared to CAT and APx (Foyer and Noctor 2005). In the chloroplasts, the $O_2^{\bullet -}$ produced is scavenged efficiently by SOD associated with the stromal face of the thylakoid membrane while H_2O_2 is reduced in a reaction catalyzed by APx. On the other hand, carotenoids and tocopherols are the main non-enzymatic antioxidants that neutralize $^1O_2$ in chloroplast (Khorobrykh et al. 2020).

The over-generation of ROS disrupts the equilibrium between ROS accumulation and degradation, causing oxidative damage to biomolecules (Hasanuzzaman et al. 2020). Although under oxidative stress biomolecules like lipids, proteins, DNA and carbohydrates become reversibly or irreversibly modified, many of the ecotoxicological studies have focused on the damage to lipids known as lipid peroxidation (LPO), as shown in Table 2.2. In the membrane phospholipids, the most susceptible sites for ROS attack are the carbon (C) atoms and the ester linkage between fatty acids and glycerol. Moreover, $^1O_2$ and $OH^{\bullet}$ attack the polyunsaturated fatty acids in the plasma membrane. After several steps, aldehydes like malondialdehyde (MDA), acrolein and 4-hydroxy-2-nonenal are formed, which are all oxidative stress markers in plants. Products of oxidative damage to nucleotides of DNA such as 8-hydroxyguanidine (8-OHdG) can also be monitored as indicators of oxidative stress. Extreme LPO results in loss of membrane function and thus, cellular organelles and the cell

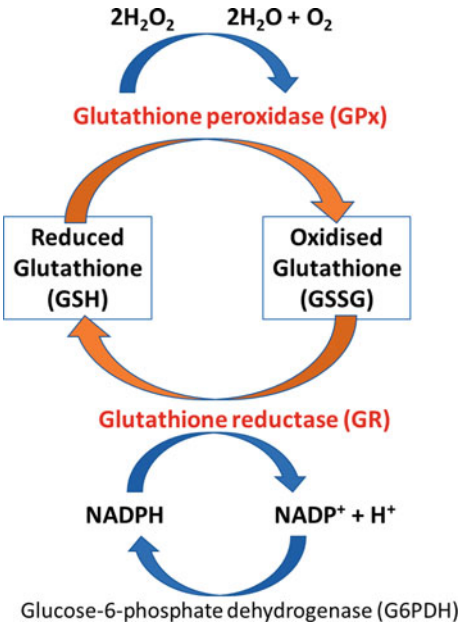

Fig. 2.2 Mechanism for reduction of hydrogen peroxide by the activity of glutathione peroxidase (GPx) and other enzymes involved in the cycling of glutathione between its oxidized state (GSSG) and reduced state (GSH)

Table 2.2 Biochemical biomarkers used to evaluate effects in aquatic macrophytes

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Metals, metalloids and organometals (METs)					
Antioxidant enzymes, MDA	*Potamogeton pusillus*	Cu	5, 20, 40, 100 μg/L - 1, 3, 7 d	↑ GPx, GR, POD, MDA	Monferrán et al. (2009)
PCs	*Halimione portulacoides*	Hg	2.5–15 mg/Kg in field sediment	↑ PCs	Válega et al. (2009)
CAT, POD, MDA, Carbonyl content	*Lemna minor*	Cu Cd	2.5 μmol/L; 5 μmol/L each one and their mixture - 4, 7 d	↑ CAT, POD, MDA, Carbonyl content	Cvjetko et al. (2010)
GSH, PCs	*Kandelia candel, Bruguiera gymnorrhiza*	Cd Pb Hg	T1: 0.1 mg/L Cd + 1 mg/L Pb + 0.1 mg/L Hg. T2: 5 × , T3: 10 × , T4: 15 × higher than T1 - 30 d	↑ GSH, PCs	Huang and Wang (2010)
PCs	*Wolffia globosa*	As(V)	1, 5, 10, 30, 50, 100 uM - 5 d	↑ PCs	Zhang et al. (2012)
H_2O_2, $O_2^{\bullet -}$, NAD(P)H-oxidase, MDA, Protein oxidation, GSH, GSSG, Antioxidant enzymes	*Salvinia natans*	Al	240, 360, 480 μM - 7 d	↑ H_2O_2, $O_2^{\bullet -}$, NAD(P)H-oxidase, MDA, Protein oxidation, GSH, POD, APx, CAT, GR, SOD	Mandal et al. (2013)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
ASC, Non-protein thiols, PCs, PC synthase, tGSH	*Najas indica*	As	10–250 μM As V, 1–50 μM As III - 1, 2, 4, 7 d	↑ ASC, tGSH, Non-protein thiols, PCs	Tripathi et al. (2014)
ASC, Cys-y-Glu, PCs, PC synthase, Total protein	*Elodea canadensis* *Salvinia natans* *Lemna minor*	Cu Zn Cd	4.1 mg/L Cu + 4.3 mg/L Zn + 7.3 mg/L Cd - 6 d	↓ ASC and Total protein in *E. canadensis* ↑ ASC in *L. minor* and *S. natans*	Torok et al. (2015)
Antioxidant enzymes, Soluble proteins, MDA	*Eichhornia crassipes*	Cd	5, 10, 15, 20 mg/L - 21 d	↓ Soluble proteins ↑ MDA	Das et al. (2016)
Antioxidant enzymes	*Elodea nuttallii*	Hg	77 ng/L, 77 μg/L - 1 d	↓ POD ↑ SOD	Regier et al. (2016)
Antioxidant enzymes, GSH, GSSG, PCs	*Halimione portulacoides* *Sarcocornia perennis* *Spartina marítima*	Zn Pb Cu As Cd	500 μg/g Zn 400 μg/g Pb 70 μg/g Cu 50 μg/g As 1 μg/g Cd (field)	↑ PC2 with Zn, Pb and As ↓ Total PCs with Zn, Pb and Cu	Negrin et al. (2017)
Antioxidant enzymes, MDA, GSSG, GSH, tGSH, PCs	*Suaeda maritime*	As	200 and 400 μM - 14 d	↑ GSSG, GSH, tGSH; CAT ↓ SOD, POD, APx, GR, PCs	Panda et al. (2017)
PCs	*Ceratophyllum demersum* *Myriopyllum spicatum*	Hg	1–4 ng/L (field)	↑ PCs	Turull et al. (2017)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
H_2O_2, $O_2^{\bullet -}$, MDA	*Hydrilla verticillata*	Cu	0.01, 0.05, 0.1 mg/L - 5 d	↑ H_2O_2, $O2^{\bullet -}$, MDA	Shi et al. (2017)
Antioxidant enzymes, Free proline, G6PDH, MDA	*Myriophyllum alterniflorum*	Cd	6.7 μg/ L - 27 d	↑ Free Proline, G6PDH, SOD, CAT, APx, MDA	Decou et al. (2018)
Antioxidant enzymes, MDA	*Vallisneria natans*	Mixture As(III)/As(V)	As(III) 100 μg/L + As (V) 200 μg/L - 3, 7, 14 d	↑ POD, SOD, CAT, MDA	Li et al. (2018)
Antioxidant enzymes, MDA	*Limonium brasiliense*	Pb	45, 90 μM - 30 d	↑ APx, POD, SOD, MDA	Idaszkin et al. (2019)
H_2O_2	*Myriophyllum alterniflorum*	As	100 ug/L As (V) - 21 d	↑ H_2O_2	Krayem et al. (2019)
G6PDH, MDA, α-Tocopherol	*Myriophyllum alterniflorum*	Cu	5, 10, 25, 50, 100 μg/L - 27 d 0.5, 1, 4, 7, 10 μg/L - 27 d	↑ MDA ↓ α-tocopherol	Decou et al. (2019)
PCs, tGSH	*Zostera marina*	Cu Cd	0.8, 2.4 mM Cu - 6 d 0.89, 8.9 mM Cd - 6 d	↑ PCs and tGHS	Greco et al. (2019)
Total ROS, MDA, tGSH	*Rhizophora mucronata*	Cu Zn	Cu: 200 mg/L - 1, 5 d Zn: 200 mg/L - 1, 5 d	↓ tGSH	Nualla-ong et al. (2020)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Antioxidant enzymes, PCs	*Oryza sativa*	Cd	10 μM 6, 12 h, 1, 2, 4 d	↑ SOD, CAT, POD, PCs	Chen et al. (2021)
Polycyclic aromatic hydrocarbons (PAHs)					
Antioxidant enzymes, tGSH, GSSG, $O_2^{\bullet -}$, MDA	*Ceratophyllum demersum*	Phenanthrene	0.02, 0.04, 0.06, 0.08, 0.1 mg/L - 10 d	↓ SOD, tGSH ↑ CAT, GST, POD, GSSG, MDA	Yin et al. (2010)
Current use pesticides (CUPs)					
Antioxidant enzymes, MDA	*Myriophyllum quitense*	Azoxystrobin	0.1, 1, 10, 50, 100 μg/L - 1 d	↓ CAT, POD ↑ MDA	Garanzini et al. (2015)
NADPH CYP450 Reductase, p-nitroanisole O-demethylase	*Oriza sativa*	Atrazine	0.4 mg/L - 2 d	↑ NADPH CYP450 Reductasa, p-nitroanisole O-demethylase	Tan et al. (2015)
Antioxidant enzymes, H_2O_2, MDA,	*Lemna minnor*	Glyphosate	5, 10, 100, 500 mg/L - 45 min	↑ CAT, APx, H_2O_2, MDA	Gomes and Juneau (2016)
Antioxidant enzymes, MDA	*Potamogeton pussilus*	Chlorpyrifos	0.0035; 0.0105, 0.0315, 0.0945 μg/L - 4 d	Leaf: ↑ POD, GPx, SOD and ↓ MDA Root: ↓ SOD, MDA	Bertrand et al. (2017)
Antioxidant enzymes	*Myriophyllum quitense*	Endosulfan	5, 10 ug/L - 1 d	↓ POD, CAT ↑ GST	Garanzini et al. (2019)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Antioxidant enzymes, MDA	*Oriza sativa*	Fenclorim (FEN) Pretilachlor (PRE)	10 μM FEN 10 μM PRE 10 μM FEN + 10 μM PRE - 3 d	FEN: ↑ POD, GST PRE: ↑ SOD, CAT, POD, MDA	Hu et al. (2020)
Pharmaceuticals and personal care products (PPCPs)					
Antioxidant enzymes	*Typha* spp.	Ibuprofen	0.5, 1, 2 mg/L - 7, 14, 21 d	↑ SOD with time/dose ↑ CAT at 0.5 mg/L, 7 d ↑ POD at 0.5 mg/L, 7 d	Dordio et al. (2011a, b)
Antioxidant enzymes	*Typha* spp.	Carbamazepine (CBZ) Ibuprofen (IBU)	0.5, 1, 2 μg/L - 7, 14, 21 d	CBZ: ↑ SOD, CAT, GST, GPx ↓ ↑ IBU: SOD, CAT, POD	Dordio et al. (2011b)
Antioxidant enzymes	*Phragmites australis*	Ciprofloxacin Oxitetracycline Sulfamethazine	Mixture: 0.1, 1, 10, 100, 1000 μg/L of each one - 14, 28, 42, 56 d	↑ POD, ↓ CAT, SOD	Liu et al. (2013)
Antioxidant enzymes, Glycosyl transferase	*Typha* spp.	Diclofenac (DCF)	1 mg/L - 1, 3, 7 d	↑ POD, APx, GR, GST, Glycosyl transferase	Bartha et al. (2014)
APx, ASC, H_2O_2	*Lemna minor*	Ciprofloxacin	750, 1050, 2250, 3050 μg/L - 5 d	↑ CAT, APx, ASC H_2O_2	Gomes et al. (2017)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Antioxidant enzymes	*Lemna minor*	Diclofenac (DCF) Salicylic acid (SA)	4, 20, 100 µg/L - 6, 10, 14 d	DCF: ↑ GST, ↓ CAT SA: ↑ CAT, APx, ↓ GST	Alkimin et al. (2019a, b)
CAT	*Lemna minor* *Lemna gibba*	Chlorpromazine (CPZ) Paracetamol (PCM) Diclofenac (DCF)	CPZ: 0.032, 0.16, 0.8, 4, 20 µg/L PCM: 0.2, 1, 5, 25, 125 µg/L DCF: 0.16, 0.8, 4, 20, 100 µg/L - 4 d	*L. minor*: ↑ CAT with with CPZ, PCM and DCF *L. gibba*: ↑ CAT with PCM	Alkimin et al. (2019a, b)
CAT, MDA	*Lemna minor, Lemna gibba*	Ciprofloxacin	5, 13, 31, 78, 195 µg/L - 5 d	*L. minor*: ↑ CAT *L. gibba*: ↓ MDA	Nunes et al. (2019)
Antioxidant enzymes	*Spirodela polyrhiza*	Ofloxacin	10, 50, 100, 500, 1000 µg/L - 7 d	↑ SOD, APx	Singh et al. (2019)
Antioxidant enzymes, MDA, Carbonic anhydrase	*Lemna minor*	Ketoprofen	0.24, 1.2, 6, 30 µg/L - 4 d	↑ GST, CAT ↓ Carbonic anhydrase	Alkimin et al. (2020a, b)
Antioxidant enzymes	*Lemna minor* *Lemna gibba*	Clotrimazol	0.008, 0.04, 0.2, 1, 5 µg/L - 4 d	*L. minor*: ↑ CAT, ↓ GST *L. gibba*: ↑ CAT, GST	Alkimin et al. (2020a, b)
Antioxidant enzymes, H_2O_2, MDA		Amoxicillin (AMX) Enrofloxacin (ENR) Oxytetracycline (OXY)	AMX: 2 µg/L ENR: 2 µg/L OXY: 1 µg/L AMX + ENR AMX + OXY ENR + OXY AMX + ENR + OXY - 14 d	↑ CAT, APx, ↓ H_2O_2 with AMX ↑ CAT, APx, ↓ H_2O_2 with ENR ↑ H_2O_2, APx, MDA, ↓ CAT with OXY	Gomes et al. (2020)

(continued)

Table 2.2 (continued)

Biochemical biomarker	Plant species	Stressor	Exposure concentration - time	Biomarker responses	Reference
Antioxidant enzymes, MDA	*Oriza sativa*	Ciprofloxacin	0.5, 1,2,4,8 mg/L - 21 d	↑ SOD, POD, CAT, MDA	Hu et al. (2021)
Other xenobiotics					
Antioxidant enzymes, MDA	*Eichhornia crassipes* *Cyperus alternifolius*	Perfluorooctane sulfonate	1, 100, 10,000 μg/L - 42 d	↑ CAT, MDA in both sps. ↑ POD in *C.a*	Li et al. (2020)
Antioxidant enzymes, MDA, soluble proteins, carbohydrates	*Spirodela polyrhiza*	Fluoride (FLU) Diethyl phthalate (DEP) Diallyl phthalate (DAP)	FLU: 50 mg/L DEP and DAP: 75, 150, 300 mg/L individually and their single and binary combination - 1, 3, 5, 7 d	↓ Soluble proteins, carbohydrates ↑ SOD, CAT, APx, GR, MDA	Sharma et al. (2019)
Antioxidant enzymes, MDA	*Salvinia cucullata*	Glyphosate (GLY) Fluorescent polystyrene microplastics (PS-MPs)	GLY: 5, 25, 50 mg/L - 7 d PS-MPs: 3, 15, 75 mg/L PS-MPs + GLY (mg/L): 3 + 5; 15 + 25; 75 + 50	GLY: ↑ SOD, MDA, ↓ CAT PS-MPs: ↑ SOD, CAT, APx, PS-MPs + GLY: ↑ SOD, CAT, MDA	Yu et al. (2021)

APx: ascorbate peroxidase; ASC: ascorbic acid; CAT: catalase; Cys-y-Glu: Cysteine- y-glutamylcysteine; d: days; h: hours; H_2O_2: Hydrogen peroxide; G6PDH: glucose-6- phosphate dehydrogenase; GSSG: oxidized glutathione; GSH: reduced glutathione; tGSH: total glutathione; GST: glutathione-S-transferase; MDA: malondialdehyde; min: minutes; MTs: metallothioneins; PCs: phytochelatins; POD: guaiacol peroxidase; SOD: superoxide dismutase; $O_2^{\bullet -}$: superoxide radical anion; Total ROS: total reactive oxygen species

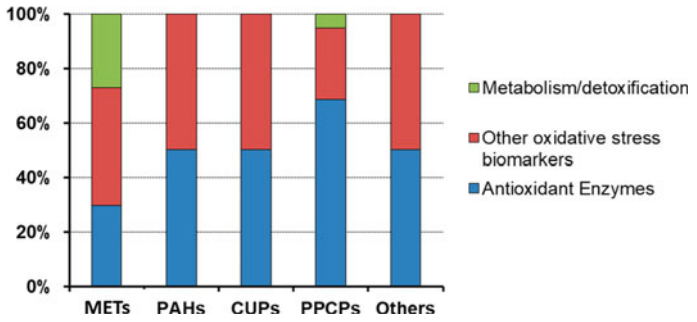

Fig. 2.3 Percentage of the different types of biochemical biomarkers used to evaluate the effects of exposure to each group of contaminants: METs, PAHs, CUPs, PPCPs and other xenobiotics (e.g., PFASs, solvents and plasticizers)

membrane disintegrate, while there is also malfunctioning of enzymes, and damage to DNA and RNA (Ahmad et al. 2019; Hasanuzzaman et al. 2020).

Recent literature indicates that there is a sharp threshold for ROS levels that are beneficial or toxic, depending on the plant species, their growth stages and type of abiotic stresses, stress intensity and duration (Hasanuzzaman et al. 2020). Huang et al. (2019) pointed out that it is very difficult to distinguish between the cytotoxic and signaling events that are induced by a particular ROS species. Therefore, caution should be applied in interpreting data on biomarkers of oxidative stress in an ecotoxicological context. In general, ROS production tends to be greatly increased by pollution as a stressor (Dordio et al. 2011b). As can be seen in Fig. 2.3, the levels of antioxidant enzymes are the most widely studied biomarkers among the biochemical group, although other biomarkers of oxidative stress such as levels of total glutathione (tGSH), GSSG and GSH are also monitored. The products of lipid peroxidation (e.g., MDA) and oxidative damage to DNA (8-OHdG) are also widely monitored (Fig. 2.3). From our point of view, integration of batteries of biochemical biomarkers with physiological biomarkers is recommended for studies of responses in aquatic macrophytes to environmental stressors.

2.4 Detoxification/biotransformation

Concerning metals and metalloids, primary detoxification refers to the processes to complex/chelate metal ions with cellular components (cell wall), gene-encoded polypeptides such as metallothioneins (MTs), enzymatically synthesized peptides like phytochelatins (PCs) and organic acids such as citrate and malate (Shrivastava and Srivastava 2021). The cysteine-rich group of polypeptides known as MTs regulate cellular homeostasis for both essential cations (e.g., Zn^{2+}) and non-essential cations (e.g., Cd^{2+}) in plants and animals through the processes of cytosolic metal binding, vacuolar sequestration and metal efflux through the plasma membrane. The

expression of MTs is rapidly activated under conditions of high cation concentrations and are expressed in metal-sensitive organisms to provide tolerance toward treatment with Cd, Cu and Zn (Zhou et al. 2014; Ur-Rahman et al. 2020). Although MTs are primarily involved in the homeostatic regulation of essential cations, such as copper and zinc, they can also play a protective role by detoxifying non-essential metals (Talebi et al. 2019). Another group of Cys-rich peptides, which are also metal and metalloid chelators in plants is PCs (Yadav 2010). As an example, the process of arsenic detoxification in plants involves the formation of an As(III)-PC complex in the cytosol followed by sequestration in the vacuoles with the help of protein transporters. Indeed, this is the tolerance strategy adopted by plants to alleviate As-induced oxidative stress (Bali and Sidhu 2021). However, for some cations like Hg, the tolerance strategies of the plant are likely to involve cell wall immobilization in the roots as a dominant mechanism of resistance, rather than chelation in the cytosolic fraction (Válega et al. 2009).

For organic contaminants, an important group of biochemical biomarkers in plants are the enzymes and cofactors associated with biotransformation processes by which a compound is converted into more water-soluble and often less toxic metabolites that are easier to eliminate than the parent compound (Rand 1995). For aquatic macrophytes, the herbicide detoxification systems have been traditionally studied. Biotransformation processes consist of three metabolic phases: (i) phase I reactions catalyzed by CYP450 monooxygenases; (ii) phase II reactions consisting of the conjugation with biomolecules, catalyzed by glutathione-S-transferases (GST) to form glutathione conjugates and glycosyl transferases to form conjugates with glycosides; and (iii) phase III processes involving membrane-associated transporters that carry metabolites across cell membranes (Benekos et al. 2010). In addition, specific metabolites of important herbicides like atrazine have been characterized (Tan et al. 2015; Qu et al. 2018; Pérez et al. 2022). However, investigations of CYP450 and phase II enzyme systems in plants exposed to contaminants are relatively scarce (Brain and Cedergreen 2008). Hence, more research is needed on the biotransformation of all classes of organic pollutants by aquatic macrophytes. An example of these types of studies is the work by Bartha et al. (2014) on *Typha latifolia* exposed to diclofenac, where the metabolism of this anti-inflammatory drug through phase I mechanisms was observed to produce 4-OH diclofenac at 24 h after treatment in higher amounts than diclofenac itself. In addition, phase II conjugates with glycoside (4-O-glucopyranosyl-oxydiclofenac) and with glutathione (4-OH-glutathionyl-diclofenac) were detected. Recently, analysis of the plant transcriptome has helped to understand gene expression and mechanisms for detoxification and metabolism of toxic compounds in plants (Lu et al. 2013). More about it is described in the section on molecular biomarkers.

2.5 Genotoxicity Biomarkers

Genetic information is encoded in the molecular structure of the nucleic acids that make up the structures of DNA and RNA. The vehicles of genetic information within the cell are the chromosomes, which are made up of DNA and associated proteins. The integrity of DNA is under constant attack by radiation, exposure to chemicals and spontaneous changes resulting from the infidelity of replication (Pearce 2009). Genotoxicity is a broader term that refers to the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome, such as the spindle apparatus and topoisomerase enzymes (Maurici et al. 2005). Most studies with plants have focused on: (i) visualizing chromosomal aberrations during anaphase or telophase, (ii) observing the frequency of micronuclei in meiotic cells, such as in the *Tradescantia* bioassay, or (iii) using the comet assay to evaluate the extent of DNA strand breakage. As illustrated in Fig. 2.4, many of these responses can be visualized during metaphase through changes in chromosomal number (polyploidy or aneuploidy) or the formation of multi-nucleated cells or micronuclei, or during telophase and anaphase as changes in the structure of the chromosomes (i.e., clastogenicity). Because of the relative simplicity of using these microscopy techniques with plant cells, the numbers of various chromosomal aberrations observed during anaphase or telophase (CAAT) in rapidly dividing somatic cells have been widely used as biomarkers of genotoxicity in aquatic macrophytes (Table 2.3). The microscopy techniques can be used to examine rapidly dividing plant tissues, such as the tips of growing roots and shoots.

As an alternative to quantify genotoxicity, DNA fragmentation can be measured by the Comet assay (i.e., single-cell gel electrophoresis assay). One of its advantages is that it requires nucleoids but not proliferating cells, and therefore it is applicable to any cell line or tissue from which a single-cell suspension can be obtained (Koppen et al. 2017). Cells embedded in agarose on a microscope slide are lysed to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in the development of structures resembling comets is observed by fluorescence microscopy. The intensity of the comet "tail" relative to the head reflects the number of DNA breaks because the loops of DNA containing a break lose their supercoiling and can migrate more rapidly toward the anode during electrophoresis. Several other methods have been developed and used in the last three decades to test the effects of genotoxic chemicals in plants, from the traditional *Allium* CAAT assay (Fiskesjo 1985) to the Comet assay (Koppen and Verschaeve 1996).

Due to its simplicity and sensitivity, and the small number of cells required to obtain robust results, the Comet assay has been widely applied in model plant species, such as *Allium cepa*, *Nicotiana tabacum*, *Vicia faba* or *Arabidopsis thaliana*, and the number of plant species tested has increased in recent years (Santos et al. 2015). It is noteworthy, however, that all the mentioned species are terrestrial, while only a few studies have applied this method to aquatic plants. As an example, Radić et al. (2013) studied the genotoxic potential of polluted surface water contaminated

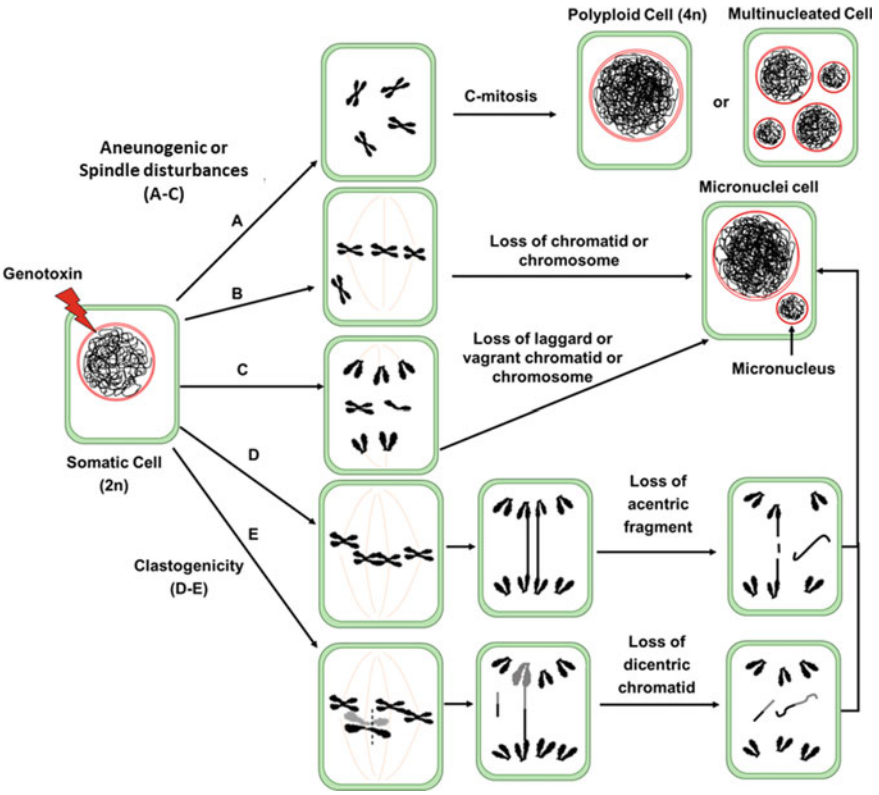

Fig. 2.4 Mechanisms of chromosome aberration formation in plant somatic cells. Aneunogenic or spindle disturbance mechanism (A–C), Clastogenic mechanism (D–E). **A** C-mitosis (or), modified mechanism of mitosis division induced by partial or total inactivation in spindle formation. This mechanism can result in polyploid cell or multinucleated cell. **B** Non-congregated chromosome occurring when a whole chromosome cannot insert in the spindle. **C** Laggard or vagrant chromatid or whole chromosome occurring when a chromatid or a whole chromosome cannot insert in the spindle. In either case (B and C) chromatid or whole chromosome, could form a micronucleus. **D–E** Chromosome bridge formation (D) where the genotoxin breaks the telomeres and two sticky chromosome fragments merge; (E) the genotoxin can break both extremes of one chromosome and these can merge; in both cases, it can be broken during migration in anaphase-telophase, resulting in the loss of an acentric fragment and dicentric chromatid, which finally could form a micronucleus in the daughter cell

by the effluents of a fertilizer factory using a battery of physiological, biochemical and genotoxicity biomarkers in *Lemna minor*. The authors reported that DNA damage measured through the Comet assay, among other biomarkers displayed a high sensitivity to pollutant levels and that the genotoxicity observed from in situ exposure was the result of numerous interactions between contaminants themselves and environmental factors, stressing the use of realistic exposure conditions.

Table 2.3 Genotoxicity biomarkers used to evaluate toxic effects in aquatic macrophytes

Genetic biomarker	Plant specie	Stressor	Exposure concentration - time	Biomarker responses	Reference
Metals, metalloids and organometals (METs)					
DNA fragmentation	*Lemna minor*	Cu Cd	2.5 µmol/L; 5 µmol/L each one and their mixture - 4, 7 d	↑ DNA fragmentation with Cd and Cu + Cd	Cvjetko et al. (2010)
DNA fragmentation	*Salvinia natans*	Al	240, 360, 480 µM - 7 d	↑ DNA fragmentation	Mandal et al. (2013)
CAAT	*Elodea canadensis*	Metals Radionuclides	Contaminanted sediments	↑ CAAT	Zotina et al. (2015)
Current use pesticides (CUPs)					
CAAT, AM	*Bidens laevis*	Endosulfan	0.02, 0.5, 5, 10, 50, 100 µg/L - 2 d	↑ CAAT, AM	Pérez et al. (2011)
DNA fragmentation	*Myriophyllum quítense*	Azoxystrobin	0.1, 1, 10, 50, 100 µg/L - 1 d	↑ DNA fragmentation	Garanzini and Menone (2015)
CAAT, AM, Cm	*Bidens laevis*	Imidacloprid	1, 10, 100, 1000 µg/L - 1 d	↑ CAAT, AM	Lukaszewicz et al. (2019)
CAAT, AM	*Bidens laevis*	Tebuconazole	1, 10, 100 µg/L - 2 d	↑ CAAT, AM	Moreyra et al. (2019)
CAAT, AM	*Bidens laevis*	Azoxystrobin	0.1, 1, 10, 50, 100 µg/L- 2 d	↑ CAAT, AM	Pérez et al. (2019)

AM: abnormal metaphases; CAAT: chromosomal aberrations in anaphase-telophase; d: days; Cm: C-mitosis

2.6 Molecular Biomarkers

Whereas most traditional biomarkers focus on measures of organism physiology or biochemistry, advances in molecular biology are extending biomarkers to the level of the genes (i.e., ecotoxicogenomics). With the ongoing rapid developments in modern molecular techniques, such approaches have been suggested as the way forward in biomarker development (Forbes et al. 2006). The techniques of genomics and transcriptomics have great potential in ecotoxicology as the basis for biomarker assays. For example, DNA microarray assays allow the measurement of changes in gene expression when organisms are exposed to chemicals such as metals (Magrini et al. 2008) or the PAH naphthalene (Peng et al. 2011), but all of these methods have been developed with the terrestrial plant, *A. thaliana.* The pattern of genomic response may give critical evidence about the MoAs and these molecular biomarkers have great potential for screening for toxic effects in the environment (Walker 2009). For example, Tan et al. (2015) evaluated the expression of CYP450 genes in rice

exposed to atrazine; observing 21 genes up-regulated and 29 genes down-regulated in the roots, while among the 29 genes in the shoots, 15 were up-regulated and 14 were down-regulated. In addition to the expression of enzymes from phase I metabolism, expression of phase II enzymes has been used to evaluate the effects of the herbicide fenclorim in rice, showing the up-regulation of GST, glycosyl transferase, aminotransferase and aminodehydrogenase (Hu et al. 2020). The up-regulation of genes involved in lipid biosynthesis, cell wall formation, PSII has also been reported in aquatic plant species exposed to PFOS (Li et al. 2020). But most of the recent work on molecular biomarkers in aquatic macrophytes has been developed to evaluate metal exposures (Table 2.4). Among them, expression of genes for proteins involved in detoxification (e.g., MTs, PC synthase) or antioxidant enzymes (e.g., APx, CAT) predominate (Talebi et al. 2019; Greco et al. 2019; Nualla-ong et al. 2020; Chen et al. 2021).

On the other hand, the field of molecular biology has rapidly incorporated epigenetic studies to evaluate organism-environment interactions that can result in chronic effects. Knowledge of epigenetic mechanisms provides the potential for a comprehensive evaluation of multigenerational and heritable effects from environmental stressors (Brander et al. 2017). In this sense, we have included epigenetic modifications that modulate transcriptionally silent or active chromatin by reversible methylation/demethylation processes as another type of molecular biomarker. These processes may be involved in metal tolerance in several plant species. Indeed, Greco et al. 2019 showed that both Cu and Cd induced the overexpression of the DNA methyltransferases such as chromomethylase 3 (CMT3) and methylase 2 (DRM2) in the seagrass *Zostera marina.* Both genes were significantly upregulated in all metal treatments, but this overexpression was to different magnitudes, which could suggest metal-specific methylation strategies. Shi et al. (2017) employed a proteomics approach to observe that excess Cu significantly induced the expression of DNA methylation-related proteins in *H. verticillata.* It is remarkable that plants are by far the most extensively studied group of living organisms when it comes to epigenetics (Brander et al. 2017) but much more study is necessary before a battery of biomarkers can be recommended for field studies.

2.7 Hypothetical Integrative Models of Biomarker Responses

In order to integrate the information compiled from our review of the literature, we developed hypothetical models of integrative biomarkers that have been used in bench-scale bioassays to screen for responses to four different classes of pollutants; that is, for exposure to copper (metal), atrazine (herbicide), ciprofloxacin (antibiotic) and PFASs (industrial chemicals). These models highlight the importance of using batteries of biomarkers, and also identify the most sensitive methods that could be used to evaluate the effects of chemicals on aquatic macrophytes. In particular, batteries of these biomarkers could be used in field studies to screen for responses by aquatic macrophytes to exposure to complex mixtures of pollutants, such as

Table 2.4 Molecular biomarkers used to evaluate toxic effects in aquatic macrophytes

Molecular biomarker	Plant specie	Stressor	Exposure concentration - time	Biomarker responses	Reference
Metals, metalloids and organometals (METs)					
Gene-Exp	*Elodea nuttallii*	Hg UV radiation	77 ng/L and 77 µg/L - 1 d	Dysregulated Gen-Exp involved in photosynthesis, energy metabolism, lipid metabolism, nutrition, and redox homeostasis	Regier et al. (2016)
Gene-Exp 8-OHdG	*Hydrilla verticillata*	Cu	0.05 mg/L - 5 d	↑ Gen-Exp of proteins involved in DNA methylation ↑ 8-OHdG	Shi et al. (2017)
Gene-Exp	*Zostera marina*	Cu Cd	0.8, 2.4 mM Cu - 6 d 0.89, 8.9 mM Cd - 6 d	↑ Gen-Exp of MTs, GR, APx, CAT, DNA methyltransferases CMT3, DRM2	Greco et al. (2019)
Gene-Exp	*Azolla pinnata* *Azolla filiculoides*	Cu Zn Ni Cd	0, 10, 50, and 500 mM of each one - 1, 2, 3 d	↑ Gen-Exp of MTs, PC synthase	Talebi et al. (2019)
Gene-Exp	*Rhizophora mucronata*	Cu Zn	Cu: 200 mg/L - 1 and 5 d Zn: 200 mg/L - 1 and 5 d	↓ Gen-Exp of PC synthase with Cu at 5 days ↓ Gen-Exp of PC synthase with Zn at 1 day and recovery at 5 days	Nualla-ong et al. (2020)
Gene-Exp	*Oryza sativa*	Cd	10 µM 6, 12 h, 1, 2, 4 d	Gen-Exp of SOD, CAT, APx, POD, APR, GlyA, SAT, GDH, MRP, GST	Chen et al. (2021)
Current use pesticides (CUPs)					
Gene-Exp	*Oriza sativa*	Atrazine	0.4 mg/L - 2 d	Modification of Gene-Exp patterns of CYP450	Tan et al. (2015)

(continued)

Table 2.4 (continued)

Molecular biomarker	Plant specie	Stressor	Exposure concentration - time	Biomarker responses	Reference
Gene-Exp	*Oriza sativa*	Fenclorim (FEN), Pretilachlor (PRE)	10 μM FEN; 10 μM PRE; 10 μM FEN + 10 μM PRE - 3 d	FEN: ↑ Gen-Exp of genes involved in phase I metabolism (P450, Oxygenese, Peroxidase, Glycosyl hydrolase, Lipase/thioesterase), and phase II (GST, Glycosyl transferase, aminotransferase, aminodehydrogenase)	Hu et al. (2020)
Other organic xenobiotics					
Gene-Exp	*Eichhornia crassipes* *Cyperus alternifolius*	Perfluorooctane sulfonate	10 mg/L - 42 d	↑ Gen-Exp of genes involved in lipid biosynthesis, cell wall formation, hormones, proteins of PSII ↓ Gen-Exp of Mn-SOD in *E. crassipes*	Li et al. (2020)

APR: adenosine 5'-phosphosulfate reductase; APx: ascorbate peroxidase; CAT: catalase; CMT3: chromomethylase 3; CYP450: cytochrome-P450; d: days; DRM2: methylase 2; GDH: glutamate dehydrogenase; Gen-Exp: gene expression; GlyA: glycine hydroxymethyltransferase; GR: glutathione reductase; GST: glutathione-S-transferase; h: hours; Mn-SOD: manganese-superoxide dismutase; MRP: Multidrug Resistance-associated Protein; MTs: metallothioneins; PC: phytochelatin synthase; POD: guaiacol peroxidase; SAT: serine O-acetyltransferase; SOD: superoxide dismutase; 8-OHdG: 8-hydroxy-2'-deoxyguanosine

municipal and industrial wastewaters, runoff from mine tailings, landfill leachate, etc.

2.8 Biomarker Responses to Copper

Based on our analysis of the available literature, copper is the most widely studied metal for effects on macrophytes. While copper is considered an essential metal for plants, when present in excess it is toxic and causes alterations to the vital process of photosynthesis (Krayem et al. 2021). The main biomarker responses observed for copper were a decrease in pigment contents, mainly Chl and Phae, in conjunction with Chl F reduction (Table 2.1). As shown in Table 2.2 and in Fig. 2.5, exposure to copper also triggers: (1) oxidative stress, demonstrated by increased ROS levels (H_2O_2, $O_2^{\bullet -}$), changes in contents of α-tocopherol, increased antioxidant enzyme activities and changes in their gene expression; and (2) oxidative damage, evidenced by formation of aldehyde products of lipid peroxidation (MDA) and DNA damage

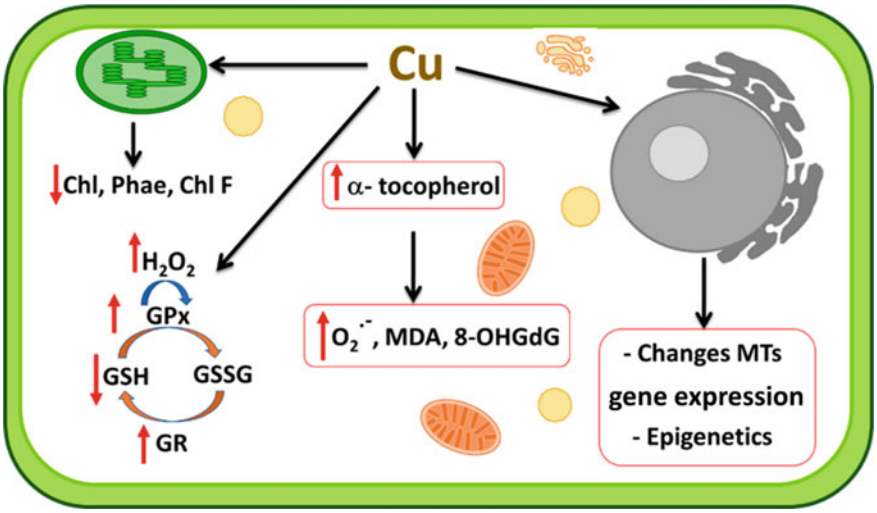

Fig. 2.5 Hypothetical integrative model of the biomarker responses in aquatic macrophytes exposed to copper (Cu). Black arrows indicate the site of action of Cu and their subsequent biomarker responses. Red arrows indicate increased levels or enzymatic activation (up-arrow) and decreased levels (down-arrow). 8-OHdG: 8-Hydroxy-Guanidine; Chl: chlorophyll; Chl F: chlorophyll a fluorescence; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione oxidized state; GSSG: glutathione reduced state; MDA: malondialdehyde; MTs: metallothioneins; Phae: phaeophytins; POD: guaiacol peroxidase; ROS: reactive oxygen species

(formation of 8-OHdG and fragmentation). Likewise, Cu induced changes in the expression of the genes for MTs and epigenetic modifications, as in DNA methylation enzymes. Moreover, it has been demonstrated that Cu has a detrimental effect on hormonal balance, inducing changes in phytohormone levels of plants (Krayem et al. 2021) but more research to use them as biomarkers of toxicity in aquatic macrophytes is required (Nguyen et al. 2021).

2.9 Biomarker Responses to Atrazine

Herbicides are the most widely studied CUPs, as they were developed to target plant considered as pests, and so aquatic macrophytes are vulnerable to these negative effects by similar MoAs. Figure 2.6 shows that, as mentioned earlier in the section on physiological biomarkers, when a photon of light reaches the chloroplast, there are three possible fates: (1) photosynthesis process, (2) re-emission as Chl F; (3) heat dissipation (NPQ). However, when a macrophyte is exposed to atrazine, an immediate reduction in Chl F and ΦPSII is observed, which indicates that this herbicide induces a block in the photosynthetic process of electron transport at PSII level. The blocking occurs because atrazine attaches at the PQ binding site, and the Chl a* cannot return

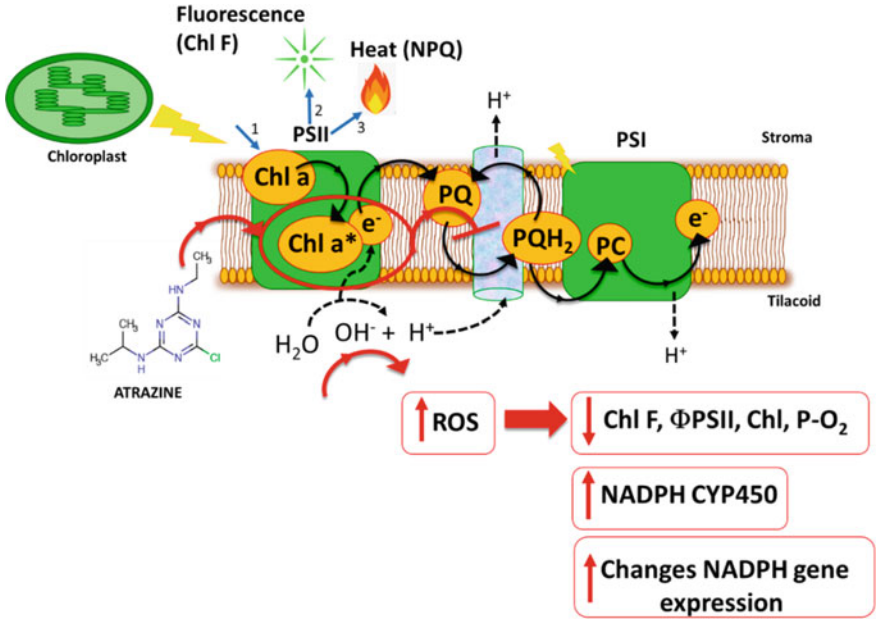

Fig. 2.6 Hypothetical integrative model of the biomarker responses in aquatic macrophytes exposed to atrazine. Blue arrows indicate the fate of photon: (1) photosynthesis process, (2) re-emission as Chl F; (3) heat dissipation. Black arrows indicate the normal electron transport chain. Red arrows indicate the block of atrazine in PSII and the subsequent biomarker responses. Chl: chlorophyll; Chl a: chlorophyll a; Chl a*: excited chlorophyll a; Chl F: chlorophyll a fluorescence; e-: electron; NADPH: nicotinamide adenine dinucleotide phosphate; NADPH CYP450: nicotinamide adenine dinucleotide phosphate cytochrome-P450; NPQ: non-photochemical quenching; P–O_2: photosynthetic oxygen production; PQ: plastoquinone; PQH_2: plastoquinol; PC: plastocyanin; ΦPSII: quantum yield efficiency of photosystem II; PSI: Photosystem I; PSII: Photosystem II; ROS: reactive oxygen species

to its ground state (Chl), consequently interrupting the flow of electrons between the PSII and PSI. This imposes a high energy load on Chl molecules, leading to their destruction, and a subsequent reduction in P–O_2, and ultimately inhibition of photosynthesis.

2.10 Biomarker Responses to Ciprofloxacin

We might expect that there are a number of physiological and biochemical targets for the effects of antibiotics in plants because of the bacterial ancestry of the plastids and mitochondrial organelles present in the cells of vascular plants (Brain et al. 2009). In this case, the model on biomarker responses was based mainly on studies from exposure of *Lemna* spp. to the antibiotic, ciprofloxacin. The main responses observed

are linked to effects on both the chloroplastic and mitochondrial electron transport chains (Fig. 2.7). These studies showed that ciprofloxacin blocks the photosynthetic process at the electron transfer between PSII and PSI. Thus, modifications in some biomarkers associated with the photon fate, such as the reduction in NPQ, increase in qP and finally a decrease in the ΦPSII are observed. Likewise, ciprofloxacin induced modification in the PQ redox cycle. In the mitochondrial electron transport, activities in Complex I–IV, mainly at Complex I level, decrease, and there are modifications to the NADH content and triggers to ROS formation. This process results in a decrease in Complex II–IV and reduction in ETR. Ciprofloxacin induced oxidative stress, resulting in increased antioxidant defenses such as ASC content, and damage to biomolecules (increased MDA).

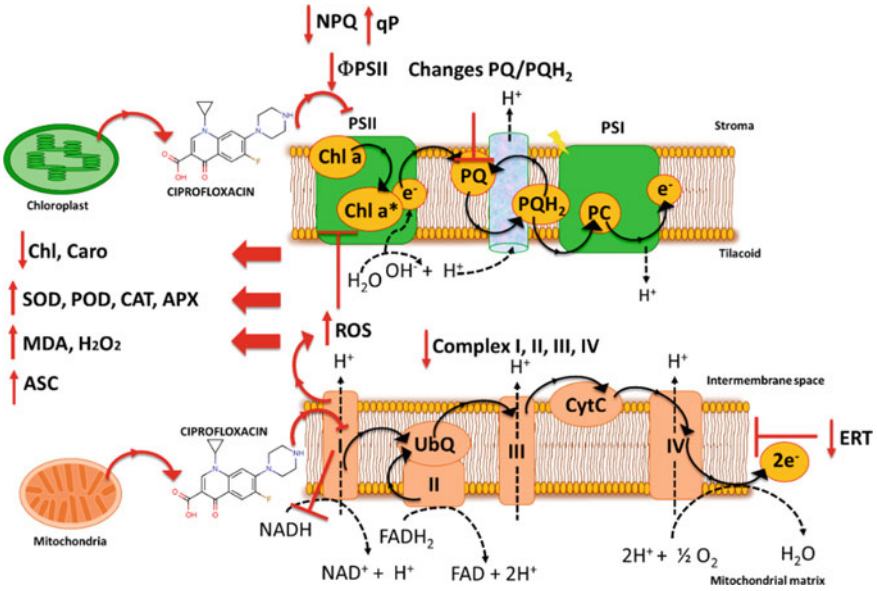

Fig. 2.7 Hypothetical integrative model of the biomarker responses in aquatic macrophytes exposed to ciprofloxacin. Black arrows indicate the normal electron transport chain. Red arrows and lines indicate the ciprofloxacin effects. APx: ascorbate peroxidase; ASC: ascorbic acid; Caro: caroteonids; CAT: catalase; Chl a*: excited chlorophyll a; Chl F: chlorophyll a fluorescence; Chl: chlorophyll; CytC: cytochrome C; e-: electron; ETR: electron transport rate; MDA: malondialdehyde; NADP: nicotinamide adenine dinucleotide phosphate; NPQ: non-photochemical quenching; PC: plastocyanin; POD: peroxidase; PQ: plastoquinone; PQH_2: plastoquinol; PSI: photosystem I; PSII: photosystem II; qP: photochemical quenching; ROS: reactive oxygen species; SOD: superoxide dismutase; UbQ: ubiquinone; ΦPSII: quantum yield efficiency of photosystem II

2.11 Biomarker Responses to PFAS

PFAS compounds include a very large class of chemicals used in many industries worldwide as additives to food packaging, stain repellents, waterproof products and in flame-fighting foams. The most widely studied compounds in toxicity studies are the long-chain perfluorinated polymers, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). These compounds are used in a variety of industrial and domestic products, but they also can be formed by biotransformation, as PFOS is the final product of microbial degradation of perfluoroalkyl acid (Li et al. 2020). Recent studies have demonstrated the negative effects of these chemicals on macrophytes (Pietrini et al. 2019; Li et al. 2020). As summarized in Fig. 2.8, PFOA mainly induces transient negative effects on mitochondrial and photosynthetic electron transport chains, while PFOS cause changes in pigment content and detoxification enzyme activities, as well as lipid peroxidation. Moreover, PFOS induced modification in the gene expression, such as up-regulation of proteins involved in the PSII and down-regulation of SOD.

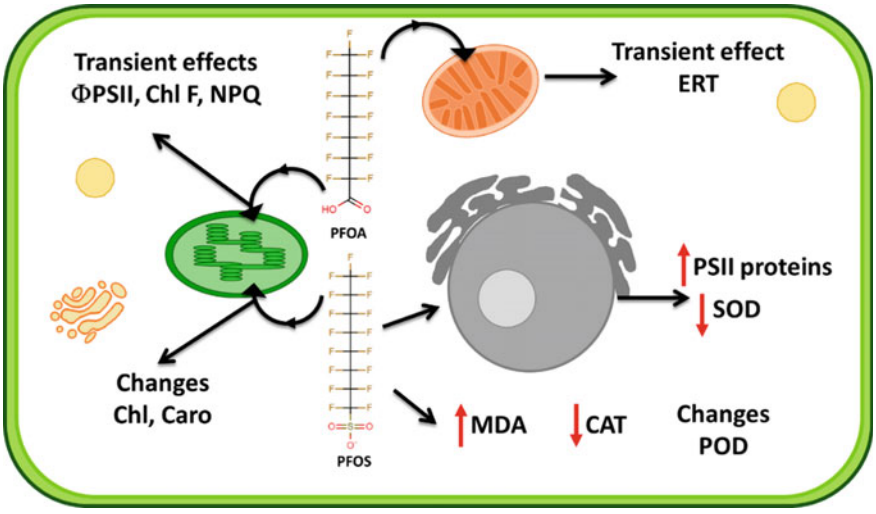

Fig. 2.8 Hypothetical integrative model of the biomarker responses in aquatic macrophytes exposed to PFASs. Caro: caroteonids; CAT: catalase; Chl F: chlorophyll a fluorescence; Chl: chlorophyll; ETR: electron transport rate; MDA: malondialdehyde; NPQ: non-photochemical quenching; PFOA: perfluorooctanoic acid; PFOS: perfluorooctane sulfonate; POD: peroxidase; PSII proteins: proteins involved in photosystem II; SOD: superoxide dismutase; ΦPSII: quantum yield efficiency of photosystem II

2.12 Conclusions

As can be seen from the information collected in this review, most of the work on the effects of chemical contaminants on aquatic macrophytes has been done under laboratory conditions while field studies of biomarker responses are scarce. In addition, many studies test the effects of toxicants at concentrations over the range of realistic levels which indicate that there is still too much work ahead for applying biomarkers for in situ biomonitoring. However, it is encouraging that biomarkers have applied to study the MoAs and to characterize the toxicity of all types of pollutants, including contaminants of emerging concern, such as PPCPs and PFASs. However, there are many challenges to overcome before biomarkers can be widely applied in field studies. Firstly, those biomarkers selected should be sensitive to responses at environmentally relevant concentrations of the pollutants. Studies must also take into consideration the interplay between contaminants and other abiotic and biotic variables that might have confounding effects in order to better understand the effects of multiple stressors in aquatic environment (Regier et al. 2016). The topic of confounding factors has been addressed since the beginning of the definition of biomarkers (Huggett et al. 1992; Peakall 1994) but not much progress has been done related to aquatic macrophytes in this matter. From all the papers included in this review, only one study by Regier et al. (2016) included an evaluation of the effects of a single chemical stressor plus an environmental variable (Hg + UV radiation). This is evidence of the critical information gap regarding the effects on macrophytes of a combination of chemical and other environmental stressors. Although the present work did not include the effects of abiotic stressors, it is important to take into account that macrophytes often undergo distinct environmental stress conditions, like changes in salinity, extreme temperatures, altered nutrient availability, drought and ultraviolet irradiation. These changes mean that macrophytes must strike an equilibrium between growth, development and survival, and as a result, adapt to stressful surroundings by reprogramming metabolic pathways and gene expression, beginning from perception of stress and concluding with particular transcriptional modifications (Hilal et al. 2019). Therefore, waterborne pollutants add more complexity to the adjustments that plants need to make to adapt to a changing environment and consequently, care should be taken in interpreting data gathered on toxicity.

The lack of studies focusing on marine or estuarine species versus freshwater macrophytes was noted. Recently, physiological, biochemical and molecular biomarkers have been studied together in the seagrass *Z. marina* exposed to cadmium and copper (Greco et al. 2019), but for most of the other marine species, only one class of biomarker has been studied, particularly those related to oxidative stress exerted by metals, such as in the halophytes from salt marshes, *Limonium brasiliense*, *Suaeda maritima*, *Halimione portulacoides*, *Sarcocornia perennis* and *Spartina maritima*, or in the mangrove species *Kandelia candel*, *Bruguiera gymnorrhiza* and *Rhizophora mucronata* (Table 2.2). Hence, there is an information gap concerning batteries of biomarkers for marine macrophytic plant species, mainly for organic contaminants. Concerning freshwater species, as can be seen in the tables, the duckweed species,

L. gibba and *L. minor* are the main species that have been used in ecotoxicological studies, followed by *M. spicatum* and *M. alterniflorum* from the Northern Hemisphere, and *M. quitense* from the Southern Hemisphere. Our survey of the literature is consistent with the review by Vonk and Kraak (2020), as these authors reported data for 109 freshwater taxa belonging to 66 genera that were tested for herbicide toxicity and the most frequently selected genus for these studies was *Lemna.*

It is noteworthy that traditional biomarkers like photosynthetic pigment contents are still the most widely used, which reflects the vital process of photosynthesis in aquatic macrophytes. However, we recommend that these biomarkers be studied together with other biochemical, genotoxicity and molecular biomarkers in order to identify the most sensitive endpoints. In this sense, applications of gene expression have shown promising results, allowing the measurement of significant responses at environmentally relevant concentrations (Cosio 2020). Other optional novel biomarkers include those related to the mitochondrial electron transport chain (Gomes et al. 2017; Pietrini et al. 2019) and the non-destructive biomarker of Chl F (Pérez et al. 2019).

Overall, this review of the literature that has been published since 2008 on biomarker responses in aquatic macrophytes shows that there are many promising research questions to be explored in this field. The goal of the research should be to identify a suite of sensitive and robust biomarkers that can be used in field studies to evaluate effects in aquatic macrophytes exposed in situ to a range of environmental pollutants.

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Chapter 3
Metal(loid)s in Macrophytes from the Americas

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Abstract Pollution from metals and metalloids is a major concern due to the persistence of these elements in the environment and the impacts on ecological and human health. Evaluation of the distribution, bioaccumulation and toxicity of metal(loid)s in aquatic organisms has been studied for many years. In consequence, research on plants with a high tolerance to metals, and therefore, of use as bioindicators of theses contaminants, has become a subject of interest in recent years. This chapter presents a brief analysis of the bioaccumulation patterns and physiological responses of aquatic plants to pollution from metals and metalloids, with a focus on describing what we have learned from our studies conducted in the laboratory and in the field with the emergent macrophyte, *Potamogeton pusillus* and with the mangrove species, *Avicennia schaueriana*, *Laguncularia racemosa* and *Rhizophora mangle*, all of which are native to South America. Furthermore, the capacity of *P. pusillus* to be used for active and passive biomonitoring in aquatic ecosystems highly impacted by environmental degradation is discussed. In addition, there is a discussion of the bioaccumulation and translocation of metals from interstitial water and sediment to the roots and leaves of mangroves inhabiting estuaries in Brazil with different levels of pollution, correlating metal bioaccumulation with differences in macrophyte anatomy. Finally, the use of stable isotopes in mangroves as biomarkers of environmental pollution is demonstrated.

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3.1 Introduction

In recent years, there has been increasing evidence of the widespread occurrence of contamination by metals and metalloids in aquatic ecosystems from all around the world. These elements, unlike organic contaminants, which can be degraded to less harmful products by biotic or abiotic processes, are non-degradable and persistent, with high toxicity, and can be bioaccumulated in aquatic organisms, resulting in sublethal concentrations affecting the biota. In some cases, these elements can even be biomagnified in the food chain, thus threatening human health (Cordoba-Tovar et al. 2022).

There are different sources of metals and metalloids in the environment. These can be of natural origin (geogenic) or derived from human activities (anthropogenic). The most important natural sources are mineral weathering, erosion and volcanic activity, while anthropogenic sources include mining, smelting, electroplating, pesticide and fertilizer use, as well as biosolids from agriculture, sludge dumping from industrial and domestic sources, among others (Shi et al. 2022). Although some metal(loid)s can be strongly adsorbed onto the suspended particles and sediments, they can be released into the water under suitable conditions of pH and redox potential, leading to further contamination (Wang et al. 2022). Some metals, including cadmium (Cd), zinc (Zn), lead (Pb), chromium (Cr), nickel (Ni), copper (Cu), vanadium (V), platinum (Pt), silver (Ag), tin (Sn) and titanium (Ti) are highly toxic to aquatic organisms (Zaynab et al. 2022).

Many aquatic ecosystems in South America are contaminated with metals and metalloids. Their distribution is quite variable, reflecting contamination from both point and non-point sources. For example, the upper and middle reaches of the Cachapoal River in Chile are characterized by elevated concentrations of Cu, Mo, As, Pb, Cr that reflect inputs of material from mining activities (Lacassie and Ruiz-Del-Sola 2021). Also, the Río de La Plata between Argentina and Uruguay shows strong features of sediment retention, favoring pollutant accumulation. Sediments from Montevideo Bay are highly polluted with Zn, Pb, Cu, Cr and mercury (Hg) and moderately contaminated with Ni and Ag (Barletta et al. 2019). The presence of metals was also documented in the main water bodies of the Province of Córdoba, Argentina, in the Suquía, Xanaes and Ctalamochita Rivers and in the San Roque, Los Molinos and Río Tercero Reservoirs, which is mainly associated with urban pollution (Contardo-Jara et al. 2009; Monferrán et al. 2011, 2016a, b; Griboff et al. 2017, 2018b, 2020; Bertrand et al. 2019). Moreover, high levels of aluminum (Al), Cr, manganese (Mn), iron (Fe), Ni, Cu, Zn, arsenic (As), selenium (Se), Ag, Cd, Hg and Pb were found in water and sediment in two neotropical estuaries located in the State of Espírito Santo, Brazil, namely, Vitória Bay and Santa Cruz, which are areas affected by different pollution sources and marine processes (Souza et al. 2014a, 2015).

The accumulation of metals and metalloids in biota depends on the chemical properties of each element, the characteristics of individual organisms and the role played by different organs and tissues in the processes of absorption, regulation, storage and excretion. Some essential elements, such as Zn, Fe and Cu are necessary

for cellular functions, but exposure to high and/or prolonged doses of these elements can lead to neurotoxic, genotoxic and carcinogenic alterations (Geng et al. 2019). Exposure to high concentrations of metals and metalloids can induce oxidative stress by producing reactive oxygen species (ROS), resulting in DNA damage, lipid peroxidation, depletion of protein sulfhydryl groups, impairment of cell signaling, altered Ca homeostasis, changes in expression of the Ca regulation gene, etc. In addition, they can replace essential metals in pigments or enzymes, disrupting their normal function (Kolarova et al. 2021).

To detect environmental pollution by using biological materials as indicators is a reliable and simple alternative to conventional sampling methods. The distribution and condition of many aquatic macrophytes are often correlated with water quality. Some macrophytic species can accumulate considerable amounts of metals in their tissues. Therefore, aquatic macrophytes stand out as having the potential to be useful indicators of metal contamination in the aquatic environment (i.e., biomonitors, bioindicators) as documented by Farias et al. (2018), due to their high tolerance to metal pollution, convenience for sampling and easy culturing in the laboratory. As we document in this chapter, our previous studies have shown that monitoring of the macrophyte species, *Potamogeton pusillus*, *Avicennia schaueriana*, *Laguncularia racemosa* and *Rhizophora mangle* can be an effective tool for studying contamination by metals and metalloids in both fresh and brackish waters.

Potamogeton pusillus is an aquatic plant of 20–30 cm length, with thin and branched stems (Fig. 3.1). Its leaves are shaped like a ribbon, flattened and subphyliform when the plant is young, and they are between 3 and 6 cm long and 0.6–1 cm wide, hyaline, with 1–3 vascular bundles and air channels in the central region of the sheet. These freshwater plants have a fully developed root system that is completely submerged in the river sediment. It is considered a sentinel native macrophyte, having ecological importance within sub-tropical aquatic ecosystems, providing shelter and habitat for young fishes and other aquatic animals (Novara 2003. http://www.iucnredlist.org). This species is present in aquatic environments that are moderately to highly polluted in the main rivers of the state of Córdoba in Argentina, which makes this macrophyte very useful as a biomonitoring species (Harguinteguy et al. 2016; Bertrand et al. 2019).

Avicennia schaueriana (Stapf & Leechm. ex Moldenke) of the plant family of Acanthaceae, *Laguncularia racemosa* (L., C. F. Gaertn) of the Combretaceae family and *Rhizophora mangle* (L.) of the Rhizophoraceae family are three true mangrove species (Tomlinson 2016). *A. schaueriana* has extensive underground roots, supporting pneumatophores and absorption roots. Its bark is variably rough, dark, rigid and fissured. It has an entire leaf blade, ranging from ovate to elliptical. The leaf blade is leathery or slightly fleshy, with inconspicuous veins and a prominent mid-rib below (Fig. 3.2). It has salt glands on both sides of the leaf blade and water storage parenchyma underlying the adaxial surface of the epidermis. *L. racemosa* has extensive underground roots, cable-like, supporting pneumatophores and absorbing roots. Its bark is rough, fissured and grayish. Its branches have numerous slightly prominent lenticels. Its leaf blade is somewhat fleshy, elliptical to oblong (Fig. 3.2). They have leaves with a petiole containing a pair of extrafloral nectaries

Fig. 3.1 *Potamogeton pusillus* in the Suquía River at La Calera, Córdoba, Argentina (A). *P. pusillus* leaves shaped like a flattened ribbon (B and C)

on their adaxial surface. The leaf blade has salt glands distributed on the abaxial and adaxial surfaces of the epidermis. A cross-section of the blade reveals a water storage parenchyma in its middle portion. Finally, *R. mangle* presents rhizophores with positive geotropism, responsible for providing stability in the sediment, which, when in contact with the soil, form the roots. Its leaves are entire, elliptical, glabrous and with numerous cork warts on the abaxial surface, visible on older leaves as dark spots (Fig. 3.2). They have evident but not prominent veins. Its seeds are viviparous, germinated by the extension of the hypocotyl, with propagules measuring about 20–30 cm. The analysis of oxygen isotopes ($^{18}O/^{16}O$) in *R. mangle* showed that this mangrove plant use surface soil and seawater rather than groundwater as a water source, even when the water has a high salinity (Lin and Sternberg 1994).

This chapter describes the responses of these structurally and physiologically different macrophyte species that grow in freshwater and under saline conditions when they are exposed to metals and metalloids both in the laboratory and in the environment under natural conditions.

Fig. 3.2 Mangroves from Santa Cruz estuary, State of Espírito Santo, Brazil (A). Leaves of mangrove plants: *Laguncularia racemose* (B), *Avicennia schaueriana* (C) *and Rhizophora mangle* (D)

3.2 Laboratory Studies

3.2.1 Accumulation of Metal(loid)s in P. pusillus *and Impact on Biochemical Parameters*

We have carried out several laboratory studies with *P. pusillus* to assess bioaccumulation and effects from exposure to different metals. The concentrations used in the laboratory experiments were selected according to environmentally relevant data found in the literature (Cheung et al. 2003; Smolders et al. 2003; Monteiro et al. 2010; Hashem et al. 2020; Francisca et al. 2006).

In one series of experiments, we collected macrophytes from a reference site, placed them into a 40 L tank containing 10% Hoagland's solution and sediment (1/4) from the same sampling area and grew them for two weeks under a light/dark photoperiod of 14 h:10 h before starting the exposures (Monferrán et al. 2012a). For exposure to metals and metalloids, organisms were relocated into 1 L beakers (three plants per beaker, 5–8 g wet weight-w.w. per liter) containing 10% Hoagland's solution prepared without the element to be tested. After the exposure time, which depended on the element studied in each case, plants were washed three times with ultra-pure

water, frozen with liquid nitrogen and kept at −80 °C until analysis. Concentrations of metals and metalloids in exposure media were measured by inductively coupled plasma-mass spectrometry (ICP-MS) and accumulation in plant tissues was determined by atomic absorption spectroscopy (AAS) after digestion of the samples with aqua regia (Monferrán et al. 2012a), with the exception of Hg and As, which were also analyzed by ICP-MS.

A laboratory bioassay testing the kinetics of Cu^{+2} and Cr^{+6} uptake by *P. pusillus* from water solutions (individual exposure) demonstrated that accumulation of these metals is in a concentration and time-dependent manner, where the most significant increase in concentration observed was at 5-day exposure, although the metal content continued to increase gradually up to 15 days. Time-dependent (kinetic) studies on the uptake of metals by aquatic plants have shown an initial rapid accumulation phase, followed by a slower linear phase. The initial phase represents a rapid, reversible, metal binding process (i.e., biosorption), while the subsequent slower phase is governed by metal transport across the plasma membrane into the plant cytoplasm (i.e., bioaccumulation), as described by Monferrán et al. (2012a).

Another experiment was conducted to assess the effect of Cu^{+2} on the bioaccumulation of Cr^{+6} by *P. pusillus*. These assays showed that the presence of Cu^{+2} drastically increased the phytoextraction of Cr^{+6}, particularly at the lowest Cu^{+2} concentrations of 0.1 mg/L and 0.5 mg/L, where the phytoextraction of Cr^{+6} by the plant rose 3.5-fold when Cu^{+2} concentration was increased from 0 to 0.5 mg/L, keeping Cr^{+6} concentration constant. These observations are clear evidence of enhanced phytoextraction of Cr^{+6} by *P. pusillus* from binary solutions containing Cu^{+2}.

The accumulation of both metals in the plant resulted in toxic effects. This was seen when NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values were calculated, based on changes in chlorophyll-a (Chl-a) and protein contents in *P. pusillus* exposed to different concentrations of Cu^{+2} and Cr^{+6}. The NOEC for Chl-a was 0.5 mg/L for Cu^{+2} and 2 mg/L for Cr^{+6} (Monferrán et al. 2012a). This indicates that Cu^{+2} is more toxic to the plant than Cr^{+6}. The same trend was found when protein contents were used to calculate the NOECs, which were 1 mg/L for Cu^{+2} and 2 mg/ L for Cr^{+6}. This indicated that Chl-a is the more sensitive toxic endpoint for Cu^{+2} toxicity than the protein level (i.e., NOEC = 0.5 mg/L for Chl-a and NOEC = 1 mg/L for proteins), while Cr^{+6} showed the same NOEC for both toxic endpoints.

We also demonstrated that *P. pusillus* was able to accumulate significant concentrations of Hg after 7, 14 and 20 days of hydroponic treatment (Griboff et al. 2018a). The maximum rate of metal accumulation was found after day 7 in a treatment with 2 mg/L Hg, when 96% of the total accumulated metal was taken up by the plant (2,372 μg/g dw). Metal accumulation continued through days 14 and 20, although the bioaccumulation rate was lower than that reported for day 7. Thus, Hg content was 2,034 μg/g dw (83%) after a 14-day exposure, and 2,465 μg/g dw after a 20-day exposure. It is worth mentioning that bioaccumulation also occurred at lower Hg concentrations (i.e., 0.1; 0.5 and 1 mg/L) but at lower rates in comparison with the bioaccumulation observed during exposures at 2 mg/L.

When the capacity of *P. pusillus* to accumulate As was evaluated, we observed that the accumulation of As^{+3} and As^{+5} by *P. pusillus* increased as the exposure concentration increased, but it did not increase as the exposure time increased. Specifically, the concentration of As accumulated by *P. pusillus* when it was exposed for 7 days at different As^{+3} or As^{+5} concentrations was the same or not statistically different from treatments with exposure for 14 or 20 days to the same concentrations (Griboff et al. 2018a). This plant accumulated more As when it was exposed to As^{+3} (281 μg/g dw) relative to accumulation when exposed to relative to As^{+5} at the same concentration (117 μg/g dw). These results are important, given that As^{+5} is more toxic than As^{+3}.

NOEC and LOEC values were calculated for the toxic endpoints of Chl-a and protein content in *P. pusillus* exposed to different concentrations of As^{+3}, As^{+5} and Hg over 15 days. NOEC for chlorophyll-a was 0.1 mg/L for As^{+3}, As^{+5} and Hg (Griboff et al. 2018a). These experiments indicated that the As species were more toxic to the plant than Hg, taking into account that the concentrations of Hg accumulated (46 μg/g dw) at 0.1 mg/L of exposure were much higher than that of As (10 and 6 μg/g dw for As^{+3} and As^{+5} exposure, respectively) exposed in treatments at the same concentration. No statistically significant differences in protein levels were observed in the plants exposed to As^{+3}, As^{+5} or Hg compared to the control group. Comparing these results, it appears that Chl-a is a more sensitive endpoint for the toxic effects of As^{+3}, As^{+5} and Hg than the proteins level (NOEC = 0.1 mg/L). In our experiments, significant damage to the macrophyte pigments were observed in *P. pusillus* after exposure to As^{+3}, As^{+5} and Hg (Griboff et al. 2018a). These changes reflect the diversity of the disorders to cellular metabolism generated by exposure to these elements. Loss of photosynthetic pigments is a common response of plants to environmental stressors such as heat, diseases and pollution.

P. pusillus accumulated large amounts of Pb (2,470 μg/g dw) after exposures of the plants for 10 days to Pb^{+2} at a concentration of 2.0 mg/L, with removal of 74–92% of this metal from solution. In addition, *P. pusillus* accumulated large amounts of Cd^{+2} (2,045 μg/g dw) after exposure of the plants for 10 days to 2.0 mg/L of Cd^{2+}, with removals from solution of 89 to 91% (Rivela Fretes et al. 2021a, b). The accumulation of Pb in *P. pusillus* did not result in changes to the content of Chl-a and b, malondialdehyde (MDA) and sugars in all treatments. However, the content of carotenes increased relative to the control treatment for the plants exposed to 0.5 mg/L of Pb for 7 days. Carotenoids belong to the plant's non-enzymatic antioxidant defense system and in fact play a key role in protecting the photosynthetic system from the effects of excess metals. Carotenoids trap and then scavenge ROS (Krayem et al. 2021). In contrast, the accumulation of Cd resulted in changes in levels of Chl-a and Chl-b. Chlorophyll-a and b decreased as Cd concentration increased and the NOEC for Chl-a and b toxic endpoints was 1 mg/L. The content of carotenoids, sugars and MDA were not affected by exposure to Cd in all treatments.

Finally, to determine the potential use of *P. pusillus* as a bioindicator of aquatic contamination with Zn, the macrophyte was experimentally exposed to this metal and the response of biomarkers of exposure and effect were evaluated (Bertrand et al. 2016). In this study, both the biomarkers and the accumulation of Zn were evaluated in different tissues of the plant (leaf, stem and root). The experimental treatments were: Control (not metal exposed) and plants exposed to 5, 50 and 500 μg/L Zn. The

biomarkers of toxicity monitored were hydrogen peroxide (H_2O_2) concentration, lipid peroxidation measured as thiobarbituric acid-reactive substances (TBARs), antioxidant enzymes activities and concentrations of chlorophyll (Chl) and pheophytins (Pheo) concentrations, consistent with Bertrand et al. (2016). In these experiments, Zn bioaccumulation and biological responses (i.e., oxidative stress biomarkers and pigments) indicated a differential response pattern among leaf, stem and roots in *P. pusillus*. This macrophyte showed rapid Zn accumulation, with a significant increase in tissue concentrations in treatments with 50 μg/L in the leaf and in treatments with 5 μg/L in the stem and root. Although Zn accumulation in the leaf occurred at higher exposure concentrations than in the other plant tissues, the bioaccumulation in the treatment with 500 μg/L was greater than those in stem and root. Specifically, the Zn concentration in leaf exposed at 500 μg/L was five times higher than in the control treatment (i.e., leaf in control = 198 ± 34 μg/g d.w.; leaf in 500 μg/L treatment = 1,063 ± 208 μg/g d.w.), while Zn concentrations in other plant tissues increased only three times relative to the control (i.e., stem in control = 177 ± 56 μg/g d.w.; stem in 500 μg/L treatment = 676 ± 165 μg/g d.w.; root in control = 146 ± 83 μg/g d.w.; root in 500 μg/L treatment = 554 ± 120 μg/g d.w.).

The induction of cellular changes often goes along with the bioaccumulation of metals in higher plants, some of which directly contribute to metal tolerance of plants. In *P. pussilus*, even though a significant rise in H_2O_2 was observed in leaf and root in treatments with Zn at 5 μg/L, no significant variations in TBARs concentration were detected in any of the plant tissues (Bertrand et al. 2016). Higher levels of H_2O_2 were measured in the root from the Zn treatment at 500 μg/L, at levels three times greater than in the control condition (i.e., root in control = 0.33 ± 0.06 mg/g H_2O_2 w.w.; root in 500 μg/L treatment = 0.92 ± 0.19 mg/g H_2O_2 w.w.). When this macrophyte was exposed to Zn, the levels of Chl-a in the leaf decreased significantly in the 500 μg/L treatment (i.e., leaf in control = 399 ± 35 μg/g w.w.; leaf in 500 μg/L treatment = 191 ± 55 μg/g w.w.). However, no significant differences between the control and other treatments were observed in the Chl-b, Pheo-a or Pheo-b levels measured either in the leaf or the stem. Among pigments, Chl-a is well-known to be the most sensitive to oxidative stress. Therefore, the decrease in Chl-a concentrations in the leaf could be related to the increased H_2O_2 levels in the same plant tissue. In the stem, concentrations of Chl-a remained constant. The Zn concentration at which a significant effect on pigments is detected is species-dependent. In *P. pectinatus*, significant Chl-a loss was observed in the treatment with 6.5 mg/L Zn after 24 h exposure (Tripathi et al. 2003), while similar changes in *Ceratophyllum demersum* required concentrations higher than 13 mg/L Zn to affect the photosynthetic system, including the pigments (Aravind and Prasad 2004).

Regarding antioxidant enzyme activities, there was significant inhibition of glutathione peroxidase (GPx) activity in the leaf and root in the Zn treatment with 5 μg/L, the lowest Zn concentration tested, while in the stem, the enzyme activity increased in the treatments with 5 and 50 μg/L. The activity of glutathione reductase (GR) in leaf diminished in the 500 μg/L treatment, while in the root, the same effect was observed at 50 and 500 μg/L (Bertrand et al. 2016). The capacity of Zn to inhibit the activity of GR in plants has been reported by other authors (Schaedle and

Bassham 1977). This response could indicate a negative effect on enzymatic antioxidant mechanisms. However, no variations were observed in guaiacol peroxidase activity (POD). This lack of response could indicate that exposure concentrations were not sufficient to affect this enzyme of the antioxidant system. No change in enzymatic activity was detected for glutathione-S-transferase (GST) in microsomal or cytosolic fractions, as well as for GR in the stems.

All results considered, the leaf of *P. pusillus* showed a higher resistance to the inhibition of antioxidant enzymes in comparison to the root. The increase in H_2O_2 levels (i.e., ROS) in the leaf was not enough to activate the enzymes from the antioxidant and protective system. This could be due to non-enzymatic antioxidant mechanisms being able to neutralize the ROS. Non-enzymatic antioxidant mechanisms such as glutathione were not measured in the present study. The biomarkers of Chl-a and GR measured in leaf as well as H_2O_2 and GR activity in root were the best parameters to explain the variation in effects from Zn exposure concentrations (Bertrand et al. 2016). However, these biomarkers did not show a good capacity to predict impacts from the different exposure concentration. The levels of Zn accumulated in any tissues of *P. pusillus* were more representative of exposure concentrations.

Submerged plants have very thin cuticles through which metals in the surrounding water can readily pass. The accumulation of metals and metalloids by *P. pusillus* is selectively related to the physiological roles of these elements in the metabolism of the plant. The surfaces of submerged plants such as *P. pusillus* are usually coated with active biofilms, which consist of a complex combination of microorganisms, exudate polymers, absorbed nutrients and metabolites, and particulate materials. Biofilms have been found to exert effective control on metal pollution in aquatic systems. Since they are polyanionic, biofilms can facilitate the biosorption of metal compounds (Geng et al. 2019). In addition, some bacteria species can modify sorption of these elements by increasing the surface area of the plants or root length, or promoting biofilm formation, which can potentially increase their bioavailability (Palansooriya 2019).

The bioaccumulation of metals in *P. pusillus* is often accompanied by the induction of a variety of cellular changes, some of which directly contribute to the metal tolerance of plants. Among the variety of toxicity endpoints for the elements that we studied in *P. pusillus*, the photosynthetic apparatus and protein contents seem the most sensitive. The toxicity of metals also involves oxidative stress, followed by oxidative damage to membranes and pigments.

3.2.2 Distribution of Metal(loid)s in P. pusillus *Tissues*

Our previous studies on the accumulation Cu and Cr by *P. pusillus* showed that the amounts accumulated in the plant tissues were consistent with the concentrations of the metals in the aqueous medium, with significantly higher levels of both metals in the root and leaves than in the shoots (Monferrán et al. 2012a). The relative amounts of As and Hg increased in all studied tissues as metal concentration increased, showing

significantly higher levels of As in the root than in the shoots or leaves when the aquatic plant was exposed to all concentrations of As^{3+}. On the other hand, when *P. pusillus* was exposed to As^{5+}, the plant tissues with the highest As accumulation were the root at the lowest exposure concentration of 0.1 mg/L and the stems at the exposure concentration of 0.5 mg/L, while no statistically significant differences were observed between the plant tissues in treatments at 1 and 2 mg/L. Finally, when *P. pusillus* was exposed to Hg, it was observed that the highest levels of Hg were found in leaves relative to the shoots or roots at all concentrations evaluated (Griboff et al. 2018a).

When *P. pusillus* was exposed to Cd at concentrations of 0.5 and 1 mg/L, no significant differences were observed in accumulation in the different parts of the plant. However, in a treatment at 2 mg/L of Cd for 10 days, the leaves showed higher accumulation for this metal relative to the stem and root. The *P. pusillus* tissue that accumulated the highest Pb concentrations was the root (Rivela Fretes et al. 2021a, b).

As was described earlier, *P. pusillus* exposed to Zn at 5 and 50 μg/L showed no significant differences in Zn concentration accumulated in the different sections of the plant. However, in a treatment with 500 μg/L Zn for 4 days, the leaves showed a higher accumulation for this metal relative to the stem and root.

Submerged plants have considerable potential to accumulate metals from the surrounding environment. The leaves and roots provide physical support for biofilms, which facilitate both facultative anaerobic and anaerobic microorganisms to absorb nutrients. In addition to the nutrients required by living organisms, plants and biofilms also accumulate non-essential elements (e.g., Cd, Cr and As). The epiphytic biofilms adsorb/absorb metals and transport them to the leaves (Geng et al. 2019).

Roots are the main tissue for the accumulation of various metals by aquatic plants. The sequestration, immobilization and accumulation of metals in the root may be due to the process of rhizofiltration, which is commonly observed in aquatic plants. Roots exudates in the rhizosphere may also cause settling of metals onto the root surface. Moreover, metals can be actively absorbed into root cells via plasmalemma, adsorbed onto cell walls via passive diffusion or moved acropetally in the roots of aquatic macrophytes. Besides, ion exchange with the surrounding solution may also take place rapidly in the "free space" (apoplasm) of the root, which facilitates the penetration process without passing through living membranes (Tibbett et al. 2021).

Metal accumulation in leaves may be largely attributed to ion exchange within this tissue and the surrounding solution and also via passive transport of ions into the peripheral region. Aquatic macrophytes, with a well-developed root/rhizome system and totally submerged foliage, extract elements mostly from sediments. However, uptake by leaves becomes important when the metal concentration in the surroundings is high or when metals are bound to not readily available compounds in the sediment (Rezania et al. 2016).

Stems of *P. pusillus* accumulated much less metals than leaves or roots. This could be due to its lower volume in relation to a large surface area for uptake in leaves. It has been demonstrated that the ratio of total volume to surface area differed significantly between leaves and stems in *P. natans* (Rezania et al. 2016). Additionally, leaves have

lower water content than stems, indicating that leaves contain more dry material to which metals can bind. Furthermore, the organic matter content may influence the binding capacity, since metals have a high affinity for organic material. The organic matter consists largely of cell walls containing pectin, which contain a number of negative-charged polygalacturonic acid sites, allowing cation exchange and thus, metal absorption (Rezania et al. 2016).

3.2.3 Accumulation and Translocation of Metal(loid)s in Mangrove Plants

Mangrove ecosystems in tropical and subtropical intertidal zones play a key role in maintaining the coastal ecological balance and species diversity (Souza et al. 2015). Ecotoxicological studies with mangrove plants can be carried out by growing the plants in a greenhouse. In experiments described by Arrivabene et al. (2016), propagules of *A. schaueriana*, *L. racemosa* and *R. mangle* were collected directly from the mother plant in an ecological reserve, transported to a greenhouse and cultivated in pre-cleaned PVC pots (2.8 L each) containing washed sand. Sand pots were stored in receptacles containing Hoagland's nutrient solution, with 0.25 ionic strength and a salt content of 7 g/L. The level of the nutrient medium in the substrate was approximately 3 cm during plant growth, and approximately 7 cm during exposures, simulating mangrove swamp conditions. The nutrient medium was covered with a black PVC film to prevent photo-oxidation. Propagules were developed during eight months and afterward plants were used for metal exposure. Initially, exposures were performed by adding 0 (control), 10, 20 and 100 mg/L $Fe(II)SO_4$ (to simulate the bioavailable form of Fe), disodium EDTA and MES buffer (1 mM, pH 6) to the nutrient medium (which already contained 0.53 mg L^{-1} Fe as $FeCl_3$). Iron concentrations of 10 and 20 mg/L were selected as they are close to values found in the interstitial water during field studies and the highest concentration (100 mg/L) was selected to simulate a more toxic condition, with iron levels exceeding current environmental levels. Sets of five independent plants from each species (randomly selected) were exposed to different Fe concentrations over a period of eight weeks. After exposure, plants were harvested and analyzed.

In the experiments conducted according to this protocol, it was found that the three plant species were capable of bioaccumulating Fe in their tissues (Arrivabene et al. 2016). L. *racemosa* showed dose-dependent bioaccumulation in root and in iron plaque, in addition to an inhibitory behavior with secretion of Fe through salt glands. This species was judged to be the most appropriate mangrove for biosensing the amount of iron present in estuarine/marine environments due to environmental pollution. A significant decline in translocation factors between aerial parts of the plant and the root was evident, mainly in *R. mangle* and *A. schaueriana*, indicating the impact on the plant transport mechanism induced by high concentrations of added Fe(II). Changes in plant anatomy and histochemistry were not as evident as those observed with bioaccumulation and translocation. Iron plaque proved to be an

important site of accumulation of the metal, functioning as a barrier to entry into the plant. Furthermore, Fe elimination was observed by salt glands located in leaves of *A. schaueriana* and *L. racemosa*, although there was no greater Fe elimination in plants subjected to higher doses of Fe in the substrate (Arrivabene et al. 2016).

Using the same experimental design, a study was carried out to evaluate the effects of salinity on the bioaccumulation of Cr, As, Hg and Pb and on the anatomical, physiological and biochemical characteristics of *L. racemosa* and *R. mangle* (Campos 2018). Exposures to these metal(loid)s occurred by adding 28 μg/L of Cr_2O_7, 2 μg/L of As_2O_3, 10 μg/L of $HgCl_2$ and 10 μg/L of $PbCl_2$ into Hoagland's nutrient solution. After 12 weeks of treatment, samples were collected for analysis. The results showed that *L. racemosa* was more responsive to the sublethal toxic effects of Cr, As and Hg than *R. mangle*, especially in the root. In *L. racemosa*, the accumulation of Cr, As and Hg in the root changed stomatal density, stomatal conductance and the vascular bundle area of the mid-rib. Therefore, considering the species studied, *L. racemosa* proved to be most suitable as an environmental bioindicator for the presence of these elements (Campos, 2018).

3.3 Field Studies

3.3.1 Potamogeton pusillus *as a Bioindicator of Elemental Contamination*

Biomonitoring can be conducted by sampling organisms living in an investigated area (i.e., passive biomonitoring), or by exposure of organisms collected from a reference site or from a laboratory culture translocated to the investigated area (i.e., active biomonitoring). Both approaches were applied with *P. pusillus* in our field studies conducted in Córdoba Province: passive biomonitoring in the Suquía River and active biomonitoring in the Ctalamochita River.

3.3.1.1 Suquía River, Córdoba Province, Argentina

The Suquía River basin is the main source of drinking water for the city of Córdoba in Argentina. In recent times, the use of agrochemicals in nearby lands and discharges of metals and poorly treated domestic waste have resulted in increased pollution of its waters. In order to assess whether *P. pusillus* reflects different degrees of pollution generated by anthropogenic sources, the concentrations of a range of metals, metalloids and other elements were evaluated in *P. pusillus*, matching this information with the concentrations in corresponding water and sediment samples from the river basin. The elements studied included Ag, Al, As, gold (Au), barium (Ba), beryllium (Be), bismuth (Bi), boron (B), calcium (Ca), Cd, cerium (Ce), cobalt (Co), Cr, Cu, dysprosium (Dy), europium (Eu), erbium (Er), Fe, gallium (Ga), gadolinium (Gd), hafnium (Hf), Hg, holmium (Ho), potassium (K), lanthanum (La), lithium (Li), lutetium (Lu),

magnesium (Mg), Mn, molybdenum (Mo), palladium (Pd), praseodymium (Pr), Pt, sodium (Na), neodymium (Nd), Ni, Pb, rubidium (Rb), Se, samarium (Sm), strontium (Sr), terbium (Tb), thorium (Th), thallium (Tl), thulium (Tm), uranium (U), V, yttrium (Y), ytterbium (Yb) and Zn. Sample preparation and analyses of samples were carried out according to Monferrán et al. (2011, 2012a). Plants were collected during the wet season at two stations along the Suquía River basin, having different degrees of pollution and anthropogenic impacts. One area was located upstream from the provincial capital city of Córdoba, representing a site with low population impact and with less pollution according to previous studies. The second monitoring station was located downstream from Córdoba city. This area is primarily affected by the input of pollutants from domestic sewage, in addition to massive urbanization and intensive agriculture downstream from the city.

Considering the concentrations of the target elements in both water and sediments, most of the elements were at lower concentrations upstream from Córdoba city. Some elements (i.e., Al, B, Ba, Ce, Cr, Cu, Fe, Ga, Hg, Mn, Ni, Pb, Pd, Rb, Rh, Sb, Sn, Sr, V, Y and Zn) were present in sediments and water at significantly higher concentrations downstream of Córdoba, originating from sewage discharge. Chemometrics demonstrated good matching between metal and trace element concentrations found in water and sediment with those observed in aquatic plants collected at each monitoring site, indicating the accumulation of these pollutants from both water and sediment to the plant (Monferrán et al. 2012b). These results demonstrate the capacity of *P. pusillus* to be used as an effective bioindicator of aquatic pollution.

3.3.1.2 Ctalamochita River, Córdoba Province, Argentina

An active biomonitoring approach was used to evaluate the capacity of *P. pusillus* to reflect environmental quality. Plants were translocated from a pristine reference site and exposed during two different seasons at seven sites in the river (i.e., S1–S7) with different land uses, where variations in pollution could be expected due to the impacts of different sources (Fig. 3.3). Before the exposures, individuals of *P. pusillus* were acclimated during two weeks in glass aquaria filled with 10% Hoagland's solution, sediments (1/4) from the same sampling area, and maintained at 25 ± 1 °C under a natural light: dark regime. Then, acclimated plants were transported in tanks to the monitoring area. Perforated plastic envelopes containing groups of 24 macrophytes were deposited at each site. Envelopes were maintained at a water depth of 0.5–0.7 m, simulating environments usually colonized by *P. pusillus*. The plants were exposed for four days. Studies were performed in two seasons, reflecting the rain seasonality and temperature variation for the Ctalamochita River basin; that is, cold in July (CP) and warm in December (WP), according to previous studies (Bertrand et al. 2018). After exposure, macrophytes were collected, counted, washed with ultrapure water, flash frozen in liquid nitrogen and stored at -80 °C until analysis. During each monitoring campaign, water and sediment samples were also collected. A water quality index (WQI) was calculated with physicochemical and bacteriological data from water samples (Pesce and Wunderlin 2000). Residues of 13 pharmaceuticals were also measured in water samples and 20 elements, including 17 metals and three

metalloids were quantified in collected water and sediment samples, according to methods described by Valdés et al. (2014) and Bertrand et al. (2018), respectively.

The WQI displayed spatio-temporal variations along the basin, with lower values measured during the WP when compared to the CP (Bertrand et al. 2019). In most cases, a decrease in water quality could be observed at those sites downstream of cities (S2, S4, S6), with S6 being the site with the lowest WQI in both monitored periods. The presence of pharmaceutical compounds in water along the basin (e.g., atenolol and carbamazepine showed the highest levels), as well as metal(loid)s in water (Pb, Al, As, B, Hg) and in sediments (Hg) surpassing local and international environmental guidelines were evidence of discharges of inadequately treated sewage and, possibly, industrial wastewater. In *P. pusillus*, the levels of metals and metalloids measured in plant tissues showed the following order: stem < root < leaf in the CP and the inverse pattern during WP; leaf < root < stem. During the CP, the maximum accumulated concentrations in leaf and root occurred in plants at S4, thus exceeding by 7 and 10 times, respectively, the accumulation values of plants at the reference site, S1. On the other hand, maximum accumulation levels in the stem were observed in plants from S5 (i.e., the site with moderate industrial activities), representing a doubling over levels at S1. In WP, the maximum concentrations of metal(loids) in leaf and

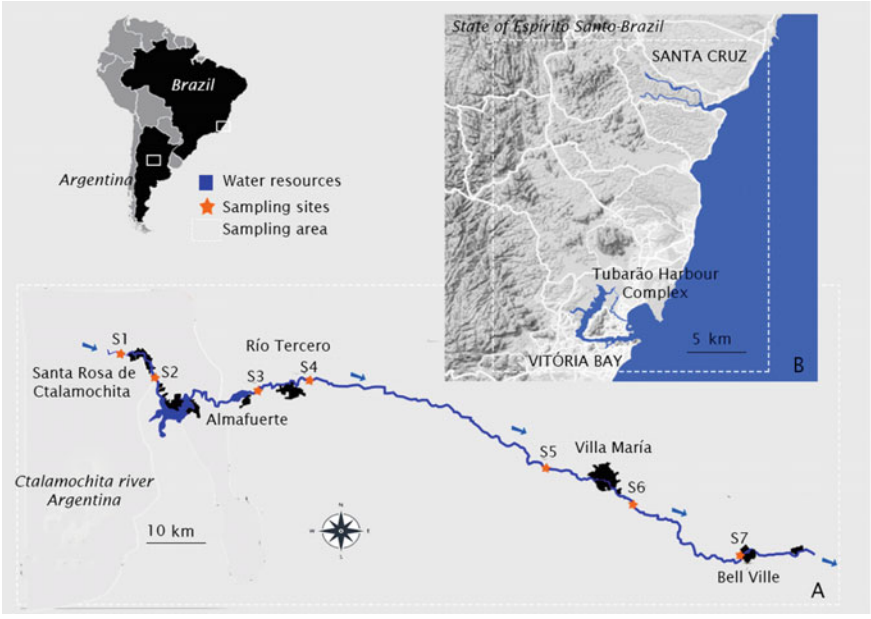

Fig. 3.3 Field study locations that include: (A) Active biomonitoring sites for *P. pusillus* in the Ctalamochita River in Córdoba Province, Argentina; Note that S1 was considered a reference site due to low anthropogenic activities upstream (B) Monitoring sites for mangroves in the State of Espírito Santo in Brazil illustrating the sampling points located in Santa Cruz, Vitória Bay and Tubarão Complex

root were observed in plants from S3, where there is low urban but high agricultural activities, doubling the values in plants from S1. In contrast, the stems showed the highest concentrations of total metal(loids) accumulated in plants from S4 and the lowest in plants from S6. In general, the accumulation of target elements in leaf and stem showed a significant correlation with elemental concentrations in water, for both monitoring periods (i.e., significant for Al, As, Ba, Cr, Pb, Sr and V). In contrast, the accumulation of elements in roots was correlated with the elements measured in the bioavailable and pseudo-total fractions of the sediments (i.e., significant for Cd, Co, Pb, V and Zn).

Throughout the study, biomarker responses in *P. pusillus* showed sensitivity under different environmental scenarios and indicated the most contaminated sites. In CP, Chl-a, Chl-b, Pheo-a and Pheo-b levels decreased significantly or showed a tendency to decline at sites S2, S4, S5 and S6, relative to the reference site (Bertrand et al. 2019). However, this trend was not so clear in the WP. Regarding antioxidant enzymes, different patterns of response were observed at each monitoring site, depending on the season of sampling, of the tissue or the biomarker measured. When comparing the antioxidant enzyme responses in plants from S2 to S7 with plants from S1, a greater number of significant responses were observed in plants deployed in WP than for plants deployed in CP, and they were observed in both leaf and root. Inhibition and/or induction of GPx, POD and SOD activities indicated significant levels of oxidative stress in P. *pusillus* translocated to the Ctalamochita River, particularly at the urban sites (S4 and S6) as well as at those sites with intense industrial and agricultural activities (S5 and S7, respectively).

The individual interpretation of biomarkers in field studies is complex due to the different patterns observed for each of them. Therefore, an integrative biomarker response index (IBR) was used as a tool to integrate and interpret responses obtained along the basin to achieve a comprehensive understanding of the biomonitoring response (Bertrand et al. 2016). During both monitoring periods, the stressor response in *P. pusillus* increased along the basin from S1 to S5 or S6, with a slight or strong decline at S7. The higher values of IBR in plants deployed at S5 and S6 could be associated with an increased complexity of the pollutant mixture originating from multi-sources discharges into the river at both sites. The higher conductivity and salinity observed in the lower basin could be responsible for the IBR decrease in plants at S7, since those physicochemical parameters were described to promote variations in the speciation and bioavailability of pollutants (Luoma and Rainbow, 2008). Through our results, there is strong evidence of the potential to use *P. pusillus* as a biomonitor of pollution hotspots in aquatic ecosystems using both passive and active biomonitoring approaches.

3.3.2 Mangrove Plants as Bioindicators of Metal Contamination

Field studies to measure the concentrations of metals in the abiotic medium and in mangrove plants can be carried out to assess the health of estuarine and marine

ecosystems. Our field studies in southeastern Brazil (Fig. 3.3) conducted in contaminated and pristine mangrove areas provide an example of how this can be done. Using *A. schaueriana*, *L. racemosa* and *R. mangle*, it was possible to evaluate the accumulation of 28 metals, metalloids and other elements (i.e., Ag, Al, As, B, Ba, Bi, Cd, Ce, Cu, Cr, Fe, Hg, La, Mn, Ni, Nb, Pb, Rb, Se, Sn, Sr, Ta, Ti, V, W, Y, Zn and Zr) in sediment, interstitial water, roots and leaves, in addition to some anatomical responses of these plants to pollutants and different physical conditions (Arrivabene et al. 2014; Souza et al. 2014a, b, 2015). The studies showed that the elements accumulate in different concentrations in sediment and interstitial water close to the rhizosphere of each species. Our studies also indicated that there is a differential in the bioaccumulation of these elements between the three study species. In general, the elements showed preferential accumulation in roots, but some of the elements were more easily translocated to the shoot, such as Cu, Ag, B and Mn (Arrivabene et al. 2014; Souza et al. 2014a, b, 2015). Comparing the three species, *A. schaueriana* was the mangrove plant that generally accumulated higher levels of the elements in their tissues.

The three mangrove species also showed adaptive plasticity by changing their root anatomy in response to the pollutants, where air gap area, cortex/vascular cylinder ratio, periderm thickness and lignification of the periderm were some of the parameters directly related to the level of environmental contamination. Multivariate analysis revealed that among more than 60 parameters evaluated (between physical, chemical and biological parameters), only 6 to 15 parameters (6 for *A. schaueriana*, 13 for *R. mangle* and 15 for *L. racemosa*) were necessary to identify the study areas according to the anatomical responses, with 100% correct classification. For *L. racemosa*, the multivariate analysis indicated that the cortex/vascular cylinder ratio of pneumatophores, periderm of pneumatophores and air gap area of absorption roots were the parameters showing the maximal discriminating power. For *A. schaueriana*, anatomical parameters evaluated in roots were also the most important for such differentiation. Thus, it is clear that the condition of the plant can indicate differences in pollution between mangrove areas to complement analytical measurements from sediment and interstitial water.

Regarding pollution by sources of atmospheric metals in dust, the leaf structure may or may not favor metal accumulation on the leaf surface. Leaves such as those of *A. schaueriana* and *L. racemosa*, which have salt glands, tend to accumulate dust that easily adheres to the saline secretions deposited on the leaf surface, while glabrous leaves with a large amount of epicuticular wax, such as of *R. mangle*, accumulate less dust (Arrivabene et al. 2015). Leaf analysis of these three mangrove species showed that dust from mining activities deposited on the leaf surface was not capable of generating morphological and anatomical changes. Although gas exchange was not evaluated, it was observed by scanning electron microscopy that the dust particles were large enough to obstruct the stomatal pore and had the potential to alter gas exchange rates. The dust was largely made up of Fe, but it also contained Al, Mn, Zn, Sr, Cr, Ni, V, Cu, Pb, Rb and As. Furthermore, the chemical analyses of Fe in the leaves and in the substrate suggest that there is foliar absorption of this element (Arrivabene et al. 2015).

3.3.3 *Stable Isotopes in Mangrove Plants as Bioindicators of Environmental Pollution*

Stable isotopic studies to assess the sources of environmental pollution were carried out by Souza et al. (2018) over eight trophic levels, including mangrove plants (*R. mangle, L. racemosa* and *A. schaueriana*). Analysis of isotopes of Sr ($^{87}Sr/^{86}Sr$) showed the influence of marine water on mangrove plants, which also demonstrate the potential for using these plants for estuarine/marine monitoring programs. Lin and Sternberg (1994), based on the analysis of oxygen isotopes ($^{18}O/^{16}O$) in *R. mangle*, also showed that mangrove plants use surface soil and seawater rather than groundwater as a water source, even when the water has a high salinity. Mangrove plants also proved to be good bioindicators of environmental contamination by particulate matter through the analysis of Pb stable isotopes ($^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$), which have higher values in plants located in areas with metallurgical activity as the main contamination source, such as at the Tubarão Complex (Fig. 3.3). Moreover, field research measuring stable nitrogen isotopes, such as $\delta^{15}N$, in mangrove plants, can show the anthropogenic impact of fertilizers (Souza et al. 2018). Changes in the ratios of $\delta^{15}N$ were associated with nitrogen enrichment from fertilizers in the mangrove plants from Vitória Bay when compared with those in Santa Cruz estuary, which is in close proximity (Souza et al. 2018). According to Tanu et al. (2020), the nitrogen ratios in mangroves from less contaminated site are typically lower than the ratios in mangroves from contaminated sites, highlighting how mangroves can be a powerful tool for anthropogenic disturbances. Therefore, the measurement of $\delta^{15}N$ ratios in mangroves allowed us to define Santa Cruz as a quasi-pristine mangrove ecosystem ($-2‰$ $\delta^{15}N$, according to Souza et al. 2018) and this site was used in this study as a reference location to understand metal/metalloid dynamics under natural conditions. Conversely, the higher $\delta^{15}N$ values for mangroves from Vitória Bay represented an anthropogenically impacted site. Consequently, a comparison between $\delta^{15}N$ in these estuarine plants is instructive. Due to the close proximity of the study sites (i.e., just 70 km), the original ecosystems should have been similar but current differences in stable isotopes can be attributed to the intensive development around Vitória Bay (Souza et al. 2018).

3.4 Conclusions

This chapter highlighted the capacity of macrophytes, which grow under different environmental conditions, to adapt to stress caused by exposure to metal(loids), with specific examples from laboratory and field studies with *P. pusillus*, *A. schaueriana*, *L. racemosa* and *R. mangle*. We observed that each of these macrophyte species adapted differently, taking into account their physiology or structure. For instance, *A. schaueriana* showed adaptive changes, leading to reduced amounts of metals and metalloids in roots by limiting the uptake and/or increasing the translocation of the elements, or both. However, it is not fully understood what triggers these anatomical

and physiological changes to adapt to the presence of environmental contaminants by preventing the absorption of potentially toxic metals and metalloids. Future studies, evaluating gene expression and epigenetic factors could help to elucidate the research questions arising from the current results.

Analysis of Sr isotopes ($^{87}Sr/^{86}Sr$) in *A. schaueriana*, *L. racemosa* and *R. mangle* demonstrated that they use seawater rather than groundwater as a source of water. Although this was already demonstrated by other authors in *R. mangle* using oxygen isotopes, these studies are the first to demonstrate this mechanism in *A. schaueriana*, *L. racemosa* and *R. mangle* using other isotopes (i.e., $^{87}Sr/^{86}Sr$). These results confirm the high potential of these plants to be used for estuarine/marine quality biomonitoring programs.

Studies carried out on *P. pusillus* exposed to metal(loid)s indicated that this macrophyte species can be used as a bioindicator for these elements, but also, this species shows potential for removing them from solution (e.g., industrial and domestic wastewaters), particularly for those aqueous solutions with high Hg concentrations. Our results are of note since previous studies reported lower Hg accumulation for different plant species than those reported in our work with *P. pusillus*. Further work is in progress to understand the molecular and biological mechanisms by which *P. pusillus* can accumulate large amounts of Hg in its tissues without showing great physiological damage.

Our results could help to understand the responses of different kinds of macrophytes to metal exposure. This will contribute to a more precise risk assessment, helping to predict and prevent toxic effects in these species. These studies will also guide regulatory decisions for the development of national and international plans for conserving biodiversity and protecting wetlands.

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Chapter 4
Global Perspective for the Use of Aquatic Macrophytes in Regulatory Risk Assessment for Contaminants

Verena Sesin, Judith L. Arnolds, and Gertie H. P. Arts

Abstract Macrophytes (aquatic plants) perform key structural and functional roles in aquatic and semi-aquatic ecosystems, and they also provide important ecosystem services for humans. It is therefore pertinent that macrophytes are considered in the ecological risk assessment (ERA) for chemicals and other contaminants that could impact their services. Macrophytes can display a range of morphologies and growth forms, and depending on those, require water, sediment (including pore water), and/or air to thrive in their environment; this diversity must be considered in ERAs. This chapter provides an overview of the use of macrophytes for ERAs as part of regulatory procedures. For several decades, free-floating *Lemna* spp. have been used as a "default" standard test species in phytotoxicity assays and ERA. During the last 15 years, additional species as well as toxicity endpoints beyond morphology and biomass have been included in regulatory approaches for potential contaminants of concern. Furthermore, increasingly complex, "higher-tier" ecological effects assessment approaches were developed, including species sensitivity distributions, microcosm and mesocosm studies, and modeling approaches. This chapter summarizes these developments and provides a global perspective on macrophyte use for risk assessments. It concludes with three recommendations for future ERAs with macrophytes: to educate young scientists in and raise awareness of ERA frameworks and testing methods for macrophytes, on a global scale; to fill knowledge gaps in the toxicity assessment with focus on submerged and emergent species and local species or varieties and climates; and to consider the complexity of stressor exposures and ecological contexts.

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4.1 Introduction to Aquatic Macrophytes as Relevant to Risk Assessment

4.1.1 Macrophyte Growth Forms

Macrophytes (aquatic plants) are growing in or near water and might be growing with upright positions above the water surface (e.g., sediment-rooted, emergent), below the water surface (e.g., sediment-rooted, submerged, or non-rooted, submerged) or floating (e.g., rooted, floating-leaved, or free-floating) (see Fig. 4.1). Several examples of macrophytes include coontail (*Ceratophyllum demersum* L.), cattail (*Typha* L.), waterthyme (*Hydrilla verticillata* (L.f.) Royle), common water hyacinth (*Pontederia crassipes* Mart.), and duckweed (*Lemna* L.). Aquatic ecosystems provide essential services and macrophytes perform a key role in their functioning (Jackson et al. 2001; Maltby et al. 2010; Borst et al. 2018; Temmink et al. 2021). Abiotic and biotic factors influence the natural occurrence and abundance of macrophytes, and thereby affect ecosystem services (Temmink et al. 2021). Relevant abiotic conditions include water transparency (i.e., light availability), water temperature, carbon species, nutrient enrichment availability in surface water and sediment, water movement, and sediment and water phytotoxicity. Biotic factors that influence plant occurrence, distribution, and growth include herbivory and bioturbation by water birds, large fish, and crayfish (Lamers et al. 2013; Dar et al. 2014; Bakker et al. 2016; Temmink et al. 2021).

Macrophytes are adapted to growing in water-saturated sediments. The major difference between water-saturated and well-drained sediments is oxygen availability. The pore spaces are filled with air with a relatively high oxygen content in well-drained soils. Microorganisms that inhabit the soil and roots of plants growing in the soil are able to get oxygen directly from their surroundings. In water-saturated sediments, pore spaces are water-filled, and because of the slow rate of oxygen diffusion in water, the water-saturated sediments become anaerobic. The root systems of macrophytes growing in water-saturated substrates therefore must use oxygen from their aerial parts via internal transport. These macrophytes are morphologically adapted to grow in water-saturated sediment through large internal spaces for transportation of oxygen and rhizomes (Brix 1994). Some macrophytes are also able to radiate oxygen from their roots to the root environment to oxidize the sediment around the root tips.

4.1.2 Macrophyte Plasticity

The successful distribution of aquatic plants in new environments is often linked to multiple introductions and a diverse gene pool that facilitates adaptation to variable environmental conditions (Riis et al. 2010). However, there are two distinctive adaptive mechanisms that improve the survival, reproduction, and dispersal of plant

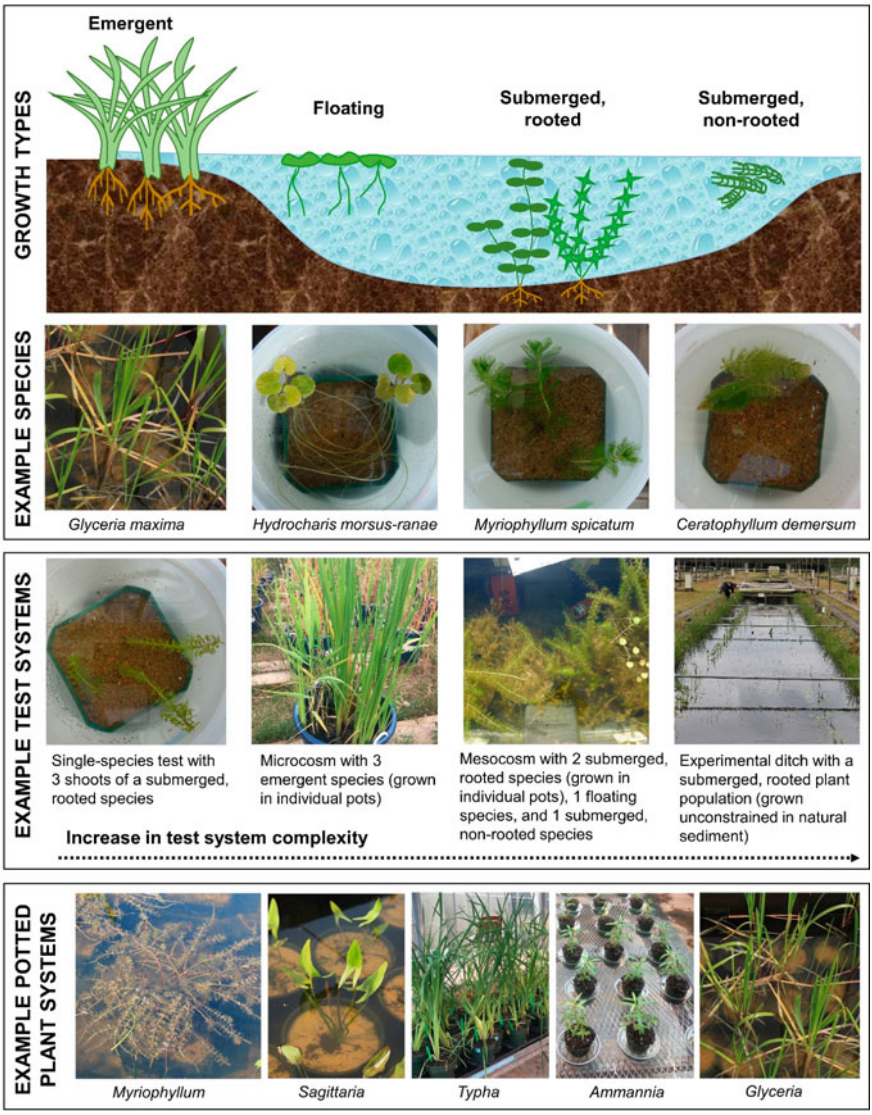

Fig. 4.1 Overview of macrophyte species used for phytotoxicity testing and ecological risk assessments. The figure displays examples of different growth forms and respective species, test systems of varying complexity, and potted plant systems

species: phenotypic plasticity and local adaptation (i.e., the capacity of a species to rapidly adapt genetically by virtue of a diverse gene pool) (Ward et al. 2008; Riis et al. 2010).

The capacity of a given genotype to express different phenotypes in different environments is called phenotypic plasticity (Sultan 2000). Plants are capable of rapidly

changing their phenotypic characters if phenotypic plasticity is the primary adaptive mechanism for plants to spread into a range of habitats. The change is caused by environmental conditions in the habitat (Ward et al. 2008; Riis et al. 2010). Phenoplastic species can change their physiology or morphology in response to variations in environmental conditions (Schlichting 1986). The number of introductions of a species is essential in determining whether phenotypic plasticity or local adaptation is the most adaptive mechanism for invasive plant species (Kawecki and Ebert 2004). A morphologically plastic plant can display either competitor or stress-tolerant phenological traits, depending on the environmental conditions (Kautsky 1988; Garbey et al. 2004). One of the most important environmental conditions determining plasticity in aquatic systems is disturbance (Barrat-Segretain 2001).

4.1.3 Role of Macrophytes in Aquatic Ecosystems

Macrophytes are important components of aquatic and wetland ecosystems (Lesiv et al. 2020; Rejmankova 2011; Thomaz 2021) and play a diverse role in determining the structure and function of these systems through, for example, oxygenation of water, productivity, and nutrient recycling (Meena and Rout 2016; Ceschin et al. 2020). Macrophytes are involved in ecosystem processes such as biomineralization, transpiration, and sedimentation. Among biotic components of aquatic ecosystems, higher aquatic plants are one of the main factors of the formation and regulation of water quality and oxygen content in natural water (Rejmankova 2011). They are primary producers and provide food to invertebrates, fish, and birds, as well as organic carbon for bacteria. Macrophytes are at the bottom of herbivorous and detritivorous food chains, and their stems, roots, and leaves serve as substrate for periphyton, and as shelter for several invertebrates and different stages of fish, amphibians, and reptiles (Timms and Moss 1984; Rejmankova, 2011). Certain macrophytes are valuable for their direct contributions to human societies by providing food, biomass, and building materials (Egertson et al. 2004; Bornette and Puijalon 2011; Rejmankova, 2011). Moreover, macrophytes can accumulate heavy metals and other toxic substances from water bodies and play an important role in bioindication and phytoremediation (Kurilenko and Osmolovskaya 2005; Ceschin et al. 2020, 2021; Kumar et al. 2022).

The occurrence and growth forms of macrophytes influence the biogeochemical processes and movements in the water column and sediments. Submerged macrophytes play an important role in maintaining good water quality and high biodiversity in shallow ecosystems, and act as biofilters. Sediments represent an important source of nitrogen and phosphorus for rooted aquatic macrophytes (Barko & Smart 1981). The accumulation of nutrients in an aquatic system causes eutrophication which results in substantial growth of macrophytes and weeds. Submerged macrophytes could play a role in alleviating the adverse effects of phosphorus resuspension and release from bottom sediments. Particles resuspended from the bottom could increase turbidity and deteriorate the underwater light field. Resuspension processes influence nutrient flux at the sediment-water interface and in the water column, and

then affect primary production by macrophytes (Zhu et al. 2015). Submerged macrophytes can also prevent the growth of algal blooms through the reduction of nutrients, allelopathy, and shading (Dhote 2007; Lv et al. 2019).

4.1.4 Ecosystem Services Provided by Macrophytes

The Millennium Ecosystem Assessment (MEA 2003, 2005) describes ecosystem services as "the benefits people obtain from ecosystems." These benefits can be classified into four broad categories: supporting, provisioning, regulating, and cultural services. Supporting services include soil and sediment formation, photosynthesis, primary production, nutrient cycling, water cycling, and provisioning of habitat. Regulating services include climate regulation (e.g., through carbon sequestration), water regulation, erosion regulation on shores, water purification, waste treatment, disease regulation through filtration of pollutants and pathogens, pest regulation, and biological control. Provisioning services include food, fiber, genetic resources, and environmental monitoring. Lastly, cultural services include educational value and cultural heritage value (MEA 2003, 2005; Dhote and Dixit 2009; Thomaz 2021; Kumar et al. 2022). The multiple benefits provided by macrophytes are often associated with ecosystems such as wetlands and shallow lakes (Taillardat et al. 2020).

4.2 Current Use of Macrophytes in Ecological Risk Assessments

4.2.1 Overview and Rationale for Ecological Risk Assessments

Effective environmental protection strategies face diverse ecological issues, including climate change, loss of biodiversity, and ubiquitous pollution by anthropogenic substances (Hope 2006). Ecological risk assessments (ERAs) use scientific knowledge and tools to generate informed conclusions that can support environmental decision-makers in designing effective protection strategies (Suter 2006). The ERA process is designed to evaluate how likely adverse ecological effects occur following exposure to one or more environmental stressors (Suter 2006; Quanz et al. 2020), with the goal to generate transparent, objective, and reliable information for decision-makers. Frameworks to guide this process are established by government authorities, including in Europe (e.g., European Chemicals Agency, European Food Safety Authority), North America (e.g., United States Environmental Protection Agency, Canadian Pest Management Regulatory Agency), Asia (e.g., Fan et al.

2019), Africa (e.g., Utembe and Gulumian 2015), Australia (e.g., Australian Government 2021), and across countries (e.g., Organisation for Economic Co-operation and Development, International Organization for Standardization). While there is no single internationally accepted framework (Quanz et al. 2020), most jurisdictions follow similar principles.

The three general principles of ERAs are problem formulation, analysis of exposure and effects, and risk characterization (Hope 2006; Suter 2006) (Fig. 4.2). Problem formulation specifies the issue to be solved, defines environmental components to be protected, and outlines a plan to obtain the necessary data to perform the assessment (Hope 2006; Suter 2006). The analysis of exposure and effects characterizes the spatio-temporal fate as well as interactions of a stressor in the environment, and then assesses the response of environmental components to exposures of realistic durations and magnitudes (Hope 2006; Suter 2006). Finally, risk characterization integrates all obtained information and estimates the ecological risks (Hope 2006; Suter 2006).

The ERA process consists of several levels, so-called tiers, that range from lower-tier screening methods employing hazard quotients calculated from laboratory-derived exposure and effect data, to higher-tier approaches such as ecological modeling, mesocosm and field studies, and weight-of-evidence analysis (Hope 2006; Suter 2006). The progression from lower to higher tiers increases complexity and costs but also results in higher accuracy, realism (risk-based), and predictive power for environmental decision-making (Solomon et al. 2008). Risk assessments are designed to be iterative, where decisions are refined through the acquisition of additional data (Solomon et al. 2008). The process starts with a lower-tier assessment, which if it suggests a potential risk, triggers a higher-tier assessment to further investigate the nature and extent of the risk. This progression promotes efficient use of resources while ensuring that risks are sufficiently characterized for an informed decision (Hope 2006). Scientific ERAs thereby represent an important part of regulatory environmental protection decisions that ultimately consider multiple factors, including economic benefits associated with an activity that results in environmental stress, possible human health risks, and the options for impact mitigation and management.

4.2.2 Macrophyte Use in Ecological Risk Assessments

Macrophytes play an important role in aquatic ecosystems, contributing to structural complexity, biogeochemical cycles, and overall productivity of waterbodies (Carpenter and Lodge 1986; Thomaz and Da Cunha 2010; Lewis and Thursby 2018), as outlined in previous sections. Risk assessments intend to ensure that these ecosystem services are not compromised by any activities that directly or indirectly affect macrophytes and their habitat. Macrophytes are therefore considered in risk assessments and monitoring of water quality including nutrient loading and eutrophication (Delmail 2014; Szoszkiewicz et al. 2020), wastewater discharges

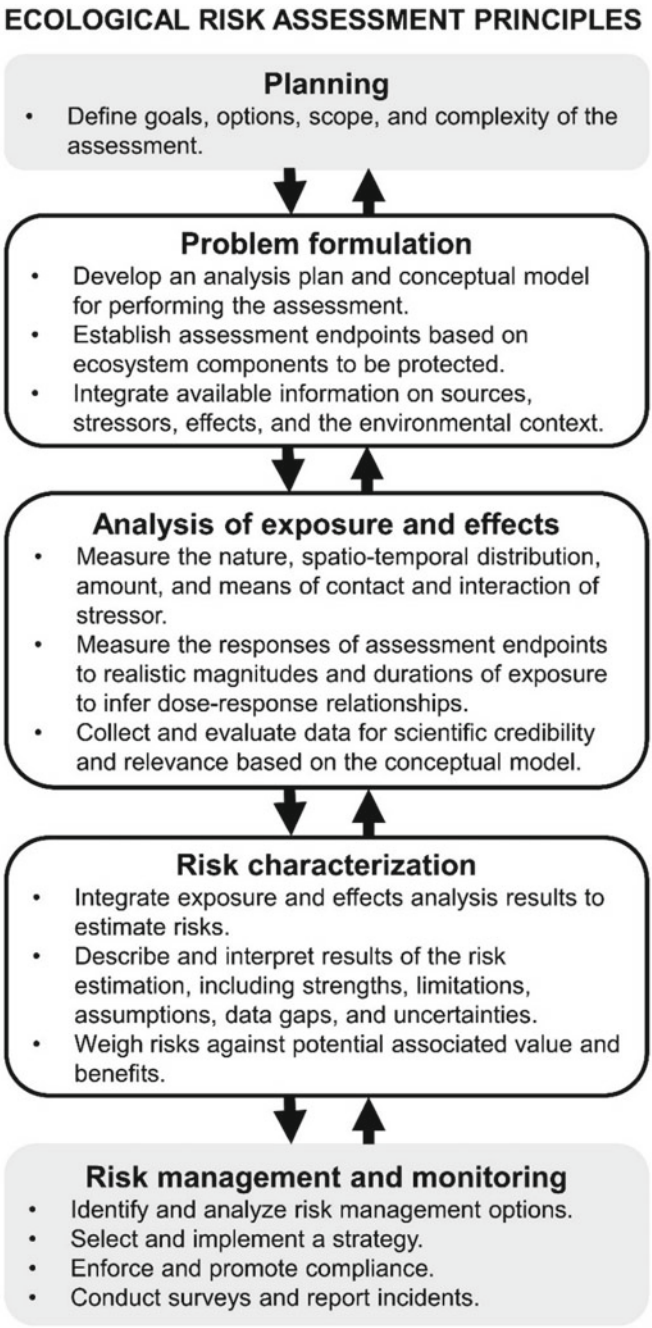

Fig. 4.2 General principles of ecological risk assessments including the core steps of problem formulation, analysis of exposure and effects, and risk characterization, as well as pre-assessment planning and post-assessment risk management and monitoring. The arrows indicate that this is an iterative process. Modified from Hope (2006), Suter (2006), and Health Canada (2021c)

(EPA Victoria 2009), contaminated sites (Government of Canada 2012), nuclear facilities and activities (CSA 2022), and chemicals that may be released into the environment (Australian Environment Agency 2009; ECHA 2017).

One important stressor to macrophytes that typically warrants ERA is pesticide use. Pesticides are anthropogenic chemicals designed for the control of pest organisms, including weeds, insects, rodents, and fungi. Pesticides are applied in agricultural, industrial, urban, and residential areas, in both terrestrial and aquatic settings. Their use is regulated through a complex registration process and tiered ERA performed by many government authorities worldwide, including Australia, Brazil, China, Europe, India, Japan, South Africa, and the United States, and efforts toward global harmonization of procedures and standards are ongoing (Handford et al. 2015). Macrophytes are an integral part of ERAs for pesticides in Canada (Health Canada 2021a), the United States (US EPA 2017), and Europe (EFSA PPR 2013). Pesticides with an herbicidal mode of action, such as herbicides, plant growth regulators, and certain fungicides, must undergo ERA on macrophytes prior to their registration (European Commission 2013; Health Canada 2021b; US EPA 2021).

4.2.3 Phytotoxicity Assessment Using Standardized Test Protocols

A key step in macrophyte ERA is phytotoxicity assessment, which determines the hazards posed by a stressor (e.g., pesticide, heavy metal, pharmaceutical, plastic) to selected non-target macrophyte species using a range of realistic exposure concentrations (e.g., Health Canada 2021a). Hereby, in lower-tier assessments, government agencies frequently rely on data from phytotoxicity studies following standardized test protocols which are designed to produce reliable and reproducible data for regulatory decisions (Taylor and Scroggins 2013; Rudén et al. 2017). The development of a standardized test protocol can be spearheaded by government agencies, industry, scientific societies, or any group of scientists, to address a lack of data in risk assessments (OECD 2009; Taylor and Scroggins 2013). Development procedures involve the selection of a suitable test species, establishing technical procedures of how to conduct the test, and validating that the method produces consistent results across laboratories (Taylor and Scroggins 2013). The final test protocol is typically published by an internationally recognized organization, such as the Organisation for Economic Co-operation and Development (OECD) or the American Society for Testing and Materials (ASTM) International. A selection of internationally recognized, standardized macrophyte tests are listed in Table 4.1. Development of standardized protocols has primarily focussed on testing on the laboratory to greenhouse scale, although a few testing guidelines have been proposed for higher-tier testing such as mesocosms (e.g., OECD 2003; Coors et al. 2006; EFSA PPR 2013); some of these have been standardized among continents, such as the *Myriophyllum*

spicatum water-sediment test protocol (OECD 2014b) and the *Lemna* sp. growth inhibition test (OECD 2006). Standardization of test protocols aims to harmonize ERAs across countries (Taylor and Scroggins 2013) and continents, which can increase international cooperation as well as save costs and resources.

Table 4.1 Examples of internationally recognized, standardized macrophyte tests, listing key features of the tests: macrophyte species, duration of the test, exposure type, and assessment endpoints

Publisher	Year	Protocol	Macrophyte	Duration	Exposure type	Endpoint(s)	Reference
Floating species							
ASTM	2013	E1415-91	*Lemna gibba*	7 days	Aqueous	Growth inhibition	ASTM (2013)
EC	2007	EPS 1/RM/37	*Lemna minor*	7 days	Aqueous	Frond number and frond dry weight	EC (2007)
ISO	2005	ISO 20079	*Lemna minor*	7 days	Aqueous	Growth rate based on frond number, frond area, chlorophyll, and dry weight	ISO (2005)
OECD	2006	TG 221	*Lemna gibba*, *Lemna minor*	7 days	Aqueous	Frond number, total frond area, and fresh and dry weight	OECD (2006)
US EPA	2012	OCSPP 850.4400	*Lemna gibba*, *Lemna minor*	7 days	Aqueous	Growth rate and yield based on frond number, frond area, and frond dry weight	US EPA (2012)
Submerged species							

(continued)

Table 4.1 (continued)

Publisher	Year	Protocol	Macrophyte	Duration	Exposure type	Endpoint(s)	Reference
ASTM	2017	E1913-97	*Myriophyllum sibiricum*	14 days	Aqueous	Plant growth, shoot length, root number and length, fresh and dry weight, oxygen production, membrane permeability, and chlorophyll and carotenoid content	ASTM (2017)
ISO	2013	ISO 16191	*Myriophyllum aquaticum*	10 days	Sediment	Growth rate based on fresh weight, length, and number of new shoots and roots	ISO (2013)
OECD	2014	TG 238	*Myriophyllum spicatum*	14 days	Aqueous	Growth rate and yield based on shoot length, shoot fresh, and dry weight	OECD (2014a)
OECD	2014	TG 239	*Myriophyllum spicatum*	14 days	Aqueous, sediment	Growth rate and yield based on shoot length, shoot fresh. and dry weight	OECD (2014b)

ASTM: American Society for Testing and Materials International; EC: Environment Canada; ISO: International Organization for Standardization; OECD: Organisation for Economic Co-operation and Development; US EPA: United States Environmental Protection Agency

4.2.4 Standard Test Species for Phytotoxicity Assessment

It is not practical for an environmental assessment to analyze all species within the area under consideration; therefore, a set of representative "reference" animals and plants, in some sectors also referred to as "surrogates", is typically used (Charrasse

et al. 2022). Standard test species are species for which standardized test protocols have been developed. Typically, these species are model organisms, which are species that have been extensively studied to understand biological processes. To assess the suitability of a species for standardized testing, a set of criteria are routinely considered (Table 4.2). These criteria ensure that testing can be easily and reliably performed across facilities, producing ecologically meaningful results to inform environmental protection decisions. Ideally, phytotoxicity assessment is performed with a range of macrophytes representing the community, because no species is consistently the most sensitive to stressors (Fairchild et al. 1998; Arts et al. 2008; Lewis and Thursby 2018). Depending on the stressor mode of action and relevant exposure pathways, testing should include macrophytes of differing morphology and growth forms (e.g., free-floating and floating-leaved; sediment-rooted, submerged; non-rooted, submerged; sediment-rooted, emergent) (Fig. 4.1). Moreover, species should be chosen to reflect realistic environmental conditions, such as freshwater or saltwater, temperate or tropical environments. Marine macrophyte species are often neglected in toxicity testing (Vonk and Kraak 2020), although saltwater macrophytes can be more sensitive to several stressors, including cadmium, copper, diuron, and irgarol, compared to freshwater species (Lewis and Thursby 2018). Moreover, tropical species are commonly underrepresented in phytotoxicity studies, although they can be more sensitive than temperate species to some stressors (Binet et al. 2018; Mooney et al. 2019). When selecting a test species, consideration should also be given to the differing sensitivities of ecotypes (Kanoun-Boulé et al. 2009), as well as genotypic, intraspecific variation (Roubeau Dumont et al. 2019).

The internationally most commonly used standardized test species are free-floating, non-rooted *Lemna* spp. and submersed-rooted *Myriophyllum* spp. (see also Table 4.1). These standard tests typically quantify growth and biomass changes following 7–14 days of static exposure to a range of concentrations of a stressor. To address a lack of test protocols for emergent species, a guideline is currently under development for a 14-day test with *Glyceria maxima* (Hartm.) Holmb. (Davies et al. 2019). Another proposed emergent macrophyte is *Typha* that fulfilled many of the selection criteria (Sesin et al. 2021), although test methods are not yet developed. Several other macrophyte species and test procedures have been proposed for standardized testing. These proposals include a 48-h phytotoxicity test method using root-regrowth as a sensitive endpoint for *Lemna* spp. (Park et al. 2013) (updated to 72-h in Park et al. 2022 and ISO/DIS 4979), a 7-day test with the macrophyte *Salvinia natans* (L.) All. (Cui et al. 2020), and a bioassay with the tropical, marine seagrass *Halophila ovalis* (Wilkinson et al. 2015). Further unpublished testing protocols have been developed in research centers and by industry (Maltby et al. 2010), covering a range of floating species belonging to various genera including *Azolla*, and submerged species of the genera *Egeria*, *Elodea*, and *Ceratophyllum*, as well as emergent species of the genera *Sparganium*, *Sagittaria*, and *Phragmites* (Table 4.3); however, standardized test methods are not yet available for these species, but tests are based upon existing protocols such as the *Myriophyllum spicatum* water-sediment test (OECD 2014b) for submerged macrophytes or the *Glyceria maxima* water-sediment test (in development) for emergent macrophytes (Arts et al. 2022).

Table 4.2 Criteria to select macrophyte standard test species for phytotoxicity assessment (summarized from Powell et al. 1996; Maltby et al. 2010; Sesin et al. 2021)

Criterion	Explanation
Ecological relevance	The macrophyte should be relevant to the ecosystem and stressor exposure under investigation. Selection considers a species' role and importance in the ecosystem, geographical relevance (e.g., temperate or tropical areas), as well as its morphology and physiology
Suitability for different exposure pathways	Macrophytes can be exposed via different exposure routes depending on their growth form (e.g., emergent, submerged, floating). Selection considers if a species is likely to be exposed to the stressor via realistic routes (e.g., soil, water, air, spray drift, sediment, pore water)
Availability of material	Macrophyte material should ideally be available year-round to allow for continuous, timely testing. Selection considers the availability of material from natural populations as well as whether stock cultures can be established for continuous supply
Ease of cultivation	Standardized testing relies on protocols that minimize cost, workload, and space. Selection considers if a species can be cultivated under controlled conditions such as a growth chamber or greenhouse, and if cultivation is straightforward with high return of usable test material
Uniform growth	Macrophytes with low inherent variability in morphology and biomass are preferred as this can facilitate the statistical discernment between natural and stressor-related changes that are measured in the test. Selection considers the variation in these growth parameters to ensure it is acceptable for the test design (e.g., sample size). This criterion is transferrable to non-growth-related endpoints that may be assessed
Appropriate assessment endpoints	Endpoints are measured variables that reflect the performance of the macrophyte during the test. Selection considers if the endpoints are toxicologically sensitive to the stressor, exhibit low variability within treatments, and are biologically meaningful (i.e., useful for interference of effects on the individual to community level)

(continued)

Table 4.2 (continued)

Criterion	Explanation
Sensitivity toward stressors	The test species is ideally among the most sensitive species toward the stressor, so that the test results are protective of other co-occurring macrophytes. Selection considers the relative sensitivities of species, taking into account any "safety factors" to account for uncertainty that may be applied during ecological risk calculation or to extrapolate to other macrophyte species

Moreover, experimental conditions generally adopted in freshwater toxicity tests with macrophytes were recently summarized in a review by Ceschin et al. (2021).

4.2.5 Tier 1 (Lower-Tier) Phytotoxicity Tests with Macrophytes

Tier 1 tests are short-term, laboratory-based, and single-species phytotoxicity tests used to screen for major toxic effects. Typically, these lower-tier tests employ standardized test protocols as outlined in Table 4.1. Various potential stressors have been tested for their phytotoxicity using simple testing approaches that follow, or are modified from, standardized test protocols; these stressors include heavy metals, pharmaceuticals and personal care products, pesticides, hydrocarbons, surfactants, and plastics (summarized in Ceschin et al. 2021). Of all aquatic plants used in ecotoxicity testing, the majority (60%) are microalgae (Ceschin et al. 2021). *Lemna* spp., *Myriophyllum* spp., and *Hydrilla* spp. collectively account for one third (33%) of test species (Ceschin et al. 2021).

The small size, simple anatomy, and ease of culturing make *Lemna* spp. ideal test organisms for ecotoxicological investigations (Mkandawire et al. 2014). However, *Lemna* spp. are not appropriate test organisms in all cases and additional testing options with other macrophytes are needed. The AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) workshop (Maltby et al. 2010), held in 2008, triggered the development of test protocols for sediment-rooted aquatic macrophytes. Namely, the AMRAP workshop concluded upon regulatory concerns that risk assessments solely based on *Lemna* spp. and algal data at Tier 1 might underestimate the risk of plant protection products to aquatic macrophytes. One concern was that *Lemna* spp. are monocotyledonous species, while herbicides might also and sometimes specifically target dicotyledonous species (e.g., 2,4-D; Belgers et al. 2007). Moreover, concern was raised that *Lemna* spp. may not be sensitive to pesticides that form residues in sediment; because of considerable knowledge and experience with *Myriophyllum spicatum* L., this species was recommended as an additional Tier 1 test species (Maltby et al. 2010). After extensive test development and ring-testing among

Table 4.3 Macrophyte species previously used in laboratory studies that are potentially suitable for toxicity tests. Common names may not reflect all names used globally

Growth type	Species	English common name(s)
Floating	*Azolla**	Water fern
	*Lemna**	Duckweed
	Salvinia	Watermoss, kariba weed
	*Spirodela**	Duckmeat, duckweed
Submerged, non-rooted	*Ceratophyllum**	Coontail, hornwort
	Chara	Stonewort
Submerged, rooted	*Callitriche*	Water-starwort
	*Egeria**	Waterweed
	*Elodea**	Waterweed, pondweed
	Hydrilla	Hydrilla, water thyme
	*Heteranthera**	Mud plantain, ducksalad
	Hottonia	Water violet, featherfoil
	Hygrophila	Swampweed, starhorn
	*Lagarosiphon**	Oxygen weed, African elodea, curly waterweed
	*Myriophyllum**	Water milfoil
	Najas	Water nymph, naiad
	Potamogeton	Pondweed, ribbonleaf
	Ranunculus	Water crowfoot
	*Vallisneria**	Eelgrass, tape grass, vallis
Emergent	*Glyceria**	Sweet-grass, mannagrass
	Phragmites	Common reed
	Sagittaria	Arrowhead, duck potato, katniss, swamp potato, tule potato
	Sparganium	Bur-reed
	Typha	Bulrush, cattail, reedmace, cumbungi

The asterisk identifies species that are available from commercial suppliers, although import licences may be required. Table modified from Maltby et al. (2010)

several laboratories, a sediment-water guideline was published (OECD 2014b) and the test was adopted by the European Aquatic Guidance document (EFSA PPR 2013) and included in the data requirements of the pesticide regulation in Europe (EC 2013). These data requirements specifically include additional aquatic macrophyte species tests to be undertaken on a dicotyledonous species, such as *M. spicatum, M. aquaticum,* or a monocotyledonous species, such as the aquatic grass *G. maxima*, as appropriate. Research has shown that *G. maxima* is a suitable candidate for testing grass-specific herbicides (Mohr et al. 2015). The need to perform studies with rooted, submerged, and emergent macrophytes is to be discussed with the national competent authorities.

The *M. spicatum* water-sediment test (OECD 2014b) uses an artificial sediment (OECD 2004) with an overlying Smart and Barko test medium (Smart and Barko 1985). The sediment is enriched with nutrients (phosphorus and nitrogen) (OECD 2014b) to enable optimum growth of the macrophytes, while the overlying Smart and Barko medium only includes carbon. The protocol can be used as a blueprint for testing other sediment-rooted macrophytes in the laboratory, such as *Elodea nuttallii* (Planch.) H. St. John and *Elodea canadensis* Michx. In addition, exposure via the sediment may be simulated by spiking the artificial sediment with a test chemical and transplanting plants into this spiked sediment. As stated above, a *G. maxima* test is under development and has been ring-tested since 2016 (Arts et al. 2022).

Submerged macrophytes are easy to propagate, as each side shoot can develop new roots and can grow into a new shoot. Emergent macrophytes are different: mother plants need to be propagated to develop enough young shoots of similar length and leaf number to perform a test. Besides *G. maxima* as a potential emergent test species, *Typha* species turned out to be promising (Sesin et al. 2021). *Typha* spp. are increasingly used to assess the phytotoxicity of pollutants. *Typha* is easy to grow and suitable for water, soil, and air exposure tests. It enables a suite of morphological and physiological toxicity endpoints to be measured (Sesin et al. 2021). A drawback might be that *Typha* species have an ability to hybridize, which might be an issue in certain geographical regions. No species within the *Typha* genus is consistently the most sensitive to a range of stressors although comparable data is currently limited (Sesin et al. 2021). Selection of a *Typha* test species may therefore be based on local availability, and on the feasibility to propagate enough young shoots with an initial variability low enough to perform a toxicity test following regulatory requirements. This latter issue is relevant for all emergent macrophytes for use in laboratory toxicity tests.

4.2.6 Higher-Tier Phytotoxicity Tests with Macrophytes

In the risk assessment for pesticides, microcosm and mesocosm test systems can be used as a suitable reference tier. Maltby et al. (2010) stressed that the required endpoints for macrophytes need to be studied as naturally as possible, considering competition, predation, and natural stressors. Microcosms and mesocosms enable this type of studies as species are considered within their community. Microcosm and mesocosm studies with aquatic macrophytes can be performed as two different test designs: one option is the inclusion of the macrophytes as free-growing natural populations; the second option introduces the macrophytes as potted plants (Fig. 4.1). The first design limits the number of macrophyte species that can be studied, because free-living populations require a large surface area. It is only achievable in larger mesocosms such as experimental ditches. The second design with potted plants excludes below-ground competition between the rooted macrophyte species. Both approaches might be combined in a mesocosm when it is divided into two parts: one part is reserved for free-growing populations and the other part for potted plants

(e.g., in the form of bioassays). Both design options allow the study of effects on free-living algae populations, phytoplankton and periphyton. Maltby et al. (2010; see Table 3.3 therein) gives an overview of advantages and limitations of assessing phytotoxicity in microcosms and mesocosms using potted plants *versus* plants rooted in natural sediment.

The earliest mesocosm studies were performed in the United States in the 1980's (Graney 1990; Solomon et al. 1996). There was only a short time window from 1988 to 1992 in which the United States Environmental Protection Agency (US EPA) required aquatic field studies (Boyle and Fairchild 1997). Afterward, the interest for mesocosm studies declined, partly due to difficulties in interpretation; however, there is a recent revival of the interest (see recent sessions and presentations in SETAC scientific congresses). Microcosm and mesocosm studies targeted for aquatic macrophytes have been comparably rare. An overview of mesocosm studies in ecotoxicology by Caquet et al. (2000) only mentions aquatic macrophytes as structural elements for other groups of organisms, but not as a sensitive or vulnerable taxonomic group to be studied. Studies performed in the 1990s addressed the effects of linuron, an herbicide, on primary producers including aquatic macrophytes in experimental ditches (Brink et al. 1997; Cuppen et al. 1997). Fairchild and Sappington (2002) studied the fate and effects of the triazinone herbicide metribuzin in experimental pond mesocosms including the effect on the inhabiting macrophytes. Mohr et al. (2007, 2009) performed studies in experimental pond and stream mesocosms. An alternative is to use bioassays with aquatic macrophytes in mesocosms (Coors et al. 2006). Bioassays are in situ tests and include the use of, for example, potted plants (Fig. 4.1).

The AMRAP workshop and its publication (Maltby et al. 2010) drew renewed attention to the inclusion of aquatic macrophytes in the risk assessment for pesticides and stressed the need for a proper higher-tier risk assessment for aquatic macrophytes. It is only recently, after the publication of the Aquatic Guidance document in Europe (EFSA PPR 2013) and a publication about the Minimum Detectable Difference approach for mesocosm studies (Brock et al. 2015), that potted-plant studies are presented by regulators as the only way forward for higher-tier aquatic macrophyte mesocosm studies to meet the European regulatory requirements of the inclusion of eight sensitive species.

Mesocosm studies were also performed in tropical climates (Daam et al. 2009a, b). The comparison of microcosm and mesocosm studies between temperate and tropical climates does not generate an unambiguous conclusion (Daam and van den Brink 2010). Pesticide dissipation rates and vulnerability of freshwaters do not appear consistently higher or lower in tropical regions compared to their temperate counterparts (Daam and van den Brink 2010). However, differences in fate and effects may occur for individual pesticides and taxa. Moreover, intensive agricultural practices in tropical countries lead to a higher input of pesticides and spread of contamination over watersheds (Daam and van den Brink 2010).

4.2.7 Ecotoxicological Endpoints for Macrophytes

Endpoints are explicit expressions of environmental values that environmental managers wish to protect (CSA 2022). An ecotoxicological endpoint can be defined as a variable reflecting macrophyte performance and development during and after exposure to a toxic compound. Several different categories of endpoints can be distinguished (Arts et al. 2008). Assessment endpoints are directly related to environmental management goals but they are typically stated in terms of population and community attributes (e.g., population success, community success, diversity) (CSA 2022). However, it is not always practical to quantify those attributes; therefore, more readily measurable or predictable surrogates, so-called measurement endpoints, can be selected (CSA 2022). For instance, for an assessment endpoint of "probability of greater than 10% reduction" in recruitment, the related measurement endpoints could be "% mortality" in exposed habitats (CSA 2022). Measurement endpoints should be defined in terms of survival, growth, or reproduction (CSA 2022). Examples for plants include biomass, frond number, number of leaves, area of leaves, shoot height, fresh weight (related to growth), mortality (related to survival), and number of new ramets, or number of seeds (related to reproduction). When defining measurement endpoints, priority should be given to those that are closely linked to assessment endpoints; for example, survival, growth, and reproduction are generally considered to be closely linked to population success (CSA 2022).

Assessment endpoints are used in the formal risk assessment. These may include the LOEC (Lowest Observed Effect Concentration), NOEC (No Observed Effect Concentration), or ECx (effect concentration for x% of the test population). For primary producers, both growth rate (ErC_{50}) and yield/biomass endpoints (EyC_{50} or EbC_{50}) are assessment endpoints. ErC_{50} is the preferred endpoint for primary producers (EFSA PPR 2013; OECD 2006, 2014b) and is a protective endpoint in most cases (Van Wijngaarden and Arts 2018). In order to use growth rate as an endpoint, exponential growth in the control plants should be demonstrated (EFSA 2015, 2019). Growth rate endpoints are independent of test duration, while yield or biomass endpoints decrease with test duration (Bergtold and Dohmen 2011). This is a consequence of mathematical calculation and not sensitivity (EFSA PRR 2013).

Effects of pesticides, other organic chemicals, as well as other pollutants on macrophytes generally do not cause mortality if environmental concentrations are applied (Maltby et al. 2010). Only at very high doses, macrophytes cannot survive. This means that endpoints for aquatic macrophytes are sub-lethal by nature (Arts et al. 2008). A range of endpoints is available to test the response and fitness of macrophytes. However, the endpoints included in toxicity tests should meet a number of criteria. They need to be sensitive to the stressor(s), exhibit low variability, and allow for easy measurement in standardized laboratory tests (Arts et al. 2008). In the *M. spicatum* test protocol (OECD 2014b), measurement endpoints are shoot fresh weight, total shoot dry weight, and total shoot length. In the ring-tests performed for the validation of this test protocol, these endpoints performed best in terms of achieving a low variability and appropriate sensitivity. These endpoints might slightly differ per plant species and growth form. For example, for the *G. maxima* protocol that is currently in development, shoot height was not an appropriate and sensitive

endpoint and was replaced by total leaf length (Davies et al. 2017; Arts et al. 2022). Root endpoints were considered in some studies and are a sensitive endpoint (e.g., Belgers et al. 2007); however, limitations include potential high variability (Arts et al. 2008) and difficulty to continuously measure if plants are grown in soil (Sesin et al. 2020).

The following are examples of endpoints used for various contaminants: growth rate and biomass endpoints were used to assess toxicity of heavy metals, pharmaceuticals, pesticides, surfactants, and plastics (Ceschin et al. 2021). Measurements of enzymatic activity were performed to assess toxicity of heavy metals, pharmaceuticals, hydrocarbons, and pesticides (Ceschin et al. 2021). Antioxidant enzymes (e.g., superoxide dismutase, catalase, peroxidase) scavenge reactive oxidant species and thereby prevent oxidative damage, and can serve as biomarkers for exposure, particularly for stressors that target the photosynthetic chain by disrupting electron flow (Brain and Cedergreen 2009). Chlorophyll fluorescence was measured to assess toxicity of heavy metals, pharmaceuticals, and surfactants (Ceschin et al. 2021). Chlorophyll and carotenoid pigments absorb light energy for photosynthesis; stressors can affect their content and composition (Brain and Cedergreen 2009). Moreover, a review by Sesin et al. (2021) summarized morphological and physiological endpoints that can be used for ecotoxicological tests for various stressors (e.g., chemicals, heavy metals, carboxylic acids, xenobiotics, pharmaceuticals, persistent organic pollutants, wastewater, and algal toxins) with the emergent macrophyte *Typha* spp.

4.2.8 Sensitivity of Macrophyte Species and Endpoints

Macrophyte species might differ in their sensitivity to pollutants. We already discussed, as an example, the potential differences between monocotyledonous and dicotyledonous macrophytes in sensitivity to specific herbicides. Depending on the endpoint, sensitivity can vary greatly within a species, and pollutant- and species-specific endpoints should be considered in ERA (Berghahn et al. 2007; Dumont et al. 2019). Giddings et al. (2013) state that endpoints might differ in sensitivity by a factor of 10–1000. These authors compared the sensitivity of different aquatic primary producers (macrophytes and algae) to a series of herbicides by using the species sensitivity distribution (SSD) approach. They used the lowest reported reliable EC_{50} for each species after calculation of the geometric mean of identical measurement endpoints as recommended by Brock et al. (2011). This methodology gives insight into the sensitivity of standard test species used in the risk assessment for pesticides compared to other algae and macrophyte species. They found that no single species consistently represents the most sensitive aquatic plant species. For 12 of 14 chemicals, *Lemna gibba* L. and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) algae (i.e., the algae used in the risk assessment under the United States *Federal Insecticide, Fungicide, and Rodenticide Act*; *Pseudokirchneriella subcapitata*, *Anabaena flos-aquae*, *Navicula pelliculosa*, and *Skeletonema*

costatum) included an EC_{50} near or below the lowest macrophyte EC_{50} and the macrophyte HC_5 (i.e., hazardous concentration for 5% of species). For the other compounds, *M. spicatum* was the most sensitive species of all aquatic plants considered. Overall, these results support the usefulness of testing *L. gibba*, *M. spicatum*, and the FIFRA algae for assessing pesticide risks to aquatic primary producers.

4.3 Global Examples of the Use of Macrophytes in Regulatory Risk Assessment

4.3.1 North America

Macrophytes are an important part of pesticide risk assessments in Canada that applies a tiered ERA approach (Health Canada 2021c). An initial screening level identifies non-target organisms for which there may be a potential risk. The screening uses conservative exposure scenarios and sensitive toxicity effects endpoints. A risk quotient (RQ) is calculated by dividing the exposure estimate by an appropriate toxicity value, which is then compared to the level of concern (LOC). If the RQ is equal to or greater than the LOC, then a refined risk assessment is warranted to further characterize the risk. The refined assessment considers more realistic exposure scenarios and different toxicity endpoints. Refined methods include exposure modeling, monitoring data, mesocosm or field study data, and probabilistic approaches. The refinement process continues until either the risk is judged to be adequately characterized, or no further refinement is possible due to limited available data.

Currently, testing with aquatic vascular plants in Canadian pesticide risk assessments is only required if there is potential for freshwater exposure. From a regulatory perspective, testing with macrophytes of the genus *Lemna* is sufficient to meet the requirements (Sauvé 2012; Whiteside 2017), although data from other macrophytes are considered in assessments, if available. For example, the re-evaluation of the pesticide glyphosate (HC PMRA 2015) included data from the floating *Nymphaea odorata* Aiton, and emergent *Pontederia cordata* L. and *Carex comosa* Boott, all of which turned out to be more sensitive than *Lemna* spp. to the formulated product as compared by their respective EC_{50} values.

Macrophytes are also an important consideration in ERAs for nuclear facilities and activities regulated by the Canadian Nuclear Safety Commission (CNSC). The ERAs evaluate exposure and effects on representative biota and valued ecosystem components (CNSC 2020; CSA 2022), which in many cases include aquatic plants. Moreover, the assessments must specifically consider vulnerable, threatened, and endangered species, including plants, listed under the Government of Canada's *Species at Risk Act* as well as corresponding provincial and territorial statutes and regulations (CSA 2022).

The United States use a similar approach to Canada for most pesticide risk assessments. For aquatic macrophytes, the screening level RQ is routinely based on the

lowest EC_{50}, although other toxicological endpoints may be used if they can be linked to assessment endpoints in a reasonable and plausible manner (US EPA 2022a). Typically, *Lemna gibba* is used in Tiers 1 and 2 (US EPA 2022b). In Tier 3, aquatic field tests are performed on a case-by-case basis if macrophytes show greater than 50% adverse effects on plant growth (US EPA 2022b). The US EPA has also developed the Plant Assessment Tool to better align macrophyte exposure models to pesticide fate and transport (Moore et al. 2021). The tool can be used to estimate pesticide exposures to plants inhabiting semi-aquatic areas that are adjacent to treated sites (Hook 2020). In a refined risk assessment, probabilistic tools and methods are used to estimate the variability and uncertainty in factors that influence risk (US EPA 2022a).

The US EPA specifically considers threatened and endangered species listed under the *Endangered Species Act*. Under this Act, all federal agencies must ensure that their regulatory actions are not likely to jeopardize the continued existence of listed species or destroy or adversely modify their critical habitat (US EPA 2022a). For threatened or endangered macrophytes, the NOEC is used in the RQ calculation. However, toxicity data are rarely available for listed species, and therefore surrogate species are often used, such as *Lemna* spp. (US EPA 2004). For data-deficient species, expert knowledge can also fill gaps and support decision-making (Fitzgerald et al. 2021).

4.3.2 South America

Risk assessment, especially for pesticides, is rapidly developing in South America (Carriquiriborde et al. 2014; Casallanovo et al. 2021a, 2021b). In Brazil, the current process is mainly hazard-based, but risk assessment guidelines for aquatic, terrestrial, and soil organisms are expected to be published by regulators within the next two years (Casallanovo et al. 2021b). A workshop held in 2014 (Carriquiriborde et al. 2014) recommended including macrophytes in the first tier of the risk assessment in the form of required tests for *Lemna* spp. Brazil uses procedures adapted from the European scheme (Topping et al. 2020).

4.3.3 Europe

In Europe, a tiered risk assessment procedure for pesticides has been established and is in force (EFSA PPR 2013; European Commission 2013). For compounds with an herbicidal mode of action, the first tier requires tests with *Lemna* spp. (*L. gibba* or *L. minor*). For substances with an herbicidal mode of action for which *Lemna* spp. are not sensitive or there is expected uptake from sediment by the roots of macrophytes, toxicity testing is required with another macrophyte, either *M. spicatum* or *G.*

maxima. The regulatory endpoint used in the risk assessment is the regulatory acceptable concentration (RAC), which—for macrophytes—is the EC_{50} with an assessment factor of 10. This RAC is compared with the predicted environmental concentrations (PECs) resulting from modeling of FOCUS scenarios for the use of the specific compound under evaluation. This results in tables with conclusions about the safe or unsafe use of the compound in the different scenarios.

In the aquatic risk assessment, the second tier provides several methods to refine the risk assessment (EFSA PPR 2013). If primary producers are the most sensitive group of organisms in Tier 1, a geomean approach can be followed if more macrophyte or algae endpoints are available, but less than eight. If at least eight endpoints from macrophytes and algae are available, an SSD curve can be generated. Rules are in place which organisms can be combined in one SSD. The best approach is to make an SSD with all primary producers first. If one of the groups of primary producers (e.g., algae, diatoms, macrophytes) are more sensitive than the others, separate curves for these groups need to be generated and the HC_5 of the most sensitive curve can be used in the risk assessment. A third option in the second tier is modified exposure tests. These tests include the standard test species but have a modified, more realistic exposure. These tests can be combined with TKTD (i.e., Toxicokinetic–Toxicodynamic) modeling. For example, for *Lemna* spp. a fit-for-purpose model is available (Schmitt et al. 2013), while this is in development for *M. spicatum* (Heine et al. 2015).

The third tier includes microcosm and mesocosm studies, which are described in Sect. 4.2.6. The highest tier might be on the landscape level, including a multi-species and multi-compound approach. This landscape approach is currently under debate and development in Europe.

4.3.4 Africa

In developing countries, risk assessment on pesticides has not been adequately studied due to the situation that concentrations and fate of pesticides in the environment are often undetermined. South Africa is facing challenges with significant pressures on its freshwater and agricultural resources, which enhances the prospects of pesticide effects. A few studies have been performed in South Africa in terms of pesticide risk assessment. The majority of the work concentrated on the estuaries and rivers of the Western Cape (Bollmohr et al. 2007; Malherbe et al. 2013). The PRIMET model is currently used in South Africa to predict risk to the aquatic environment. Models that are used to predict risks must be validated through field monitoring of pesticide exposure and effects. Most studies on macrophytes have focused on these plants as invasive alien species and very little work has been done on risk assessment.

In Ethiopia, the risk assessment that is currently being implemented is based on European principles on aquatic risk assessment and the registration procedure for pesticides. Risks for aquatic organisms are calculated by using water concentrations demonstrating the 90th percentile probability of occurrence in Ethiopia. This

percentile is standard in the European Union's registration procedures for risks in the aquatic ecosystem and reflects a less strict requirement for the protection of aquatic organisms compared to humans. Pesticide toxicity to rooted macrophytes is not currently considered as a future addition to the risk assessment procedure (Teklu et al. 2015). Moreover, there is no pesticide monitoring system in place for the environment, primarily due to poor institutional capacity, and a lack of coordination on the safe use of pesticides among federal and regional governments (Negatu et al. 2021). A need to raise awareness of the public on issues of pesticide misuse was identified by scientists (Negatu et al. 2021).

4.3.5 Australia

Australia has a well-developed risk assessment process that evaluates the impacts associated with licensed activities that include various potential stressors such as radioactive substances, pesticides, and hazardous chemicals (NSW EPA 2022). For example, in the pesticide risk assessment, non-target macrophyte toxicity tests are integral to the hazard assessment (APVMA 2019). Notably, Australia uses a site-specific, "eco-regionalized" approach that recognizes the wide range of ecosystem types (e.g., tropical, temperate, arid environments) within their jurisdiction, and associated differences in water quality characteristics (Water Quality Australia 2019). As one example, an ERA of tebuthiuron in tropical Australian wetlands considered specifically tropical species including the macrophyte *Lemna aequinoctialis* Welw. (Dam et al. 2004).

4.3.6 Global Perspective on the Risk Assessment for Macrophytes

The United States, Canada, and the European Union were pioneers in developing sound risk assessment schemes (Casallanovo et al. 2021b). The procedures developed in these countries are taken as examples and adapted to other countries and their specific circumstances, such as in Brazil (Topping et al. 2020; Casallanovo et al. 2021b). However, macrophytes are often not included in risk assessment schemes, and if they are, then it is usually limited to requiring the standard test species *Lemna* spp. in the first tier of the risk assessment. Europe also considers rooted macrophytes in the risk assessment when triggered by the fate of the compound and/or the sensitivity of the standard test species *Lemna* spp., while in North America toxicity data from other macrophytes might be used in the risk assessment when available. The comparably minor role of macrophytes in ecotoxicological investigations does not

reflect the major role macrophytes play in ecosystems. Many contaminants enter ecosystems via plants which are a key link in food webs (Ceschin et al. 2021). The usefulness of macrophytes goes beyond simple toxicity tests; they can also serve as bioindicators of water quality and phytoremediation agents (Ceschin et al. 2021). Moreover, there is a potential to establish large-scale monitoring programs to verify risk assessment predictions on a global level; for example, South African scientists called for intensifying and expanding water monitoring for pesticides using chemical, toxicological, and biological techniques (Ansara-Ross et al. 2012). Lastly, risk assessments on a global scale are heavily relying on standard test toxicity data produced in Europe or North America, and there is a lack of locally adapted and indigenous species being tested (e.g., Daam and van den Brink 2010; Ansara-Ross et al. 2012) which would be most relevant to the local risk assessment context.

4.4 Conclusion and Outlook: Future Ecological Risk Assessments with Macrophytes

Macrophytes are important components of aquatic and wetland ecosystems and sustain many ecosystem services, and therefore need to be an integral part of ERAs. Yet, ERAs tend to overlook the complexity of macrophytes, their growth forms and plasticity on an individual to community level, possibly resulting in insufficient protection measures. On an individual level, macrophyte growth forms (e.g., emergent, submerged, floating) and classes (e.g., monocots or dicots) influence exposure pathways and responses to stressors. On a community level, co-occurring species can influence community dynamics through competition for light or resources. As this chapter outlined, ERA approaches have been updated to try to address these factors, such as through the addition of new single-species tests with submerged and emergent species, as well as higher-tier, multi-species testing and modeling methods.

Scientific knowledge is continuously evolving, and the scientific community regularly proposes new ERA processes and tools to align approaches with environmental reality (Topping et al. 2020). However, regulatory frameworks are rarely modernized. This causes a time-lag of incorporating the most recent scientific knowledge into regulatory decisions. In addition, the widely used tiered risk assessment process is primarily based on single-stressor, single-use assessments (Topping et al. 2020), although multiple chemical products are typically used on the landscape scale. If the goal of ERA is to protect macrophyte populations and communities and ultimately biodiversity, then the current approach can be ineffective (Frische et al. 2018; Schäfer et al. 2019; Topping et al. 2020). Moreover, regulatory progress is not equivalent on a global level, and many countries have not yet established ERA frameworks for macrophytes (e.g., South Africa, Ethiopia, and countries in Latin America, possibly also in Asia).

We have three key recommendations for ERAs with macrophytes that can be considered in the adaptation of current regulatory processes as well as in the establishment of new frameworks, which should be relevant across countries.

First, we recommend educating young scientists all over the globe in ERA frameworks, in the effects of pollutants on individual, population, and ecosystem levels, on how these can be assessed (experimental and modeling tools), and on how a risk assessment process could look like in practice (see also Fig. 4.2). Awareness needs to be raised about the diversity of species and ecosystems in the environment and how these organisms can be protected from adverse effects. Knowledge exchange could be facilitated through bilateral or multilateral collaboration and training. One recent example is the collaboration between the International Institute for Sustainable Development and the African Center for Aquatic Research and Education to strengthen freshwater science in large lakes, addressing pollution at local, regional, and global scales (IISD 2020). Education can also extend to the public, and outreach and engagement efforts can include local residents, naturalist and stewardship groups, and indigenous communities. These stakeholders already have tremendous knowledge and experience with the local environment and plant communities. Acknowledging that communication is a two-way process, stakeholders' knowledge can in turn be linked to the ERA framework and could inform the selection of macrophyte test species as well as monitoring sites, frequency, and sample types. One example is the ERA conducted for certain nuclear facilities in Canada, which is periodically reviewed and revised using site-specific knowledge and indigenous knowledge, among other sources (CNSC 2020).

Secondly, we recommend developing scientific approaches to fill the gaps in our knowledge related to risk assessment for aquatic macrophytes. We have identified the following knowledge gaps: (1) we need more understanding of the sensitivity of different macrophyte growth forms, (indigenous) species, macrophyte ecotypes, and genotypes to herbicide exposure and exposure to other contaminants such as pharmaceuticals, nanoparticles, or radionuclides. (2) We need more knowledge on how to do a proper risk assessment on a local level, especially in different climatic zones all over the globe. Compared to temperate zones, tropical zones and tropical macrophyte species are less studied. For example, the applied field rates of pesticides and associated exposure routes differ locally, influenced by the climate, crop production, and government laws and regulations, among other factors. (3) We need to develop statistical and TKTD models for rooted (submerged and emergent) macrophytes to be used in risk assessment. (4) We need to revive aquatic microcosm and mesocosm studies with aquatic macrophytes as an important intermediate step between the lower-tier risk assessment for individual species and the risk assessment at the landscape level. (5) We need to develop approaches to perform a risk assessment for aquatic macrophytes at the landscape level.

Third, while further developing risk assessment for aquatic macrophytes, we recommend that future ERAs reflect the complexity of stressors that may expose macrophytes, as well as their ecological context. Macrophytes are typically exposed to a mixture of stressors, including anthropogenic pollutants, habitat disturbances

and loss, climatic changes, and competition by invasive species. Exposure to stressors can be highly variable in temporal and spatial dimensions, and accounting for these in an assessment can increase environmental realism. Moreover, ERAs should ideally consider the ecological context, such as species interactions and community composition, as well as the landscape context, including habitat types and connectivity (Milner and Boyd 2017; Schäfer et al. 2019). As the case of South Africa shows, on a local level, invasive macrophyte species represent a significant pressure on freshwater ecosystems, and these issues should be considered in ERAs for co-occurring stressors, for example through a cumulative risk assessment. Renewed interest in microcosm and mesocosm studies is also promising in this regard. While accounting for all these factors is challenging, partly due to limited data availability, an approach that reflects the complexity and interdependence of ecosystem components, however, is needed to provide effective, long-term environmental protection.

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Chapter 5
Wild Rice (Zizania spp.) as a Model Macrophyte Toxicity Test Species for Ecotoxicological Risk Assessment

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Abstract This chapter outlines the life history of wild rice (*Zizania* spp.), assesses their ecological, sociocultural, and economic relevance, reviews the current state of knowledge around their use as a test species, and makes recommendations around their possible inclusion in ecological risk assessments. Northern wild rice (*Zizania palustris*) holds significant importance to North American Indigenous communities, is an integral aspect of wetland structure and function, and is rising in commercial demand and value due to their high nutritional content and long shelf-life. While *Z. palustris* has been used as a species in toxicity assessments, a standard test protocol has not yet been established. We performed a review to assess the utility and identify gaps in the available peer-reviewed literature for wild rice toxicity studies pertaining to methodology and experimental design. We found 11 articles reporting 22 studies that specifically examined the responses of *Z. palustris* to contaminants under controlled conditions (laboratory or mesocosm studies). The studies were evaluated for methodological reporting in five categories: (1) test organism; (2) test conditions; (3) test media; (4) experimental design; and (5) test performance. The conditions for stratification and control performance, both crucial for experimental replication and credibility, were under-reported in the literature (only 45% and 14% of studies, respectively). It was also found that conditions for seed storage were highly ambiguous or were not included at all. There were few consistent approaches between different research groups when conducting wild rice toxicity studies. We recommend that wild rice toxicity test reports incorporate experimental conditions in detail to ensure both transparency as well as to facilitate the ability of others to adopt

Throughout this chapter, wild rice is referred to as "they/them". In Anishinaabemowin (the shared language of the Algonquin, Mississauga, Nipissing, Odawa, Ojibwe, Potawatomi, and Saulteaux North American Indigenous peoples), northern wild rice, or manoomin, is grammatically referred to as "him/her/them", as opposed to "it", since they are not viewed as inanimate "resources" by the Anishinaabeg (Vizenor 2008). This important distinction in translation highlights the need for Western societies to recognize the rights of all organisms, not just humans and animals.

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Z. palustris as a toxicity test species. Overall, wild rice has potential as a macrophyte toxicity test species, but significant work is required to validate methods to ensure repeatable and reproducible data across various life stages.

5.1 Introduction

As outlined in Chap. 1, macrophytes are an essential component of nontarget toxicity characterization when assessing the risk to aquatic ecosystems; however, their widespread use in ecotoxicological testing is still relatively lacking, with most studies focusing on a narrow range of species. Many ecological risk assessments rely on a single macrophyte test species to extrapolate responses to population, community, or ecosystem-level effects for this class of organisms. This has led to concerns about the predictive capabilities of these assessments, especially under varying exposure scenarios (e.g., sediment, water column, or aerial exposure). As it pertains to primary producers, standardized algae and duckweed tests offer advantages for characterizing the effects of contaminants present in the water column (e.g., cost-effective, quick, and simple to conduct); however, they may lack ecological relevance for sediment-bound toxicants. To reduce uncertainty when characterizing the risk to nontarget organisms, representation of macrophytes with different morphologies and exposure pathways (e.g., rooted emergent species) are needed in the standard regulatory risk assessment process. As such, wild rice (*Zizania* spp.) may be a suitable candidate for inclusion into the battery of test species when assessing the risk to wetland ecosystems.

Wild rice species (*Zizania* spp.) are rooted emergent aquatic macrophytes that are indigenous to North America (except for Manchurian wild rice, *Z. latifolia*), resulting in potential exposure to toxicants bound to sediment, suspended in the water column, or deposited aerially. Additionally, studies have found that wild rice is sensitive under both laboratory and field conditions, predominantly conducted using the species *Zizania palustris* (Durkee Walker et al. 2006, 2010; Fort et al. 2014, 2017, 2020; Johnson et al. 2019; LaFond-Hudson et al. 2018; Malvick and Percich 1993; Nimmo et al. 2003; Pastor et al. 2017; Sims et al. 2012). Overall, wild rice presents the opportunity to address some of the identified gaps in macrophyte testing within North American ecotoxicological risk assessments.

To extrapolate meaningful and relevant results from toxicity tests, six criteria should be considered when selecting an appropriate test organism: (1) a group of species representing a broad range of sensitivities should be used whenever possible, as sensitivities vary among species; (2) species that are widely abundant and available should be considered; (3) species that are indigenous to or representative of the ecosystem of interest should be studied whenever possible; (4) species of ecological, cultural, or commercial importance should be included; (5) species should be amenable to routine maintenance, with techniques available for culturing and rearing in the laboratory to facilitate both acute and chronic tests; and (6) species with

adequate background information (e.g., their genetics, physiology, and behavior) may allow for test results to be more easily interpreted, and should be considered (Rand et al. 1995). In this chapter, we examine how wild rice would meet these expectations, as well as reviewing the current state of knowledge and making recommendations to promote their inclusion as an alternative test species in ecological risk assessment.

5.2 Wild Rice Life History

Wild rice species (*Zizania* spp.) are emergent aquatic macrophytes that grow in dense (often monotypic) stands, typically in freshwater riparian and littoral zones (Ahmad et al. 2018; Crow and Hellquist 2006; LaFond-Hudson et al. 2018; Myrbo et al. 2017; Pastor et al. 2017; Wetzel 1975). They are monocotyledonous flowering grasses of the Family Poaceae (Aiken et al. 1988; Crow and Hellquist 2006; Pastor et al. 2017; Terrell et al. 1997). They have also been classified as part of the Tribe Oryzeae, as there is extensive genetic colinearity and synteny between wild rice (*Zizania* spp.) and domesticated rice (*Oryza sativa*), with differences primarily occurring in the number of chromosomes (e.g., wild rice has 15 pairs, while domesticated rice has 12) and total DNA content (e.g., wild rice has two times more than domesticated rice) (Grombacher et al. 1996; Hass et al. 2003; Kennard et al. 2000; Porter 2019). There are four recognized species of wild rice within the genus *Zizania* L.; two of which are annual species, *Z. palustris* L. (northern wild rice) and *Z. aquatica* L. (southern wild rice), and the other two are perennial species, *Z. latifolia* (Griseb.) Turcz. ex Stapf (Manchurian wild rice), and *Z. texana* Hitchc. (Texas wild rice) (Ahmad et al. 2018; Aiken et al. 1988; Archibold 2003; Crow and Hellquist 2006; Duvall and Biesboer 1988; Porter 2019; Terrell et al. 1997). In this chapter, we primarily focus on northern wild rice (*Z. palustris*) and discuss the other species for context and contrasting.

Northern wild rice (*Z. palustris*) is the most prevalent of the four species, and due to their larger seed size, they have been traditionally and commercially harvested as a food source (Archibold 2003; Porter 2019). They are predominantly found in freshwater wetlands, slow-moving rivers and streams, and the shallow waters of lakes within the Great Lakes and Boreal Forest regions of Canada and the United States, as seen in Fig. 5.1 (Ahmad et al. 2018; Aiken et al. 1988; Archibold 2003; Crow and Hellquist 2006; Duquette and Kimball 2020; Fort et al. 2014; LaFond-Hudson et al. 2018; Malvick and Percich 1993; Pastor et al. 2017; Porter 2019). Southern wild rice (*Z. aquatica*) can be found along the Atlantic coastal plains of Canada and the United States, with one variety (*Z. aquatica* var. *brevis* Fassett) found in the tidal waters and tributaries of the St. Lawrence River in Quebec (Aiken et al. 1988; Crow and Hellquist 2006; Terrell et al. 1997). Manchurian wild rice (*Z. latifolia*) is widely grown in southeastern Asia, primarily as a cultivated crop (Surendiran et al. 2014; Terrell et al. 1997; Xu et al. 2010). Texas wild rice (*Z. texana*) is an endangered species that is native to a small portion of the upper San Marcos River in Texas (Porter 2019; Surendiran et al. 2014; Xu et al. 2010).

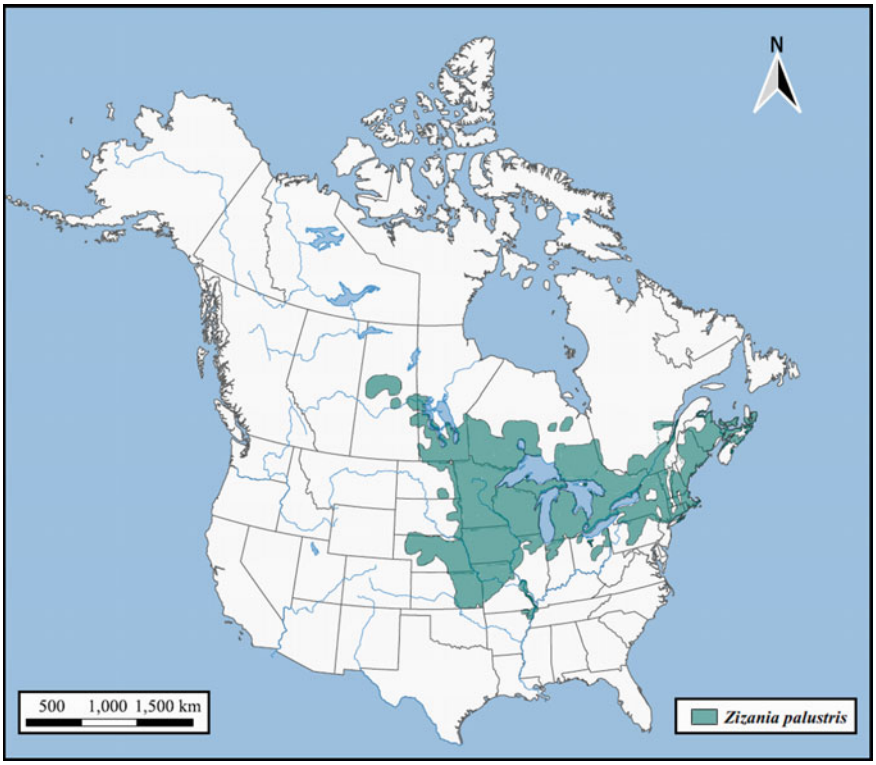

Fig. 5.1 Map of the distribution of northern wild rice (*Zizania palustris*) across Canada and the United States, excluding artificial paddies for commercial harvesting. The natural ranges of both *Z. palustris* var *palustris* and *Z. palustris* var *interior* were included and adapted from maps by Barkworth et al. (2007) and Porter (2019)

Aside from distribution and life cycle duration (annual versus perennial), spikelet anatomy is a reliable characteristic for distinguishing between the species, as the morphology of the pistillate lemmas and paleas of *Z. palustris* are coriaceous (i.e., leathery), whereas the intercostal species (e.g., *Z. aquatica*, *Z. latifolia*, and *Z. texana*) are chartaceous (i.e., papery), as described by Crow and Hellquist (2006), Duvall and Biesboer (1988), and Porter (2019). The two varieties of *Zizania palustris* are *Z. palustris* var. *palustris* and *Z. palustris* var. *interior*, both of which are commonly referred to as northern wild rice (Ahmad et al. 2018; Archibold 2003; Crow and Hellquist 2006). These can be distinguished based on height, leaf width, ligule length, and number of spikelets on the lower pistillate branches, as *Z. palustris* var. *palustris* has a height of about 0.7–1.5 m, 3–15 mm wide leaves, 3–5 mm long ligules, and 2–8 spikelets, while *Z. palustris* var. *interior* has a height of 0.9–3 m, 20–40 mm wide leaves, 10–15 mm long ligules, and 9–30 spikelets (Ahmad et al. 2018; Crow and Hellquist 2006). Wild rice (hereafter referring to the annual species *Z. palustris* and *Z. aquatica* collectively) are heterophyllus, with submerged and

floating leaves preceding mature aerial leaves and the production of aerial reproductive organs (Wetzel 1975). They typically have short roots, long, narrow blade-like leaves, hollow, cylindrical stems, a panicle at the apex for the type of inflorescence, with spikelets of the upper inflorescence branches pistillate (female), and spikelets of the lower branches staminate (male) (Ahmad et al. 2018; Crow and Hellquist 2006; Surendiran et al. 2014). With their roots only extending into the shallow depths of the sediment, there is an increased risk of exposure to sediment-bound contaminants (i.e., those that form residues near the top of the sediments) in comparison to deeper rooting macrophytes. In addition, these short roots are easily pulled up, which can be ideal when examining root and shoot endpoints directly. As annual macrophytes, they must undergo all allocation processes required to complete their life cycle within the same year as their germination, often resulting in trade-offs between seed, leaf, stem, and root development if carbon or nutrients are limited (Sims et al. 2012). Wild rice has been found to respond plastically to environmental conditions, as the morphology of wild rice typically varies between natural stands and years. However, when seeds are grown in similar conditions, the variation significantly decreases (Archibold et al. 1989; Durkee Walker et al. 2010; Sims et al. 2012). Therefore, it is important to maintain appropriate water levels (ideally 0.75–1 m) when establishing wild rice stands, as greater water depths produce plants with longer, thinner stems and fewer seed heads (Archibold et al. 1989; Archibold 2003).

With the male and female flowers separate from each other on the same stalk, wild rice cross-pollinates to reproduce, and with clusters of receptive female florets emerging prior to the male florets, the chances of self-pollination are low, as females are often pollinated before the males emerge and shed pollen (Duquette and Kimball 2020). As seeds mature, they will shatter from the panicle, falling to the bottom of the water column to overwinter in the sediments in a dormant state. However, during commercial production, seeds are harvested and stored in near freezing water to mimic overwintering in controlled settings (Duquette and Kimball 2020; Grombacher et al. 1996). Early growth stages are susceptible to being uprooted or drowned by wave action if there is too much wind or the water depth is greater than 2.5 m (Aiken et al. 1988; Archibold 2003; Porter 2019). Wild rice does require water for growth, but does not grow well in saline, alkaline, or acidic water that is low in essential nutrients, as optimal alkalinity values range from 40 to 80 mg/L and optimal pH values range from 6.9 to 7.4 (Archibold 2003). Optimal wild rice habitats have long, cold winters, as seeds will germinate at low rates if the winter is too short or too warm (Ahmad et al. 2018; Myrbo et al. 2017). Additionally, transparent surface waters in the spring and summer are ideal, as low water clarity can inhibit photosynthesis prior to emergence (Ahmad et al. 2018; Myrbo et al. 2017).

In more northern latitudes, wild rice commence their annual life cycle with seed germination in the spring (i.e., May), followed by emergence from the sediment and water column typically in June, continuing their vegetative growth throughout the summer, with flowering and seed production usually beginning in August, and then the seeds begin to shed in autumn (Fig. 5.2); the plant dies as temperatures drop at the end of the season and seeds overwinter in the sediment until the cycle begins again the following spring (Grava and Raisanen 1978; LaFond-Hudson et al.

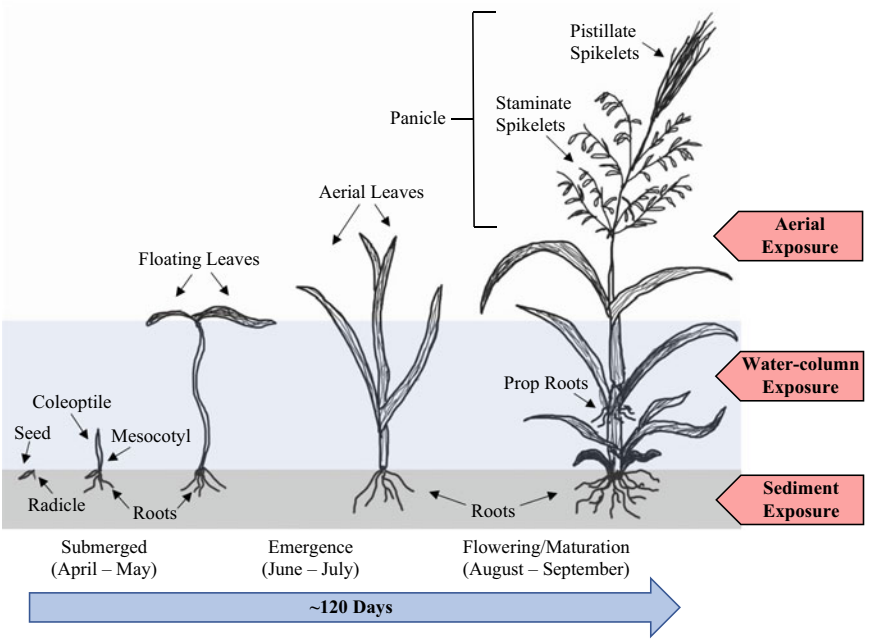

Fig. 5.2 The life cycle of northern wild rice (*Zizania palustris*). Seeds germinate in the sediment, with the development of the primary root (radicle). The mesocotyl then emerges from the sediment and elevates the coleoptile, which sheathes the emerging shoot and first leaf. This is followed by the floating-leaf stage, with further development of the roots. Next is the aerial leaf stage, where the plant begins to emerge out of the water. Then, at the mature plant stage, prop roots arise from the stem to provide additional support, and there is the development of the panicle, comprised of staminate spikelets (male florets) and pistillate spikelets (female florets). Potential contaminant exposure pathways are highlighted in red

2018; Sims et al. 2012). The plant requires approximately 120 days to reach maturity from germination (Archibold 2003). Seeds will penetrate the upper 2–5 cm of the sediment after falling through the water column, and this is where seed germination and early seedling growth (e.g., development of primary root and shoot) occurs the following spring (Pastor et al. 2017). Young plants are submerged for their first 3 to 4 weeks of growth and then long, thin leaves reach the top of the water column as they enter the floating-leaf stage (Archibold 2003). The floating leaves fix carbon into carbohydrates for root production and subsequently nutrient (e.g., nitrogen and phosphorous) uptake (Pastor et al. 2017). They will then progress to the aerial stage, with the stem emerging out of the water, and then as they enter the reproductive cycle, the panicle emerges, the stem elongates, the flowers develop for pollination, and the seeds begin to mature prior to senescence (Duquette and Kimball 2020).

5.3 Wild Rice Ecological Relevance

Wild rice plays an integral role in the structure and function of freshwater ecosystems. Emergent angiosperms (such as wild rice) are highly productive macrophytes, due to the abundance of available water and nutrients in sediments compared to floating macrophytes, and the greater availability of atmospheric carbon dioxide and oxygen compared to submerged macrophytes (Wetzel 1975). Emergent macrophytes are often found within the littoral region of small and shallow lakes, and as such are a major source of organic matter synthesis, contributing significantly to the productivity and metabolism regulation of the whole lake ecosystem (Wetzel 1975). By converting carbon dioxide and solar energy to organic matter via primary production, they provide food and habitat resources for herbivores, omnivores, and detritivores in aquatic ecosystems (Arts et al. 2008, 2010; Fairchild et al. 1998; Wetzel 1975). Wild rice is a vital food source for waterfowl, muskrats, beavers, moose, and other wildlife (Aagaard et al. 2019; Archibold 2003; Crow and Hellquist 2006; Fort et al. 2014; Myrbo et al. 2017; Pastor et al. 2017). Wild rice also provides habitat and shelter for both aquatic and terrestrial organisms, as the dense monotypic stands hide them from predators (Lewis 1995; Myrbo et al. 2017; Pastor et al. 2017). Wild rice stands are especially valuable resources for migrating waterfowl and other wetland birds, as they provide direct (e.g., consumption of seeds, flowers, young shoots, leaves, and mature stems) and indirect forage (e.g., consumption of nearby invertebrates), roosting habitat during migration, and nesting habitat for breeding (Aagaard et al. 2019). For instance, wild rice is a primary dietary constituent of mute swans (*Cygnus olor*), Canada geese (*Branta canadensis*), and red-winged blackbirds (*Agelaius phoeniceus*) (Bailey et al. 2008; Haramis and Kearns 2007; Meanley 1961), and the preferred food of soras (*Porzana carolina*), with wild rice comprising up to 94% of their fall diet (Webster 1964).

In addition to primary productivity, emergent macrophytes, such as wild rice, contribute to the biogeochemical cycling and structural complexity of aquatic ecosystems (Carpenter and Lodge 1986; Lemly et al. 1999; Lewis and Thursby 2018). The emergent leaves of wild rice reduce light availability to submerged macrophytes and algae, reduce water column circulation, and the shading provided by the leaves may reduce water temperatures (Carpenter and Lodge 1986; Lemly et al. 1999). The roots and shoots of wild rice stabilize sediments, introduce structural components (e.g., cellulose and lignin) to the detrital pool, and may enhance or reduce mineral uptake and release into aquatic ecosystems (Carpenter and Lodge 1986; Diepens et al. 2017; Fairchild et al. 1998; Lemly et al. 1999; Lewis 1995; Wetzel 1975). The roots of wild rice create a redox interface, which cycles nitrogen, sulfur, iron, and other metals (LaFond-Hudson et al. 2018). As oxygen is transported from the atmosphere to the roots, an aerobic rhizosphere develops from the radial oxygen loss of the roots, which may result in the sequestration of heavy metals due to the high adsorption capacity of iron hydroxides that may form as iron plaque on the root (Jorgenson et al. 2013).

These iron root plaques may also function to sequester nutrients, such as phosphorus, which has implications on bioremediation efforts in eutrophic systems (Jorgenson et al. 2013). Wild rice plays clear structural and functional roles, as well as in the suppling of essential ecosystem services.

5.4 Wild Rice Socio-Cultural and Economic Importance

Northern wild rice, or manoomin as it is called by the Anishinaabe First Peoples of North America, is an important food resource for Indigenous communities that has been traditionally harvested across North America for thousands of years (Ahmad et al. 2018; Aiken et al. 1988; Archibold 2003; Crow and Hellquist 2006; Fort et al. 2014; Porter 2019). Manoomin is often translated as "the good fruit" or "the good berry" in Anishinaabemowin or Ojibwemowin (David et al. 2019). They are a nutritious staple that is high in carbohydrates, proteins, vitamins (e.g., riboflavin), minerals (e.g., potassium and zinc), antioxidants, and dietary fiber, while also having a low-fat profile (Ahmad et al. 2018; Aiken et al. 1988; Fort et al. 2014; Surendiran et al. 2014). Within the traditional diet, manoomin was overall more nutritious than any other food available, and despite the labor-intensive process of harvesting and finishing, grains were seasonally abundant, and could be preserved for extensive periods of time (e.g., over the winter, when other foods are scarce) (David et al. 2019; Vennum Jr 1988). Manoomin is an integral part of the lives of the Anishinaabeg, and is often the first food given to children and the last food given to elders (David et al. 2019; Vennum Jr 1988; Vizenor 2008).

Manoomin holds strong spiritual and cultural significance and remains part of many ceremonies (as both a sacred food and medicine) and legends (Archibold 2003; David et al. 2019; Vennum Jr 1988). According to the sacred migration story of the Anishinaabeg, a prophet long ago beheld a vision from the Creator calling the Anishinaabeg to move west until they found the place "where food grows on the water" (Vizenor 2008). This journey led them to find the wild rice stands of the Great Lakes region. For generations, the Anishinaabeg of the western Great Lakes and upper Mississippi region have understood their connection to Anishinaabe Akiing (the land of the people) and the significance of manoomin as a gift from the Creator (Vizenor 2008). In the words of White Earth Tribal Historian Andy Favorite (as told by Erma Vizenor, former Chairwoman of the White Earth Nation), "Wild rice is part of our prophecy, our process of being human, our process of being Anishinaabe … we are here because of the wild rice. We are living a prophecy fulfilled" (Vizenor 2008).

Northern wild rice is connected to the identity, culture, religion, and livelihood of the Anishinaabeg (Vizenor 2008). Wild rice is still harvested using traditional methods, with one person poling a canoe through the dense aquatic stands, while another knocks ripe seeds from the stems using ricing sticks, with many seeds also intentionally knocked into the water to ensure re-seeding for the following year (Archibold 2003; Grombacher et al. 1996; Porter 2019). Other Indigenous groups,

such as the Cree and Dene, have actively managed natural and planted stands of wild rice as their livelihoods (Grombacher et al. 1996). Though, by the end of the nineteenth century, wild rice as a commodity was of interest to non-Indigenous groups. Initially, brokers sought control of processing and sales, and then farmers and other planters attempted to gain control of the industry (Archibold 2003). After repeated attempts of Indigenous communities highlighting the significance of wild rice during treaty negotiations, several federal and state laws in the United States and legislation in Canada were passed, specifying the amount that can be commercially harvested, the type of equipment used, and Indigenous involvement in wild rice production (Archibold 2003). Though, due to high commodity prices and increased commercial demand in the 1970s, artificial paddies were rapidly established to enhance production (Archibold 2003).

The majority of wild rice production now occurs in artificial paddies, and with recent interest in their health-promoting properties (e.g., high in nutrients, with antioxidant and cholesterol-lowering effects), the commercial harvesting industry holds significant economic values (Fort et al. 2014; Surendiran et al. 2014). Wild rice has been cultivated in paddies since the early 1950s, and is still undergoing domestication as a crop (Porter 2019). Wild rice was initially cultivated in Minnesota, but with the recent commercial exploitation, production of the crop has extended beyond their natural range to California, Oregon, Saskatchewan, and has been established outside of North America in Australia, Finland, and Hungary (Ahmad et al. 2018; Archibold 2003; Malvick and Percich 1993; Porter 2019). Globally, the production and demand for wild rice is continuing to rise, likely due to their unique properties, such as their nutritional values, long shelf-life, versatility in food dishes, food-processing potential (e.g., wild rice blended with precooked meat has reduced cook times and enhance nutritional properties), use of presently discarded hulls (e.g., in the adhesive, paper, and textile industries), and the ability to re-seed themselves once established, unlike other commercial crops (Ahmad et al. 2018; Archibold 2003; Porter 2019; Surendiran et al. 2014).

5.5 Review of the Current State of Northern Wild Rice Ecotoxicology

5.5.1 Background

Toxicology test methods used in studies must be reported with sufficient detail for the experimental setup and procedures to be replicated effectively by other researchers. As well, inadequate reporting in peer-reviewed literature could result in the exclusion of data from formal ecological risk assessments due to uncertainty related to data quality. These concerns surrounding reliability and completeness of methodological reporting in the ecotoxicology literature are not unusual or limited to macrophytes (Ågerstrand et al. 2011; Hanson et al. 2017). Therefore, the need for direct, precise,

and transparent methodology reporting must be a priority if northern wild rice is to be more widely adopted as a test species. Currently and to our knowledge, no standard test method exists for wild rice in toxicology studies. Therefore, contrasting between wild rice studies, and wild rice with other species, is difficult or not possible without clear methodological reporting, and ideally consistent methods across tests in general.

This section collates and summarizes current toxicity test methods for northern wild rice (as of 2021). The totality of the peer-reviewed scientific literature was systematically evaluated to outline similarities and differences in basic methodological techniques and reporting. Gaps were identified and direction is given on how to approach the growth and maintenance of this species for future testing. The effects that test compounds have on the wild rice were beyond the scope of this review. The aim was to identify areas in need of further research and standardization to effectively allow the use of *Z. palustris* in ecotoxicology.

5.5.2 Methods

5.5.2.1 Literature Search

Our focus was on studies of northern wild rice toxicity tests that were conducted in a laboratory, an indoor area (such as a greenhouse), or outdoor mesocosms (e.g., simulated wetland enclosures). The databases Google Scholar, Scopus, Web of Science, and University of Manitoba Library Services were utilized to search for articles related to wild rice ecotoxicology. Search queries commenced with "Wild rice OR Zizania palustris", and then became more specific including, "wild rice toxicology testing" and "wild rice stratification". Additional articles were also found by reviewing references in relevant wild rice articles. Alerts were set up on Google Scholar and Web of Science using key words such as "Wild Rice", "Zizania palustris", and "Wild Rice Toxicity Testing". The search was completed by April 2021. The selection criteria for the inclusion of articles in this review were:

1. Must use northern wild rice (*Z. palustris*) as test organism
2. Toxicity test conducted in a laboratory, indoor area (greenhouse), or a mesocosm
3. Written in English language only
4. Peer-reviewed article published in a scientific journal by a recognized database

For the purposes of this chapter, single published papers within a scientific journal are referred to as an article, while separate experiments conducted within an article are referred to as studies, as an article may contain multiple types of studies. The criteria used to distinguish between an article and a study revolved around if the experiments in question were: (1) conducted at separate times; (2) independent control organisms were used; and (3) if any component of the study design was changed.

5.5.2.2 Methodology Assessments

The review was organized as a list of questions in five categories: (1) test organism; (2) test conditions; (3) test media; (4) experimental design; (5) and test performance (Fig. 5.3). These categories pertained directly to elements that would allow for effective replication and data quality assessment of any experiment. Metadata were extracted from each study, covering contributing authors, the scientific journal, test compounds with their accompanying concentration, and whether the experiment was laboratory, greenhouse, or outdoor mesocosm based. Questions were generated with direction from the ASTM (American Society for Testing and Materials) International E1841-04 Standard Guide for conducting renewal phytotoxicity tests with freshwater emergent macrophytes (ASTM 2012), as well as previous reviews of data reliability for primary producer toxicity literature (Hanson et al. 2019). The guide provided key details that "must be met", and requirements that were relevant to wild rice toxicity testing design or methods were considered and incorporated. For instance, the ASTM guide requires that plant test organisms used must be the same age and collected from the same source.

Laboratory and mesocosm studies were addressed separately for certain aspects (e.g., growth chamber settings) that were not applicable across study types. The test organism section first identified the source of wild rice seeds or plants either by collection location or by supplier to satisfy the ASTM requirements (ASTM 2012). Depending upon if seeds were purchased from a supplier or harvested, storage conditions prior to and post-purchasing were collected to assess viability (Kovach and Bradford 1992). The remainder of the section focused largely on stratification techniques. Stratification is a crucial component for the germination of wild rice, as it is a process used to simulate the natural overwintering conditions necessary to break seed dormancy (Baskin and Baskin 2014).

Test conditions pertained to such elements as growth chambers and vessels used to house the plants. The photoperiod and temperature are fundamental conditions for replication of the experiment. In-depth questions on vessel structure, size, and rooting substrates were included, as the ASTM requirements outlined that the vessel should be large enough to prevent the plant from becoming root bound (ASTM 2012). Test media looked specifically at the composition of nutrient solutions used to support adequate plant growth, and what type of water source was used for dilution. A subsection was also created in the case that a nutrient solution was not used, common with mesocosm experiments, in which only water conditions were addressed.

Experimental design was related to setup procedures, such as numbers of test organisms per replicate and replicate numbers. Maintenance of the test conditions, such as if the test organisms spent time outside the growth chamber, were also covered as exposure to different surroundings can influence growth; ASTM requirements stress the importance of consistency within an experiment (ASTM 2012). The test performance category was solely focused on controls and potential contamination of the system throughout the duration of the study. Test performance criteria for

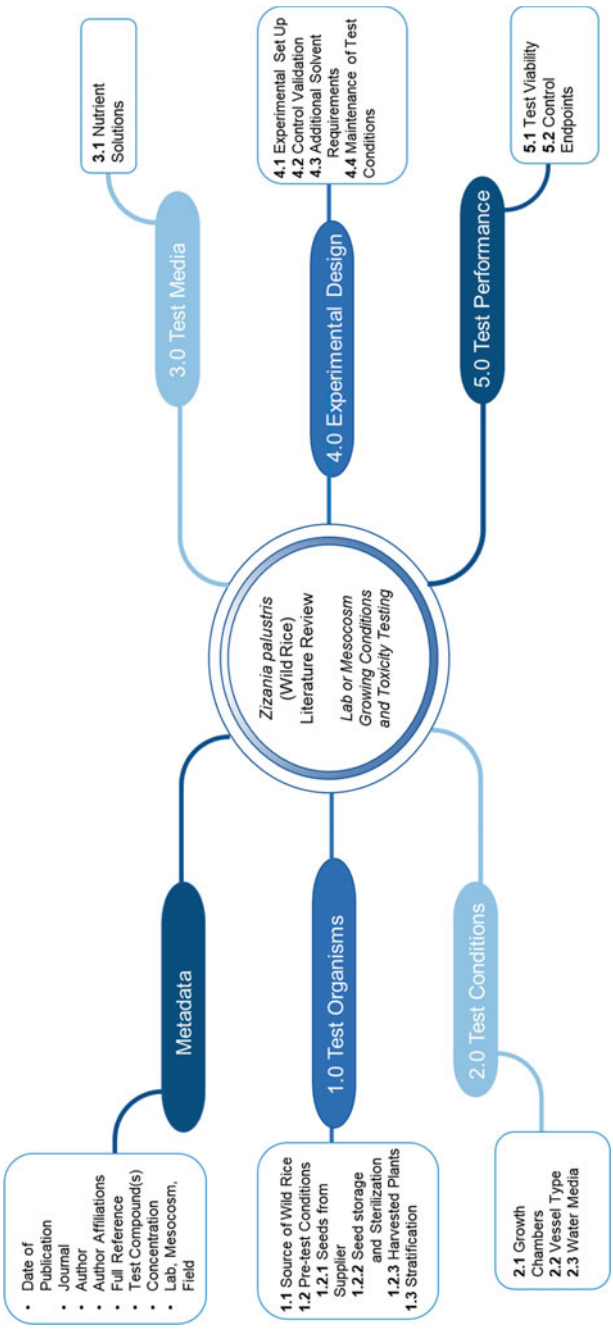

Fig. 5.3 Conceptual model of review categories and subcategories for peer-reviewed wild rice toxicity tests

controls were recorded as either qualitatively and/or quantitatively as applicable, as the success of controls provides an indicator of method viability. Specific questions concerning wild rice toxicity outcomes in response to tested compounds were not included, as this was beyond the scope of the review.

5.5.3 Results

5.5.3.1 Overview of Articles and Studies Reviewed

The search returned 11 published articles that met the inclusion criteria, and of those, 22 unique studies were identified and individually assessed. The majority of articles were published in the last decade (2011–2021). Overall, there were six outdoor mesocosm and 16 laboratory or indoor studies conducted within the total articles collected; therefore, laboratory or indoor experiments were the dominant type of experiment. Due to the nature of control in these types of experiments (laboratory or indoor vs. mesocosm), they were compared separately for certain components of study design, and the breakdown of results was presented independently.

5.5.3.2 Summary of Seed Harvest and Preparation

Half of the studies ($n = 11$) utilized northern wild rice seeds as the initial test organism, with the remaining half using seedlings or mature plants (Fig. 5.4). All studies conducted in mesocosms ($n = 6$) used seeds, and then allowed the plant to complete successive life cycles, which produced seeds fueling the successive generations. Durkee Walker et al. (2006) was the only mesocosm study to use both seeds and seedlings. The source of seeds (e.g., harvested or purchased) was not reported in all studies ($n = 3$ did not report), but of the studies that did report source, all were obtained by harvesting from natural stands ($n = 19$). Locations for the harvesting of wild rice seeds were all within the native growing range of the species, but at times vaguely stated (e.g., Central Minnesota by Malvick and Percich [1993]). None of the studies reported using commercial suppliers.

Only 27% of the studies ($n = 6$) provided information on seed sterilization or debris removal techniques. Fort et al. (2014, 2017) used a sieve with mesh to remove unwanted debris and the four studies within Nimmo et al. (2003) used deionized water to rinse harvested seeds. No indoor or laboratory studies indicated use of any sterilization techniques on the seeds to remove potential pathogens prior to experimental use.

Seed storage conditions were generally inadequately reported. Explicit information on storage conditions or time frames were limited. For example, Pastor et al. (2017) stated that storage of seeds occurred until needed for experiments, but did not include information such as temperature, light, or humidity, leading to questions about possible decline in seed viability over time. Fort et al. (2014, 2017, 2020),

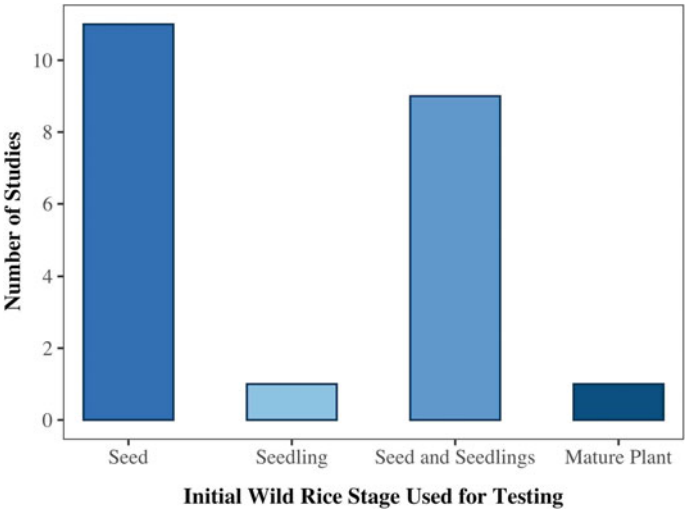

Fig. 5.4 Initial growth stage of northern wild rice (*Zizania palustris*) utilized for laboratory or indoor and mesocosm experiments from 22 ecotoxicology studies

stored seeds at 4 °C in the dark, but did not include details on storage duration, vessel type, or if water media was used in the three studies. LaFond-Hudson et al. (2018) indicated a one-year storage period, but did not include storage conditions. Storage of seed is not an experimental condition of the toxicity test itself; however, the literature should acknowledge this step, especially over long durations of times (months to years), to ensure the viability of test organisms. Ultimately, it is akin to the culturing of test organisms (along with stratification discussed below), which typically have detailed protocols that are followed. We concluded that all available studies provided insufficient information on all three of the following categories: seed acquisition, cleaning, and storage conditions.

Stratification

The conditions under which studies reported performing stratification of their northern wild rice seed is found in Table 5.1. The use of stratification was stated in 45% of the studies ($n = 10$). Of these 10 studies, 60% ($n = 6$) provided limited information, meaning they mentioned at least one component, such as duration of stratification, but failed to further expand on other necessary details to allow for replication. The four laboratory Nimmo et al. (2003) studies, which accounts for the remaining 40%, had sufficient information to replicate the process. They completed stratification by submerging a burlap sack into a lake; however, all were from the same article, and therefore, only one stratification approach was undertaken to germinate all the seeds used. Durkee Walker et al. (2006) and Sims et al. (2012) were the

Table 5.1 Summary of stratification data for the 22 studies conducted with *Zizania palustris*. Letter designations present the breakdown of studies within the article. N/R (Not Reported)

Study	Stratification performed	Temperature	Duration	Stratification vessel	Seed density or mass	Stratification location
Durkee Walker 2006*	Yes	2–4 °C	26 weeks	N/R	N/R	N/R
Durkee Walker 2010a*	N/R	N/R	N/R	N/R	N/R	N/R
Durkee Walker 2010b	N/R	N/R	N/R	N/R	N/R	N/R
Fort 2014	N/R	N/R	N/R	N/R	N/R	N/R
Fort 2017	N/R	N/R	N/R	N/R	N/R	N/R
Fort 2020	N/R	N/R	N/R	N/R	N/R	N/R
Johnson 2019*	N/R	N/R	N/R	N/R	N/R	N/R
LaFond-Hudson 2018*	N/R	N/R	N/R	N/R	N/R	N/R
Malvick 1993a	Yes	3.5 °C	12 weeks	N/R	N/R	N/R
Malvick 1993b	Yes	3.5 °C	12 weeks	N/R	N/R	N/R
Malvick 1993c	Yes	3.5 °C	12 weeks	N/R	N/R	N/R
Malvick 1993d	Yes	3.5 °C	12 weeks	N/R	N/R	N/R
Nimmo 2003a	Yes	N/R	8.5 weeks	Burlap sack	50 kg	Submerged 1.5 m from lake surface
Nimmo 2003b	Yes	N/R	8.5 weeks	Burlap sack	50 kg	Submerged 1.5 m from lake surface
Nimmo 2003c	Yes	N/R	8.5 weeks	Burlap sack	50 kg	Submerged 1.5 m from lake surface
Nimmo 2003d	Yes	N/R	8.5 weeks	Burlap sack	50 kg	Submerged 1.5 m from lake surface
Pastor 2017a	N/R	N/R	N/R	N/R	N/R	N/R
Pastor 2017b	N/R	N/R	N/R	N/R	N/R	N/R
Pastor 2017c	N/R	N/R	N/R	N/R	N/R	N/R
Pastor 2017d	N/R	N/R	N/R	N/R	N/R	N/R
Pastor 2017e*	N/R	N/R	N/R	N/R	N/R	N/R
Sims 2012*	Yes	4 °C	34.7 weeks	N/R	N/R	N/R

*Indicates a mesocosm study

only two studies conducted in mesocosms that report that the seeds were stratified before being added into the system. The remaining mesocosm studies state adding their seeds to the mesocosms in springtime without mention of stratification. Without the inclusion of information on seed harvesting (and therefore potential for natural stratification if done in early spring), nor information on stratification techniques, seeds used in a replication of this experiment may not reach sufficient temperature to break dormancy, and thus would be unsuccessful.

Stratification durations (Table 5.1) were highly variable and ranged from 60 days to approximately eight months. The maximum duration value reported, eight months, is approximate as Sims et al. (2012) indicated a date range of Fall 2008 to Spring 2009, with the sowing of seeds the following June 2009. Overall, stratification conditions were poorly reported. The average stratification temperature across the ten studies was 3.5 °C, but it was not indicated whether this was water or air temperature in six of the studies. Stratification conditions, such as vessel type, photoperiod, seed density, and media use, were also not reported in these six studies.

5.5.3.3 Test Conditions

All studies described the growth environment and housing vessels used in their experiments; the types of chambers and vessel data identified in each study are described in Table 5.2. ASTM guidelines for photoperiod with freshwater emergent macrophytes in growth chambers or greenhouses is 16 h of light (ASTM 2012). While all laboratory or indoor studies reported photoperiod values, consistent durations or justifications were not provided. Nimmo et al. (2003) used 12 h in three studies, and one from Durkee Walker et al. (2010) used a range of 10, 14, and then natural light exposure durations since they were contained in a greenhouse. All mesocosm studies were conducted outdoors, using natural light sources, but none reported use of light meters to confirm light levels.

The specifications surrounding types of test vessels, their measurements, and material type were well reported across all studies, with either the volume or dimensions of the test vessel(s) provided. Studies using sediment as substrate in mesocosm studies, as seen in Table 5.2, were all obtained from location of the water source where the wild rice seeds were harvested; however, in some of these studies, additional sand was added. It was not clear if this sand was naturally obtained or purchased from a commercial supplier. None of the laboratory or indoor experiments that used artificial substrates ($n = 4$) reported the substrate brand names or other characteristics of the materials. It should be noted that 44% ($n = 7$) of laboratory or indoor experiments failed to indicate whether a substrate was used.

Table 5.2 Summary of study design data for initial setup of all 22 studies conducted with *Zizania palustris*. Letter designations are added beside the year of an article to present the breakdown of studies within the article. N/R (Not Reported)

Study	Growth environment	Test vessels	Volume (L)	Substrate	Photoperiod	Test duration	Full life cycle	Number of test organisms/ replicate	Number of replicates/ treatments	Test compounds
Durkee Walker 2006*	Mesocosm	Stock tanks	378	Sediment & sand	Natural photoperiod	N/R	Yes	30	9	Straw litter
Durkee Walker 2010a*	Mesocosm	Stock tanks	378	Sediment & sand	Natural photoperiod	4 years	Yes	30 (thinned), N/R (not thinned)	N/R	Wild rice root and shoot litter
Durkee Walker 2010b	Greenhouse	Pots housed in pails	5 & 20	Sediment	10 h, 14 & natural photoperiod	16 weeks	Yes	1	4	Nitrogen & phosphorus
Fort 2014	Hydroponic chamber	Baskets housed in aquaria	N/R & 10	Inert mesh	16 light & 8 dark	21 days	No	60	4	Sulfate & chloride
Fort 2017	Exposed in laboratory	Baskets housed in aquaria	N/R & 10	Inert mesh	16 light & 8 dark	21 days	No	80	4	Sulfide
Fort 2020	Hydroponic tent	Baskets housed in aquaria	N/R & 10	Inert mesh	16 light & 8 dark	21 days	No	80	4	Sulfide
Johnson 2019*	Mesocosm	Stock tanks	457.5	Sediment	Natural photoperiod	4 years	Yes	N/R	6	Sulfate

(continued)

Table 5.2 (continued)

Study	Growth environment	Test vessels	Volume (L)	Substrate	Photoperiod	Test duration	Full life cycle	Number of test organisms/ replicate	Number of replicates/ treatments	Test compounds
LaFond-Hudson 2018*	Mesocosm	Pails housed in bucket	4 & 20	Sediment	Natural photoperiod	265 days	Yes	2	40	Sulfate
Malvick 1993a	Growth chamber	Pots with mesh bottom housed in buckets	0.025 & 6	Acetyl beads	16.5 light & 7.5 dark	6 weeks	N/R	27	2	Nutrient solutions
Malvick 1993b	Growth chamber	Buckets	6	N/R	16.5 light & 7.5 dark	8–9 weeks	N/R	7	5	Nutrient solutions
Study	Growth environment	Test vessels	Volume (L)	Substrate	Photoperiod	Test duration	Full life cycle	Number of test organisms/ replicate	Number of replicates/ treatments	Test compounds
Malvick 1993c	Growth chamber	Buckets	6	N/R	16.5 light & 7.5 dark	7–8 weeks	N/R	7	4, 6	Silicon
Malvick 1993d	Growth chamber	Buckets	6	N/R	16.5 light & 7.5 dark	5–6 weeks	N/R	7	5	Silicon & pathogen *B. oryzae*
Nimmo 2003a	Growth chamber	Petri dish	0.015	No substrate	12 light & 12 dark	10 days	No	1	3	Copper

(continued)

Table 5.2 (continued)

Study	Growth environment	Test vessels	Volume (L)	Substrate	Photoperiod	Test duration	Full life cycle	Number of test organisms/ replicate	Number of replicates/ treatments	Test compounds
Nimmo 2003b	Growth chamber	Petri dish	0.015	No substrate	12 light & 12 dark	14 days	No	1	8	Copper
Nimmo 2003c	Growth chamber	Petri dish	0.015	No substrate	12 light & 12 dark	14 days	No	1	12	Copper
Nimmo 2003d	Growth chamber	Petri dish	0.015	No substrate	12 light & 12 dark	14 days	No	1	8	Copper
Pastor 2017a	Growth chamber	Mason jars	0.473	N/R	All dark	11 days	No	50	3	Sulfate
Pastor 2017b	Growth chamber	Bottles	0.7	N/R	All dark	11 days	No	50	3	Sulfide
Pastor 2017c	Growth chamber	Kimax tubes	0.07	N/R	16 light & 8 dark	10 days	No	1	20	Sulfate
Pastor 2017d	Growth chamber	Bottles	0.125	N/R	16 light & 8 dark	10 days	No	7	3	Sulfide
Pastor 2017e*	Mesocosm	Stock tanks	400	Sediment & sand	Natural photoperiod	5 years	Yes	30	6	Sulfate
Sims 2012*	Mesocosm	Gusseted bags and stock tanks	30.5 & 1693	Sediment & sand	Natural photoperiod	131 days	Yes	2	N/R	Nitrogen & phosphorus

*Indicates a mesocosm study

Nutrient Solutions and Other Media

In total, 11 studies (50% of all; 69% of laboratory or indoor studies) reported using a standardized nutrient solution in their experimental procedures. Modified Hoagland's solution was the only standardized nutrient solution used among laboratory or indoor studies. All mesocosm studies indicated reliance of natural sediment from northern wild rice stands to provide nutrients instead. While each study using the modified Hoagland's solution reported it as such, the modifications (e.g., concentrations and recipes) differed between studies. For example, four of the Pastor et al. (2017) studies indicated a 1/5 strength Hoagland's solution, while Fort et al. (2014, 2017, 2020) used a modified Hoagland's solution with 25% ammonium (molar basis) in a mixture of ammonium and nitrate.

The laboratory or indoor experiments that did not use a standardized solution either had a short test duration (ten days in the case of studies a-d in Nimmo et al. 2003) or had nitrogen and phosphorus as the test compound (Durkee Walker et al. 2006) and, therefore, did not require additions to prevent nutrient deficiency. No studies autoclaved their nutrient solutions and none used an additional solvent to add a test compound, other than water. As seen in Table 5.3, pH and type of water diluent were not reported in various laboratory or indoor studies utilizing a standardized solution. These types of inconsistencies between studies of the same article were not uncommon. Mesocosm studies used either groundwater or well water for the filling of system; however, none of the studies stated if a characterization for nutrients occurred. Water volume levels used in the mesocosms were all reported.

5.5.3.4 Experimental Design and Performance

General experimental design weaknesses in the overall dataset were the lack of clarity on replicate and treatment numbers, endpoint rationales, and control performance. Three studies were missing information in regard to the number of test organisms per replicate or the replicates per treatment. Of these three studies, either the number of test organisms per replicate, or the number of replicates per treatment were indicated, but not both. All six mesocosm studies allowed their northern wild rice to complete a full life cycle and used seed production as a test endpoint.

Control Validation and Standards

Overall, 90% ($n = 20$) of the studies reported use of controls; however, of these 20 studies, only three had clearly stated control standards (i.e., expectations around performance). Reported control standards were 95% seed activation, 30% mesocotyl emergence, 90% control survival, and boron control >80% phytotoxicity (Fort et al. 2014, 2017, 2020); standards were met in all three studies. Of the 22 studies, only the same three (14%) Fort et al. (2014, 2017, 2020) experiments used a positive control, boron from boric acid, for the purpose of validating the experimental procedure and were all laboratory or indoor experiments. No citation was provided to support the

Table 5.3 Summary of laboratory or indoor study data that used standardized test solutions. Letter designations are added beside the year of an article to present the breakdown of studies within the article. N/R (Not Reported)

Study	Standardized test solution	pH	Type of water dilutant	Dissolved oxygen measured
Fort 2014	Modified Hoagland	6.1–7.2	Deionized	Yes
Fort 2017	Modified Hoagland	6.0–7.5 ± 0.5	Deionized	Yes
Fort 2020	Modified Hoagland	6.3–7.4 ± 0.3	Deionized	Yes
Malvick 1993a	Modified Hoagland	N/R	Deionized	N/R
Malvick 1993b	Modified Hoagland	N/R	Deionized	N/R
Malvick 1993c	Modified Hoagland	5	Distilled	N/R
Malvick 1993d	Modified Hoagland	5	Distilled	N/R
Pastor 2017a	1/5 Hoagland	6.8 ± 0.3	N/R	Yes
Pastor 2017b	1/5 Hoagland	6.8 ± 0.3	N/R	No
Pastor 2017c	1/5 Hoagland	6.8 ± 0.3	N/R	N/R
Pastor 2017d	1/5 Hoagland	6.8 ± 0.3	N/R	N/R

use of boron as a positive control, but its known plant toxicant properties were stated in Fort et al. (2014, 2017); though, in the 2020 study, the author's previous two experiments were cited as rationale for its use.

5.5.4 Discussion

This review was performed to assess key procedures and design gaps related to ecotoxicological experiments on northern wild rice (*Z. palustris*). In doing so, we hope to improve scientific reporting and direct future research. While relatively few articles were found in the peer-reviewed literature ($n = 11$), it is clear that key methodological components were missing across all articles for these experiments. This highlights the significant data reliability and replication issues within the field, and hinders the adoption of the species more widely within ecotoxicology.

Ideally, test methods should focus on sensitive and ecologically relevant endpoints that allow for sufficient and conservative extrapolations to the field, which may include expanding the range of standard test endpoints beyond growth and biomass measures (Hanson and Arts 2007). Growth measurements are relatively easy to quantify, have been widely applied under both laboratory and field conditions, and are

useful for integrating overall effects of toxicants on macrophytes; however, they lack specificity (Lemly et al. 1999). Responses such as reduced growth rates, or growth inhibition, do not indicate which specific sites or mechanisms are being affected by a particular toxicant. This is particularly notable for rooted macrophytes, where it can be difficult to assess if toxic responses are occurring due to sediment or water column exposure. Other common test methods include measurements of biomass (dry and wet), chlorophyll-α concentrations, chloroplast morphology, photosynthetic rate, enzyme activity, reproduction, seed germination, seedling growth, and root growth (Hanson 2013; Lemly et al. 1999). There are ranges of variability, sensitivity, and relevance within macrophyte toxicity testing endpoints, though root endpoints have been found to be among the most sensitive (Arts et al. 2008). This further highlights the need for macrophyte toxicity tests to encompass an array of endpoints to maximize protection when assessing the risk to nontarget organisms. We suggest that laboratory studies and test development with northern wild rice (*Z. palustris*) should focus on seed germination assays, as well as root and shoot endpoints as a first possible step toward a standardized toxicity test.

5.5.4.1 Major Weaknesses in Studies

Overall, the extent of stratification data was weak. If studies did report information, it was limited in terms of its completeness. With greater than 50% of the studies failing to indicate a stratification process, it prevents full replication of the designated experiment as readers could be unaware that stratification is a required process. The feasibility of the outdoor Nimmo et al. (2003) stratification technique is also a concern, and other means of this process should be still determined. While some studies alluded to the fact, the range of limited data supports the idea that no consensus of laboratory stratification procedures exists. Storage conditions, and use of a storage period, were also poorly reported, and we detected ambiguity in the entirety of the test organism information reported.

Another key methodology weakness in the overall dataset was the lack of any control performance standards. Few studies set criteria for control performance, making assay reliability highly uncertain. This is also concerning as control standards are needed to eliminate possible background effects. Therefore, none of the studies contained sufficient information to fully replicate the experiments, as either stratification, storage conditions, or control standards were absent or limited.

5.5.4.2 Recommendations for Improving the Reporting and Testing Within Northern Wild Rice Literature

To address this, we recommend those performing wild rice tests to:

1. Explicitly describe critical factors related to seed source, stratification, and seed storage.

In terms of wild rice, these two factors are crucial for seed viability and germination. The stratification technique used is particularly important to include, as no perceived standard method currently exists. The performance of various approaches will need to be assessed and contrasted to ensure selection of best practices regardless of the lab where a test is performed.

2. State control performance and whether requirements were met.
   Control performance helps to validate a study and excluding this information results in significant uncertainty in the data. Therefore, any control information in regards to experimental design should also be explicitly stated. Expectations around control performance need to be determined in order to ensure adequate test conduction.
3. Report all basic experimental conditions and design elements.
   An experimental conditions summary table, as seen in Fort et al. (2014, 2017, 2020), would be useful to readers for understanding how the study was performed. Checklists are an effective means for authors to confirm all essential information for replication is included in the paper.

5.6 Summary and Conclusions

Wild rice (*Zizania* spp.) presents themselves as a suitable candidate for inclusion into the battery of test species for risk assessment. They meet all six criteria of an appropriate test organism to varying degrees (Rand et al. 1995), as they are: (i) sensitive to a range of exposure types and contaminants; (ii) abundant and available in their natural range; (iii) indigenous to impacted ecosystems within North America; (iv) ecologically, culturally, and economically important; (v) amenable to routine maintenance in the laboratory for both acute and chronic toxicity tests; and (vi) have adequate background information on their physiology and life history.

We feel that risk assessments with wild rice will be most useful in North American contexts within their natural range and in situations where Indigenous concerns are paramount. Still, a significant amount of work is needed to advance wild rice toxicity testing by improving methods and reliability prior to wider adoption for ecological risk assessment, as noted by the results of this literature review.

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Chapter 6
Recovery of Freshwater Aquatic Macrophytes After Exposure to Herbicides and the Implications for Ecological Risk Assessment

Carlie Lau and Mark L. Hanson

Abstract Ecosystem recovery following natural disturbances is a ubiquitous and well-understood process. Freshwater macrophytes are able to colonize areas in which they have been extirpated through a number of mechanisms. Herbicides, which are widely used in agriculture globally, may pose a threat to non-target freshwater plants and result in individual-, population-, or community-level impairment of plant structure and function. The same mechanisms that allow for recovery of plants from non-anthropogenic stressors apply to impacts as a result of exposure to herbicides. Current ecological risk assessment (ERAs) frameworks for herbicide registration focus primarily on characterizing toxicity, and do not explicitly require data that allow for the understanding of potential recovery of plants following effect. There is disagreement on how recovery should be incorporated into ERA's for pesticides, and currently, there are no regulatory guidelines that provide standardized methods for plants. Numerous studies have characterized the effects of herbicides and the ability of macrophytes to recover following the cessation of exposure to plant protection products. A critical review of the peer-reviewed literature on the availability and quality of evidence for recovery of macrophytes exposed to herbicides was performed. A total of 25 recovery studies published between 1986 and 2019 were assessed. The relevance of endpoint and strength of methods for the recovery studies were evaluated with a scoring rubric based on three main categories: (1) test substance; (2) test organism and experimental system; and (3) test design, statistics, and results. Ecological relevance of endpoints was based on the association of reported endpoint to the population and community levels of effect. A total of 21 test species had been evaluated for 33 different herbicides. The most tested herbicide group was photosystem II inhibitors at 38% of studies. In total, 86% of studies reported clear evidence of recovery after transfer to clean media. Around 36% and 44% of tests from exposure and recovery phases, respectively, scored >50%

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on both strength of methods and endpoint relevance scores, which was the threshold for advising a study be used in ecological risk assessment. Laboratory studies in general may underestimate the potential for recovery as external mechanisms are fully excluded. Overall, we recommend that standard laboratory guidelines for the assessment of recovery in macrophytes be developed to improve the strength of methods and encourage improved reporting of toxicity data, and ultimately more formal inclusion in ecological risk assessment.

6.1 Introduction

Recovery of ecosystem structure and function can occur following natural disturbances, such as fires, flooding, and drought, and reflects the innate capacity of ecological systems to return through succession to previous or new stable states. The underlying mechanisms and processes driving ecosystem recovery will be the same for anthropogenic stressors, including chemical contaminants such as pesticides. The primary differences between natural disturbances and those driven by pesticides are typically the degree of impairment and the selectivity of that impairment. For example, fires tend to extirpate all extant species from the area in which the event occurs, while with pesticides, the removal of all non-target species off-field as a result application is unlikely, and typically only certain species classes are impacted due to compound mode of action.

Of the pesticides, herbicides are the most widely used class of pesticide globally in agriculture, have been commonly observed in surface waters following translocation off-field, and have modes of action that target plants explicitly, including aquatic macrophytes. Therefore, as herbicide exposure in freshwater ecosystems may cause impacts on macrophyte populations and communities, understanding how and if recovery can occur following such changes is important in characterizing fully the risk posed by plant protection products. The inability to recover from herbicide exposure represents a greater risk overall relative to scenarios where recovery is possible. This chapter will outline the concept of recovery in ecotoxicology, including:

- what recovery means for macrophytes;
- approaches by which macrophyte recovery can be assessed;
- inclusion of macrophyte recovery in ecological risk assessment; and
- a review of the current state of knowledge and evidence for recovery in macrophytes exposed to herbicides.

Finally, we will make recommendations for more effective inclusion of recovery for macrophytes in the ecological risk assessment of herbicides.

6.2 Concept of Recovery and Ecotoxicology

Recovery from natural disturbances as an ecological concept has been examined extensively, and the possible mechanisms by which an individual species may initially colonize or recolonize a habitat have been well characterized (Niemi et al. 1990). Ecological recovery extends beyond structural attributes, such as species abundance and richness, to functional elements such as overall biomass or nutrient cycling. The process of recovery, whether in terms of species structure or function, is limited to a few main drivers, most of which are heavily influenced by basic life history traits (e.g., reproductive strategies and fecundity), as well as inherent mobility and capacity for dispersal, coupled with the degree of isolation of the impacted ecosystem from unimpaired populations. Species have evolved a variety of strategies to survive transiently in unfavorable conditions such as temperature changes, shading, oxygen depletion, resource bottlenecks, and droughts (Ellis 1989). In turn, ecosystems can typically exist in several alternative stable states whereby each is characterized by different structural and functional parameters of the species that are found there at any one moment in time. When an impact occurs, the shift in structure and function can be ephemeral and followed by a return to the original state (O'Neill 1998). However, recovery may occur but to a "different" ecosystem, one that is permanently displaced, with a different structural and functional attributes, and reflect a new steady-state (Holling 1973; Scheffer et al. 2003).

As noted above, species and ecosystems all have some innate capacity to withstand and recover from disturbances, whether periodic (e.g., regional seasonal changes) or stochastic (e.g., burn events, flooding, droughts, pest and disease outbreaks). The concept of functional redundancy helps explain the stability of ecosystem processes in the face of stressors and, in part, why recovery of populations following a disturbance is possible. Functional redundancy states that a decrease in biodiversity (e.g., the loss of species) can be endured to a threshold, as long as key species and their functions are not adversely affected. Most ecosystems exhibit functional redundancy, where multiple species are able to perform and contribute to some functional attribute of the system as a whole (Walker 1992, 1995). For example, manipulation of plant communities in grassland ecosystems showed that community function, such as nutrient cycling, was stable despite the loss of significant numbers (>50%) of species (Tilman 1996). This is possible because of the redundancy in roles and functions provided by surviving species in the impacted ecosystem, allowing key biotic and abiotic needs to remain available (e.g., soil nutrients, structure, moisture) for extirpated species to successfully recolonize (Lawton 1994). These observations in support of the concept of functional redundancy underpin the idea in ecotoxicological risk assessment that some effects at the organism and population level can be allowed, provided that these effects are constrained temporally and spatially (Barnthouse 2004).

It is important to distinguish at which level of biological organization recovery is being investigated (e.g., from the molecular and physiological to the ecosystem level). From an ecological context, most studies have focused on populations and communities, as well as functional attributes, while for ecotoxicology, recovery can be and has been a phenomenon examined and observed from the molecular level (e.g., binding site) and upwards (Brock et al. 2015, 2018). Recovery of a population or community from contaminant exposure will adhere to the same mechanisms as for a natural stressor. These can be broadly categorized as internal or external mechanisms (e.g., from within or outside the disturbed ecosystem) (Caquet et al. 2007; Hanson et al. 2007). For example, internal and external recovery can be through recuperation of impaired organisms after exposure, or immigration of new individuals from other uncontaminated areas, respectively (Barnthouse 2004; Brock et al. 2018). The degree and time course for recovery will be highly context-dependent, varying by species, life stage, severity and duration of effect or exposure, time between or frequency of events, the type of impairment, and the degree of ecological isolation (Barnthouse 2004). Recovery tends to be most rapid at the lower levels of biological organization, where repair and a return to normal function can occur on the order of seconds to minutes (e.g., gene expression, enzyme activity), relative to ecosystem process. Effects that are spatially and temporally confined may be viewed as ecologically unimportant and/or fall within the natural variability of impacted populations (Domsch et al. 1983). In ecotoxicology, the definition of recovery has typically remained fairly straightforward, in that recovery, regardless of the level of biological organization, is said to have occurred once the element under question is no longer statistically different from an undisturbed or previous state (Brock et al. 2015, 2018; Hanson et al. 2007; Caquet et al. 2007). As well, it is important to note the difference between actual recovery to a pre-disturbance state and the potential to recover once the contaminant exposure has declined to a level that direct effects are no longer possible and recovery could occur (see EFSA 2016).

Regardless of the stressor type, there will be a threshold of intensity to which a stressor should be limited to prevent long-term adverse impacts on ecosystem structure and functions (e.g., beyond the inherent functional redundancy capacity). From an ecotoxicological perspective, the potential for recovery following the cessation of exposure is predicated on the biological level at which the effect is observed and upon the effect itself not being permanent (e.g., malformations in an individual) or continuing to worsen to the point where recovery is simply not possible (e.g., failure to reach sexual maturity, or outright mortality). The phenomenon of latency in ecotoxicology (i.e., when effects are observed relative to exposure) helps frame our understanding of the potential for recovery by an individual or a population and will be both contaminant- and species-specific. For example, Zhao and Newman (2006) showed that contaminants that do not cause cumulative damage and or/are cleared readily from an organism were unlikely to cause continuing mortality in amphipods (*Hyalella azteca*) upon the cessation of exposure, and therefore, surviving

individuals have the theoretical capacity to recover. Ultimately, individuals, populations, communities of macrophytes, and ecosystems have the capacity to recover following a stressor, whether anthropogenic or non-anthropogenic, assuming the putative stressor is no longer present, and the impaired ecosystem has the underlying biotic and abiotic conditions to support recolonization, internally or externally.

6.3 Recovery and Macrophytes

Aquatic macrophytes are annual and perennial plants that can be found in both standing or flowing water and are physically large (i.e., individuals are visible to the naked eye) relative to phytoplankton or periphyton (Wetzel 1975). They are frequently classified by growth form and/or basis of attachment to substrates, such as non-rooted free-floating (e.g., duckweeds *Lemna* spp.), non-rooted submerged (e.g., coontail; *Ceratophyllum* spp.), rooted submerged (e.g., milfoils; *Myriophyllum* spp.), rooted with floating leaves (lily pads; *Nymphaea* spp.), and rooted emergent (e.g., cattails; *Typha* spp.) (Hanson 2013; Wetzel 1975). Their community composition, abundance, and biomass are subject to seasonal shifts, and are therefore relatively dynamic (Henry et al. 1996). Macrophytes have a sometimes-underappreciated ecological role in freshwater ecosystems from both a structural and functional perspective. They provide food, shelter, and nurseries to waterfowl, fish, and invertebrates, nutrient cycling and sequestering, oxygen production, and stability to organic sediments and other substrates from wave action and flooding. As such, there is considerable value in characterizing both the response and recovery to anthropogenic and non-anthropogenic stressors (Carpenter and Lodge 1986; Crowder and Painter 1991; Hanson 2013).

Freshwater macrophytes have specific attributes related to their life histories and physical architecture that influence how recovery occurs following a disturbance, as well as the speed and the degree to which recovery is possible (Henry et al. 1996). In terms of recolonization of habitat from which a species has been extirpated, macrophytes employ tactics that are shared by all plants. These include seed dispersal and seedbanks, plant fragments (e.g., stems), expansion from intact parent plants (e.g., lateral growth), rhizomes, and resting or overwintering phases (e.g., turions). Species traits (e.g., those related to recolonization, such vegetative or sexual dissemination) can significantly influence the degree and likelihood of macrophyte recovery. Henry et al. (1996) examined recovery following frequent flooding events over multiple years on the Rhône River, France. The authors assessed, in part, the contributions of vegetative dissemination of plants by lateral spread without dispersion (including by extension of the root system); from stem fragments; and by specialized resting phases (e.g., turions), as well as the frequency of flowering of the species in question. They found that recovery was relatively rapid overall, with most species returning within a year, and typically early recovery was by those able to produce turions or other vegetative organs, followed by recovery via lateral spread and stem fragments. Dispersal mechanisms from un-impacted to impacted patches will include physical transport

by currents, wave action, or flow, as well as by birds (e.g., migratory waterfowl). The efficiency of dispersal is driven in part by the degree of connectivity and geographic isolation between systems (e.g., small agricultural pond versus downstream on a river). Sand-Jensen et al. (2000) examined the shifts in macrophyte composition and abundance in 13 Danish streams over the period of a century. Connectivity of systems likely explained both local abundances as well as number of occupied sites by species in streams that were subject to frequent disturbance (Sand-Jensen et al. 2000).

Coastal wetlands are subject to regular storm and flooding events of varying severity that can lead to pulses of salinity that can impair plant growth, so these ecoystems lend themselves to understanding the propensity for macrophyte recovery. Howard and Mendelssohn (1999) conducted a four-month greenhouse experiments with monocultures of four perennial emergent macrophytes species (*Eleocharis palustris*, *Panicum hemitomon*, *Sagittaria lancifolia*, and *Schoenoplectus americanus*) as potted monocultures at two levels of salinity (6 or 12 g/L), rate to reach exposure (3 days or 3 weeks), and duration of exposure (1, 2, or 3 months). Transfer to freshwater followed each exposure to allow for a 1-, 2-, or 3-month period of recovery depending on initial exposure duration. Both effect and recovery were species-dependent. Mortality (nonviable aboveground tissue) for all treatments and durations combined was 17.8% for *P. hemitomon*, 6.7% for *S. lancifolia* 2.2% for *E. palustris*, and 0% for *S. americanus*. Within a species, salinity level and duration of exposure were the main factors that influenced the degree and rate of recovery, and the degree of recovery was correlated to the severity of the initial impact, with *P. hemitomon* exhibiting the least capacity for recovery, *S. lancifolia* and *E. palustris* moderate recovery, and *S. americanus* full recovery across all treatments. Howard and Mendelssohn (1999) theorized that the capacity to recover was related to the growth strategies of each tested species. Specifically, the plants with the ability to produce rhizomes that could outlast the exposure conditions provided a mechanism for recolonization once favorable growth conditions returned.

The rate of recovery following the loss of species can be influenced in part by patch dynamics and the community composition of the borders surrounding the immediately impacted area. Barrat-Segretain and Amoros (1996) experimentally cleared macrophytes from 9 m^2 patches (subdivided into 144 plots) of a river channel and tracked recovery over a period of greater than three months. Within three weeks of removal, most plots had new macrophyte growth of several species, and by the end of the study, most plots had multiple species (5–6) and dense coverage, illustrating the relatively rapid recovery that is possible for macrophytes, especially when colonizing populations are adjacent and actively growing. Barrat-Segretain and Amoros (1996) concluded that recolonization by macrophytes in their study was driven mainly by vegetative propagation. Specifically, parent plants expanded into the disturbed system from the edges of the plots ("peripheric propagation") as an intact entity (e.g., through spreading rhizomes), or they had ramets that would break off from the parent plant and move some distance away from the edges to colonize patches from a distance. They also reported that plants could exhibit both strategies simultaneously, such as *Elodea canadensis*, while others were limited to one mechanism (e.g., *Potamogeton natans*

for intact plant expansion from the edges; *Potamogeton pusillus* recolonization by fragments or propagules).

Eutrophication of freshwater ecosystems is another common stressor that can result in the loss of macrophyte species through enhanced turbidity (typically via algal blooms) and subsequent loss of light penetration to support early plant growth. With improvements in wastewater treatment and enhanced efforts to reduce nutrient movement into surface waters generally, the process of recovery by macrophyte communities can be assessed. Baastrup-Spohr et al. (2017) took data from 1990 and 2010 and examined the relationship between changes in eutrophication status and species richness and community composition of aquatic macrophytes in 56 lakes in Denmark. Overall, they found species richness increased over the 20 years with improved water quality, and that lake species richness was significantly positively related to a decline in concentrations of chlorophyll-a and improved water transparency. In terms of species composition, there was a shift to biotic homogenization, whereby the similarity between systems increased significantly through the acquisition across lakes of the same new species. In this case, macrophyte community recovery was deemed to be ongoing, and likely lagging, in part due to lack of connectivity with un-impacted systems to facilitate recolonization.

6.4 Herbicides, Macrophytes, Recovery, and Ecological Risk Assessment

Currently, the majority of herbicides are used in agriculture for crop protection, but herbicides are also registered for forestry, invasive species control, and home uses (USEPA 1998; Gettys et al. 2014). Herbicides have a variety of modes of action that target different plant physiologies. The majority of herbicides interrupt plant-unique biological mechanisms by binding at specific sites of action. In general, there are two categories of herbicides, non-selective and selective, which has implications for assessing recovery. Early herbicides tended to be non-selective, with more selective herbicides being invented following World War II (Vats 2015). Non-selective herbicides, such as glyphosate, do not have specific targets (e.g., species or classes of plants) and are able to control many types of plants (Ross and Childs 1996). In contrast, selective herbicides are more toxic to certain plant species, typically due to the mode of action that is unique to the target (De Carvalho et al. 2009). For example, dicamba is a selective herbicide that mimics plant growth hormones and mainly targets eudicots like broadleaf weeds (Ross and Childs 1996). 2,4-dichlorophenoxyacetic acid (2,4-D) is another selective herbicide used to target broadleaf dicotyledonous weeds (Song 2014). It is a pre-emergent and post-emergent herbicide that mimics growth-regulating auxins, affecting cell division and elongation (Grossmann 2010). In both these cases, monocots would be significantly less

sensitive to the herbicide, so direct effects are minimal, and the need to assess recovery in these types of plants is not necessary.

The application period of an herbicide depends on the crop, its targets, mode of action, the geographic region, and regulatory restrictions. There are three general categories of application period for herbicides: pre-plant, pre-emergence, and post-emergence (Vats 2015). Pre-plant herbicides are applied before crops are planted or seeded to clear fields of weed species. Pre-emergence herbicides are those sprayed after planting and before seed crop germination, which do not affect the seed but will impact growing weeds. Post-emergence herbicides are sprayed after seeds have germinated and emerged and are typically selective for certain species or groups, other than the crop. These patterns of applications mean that herbicides that have migrated off fields through spray drift or runoff into surface waters are not constant or consistent through space and time, but are rather pulsed in nature, with periods of relatively high exposures, followed by declines and periods of low to no exposure (Smith et al. 2021). For example, concentrations of atrazine in United States Midwestern streams near agricultural lands with intensive atrazine application tend to occur as pulses in the streams, with mean daily concentrations below 10 μg/L (Andrus et al. 2013). After rainfall, runoff concentrations were observed to increase up to 200 μg/L, but would return to under 10 μg/L in a short period of time (Andrus et al. 2013, 2015). As a result, herbicides can present a risk to macrophyte communities where they are applied and on multi-occurrences annually, and so characterizing recovery potential helps to understand to risks from possible cumulative effects.

Concentrations of herbicides in the tissues of aquatic plants tend to track those in the surrounding water (King et al. 2016). As such, with the cessation of exposure, internal concentrations should decline and aquatic macrophytes can potentially recover, at least physiologically. This rapid response has been observed in algae where a study investigating the recovery of *Pseudokirchneriella subcapitata*, *Anabaena flos-aquae*, and *Navicula pelliculosa* found that PSII quantum yields were not significantly different from the control almost instantaneously following transfer to clean media (Brain et al. 2012a). This coupled with modes of action that target plant-specific biochemical or physiological processes (e.g., inhibit chlorophyll functioning) to impair growth in general, but rarely result in direct mortality of plants below recommended application rates means less concern around possible latent effects. From a risk assessment perspective, the ability to recover following herbicide exposure reduces the risk of sustained adverse effects on macrophyte communities as a whole, which in turn is important for preventing indirect effects on organisms that rely on macrophytes for food and/or habitat (USEPA 1998).

Current risk assessments and data registration requirements for herbicides in North America do not require recovery data for macrophytes, though an evaluation of adversity may include the potential for recovery (USEPA 1998). Typical regulatory requirements at the initial tier for the registration of herbicides include the submission of toxicity data from the free-floating macrophyte *Lemna* sp., commonly known as duckweed (Arts et al. 2010; Hanson 2013). Duckweed guidelines allow for the

characterization of both toxicity and recovery under laboratory conditions and in a reasonable time frame using fairly straightforward techniques (Brain and Solomon 2007). However, concerns have been raised about *Lemna* sp. being used as a surrogate for all macrophytes (Hanson 2013; Rentz and Hanson 2009; Wang et al. 2010). As they are monocots that lack stems, true leaves, and a sediment-interacting root system, their predictive capabilities may be limited for eudicots, rooted, and/or submerged macrophytes (Hanson and Arts 2007). Despite this, duckweed as a model organism lends itself readily to the assessment of recovery in the laboratory (see Sect. 5) in part because of the ease by which one can transfer plants to fresh media to mimic the removal of the stressor.

6.5 Review of the Current State and Quality of Evidence for Macrophyte Recovery Following Exposure to Herbicides

Previous studies have expressed concerns around the quality of ecotoxicology studies and recommended criteria to determine the reliability and relevance of data for risk assessment (Ågerstrand et al. 2014; Hanson et al. 2017, 2019; Harris et al. 2014). Previous work using objective scoring rubrics to assess the quality of toxicity tests for atrazine and primary producers reported that a large number of studies had experimental data fitting basic inclusion criteria, but only a small proportion provided sufficient details on the test substance, test organism, and test results to be considered of satisfactory quality for use in decision-making (Hanson et al. 2019). As part of this chapter, we set out to critically review the availability, reliability, and ecological relevance of macrophyte recovery data following exposure to herbicides in the peer-reviewed literature. We also examined the evidence from these studies that recovery can occur in macrophytes following exposure to herbicides. This was done, in part, to identify the data gaps and common methodological issues in order to improve the quality of future recovery studies. With sufficiently high-quality recovery data, policy makers could use the information to establish more credible guidelines and regulations, as well as to assay the overall risk posed by these compounds to macrophytes.

6.5.1 Materials and Methods

We assessed both the strength of methods and ecological relevance of endpoints from peer-reviewed recovery studies performed on primary producers exposed to herbicides. For the purposes of this exercise, we defined ‘recovery’ as measured endpoints not statistically different from control(s) at the end of a herbicide-free exposure period following a herbicide exposure phase. Scoring rubrics for strength

and relevance were developed and applied to each published study that met our inclusion criteria. The rubrics were modified from the scoring criteria of Hanson et al. (2019).

6.5.1.1 Literature Search

The search for relevant literature was performed using databases available through The University of Manitoba libraries, including Scopus. Published studies that could be acquired through the University of Manitoba libraries database were reviewed. The search terms employed were combinations of "herbicide", "recovery", "exposure", "aquatic primary producer", and "macrophytes". References in the scored papers were also reviewed for possible literature to be assessed. The inclusion criteria for scoring of studies required papers to have exposure and recovery periods (e.g., a pulse exposure period) for a single herbicide (no mixtures), reported effects on aquatic macrophytes, be written in English, and published in a peer-reviewed journal. The final search for published articles was performed on September 30th, 2019.

6.5.1.2 Strength of Methods Scoring

A strength of methods score (i.e., reliability) was assigned for each paper based on the information provided in the article and any associated supplementary files. The rubric for scoring strength was modified from the one Hanson et al. (2019) developed for primary producer toxicity tests. The rubric was used to evaluate all studies, regardless of species, herbicide, or reported endpoint. The criteria were divided into three main groups (Group 1: Test Substances—six criteria; Group 2: Test Organisms and Test System—four criteria; and Group 3: Test Design, Statistics, and Results—five criteria). The rubric and justifications for the scoring categories can be seen in Table 6.1. Performing and reporting for each criterion resulted in a score of 1, otherwise a score of zero was assigned.

The score for ecological relevance of exposures is one criterion that will be highly context-dependent (e.g., compound, time of year, geographic location). This criterion reflects the proximity of the recovery studies to a "real-world" situation and consequent relevance for the purposes of ecological risk assessment. In risk assessment, demonstrating toxicity and recovery, or the lack thereof, at ecologically relevant concentrations helps reduce uncertainty. For this review, a score of 1 was given when at least one of the tested concentrations in the recovery testing was equal to or less than 20 μg/L (i.e., an "environmentally relevant concentration"), regardless of the herbicide. The level of 20 μg/L was chosen based on available data for herbicides in surface water (in general and for the compounds tested), which indicates that most environmental exposures will be at or below this level for these compounds. There are numerous monitoring programs reporting herbicide concentrations in surface water that support using 20 μg/L as a cut-off. Schuler and Rand (2008) summarized the herbicide concentrations in South Florida's surface water from 1990–2006.

Table 6.1 Scoring criteria for method strength of peer-reviewed recovery studies of aquatic macrophytes after herbicide exposure. Modified from Hanson et al., (2019).

Assessment criteria [a]		Brief explanation	Rationale	Detailed Explanations
Group A - Test substance				
1	> 95% pure	Source and purity of test substance reported	The purity of test chemical should be high to increase the confidence of study's result by concluding the test substance was the one who caused effect on test species, instead of other impurity (e.g., adjuvants, formulants) in the test substance.	1 - If the percentage and source of test substance were reported with the percentage was greater than 95%; 0 - if purity and/or source of test source were not reported, or the purity was less than or equal to 95%
2	Measured concentrations reported – any [b]	Any analytical confirmation and reporting of tested concentrations	Confirmation of the test concentration would increase the accuracy of the reported toxicity value and ensure the toxicity effect on the test species was caused by the test chemical.	1 - If the analysis of stock/test concentration solution were reported at any stage of the experiment; 0 - if the analysis of stock/test concentration was/were not reported
3	Measured concentrations reported - individual initial	Initial concentrations in individual test units reported (pooled or separate)	Measuring the initial concentration would decrease the errors (e.g. wrong dilution, incorrect test chemical) in the result. With the initial and final concentration, time-average concentration could be calculated and apply to calculations.	1 - If initial tested/stock concentrations were reported; 0 - if initial tested/stock concentration were not reported
4	Measured concentrations reported - individual final	Final concentrations in individual test units reported (pooled or separate)	Measuring the final concentration would decrease the errors (e.g. wrong dilution, incorrect test chemical) in result. With the initial and final concentration, time - average concentration could be calculated and applied to calculations.	1 - If the test concentration(s) were measured and reported at the end of experiment; 0 - if the test concentration were not reported
5	No. of concentrations tested (exclude control) [b]	3 or more concentrations, plus control	Having at least 3 concentrations would assist the determination of concentration - response model.	1 - If number of tested concentration treatments (exclude control) was 3 or greater; 0 - If number of test concentrations treatments were fewer than 3
6	Ecological relevance	At least 1 exposure concentration at 20 µg/L or less	Based on the previous studies , the herbicide concentration in surface water in surface water often are often at or lower than 20 µg/L (e.g. Andrus et al., 2013, Environment Canada, 2011). Therefore, 20 µg/L set as environmentally relevant concentration.	1 - If at least one of the tested concentration was less than or equal to environmental concentration (e.g. 20 µg/L); 0 - if none of t he tested concentration was less than environmental concentration (e.g. 20 µg/L)

(continued)

Table 6.1 (continued)

Assessment criteria [a]		Brief explanation	Rationale	Detailed Explanations
Group B - Test organism and test system				
7	Strain/source identified	Provenance of test organism (e.g. wild - collected location; laboratory strain ID)	With the information of collect location and the test species, it would help to understand if the test species were exposed to test chemical previously. and which species were being tested.	1 - If the source of the test organisms was provided ; 0 - if no mention of the source of test organisms
8	Initial test organism characteristics described	As relevant, size, density, mass, feeding protocols	With the initial size, density, mass of test species were reported, it can be used to compare to the exposed test species to access the toxicity effect.	1 - If initial front number or cells counts were repor ted; 0 - if no information on the initial front number, cells counts, or mass of the organisms
9	Standard protocol followed	e.g., EPA, OECD, ASTM; deviations acceptable if described	The testing were standardized by followed a standard protocol and the res ult could be used to compare with other studies.	1 - If test procedures followed standard pro tocol; 0 - if the procedures were based on previous studies and/or not followed any standard protocol
10	Test conditions	Temp, light, pH; TOC if sediment	Reportin g the test condition was essential because the condition of test environment (e.g., temperature, light) could affect the growth rate and toxicity effect on primary producers.	1 - If temperature and/or light intensity, and media type were reported, and the error values of light intensity and/ or temperature was reported; 0 - if temperature, light intensity, and media were not reported without error values of temperature and /or light intensity was not reported
Group C - Test design, statistics, and results				
11	Replication [b]	Minimum of 3 replicates per exposure	The testing should be repeated to confirm the toxicity effect by the test chemical is consistent.	1 - Replicates ≥ 3; 0 - Replicates < 3
12	Statistical methods described [b]	Appropriate test reported and employed for NOEC, LOEC, and EC/LCx; appropriate controls employed	Proper statistic approach should be used to prevent incorrect result and misleading result for risk assessment.	1 - If the statistic method to calculate NOEC/LOEC/ECx/LCx or other endpoints were described and reported; 0 - if the statistic method to calculate NOEC/LOEC/ECx/LCx were not described and/or reported
13	Concentration - response	Concentration - response model and parameters provided	Providing concentration - response model to evaluate the toxicity effect and response.	1 - If concentration-response model and parameters was showed or reported in the format of formula, or graphs with the concentration -response model equation information ; 0 - If equation of concentration -response model and parameters were not showed or reported

(continued)

Table 6.1 (continued)

Assessment criteria [a]		Brief explanation	Rationale	Detailed Explanations
14	Measured response (raw) values	Key measured raw values (e.g., not % of control) reported (all or avg. with error; graph or table)	Raw values (data without transformation) should be reported to access the performance of the testing and toxicity effect of the test chemical.	1 - If raw values (average raw values is accepted but not % of control) were reported in t ables/figures; 0 - If only the % of control was reported in graph/figure and none of the raw values (includes a verage) were reported or plotted in figures
15 [b]	Control performance	Control values reported and performance criteria met	Control needed to be reported to ensure the culture met the standard performance and was healthy in the testing, and if it is showing the toxicity effect appropriately.	1 - If control values (included solvent control if solvent was used to prepare the test solution) were reported and the reported values showed to meet the control performance requirements; 0 - if control values were not reported and not mention on control performance requirement

[a] Full mark is fifteen and one mark would be given for meeting each criterion; if not met, a score of zero was assigned
[b] Total score multiplied by 0.5 if the criterion is zero

The authors reported that most herbicides in ten counties of South Florida had a maximum concentration that was lower than 20 μg/L, with a range of 0.003–18 μg/L and 90th percentile ranging from 0.003–1.91 μg/L. The exception was diuron in Hendry County, which had a maximum of 76 μg/L and 90th percentile of 0.15 μg/L (Schuler and Rand 2008). The monitoring program from Environment and Climate Change Canada in 2003–2005 showed that the majority of herbicide concentrations in surface waters across Canada were below 14.9 μg/L (ECCC 2011). Based on the reported values from these studies, it is reasonable to conclude that concentrations of herbicide in North America's surface waters are usually below 20 μg/L. Therefore, 20 μg/L was set as a generic environmentally relevant concentration in the strength of methods rubric.

There were also critical criteria that were considered integral to identifying a strong study. The critical criteria were analytical confirmation and number of tested concentrations, replication, and use of appropriate statistical methods. The critical criteria are highlighted in red in Table 6.1. To better evaluate the strength of the study, total scores were reduced if a critical criterion was not met. When criterion 2, 5, 11, and/or 12 (Table 6.1) was not met, the total score would be multiplied by 0.5 for each missed criterion. If two critical criteria were not met, the total score would be multiplied by 0.25. The total score would be multiplied by an additional factor of 0.5 if expert judgment deemed there were additional study flaws that were not captured in the standard rubric criteria. However, no further fundamental errors were found among reviewed papers, so no scores were reduced based on the judgment of the reviewer.

6.5.1.3 Ecological Relevance of Endpoints Scoring

The endpoints monitored in the studies were used to assign a score to the data for its relevance to ecological risk assessment. We worked from the assumption that the endpoints that are associated with higher levels of biological or ecological organization (i.e., population or community level) are most useful for risk assessment (Hanson et al. 2019). For each study, each reported response was assigned a relevance score. The scores for relevance for each endpoint were between 0 and 5 (Table 6.2). The greater the relevance score for the endpoint, the more the response was conceptually and objectively linked to population and community-level responses that best inform ecological risk assessment.

Table 6.2 Scoring criteria for the ecological relevance of endpoints from peer-reviewed recovery studies of aquatic macrophyte after herbicide exposure

Score	Macrophytes	Detailed explanation
0	No known linkage to survival, development, growth, and/or reproduction	If a response has no real or hypothetical linkage to higher-level effects, then it has little to no value in elucidating ecological risk
1	Biomarker response that has limited linkage to higher-level effects (e.g., genomic, proteomic, metabolomic)	While informative from a mechanistic perspective, the relevance of these responses to population, community, and ecosystem-level effects is considered very low. In many cases, the responses characterized are regular processes to detoxify or adapt to a transient stressor, which in and of themselves are not adverse
2	Biomarker responses such as enzymatic changes (e.g., nitrogenase activity) or general physiology (e.g., PSII quantum yield, chlorophyll-a concentrations) or functional responses, (e.g., rate of oxygen production)	While informative from a mechanistic perspective, the relevance of these responses to population, community, and ecosystem-level effects is considered very low. In many cases, the responses characterized are regular processes to detoxify or adapt to a transient stressor, which in and of themselves are not adverse
3	Changes in growth and development, such as biomass and growth rates	These responses are typically highly relevant to the success or sustaining populations and communities in an ecological context
4	Changes in reproduction, such as seed production, seed viability, flower production, frond number, plant number, and related metrics (e.g., growth rates)	These responses are typically highly relevant to the success or sustaining populations and communities in an ecological context
5	Mortality and/or community-level changes such as shifts in species diversity/composition	Loss of individuals is highly relevant to the success or sustaining populations and communities in an ecological context

6.5.1.4 Review QC/QA

Once the strength and relevance scores were assigned, the resulting data spreadsheet underwent a quality control and quality assurance (QA/QC) exercise. The spreadsheets (and papers) were reviewed again by a separate individual with experience in ecotoxicology studies to help ensure accuracy of interpretation and reporting.

6.5.1.5 Data Analysis

Graphs (e.g., scatter plot, bar graphs, bubble plots, and box plots) were generated with the use of R-studio (R Development Core Team 2019). Descriptive statistical analyses were performed in R studio (R Development Core Team 2019).

6.5.2 Results

6.5.2.1 Summary of Reviewed Studies

A total of 25 studies published between 1986 and 2019 met the inclusion criteria and were reviewed (Table 6.3). The number of recovery studies has steadily increased over years (Fig. 6.1).

Table 6.3 Summary of 25 peer-reviewed studies published between 1986 and 2019 on aquatic macrophytes exposed to herbicide and followed by a recovery period

Organism type	Number of peer-reviewed papers	Number of experiments	Number of species tested	Number of herbicides tested	Number of endpoints reported
Duckweed	14	44	3	27	25
Others	14	32	18	10	39
Total	**25**	**76**	**21**	**33**	**58**

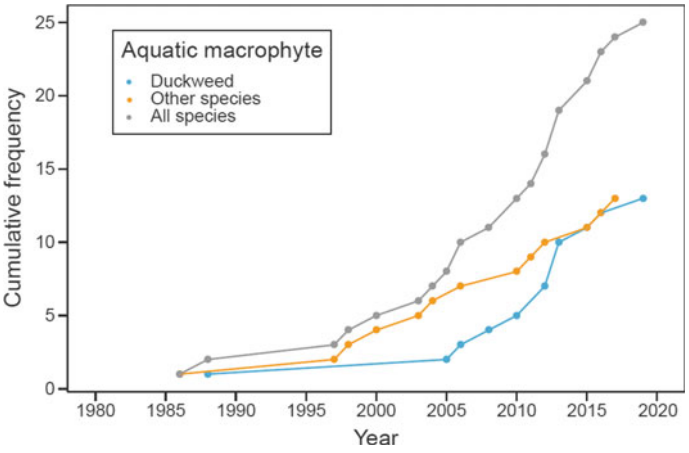

Fig. 6.1 The cumulative number of published peer-reviewed recovery studies on aquatic macrophytes (duckweed, other species, total) exposed to herbicides between 1986 and 2019. The total number of papers found was 25

Test Organism Class and Species

There were 76 unique experiments with macrophytes for 33 different herbicides and 58 distinct endpoints (Tables 6.3 and 6.4). The most commonly tested species by unique experiments were *Lemna* sp. ($n = 17$), *Lemna minor* ($n = 15$), and *Lemna gibba* ($n = 12$).

Test Substances

Figure 6.2 shows the herbicide groups tested over time. In general, there was increasing diversity of herbicide groups included in the recovery studies over time. It increased from a single herbicide group in the 1980s to eight herbicide groups in the 2010s. A total of 33 herbicides were tested in the reviewed studies, and some studies examined recoveries after multiple types of single herbicide exposure (Table 6.4). Photosystem II inhibitors were the most studied herbicide group ($n = 29$ experiments). The second most commonly tested herbicide group for macrophytes was acetolactate synthase (ALS) inhibitors ($n = 21$ experiments). The most commonly tested herbicides were atrazine (9 papers), diuron (4 papers), and metsulfuron-methyl (4 papers).

Test Duration

The mean and median exposure durations for macrophytes were 18 and 7 days, respectively ($n = 296$) (Fig. 6.3). The mean and median duration for the recovery period were 13 and 7 days for macrophytes, respectively ($n = 359$). For duckweed, the mean and median were 9 and 7 days for exposure ($n = 168$), and 8 and 7 days for recovery ($n = 176$). The mean and median for others aquatic macrophyte were 29 and 13 for exposure ($n = 128$), and 17 and 14 days for recovery, respectively ($n = 183$).

6.5.3 Strength of Method Scores

The mean and median of strength of method scores for macrophytes were 6.0 and 5.0, respectively ($n = 76$; Fig. 6.4). The mean and median of strength of method scores were 5.5 and 5 for duckweed ($n = 44$) and 6.6 and 8.0 for other species ($n = 32$). The percentage of individual tests that received a score greater than 7.5 out of 15 (i.e., > 50%) were 37% for total macrophytes ($n = 76$), 25% for duckweed ($n = 44$), and 53% for others macrophyte species ($n = 32$). The highest strength scores by each herbicide are found in Table 6.5.

Table 6.4 Summary of tested herbicides from twenty-five reviewed studies on aquatic macrophyte (duckweed, others, and total) that was published between 1986 and 2019

Herbicide group (mode of action)	Number of papers in group[a]	Number of herbicides tested	Number of duckweed experiment tested	Number of others macrophyte experiment tested	Number of total macrophyte experiment tested	Number of papers tested by herbicide	Number of experiment by species
Photosynthesis II inhibitor	16	8	14	15	29	atrazine (9), diuron (4), isoproturon (3), bromacil (1), linuron (1), simazine (1), simetryn (1), terbuthylazine (1)	*Lemna minor* (6), *Lemna gibba* (5), *Lemna* sp. (3), *Zostera capricorni* (3), *Vallisneria americana Michx* (2), *Ceratophyllum demersum* (1), *Chara globularis* (1), *Cymodocea serrulata* (1), *Elodea nuttallii* (1), *Halophila ovalis* (1), *Myriophyllum aquaticum* (1), *Myriophyllum spicatum L.* (1), *Potamogeton crispus L.* (1), *Potamogeton perfoliatus L.* (1), *Ranunculus circinatus Sibth.* (1)

(continued)

Table 6.4 (continued)

Herbicide group (mode of action)	Number of papers in group[a]	Number of herbicides tested	Number of duckweed experiment tested	Number of others macrophyte experiment tested	Number of total macrophyte experiment tested	Number of papers tested by herbicide	Number of experiment by species
Acetolactate synthase (ALS) inhibitor	8	13	18	3	21	metsulfuron-methyl (4), cyclosulfamuron (3), bensulfuron-methyl (2), thifensulfuron-methyl (2), ethoxysulfuron (1), flazasulfuron (1), flupysulfuron-methyl (1), imazamox (1), imazosulfuron (1), iofensulfuron-sodium (1), nicosulfuron (1), pyrazosulfuron-ethyl (1), rimsulfuron (1)	*Lemna* sp. (10), *Lemna gibba* (5), *Lemna minor* (3), *Myriophyllum spicatum* (2), *Elodea canadensis* (1)
Photosynthesis I inhibitor	5	3	3	2	5	Irgarol (2), paraquat (2), diquat (1)	*Zostera capricorni* (2), *Lemna gibba* (1), *Lemna minor* (1), *Lemna* sp. (1)
Acetyl coenzyme A carboxylase (ACCase) inhibitor	2	2	2	0	2	cyhalofop-butyl (1), thiobencarb (1)	*Lemna* sp. (2)

(continued)

Table 6.4 (continued)

Herbicide group (mode of action)	Number of papers in group[a]	Number of herbicides tested	Number of duckweed experiment tested	Number of others macrophyte experiment tested	Number of total macrophyte experiment tested	Number of papers tested by herbicide	Number of experiment by species
Carotenoid biosynthesis inhibitor	2	1	0	11	11	fluridone (2)	*Hydrilla verticillata* (4), *Chara* spp. and *Najas guadalupensis Magnus* (1), *Elodea canadensis Michaux* (1), *Myriophyllum spicatum* L (1), *Potamogeton nodosus Poiret* (1), *Potamogeton pectinatus L* (1), total plant community (1), *Vallisneria americana Michaux* (1)
Pigment synthesis inhibitor	2	1	1	1	2	norflurazon (2)	*Lemna minor* (1), *Vallisneria americana* (1)
Root-growth inhibitor	2	3	3	0	3	pendimethalin (1), propyzamide (1), trifluralin (1)	*Lemna minor* (3)

(continued)

Table 6.4 (continued)

Herbicide group (mode of action)	Number of papers in group[a]	Number of herbicides tested	Number of duckweed experiment tested	Number of others macrophyte experiment tested	Number of total macrophyte experiment tested	Number of papers tested by herbicide	Number of experiment by species
Shoot-growth inhibitor	2	1	2	0	2	alachlor (2)	*Lemna gibba* (1), *Lemna* sp. (1)
Mitochondrial ATP-ase activity inhibitor	1	1	1	0	1	pentachlorophenol (1)	*Lemna minor* (1)

[a]More than one herbicide was tested in some studies

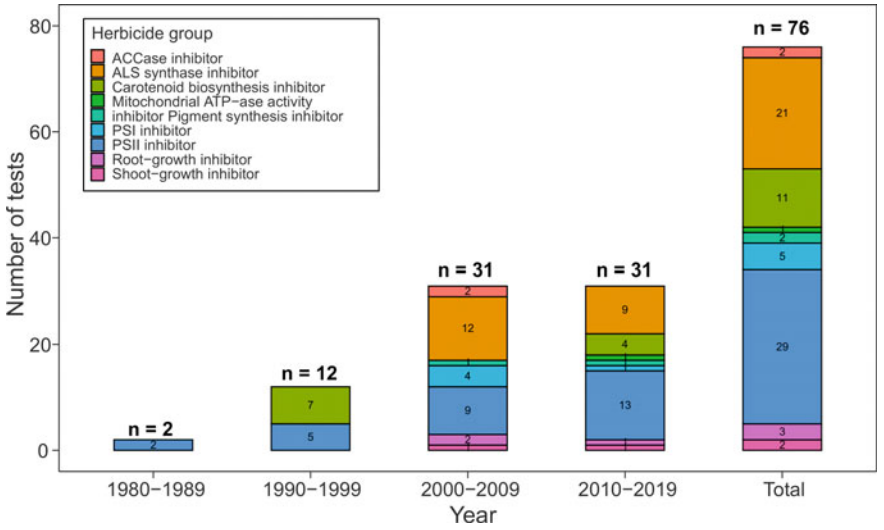

Fig. 6.2 The number of aquatic macrophyte herbicide recovery studies by herbicide group between 1986 and 2019. The *n* above each bar was the total number of unique tests in each year group, and the number in each stacked bar was the number of tests in each herbicide group in the corresponding year group

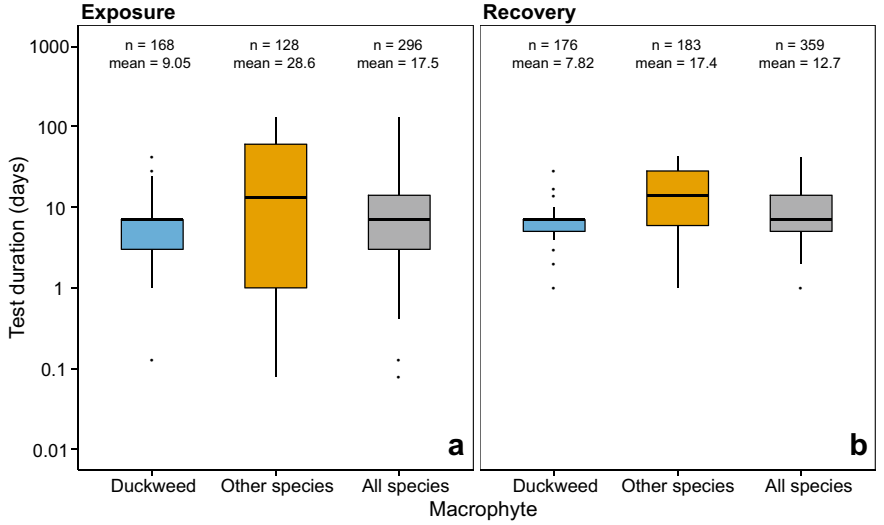

Fig. 6.3 Boxplot with (A) exposure and (B) recovery test durations on macrophytes (duckweed, others, and total) herbicide exposure recovery studies. The *n* and mean listed at the top of the boxplot were the total number and mean unique responses by time point reported

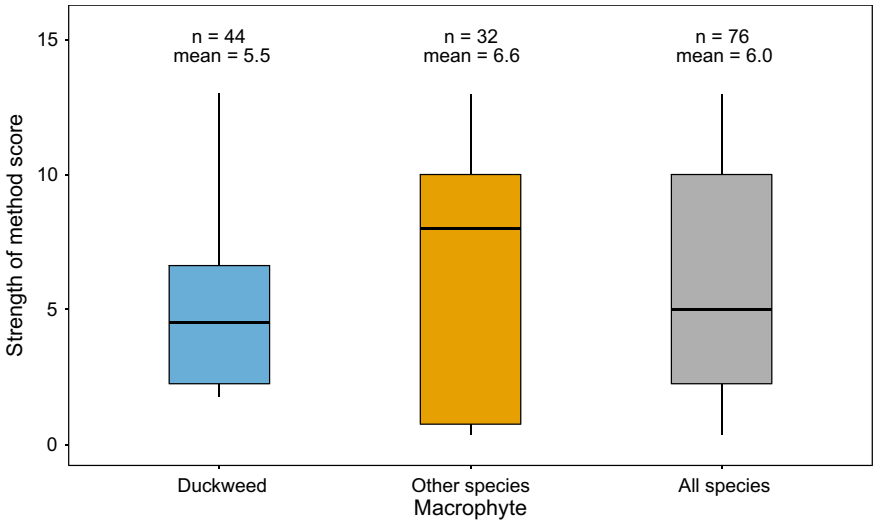

Fig. 6.4 Boxplot with strength of method score for each aquatic macrophytes (duckweed, others, and total). The *n* and mean listed above each boxplot were the total number and mean of individual test species in the studies

The scores as percentages for each criterion are found in Fig. 6.5. Criterion 8 (The initial test organism characteristics were described) was met most commonly for macrophytes (92%, $n = 76$), followed by Criterion 7 (Test organisms strain/source were identified) with 91% ($n = 76$). The criterion least likely to be met was Criterion 15 (Control criteria and performance) for macrophyte studies (18%, $n = 76$).

For critical criteria, Criterion 2 (measured concentrations) was met in 43% of macrophyte tests ($n = 76$). Criterion 5 ($\geq$ three test concentrations, excluding control) was met by 87% of macrophyte studies. Criterion 11 ($\geq$ 3 replicate in each concentration) was met by 83% of macrophytes studies. For Criterion 12 (Appropriate test statistics for NOEC, LOEC, ECx), 78% of macrophyte studies met this requirement.

6.5.4 Ecological Relevance of Endpoint Scores

The majority of endpoint relevance scores for macrophytes from exposure and recovery phases ranged between 2 and 4 (Fig. 6.6). The most common endpoint assessed was reproduction (46%, $n = 130$ for exposure; 46%, $n = 183$ for recovery). For duckweed specifically, the most commonly tested endpoint was reproduction for exposure (70%, $n = 81$) and recovery (73%, $n = 89$). The most commonly tested endpoint class for other macrophyte species was physiological for exposure (53%, $n = 49$) and recovery (43%, $n = 94$).

Table 6.5 List of reviewed studies with the highest strength of method score for macrophytes by tested herbicide group. The studies with a strength of method score (full mark of 15) greater than 7.5 marks are bolded

Herbicide group	Study	Herbicide	Test species	Endpoints	Overall score
ACCase inhibitor	Mohammad et al. 2008	Cyhalofop-butyl, Thiobencarb	*Lemna* sp.	Daughter fronds production (relative growth rate)	1.75*
ALS synthase inhibitor	Wieczorek et al. (2017)	**Iofensulfuron-sodium**	***Myriophyllum spicatum, Elodea canadensis***	**Dry weight of total shoots (growth rate), main shoot length (growth rate), side shoot length (growth rate), and total shoot length (growth rate)**	**12**
Carotenoid biosynthesis inhibitor	**Netherland** (2015)	**Fluridone**	***Hydrilla verticillata***	**Biomass, chlorophyll florescence yield, and shoot biomass**	**11**
Mitochondrial ATP-ase activity inhibitor	Boxall et al. (2013)	**Pentachlorophenol**	***Lemna minor***	**Growth rate and total frond area**	**10**
Pigment synthesis inhibitor	**Wilson and Koch** (2013)	**Norflurazon**	***Lemna minor***	**Frond production**	**12**

(continued)

Table 6.5 (continued)

Herbicide group	Study	Herbicide	Test species	Endpoints	Overall score
PSI inhibitor	Mohammad et al. (2010)	Paraquat	*Lemna gibba*	Frond number, frond number (relative growth rate)	5
PSII inhibitor	Brain et al. (2012b)	**Atrazine**	***Lemna gibba***	**Average growth rate, biomass, and frond number**	**13**
	Burns et al. (2015)	**Diuron**	***Lemna gibba, Lemna minor***	**Plant number, frond number, fresh weight, dry weight, plant number (relative growth rate), frond number (relative growth rate), fresh weight (relative growth rate), and dry weight (relative growth rate)**	**13**
	King et al. (2016)	**Atrazine**	***Ceratophyllum demersum***	**Dry mass**	**13**
Root-growth inhibitor	Knežević et al. (2016)	**Trifluralin**	***Lemna minor***	**Frond number (relative growth rate) and fresh weight (relative growth rate)**	**12**
Shoot-growth inhibitor	Mohammad et al. (2010)	Alachlor	*Lemna gibba*	Frond number, frond number (relative growth rate)	5

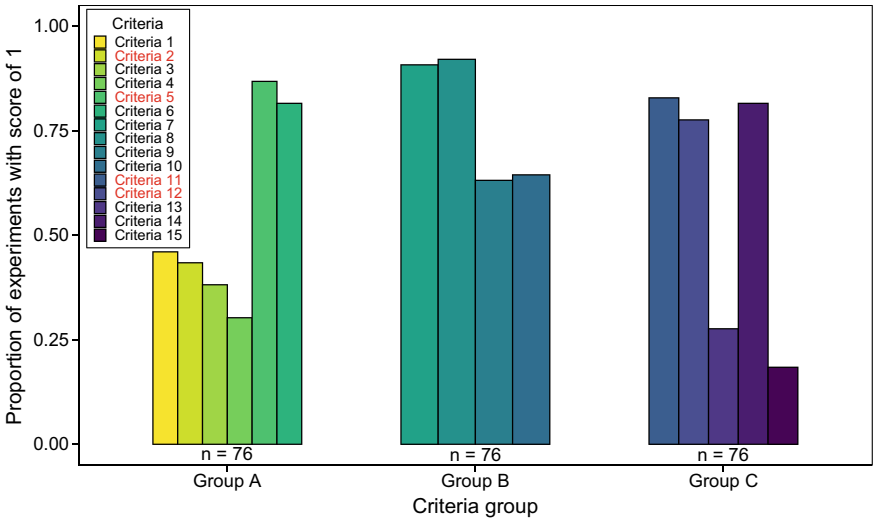

Fig. 6.5 The proportion of experiments with score of 1 for Criteria 1—15 from Group A (test substances), B (test organism and test system) and C (test design, statistics, and results) by aquatic macrophytes ($n = 76$). Criteria 2, 5, 11, and 12 were critical criteria and highlighted in red. The n is the total number of unique tests in each criteria group

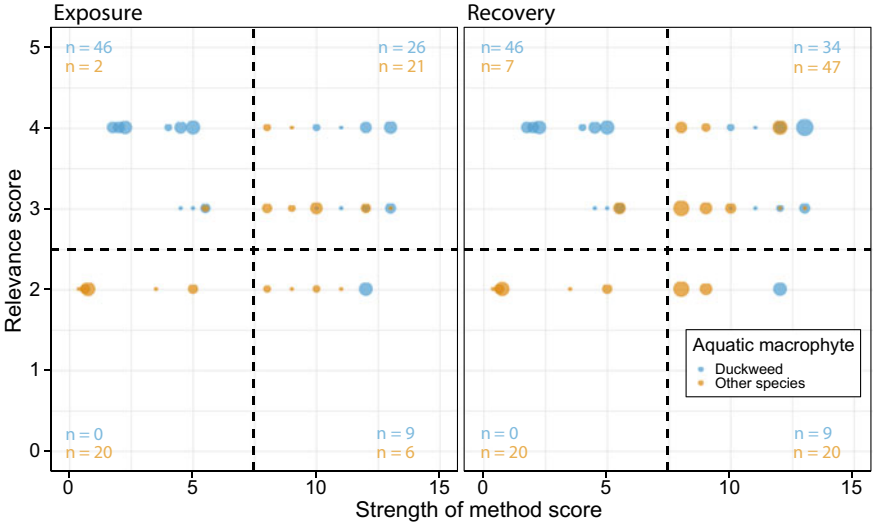

Fig. 6.6 Bubble plot with relevance score versus strength of method score from (A) exposure ($n = 130$ of total number of individual endpoint in exposure) and (B) recovery ($n = 183$ of total number of individual endpoint in recovery) period for aquatic macrophytes (duckweed and other species). The n in each corner is the total number of individual endpoints in each quadrat

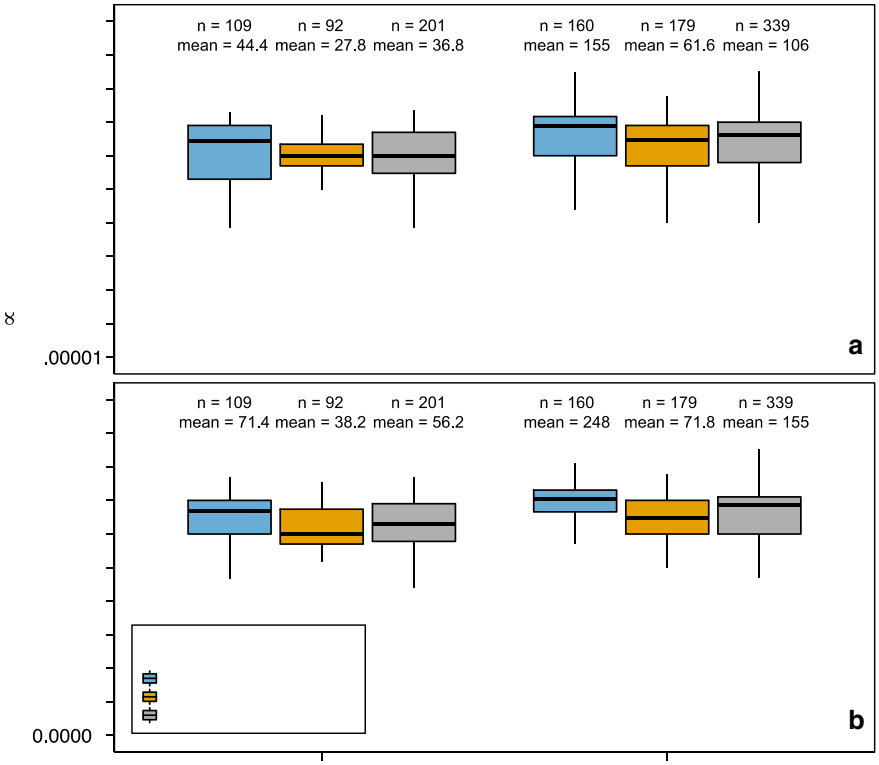

Fig. 6.7 Boxplot of (A) NOECs (no observed effect concentration) and (B) LOECs (lowest observed effect concentration) for macrophytes (duckweed, other species, all species) from the exposure and recovery periods for herbicides. The n and mean at the top of the boxplot were the total number and mean of reported NOECs and LOECs in the reviewed studies

6.6 How to Improve the Assessment of Macrophyte Recovery for Ecological Risk Assessment

Overall, recovery after herbicide exposure was observed consistently across plant species and herbicides tested as indicated by the changes in NOECs and LOECs for exposure and recovery phases, as well as a review of the statements and conclusions of the papers themselves (Fig. 6.7). We did find that many studies would fail to meet our quality threshold to be recommended for inclusion in risk assessment (see Table 6.5 for highest scoring studies). To increase the relevance and reliability of data for risk assessment, we recommend development of a guideline for recovery test procedures and encourage the use of reporting criterion to improve the reliability and relevance of recovery studies in future. The majority of tests were performed with duckweed, which is understandable considering its dominance as an organism for assessing direct toxic effects of contaminants in general, as well as the relative ease by which

recovery can be assessed with this group of plants. Despite this, there is no defined methodology for assessing recovery, which hinders cross compound and species comparisons (ECCC 2007). Standardization of methodologies is important because aspects of the protocol (e.g., the size of test chambers, amounts of nutrients, and duration of the study) can affect growth rates and result in plants reaching the carrying capacity of the test system before the study ends. For example, with duckweed tests, the rates of change in growth endpoints are the most reliable endpoints because affected fronds may have a delayed response (i.e., latency), but after a few days in clean media show normal growth rates comparable to controls. However, since they are delayed in their onset of recovery, they will not catch up to the controls in frond number or biomass as the controls had a "head start". Using rates of change over time gives a clearer and more realistic interpretation of recovery (e.g., Brain et al. 2012b).

To effectively incorporate recovery into ecological risk assessment, the decision-maker may consider the recovery of function or structural attributes via internal and external recovery mechanisms (Barnthouse 2004; Brock et al. 2018). The recovery studies in this review mainly evaluated recovery after herbicide exposure in the lab under controlled conditions. Recovery was observed in both monocot (e.g., *L. minor*) and dicot (e.g., *M. spicatum*) plants and showed that both types of physiologies have the potential to recover after exposure to herbicides. It is rare for laboratory studies to evaluate the toxicity and recovery effect with the interaction of various species from the same (e.g., intra-competition) or different (e.g., grazing pressure) trophic levels (Barnthouse 2004).

Given that laboratory tests do not allow for assessment of external recovery or other internal mechanisms of recovery (e.g., different life stages, seed banks), the true potential to recover following effects related to herbicides is likely underestimated in the available peer-reviewed literature.

Our review found less than half of individual tests provided sufficient information to achieve a score > 7.5 for strength of methods (i.e., be recommended for inclusion in risk assessment). The criterion where most of the studies lost marks was the reporting of control performance. This is consistent with Hanson et al. (2019), where many atrazine primary producer exposure studies lacked information on control performance. Reporting the control value is important for demonstrating that test organisms were healthy and meeting the requirement from standard guidelines (i.e., $\geq$ 8 times increase of *L. minor* frond number within a week), which increases the reliability of the study (ECCC 2007; Hanson et al. 2019). To further increase the number of reliable and relevant studies, journals should provide to researchers strict guidance for reporting and conducting basic toxicity studies.

The need to incorporate realistic exposure concentrations to assess recovery is highlighted in studies where unrealistic concentrations are used to cause toxicity, and no subsequent recovery is observed. In this case, the potential for recovery is not captured, nor can the responses reported be easily extrapolated to the field to make predictions related to actual herbicide exposure. In the case where toxicity occurs, but recovery also occurs at unrealistic concentrations, the uncertainty about recovery at lesser concentrations has in fact been resolved (i.e., it can occur) for risk assessment.

6.7 Conclusions and Recommendations

Macrophytes play important ecological roles in freshwater ecosystems, and have the capacity to recovery from natural stressors through a variety of mechanisms in a relatively short period of time (days to weeks) if conditions are appropriate. Herbicides present a possible risk to these organisms, so understanding the potential for recovery from an herbicide-driven effect is important for reducing uncertainty in ecological risk assessments. Most published studies on recovery by macrophytes from herbicides are lab-based and conducted with *Lemna* spp., and many have data reporting and methodological deficiencies that limit their full incorporation into risk assessments. Moving forward, we recommend that: (1) ecotoxicologists performing response and recovery tests review and implement best practices to reduce uncertainty and improve data quality and reporting (e.g., control performance) for risk assessment overall; (2) test guidelines for duckweed recovery be developed and validated in the lab as well as the field; and (3) the data on the recovery of aquatic plants be incorporated formally into the lower tiers of ecological risk assessment of herbicides where a non-continuous exposure is expected.

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Chapter 7
Vegetated Ditches for Mitigation of Contaminants in Agricultural Runoff

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Abstract The global population is expected to climb to 8.5 billion by the year 2030, and by 2050, it is projected to reach 9.7 billion individuals. Meeting the food and fiber requirements for humanity with finite land resources will require agriculture to continue to increase production while also decreasing potential impacts to natural resources. In addition to in-field conservation practices that focus on tillage reduction and planting of cover crops to prevent soil erosion, edge-of-field conservation practices that mitigate impacts of agricultural runoff are also critical to protect downstream aquatic resources. To develop edge-of-field practices that limit loss of acreage, research was initiated in the 1990s to evaluate the possibility of using vegetated agricultural drainage ditches (VADDs) to mitigate the transport of contaminants (primarily pesticides and nutrients) in runoff. This chapter includes an overview of early vegetated ditch mitigation studies conducted in the USA and the expansion of VADDs research in other countries. In this chapter, we highlight: (1) important concepts behind the use of VADDs; (2) case studies of contaminant mitigation by vegetated ditches; (3) new technologies incorporated within VADDs to further promote contaminant mitigation; and (4) challenges and future research directions. Overall, VADDs show promise for the removal of a range of pesticides and for removals of nitrogen species from agricultural runoff. Studies of phosphorus removals by VADDs show variable results, but advanced ditch designs, additional

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treatment technologies and harvesting of plants during senescence may improve mitigation results. Key parameters for removal efficiencies include plant densities, the length, and the hydraulic retention times of the VADD systems.

7.1 Introduction

Agriculture, including row crops, livestock, and aquaculture, is considered one of the major global contributors to water pollution. Runoff following irrigation or storm events may release pesticides, nutrients, veterinary pharmaceuticals, sediments, and other contaminants into downstream aquatic receiving systems, potentially affecting both ecosystem and human health (UNEP 2016; Mateo-Sagasta et al. 2017). Several in-field conservation practices have been proposed to decrease agricultural runoff, including reducing tillage, cover crops, crop rotation, and nutrient management. However, to adequately address the significant issue of agricultural runoff, edge-of-field practices should also be incorporated into farm management plans. Historically, edge-of-field practices have focused on establishing stiff grass hedges, riparian buffers, grass buffer strips, vegetated waterways, and constructed wetlands. While these practices have a common theme of utilizing vegetation to mitigate agricultural runoff, they also unfortunately require valuable acreage to be removed from production. For instance, constructed wetlands can be a successful strategy for mitigating the transport of contaminants within agricultural runoff (Li et al. 2021; Vymazal and Březinová 2015). However, the costs of lost production acreage might outweigh the environmental benefits (Ilyas and Masih 2017). With this consideration in mind, scientists with the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS) began to seek alternative edge-of-field options to minimize loss of production acreage, while maximizing agricultural contaminant mitigation potential.

7.2 Why Ditches?

Occasionally in research, the solution to a question is already present, albeit slightly hidden by other factors. While examining the agricultural production landscape for alternative edge-of-field mitigation options, one key observation was apparent: the ubiquitous presence and proximity of drainage ditches to fields (Fig. 7.1). Although these ditches are utilized for removing excess water from cropland to prevent damage, their structure led to other questions regarding their potential use (Dollinger et al. 2015). Specifically, could vegetated agricultural drainage ditches (VADDs) mitigate agricultural runoff contaminants? Ditches meet the first requirement for an alternative mitigation option of minimal to no loss of agricultural production, since they are

Fig. 7.1 Examples of vegetated agricultural drainage ditches (VADDs) in early fall (left) and early spring (right). Representative ditches were located in Washington County (left) and Lafayette County (right), Mississippi, USA. Photos courtesy of Matthew T. Moore

already present in the landscape. Ditches also are adaptable to individual farmer or producer needs through specific sizing options and variations in vegetation among climatic regions.

Another key requirement for successful edge-of-field contaminant mitigation is a similarity in function to a constructed wetland. Wetlands are generally identified according to their hydric soils, hydroperiod, and aquatic or semi-aquatic vegetation (i.e., hydrophytes). While not all drainage ditches possess hydric sediment beds, most are of sufficient texture to hold water, since they usually are comprised of sediments from adjacent upland fields. The hydroperiod of each drainage ditch is highly variable, depending on slope, soil characteristics, amount of vegetation, and other physical and geomorphological parameters. Types of ditch vegetation are dependent on regional climatic conditions, position within the ditches (e.g., on slopes or within main channel), amount of water in the ditches, etc. As evidenced above, there is no such thing as a "typical" ditch. Attempts have been made to characterize and classify ditches based on several parameters, but those efforts were generally limited to specific regions, such as the Mississippi Delta in the USA (Bouldin et al. 2004).

Prior to the 1990s, most literature with the keywords "drainage ditch" focused on ditch ecology, including macroinvertebrates, fish, and plant communities, as well as the physical impacts (e.g., sedimentation) of runoff. Several of these studies were conducted in Europe, primarily in fens of the United Kingdom and drainage ditches within the Netherlands. Meuleman and Beltman (1993) were some of the earliest proponents of utilizing vegetated drainage ditches for protecting water quality. In their seminal work, they noted the nation-wide problem of high levels of nutrients in river water in the Netherlands. They proposed routing contaminated water through a system of vegetated drainage ditches or marshlands to utilize biological, physical, and chemical processes for nutrient removal (Meuleman and Beltman 1993). Moore et al. (2001) used this initial concept to design USDA-ARS studies which focused on

pesticide mitigation. Some 20 years after these initial studies of pesticide mitigation were conducted in the USA, VADD research has been adopted in many countries around the world, including China, the Czech Republic, Germany, Finland, Italy, and Mexico (Herzon and Helenius 2008; Bundschuh et al. 2016; Moeder et al. 2017; Soana et al. 2017; Kumwimba et al. 2018; Vymazal and Březinová 2018).

7.3 Case Studies in Contaminant Mitigation

7.3.1 Pesticides

Initial USDA-ARS studies in VADDs focused on mitigation of pesticides in ditches surrounding production acreage which drained into oxbow lakes in the Mississippi Delta, USA. A 50 m stretch of a VADD in the Beasley Lake watershed, which is part of the Mississippi Delta Management Systems Evaluation Area, was dosed with a mixture of well water, the herbicide atrazine, and the insecticide lambda-cyhalothrin to simulate a storm runoff event (Moore et al. 2001). The quantities of pesticides used for dosing simulated a worst-case 5% pesticide runoff from a 0.64 cm rainfall event within a 2.03 ha contributing area. Water, sediment, and plant samples were collected at six locations within the ditch at times before, during, and after the simulated event and at locations 10 m above the inflow, at the inflow, and at 10, 20, 40, and 50 m downstream. Plant density at each sampling location was estimated, and shoot material exposed in the water column of predominant plant species (*Polygonum amphibium*, *Leersia oryzoides*, and *Sporobolus* sp.) were collected, dried, and biomass was estimated. One hour into the simulated event, 61% of atrazine concentrations measured in samples were associated with plant shoot material, while 87% of measured lambda-cyhalothrin concentrations were found in plant shoot material, indicating the importance of vegetated material for sorption of pesticides in agricultural drainage ditches (Moore et al. 2001). Using linear regression, it was determined that the concentrations of both pesticides in water could be reduced to levels below aquatic toxicity thresholds (i.e., $\leq 20\ \mu g\ L^{-1}$ atrazine and $\leq 0.02\ \mu g\ L^{-1}$ lambda-cyhalothrin) within the 50 m monitored stretch of reach, given the initial parameters for runoff and ditch flow (Moore et al. 2001).

However, a parallel ecotoxicity assessment conducted by Farris et al. (2010) indicated that toxicity persisted for 28 d post-application. Ten-day solid phase sediment exposures with larvae of the midge *Chironomus tentans* indicated persistent inhibition of survival and growth by exposures to sediments collected at all six sites in the drainage ditch. Toxicity tests with aqueous samples with the cladoceran *Ceriodaphnia dubia* and with larval fathead minnows, *Pimephales promelas*, indicated that toxicity persisted post-application at all downstream sites. Movement of the sediment-bound atrazine and lambda-cyhalothrin among lower ditch sites was reduced in comparison with the pesticide transport in aqueous samples, but still did not provide sufficient evidence to distinguish between the two pesticide effects upon

observed toxicity. While acute toxicity of sediments collected from the injection site persisted throughout the study, growth impairment also observed in *C. tentans* exposed to sediments from all downstream sites. This study using temporal and spatial sampling throughout 28 d following a simulated storm event failed to identify the duration at which acute exposures to sediment would have no sub-lethal effects in standard toxicity test organisms (Farris et al. 2010).

Information gathered from that first study was used to strengthen the design of the second VADD study in 1999 conducted by the USDA-ARS, where the pyrethroid insecticide esfenvalerate was the pesticide applied in a simulated runoff event discharging into a 650 m ditch. In the study reported by Cooper et al. (2004), the pesticide was premixed with suspended sediment as a slurry before being introduced into the ditch to better simulate a storm runoff event. Spatial and temporal samples of water, sediment, and plants (*Ludwigia peploides*, *P. amphibium*, and *L. oryzoides*; only shoot material exposed in water column) were collected and analyzed for esfenvalerate. Results indicated that 99% of esfenvalerate was associated with plant material, with pesticide half-lives in water, sediment, and plants calculated at 0.12 d, 9 d, and 1.3 d, respectively. Using the linear regression model from Moore et al. (2001), it was determined that, based on initial parameters and conditions, the concentrations of esfenvalerate could be reduced to 0.1% of its original exposure concentration within a 510 m stretch of the VADD, well before entry into downstream Thighman Lake in Sunflower County, MS, USA (Cooper et al. 2004).

The third VADD study in 2000 conducted by the USDA-ARS and reported by Bennett et al. (2005) focused again on the same ditch drainage system studied by Cooper et al. (2004), except the pesticides of interest were now the two pyrethroid insecticides bifenthrin and lambda-cyhalothrin. As in Cooper et al. (2004), a water, pesticide, and suspended sediment slurry was used to deliver the simulated runoff into the ditch. Spatial and temporal sampling of water, sediment, and plants occurred throughout the VADD. Based on mass balance determinations, plants were once again the primary sink or sorption site for both insecticides. Regression modeling determined insecticide concentrations could be decreased to 0.1% of their initial value within a 280 m reach of the 650 m VADD (Bennett et al. 2005).

Following successful experiments in the Mississippi Delta, the research by the USDA-ARS of VADDs expanded to different cropping systems through collaboration with partners in California, USA, interested in utilizing this nature-based technology to address pesticide transport in runoff from various crops, such as tomatoes and alfalfa. While initially, it seemed a simple solution to transfer the VADD technology among agricultural fields, regional differences in farmer practices and state regulations posed challenges to implementation. Whereas farmers in the Mississippi Delta maintain permanent ditches adjacent to fields, many of the ditches in the tomato and alfalfa growing regions of the Central Valley of California are temporary; dug and filled in each year after harvest. Differences in the shapes of ditch channels between the two regions posed challenges concerning hydrology and potential efficacy of pesticide mitigation. Mississippi Delta ditches were generally U-shaped with gentle sloping sides and a broad thalweg, while those in California were more V-shaped with steep slopes and minimal thalweg, owing to the type of implement commonly used

to construct temporary ditches. Vegetation, the primary component of VADD technology, was also an initial concern in transferring this technology from the Mississippi Delta to the California Central Valley. In the Mississippi Delta, vegetation was allowed to grow naturally in drainage ditches to serve as organic carbon sources for mitigation and microbial activity. Many of the plants found in these ditches are ubiquitous within the continental USA. However, California landowners saw them as nuisance vegetation that might provide habitat for other fauna. This concern was triggered by stricter environmental regulations in California. After consulting project partners in the US Environmental Protection Agency (US EPA) and the local Resource Conservation District (RCD) of the USDA, vegetation specific to California that alleviated nuisance or habitat creation concerns for landowners was chosen to be included in the experiments. Vegetation planted included *Hordeum vulgare* (barley) and *Lolium multiflorum* (annual ryegrass). The invasive weed *Chenopodium album* (lamb's quarter) was also prevalent within the vegetated ditches.

The initial California VADD experiment was conducted in Yolo County, where V-shaped vegetated and unvegetated ditches were constructed and dosed for 8 h with a simulated runoff of a slurry of suspended sediment and water containing the insecticides diazinon and permethrin, as reported by Moore et al. (2008). As with previous VADD experiments, water, sediment, and plant (*H. vulgare*, *L. multiflorum*, and *C. album*; shoot material exposed in water column) samples were collected temporally and spatially throughout the experiment. Pesticide half-lives and half-distances were calculated in each ditch. Half-lives were similar, ranging from 2.4–6.4 h, while comparisons of half-distances between vegetated and non-vegetated ditches showed the value of the vegetation in ditch systems. In the V ditches, cis-permethrin half-distances ranged from 22 m in vegetated systems to 50 m in unvegetated systems (Moore et al. 2008). Half-distances for diazinon ranged from 55 m for vegetated V ditches to 158 m for unvegetated V ditches (Moore et al. 2008).

A second VADD experiment in California utilized in situ vegetated field ditches surrounding both alfalfa and tomato fields in Yolo County to evaluate irrigation runoff for removals of the organophosphate insecticide, chlorpyrifos and the pyrethroid insecticide, permethrin. From alfalfa field irrigation runoff, the VADDs decreased the chlorpyrifos concentration by 20% between the ditch inflow and outflow, with 32% of measured chlorpyrifos mass associated with plant (*Leymus triticoides* and *Carex praegracilis*) material. A decrease of 67% in permethrin concentrations between inflow to outflow was measured in a ditch conveying irrigation runoff from a tomato field (Moore et al. 2011).

The California research project also had a separate ecotoxicity component to complement the measurements of chemical concentrations and load reduction in ditches draining irrigation water from tomato and alfalfa fields (Werner et al. 2010). One objective of this research was to validate the use of VADDs as a mitigation practice for selected organophosphate and pyrethroid insecticides. Early life stages of the fathead minnow *P. promelas* and the amphipod *Hyalella azteca* were deployed in custom-built exposure chambers within 400-m sections of two vegetated ditches, and in situ impairment of the organisms was intensively monitored during and after passage of chlorpyrifos and permethrin in ditch runoff. Both compounds are highly

toxic to aquatic invertebrates, such as standard test organisms like *C. dubia* and *H. azetca*, while less so to *P. promelas*. The predicted toxic units (TUs) from the in-stream concentrations in runoff generally agreed with the *C. dubia* 96-h LC50 values in laboratory tests but underestimated the in situ impacts seen with *H. azteca.* Sediments collected near the ditch outflow were toxic to *H. azteca*, but no significant mortality occurred with early life stages of *P. promelas* (Werner et al. 2010). Runoff containing chlorpyrifos remained highly toxic to both species and permethrin continued to elicit toxic responses from *H. azteca.* The VADD failed to reduce pesticide concentrations below the measured effective concentration, which implied an additive or greater than additive impact from the pesticides present in tomato and alfalfa field runoff. There was a modest 15% in situ reduction in toxicity to *H. azteca* at both experimental sites. In contrast, chlorpyrifos and permethrin concentrations were 23% and 50% lower, respectively, at the ditch outflow.

Scientists with the California Department of Pesticide Regulation (DPR) examined the efficacy of a vegetated irrigation ditch to mitigate runoff of chlorpyrifos associated with alfalfa irrigation (Gill et al. 2008). The ditch (2 m width × 200 m length) was planted with several native perennial grasses such as *Dactylis glomerata*, *Agroyron trichophorum*, *L. triticoides, Elymus glaucus*, and *H. brachyantherum.* For comparison, a conventional unvegetated V ditch was similarly evaluated. Results indicated that overall, chlorpyrifos concentrations were significantly lower in the outflow of the vegetated ditch than those observed in the unvegetated ditch outflow. As irrigation events continued to occur, there was a trend of a slight decrease in the removals of chlorpyrifos, but these reductions were not significantly different among the various irrigation events. The median reduction in chlorpyrifos concentration was 38% in the vegetated ditch, while only a 1% reduction was observed in the conventional unvegetated ditch (Gill et al. 2008).

Rogers and Stringfellow (2009) further investigated the mechanisms for partitioning of chlorpyrifos between plants and sediments within VADDs using a batch equilibrium method for kinetics experiments. Plants and a homogeneous sediment mixture collected from a VADD located in Stanislaus County, CA, USA, were used in controlled experiments, and a standard soil from San Joaquin, CA, USA, was utilized as a reference comparison. Plant species examined included *Triticum aestivum, Lolium* sp., *Medicago sativa, Typha* sp., and *Juncus patens*. Sorption coefficients (K_d) were more than 10 times higher in plants (570–1300 L kg^{-1}) as opposed to soil and sediment (40–71 L kg^{-1}). Plant sorption of chlorpyrifos was in the order of *Typha* sp. > *T. aestivum* > *J. patens* > *Lolium* > *M. sativa* (Rogers and Stringfellow 2009). This study was one of the first definitive evaluations into the partitioning capacity of different species of plants. This study indicated that aquatic macrophytes (e.g., *Typha* sp.) with a high internal surface area due to porous tissues may be more effective for accumulating chlorpyrifos than hollow terrestrial plant species (Rogers and Stringfellow 2009).

Several European studies on VADDs and pesticide mitigation have been conducted in Germany and Italy. Bundschuh et al. (2016) provided an extensive characterization of aquatic fungicide exposure at base flow and during rainfall events that occurred with German viticulture, contributing to catchments at a large spatial scale. The

VADDs and vegetated detention ponds containing *Phragmites australis, Glyceria* sp., *C. elata*, *Iris pseudacorus*, *Veronica beccabunga*, *Lemna minor*, *T. angustifolia*, and *Sparganium erectum* were monitored across four seasons reduced the median fungicide concentrations and their associated ecotoxicological potential by 56% and 38%, respectively (Bundschuh et al. 2016). During runoff events, the TU approach was used within the Uniform Principle of the European Union for Tier I pesticide risk assessment. Given the properties of the mitigation systems, the short-term peaks of runoff events, and physico-chemical characteristics of the targeted fungicides (azoxystrobin, boscalid, cyprodinil, dimethomorph, myclobutanil, penconazole, pyrimethanil, tebuconazole, triadimenol, and trifloxystrobin), the reductions in measured concentrations of fungicides were consistent with the acute and chronic toxicity data for aqueous samples collected during runoff and base flow. Loads of fungicide mixtures detected during base flow indicated low risks for aquatic ecosystems. Additionally, plant coverage, water depth, hydraulic retention time (HRT), flow length of the system as well as fungicide-specific partition coefficients (i.e., Log P) explained about 55% of the variability seen in detention ponds, in contrast to similar variables accounting for only 15% of the variability in the VADD systems. The importance of plant density was emphasized in these studies, as high densities contribute to greater surface areas for adsorption and for processes involving receptors and microbial degradation (Bundschuh et al. 2016).

Otto et al. (2016) utilized a field experiment on a VADD in the Po Valley of Italy to determine whether field-measured mitigation efficiencies matched predictions generated from a fugacity model. Water containing the herbicides mesotrione, *S*-metolachlor, and terbuthylazine was pumped through the VADD, and two subsequent flushes of herbicide-free water were conducted 27 d and 82 d after the original dosing event in order to assess potential herbicide wash off. Herbicide concentrations were reduced by at least half in the VADD, regardless of the flooding conditions. In the field experiment, herbicide half-distances were approximately 250 m. However, subsequent flood events indicated that these herbicides may be remobilized after initially being sorbed to plant material, although the observed herbicide concentrations were still below drinking water limits (Otto et al. 2016).

7.3.2 Nutrients

In the early 2000s, studies by the USDA-ARS began to focus on the use of VADDs to mitigate the transport of nutrients entering the Lower Mississippi River Basin, with the aim of reducing hypoxia in the receiving waters of the Gulf of Mexico. Kröger et al. (2007a, 2008) reported on the ability of VADDs to reduce both nitrogen (N) and phosphorus (P) concentrations and loads leaving production agriculture fields in monthly baseflow and under individual storm flow conditions. Two vegetated ditches (*L. oryzoides*, *Sagittaria latifolia*, *J. effuses*, and *Echinodorus cordifolius*) surrounding fields planted in continuous no-till cotton were monitored for two years for nitrate, nitrite, ammonium, and orthophosphate. During the growing

season, both nitrate and ammonium were reduced over the length of the monitored vegetated ditches while storm loads of dissolved inorganic N were reduced by 57% (0.84 kg ha^{-1} yr^{-1}) by the VADDs (Kröger et al. 2007a). Phosphorus mitigation proved to be seasonal, with the ditches alternating between a P sink and a source. Annually, the ditches reduced the maximum inorganic P load leaving the ditches by approximately 44% (Kröger et al. 2008).

Expanding upon studies by Kröger et al. (2007a, 2008), two agricultural ditches (one vegetated, one unvegetated), similar in size, landform, and location in the Mississippi Delta were utilized by Moore et al. (2010) to determine nutrient mitigation during a simulated storm runoff event. No significant differences were observed between the two ditches in reductions of nitrate, ammonium or dissolved inorganic P between the inflow and outflow. Total inorganic P loads were reduced by 71 ± 4% in the non-vegetated ditch, while there was only a 36 ± 4% reduction in the vegetated ditch (Moore et al. 2010). The reductions in phosphorus loads were not unexpected in the unvegetated ditches, since sediments can provide significant P binding potential. Kröger and Moore (2011) examined P dynamics in drainage ditch sediments across a range of sites within the Lower Mississippi River Basin. Their results indicated most drainage ditch sediments had low immediately bioavailable P, with a degree of P saturation of <20% (Kröger and Moore 2011). However, since these ditches had low P binding energy (0.34–0.60 L mg^{-1}) and low P sorption maxima (17.8–26.6 L mg^{-1}), they may not necessarily serve as P sinks. These results highlight the challenges for VADD nutrient mitigation within ditches. Results are often highly variable, depending on local conditions. Collins et al. (2016) utilized in situ mesocosms in two ditches on a cattle ranch in the Everglades region of Florida, USA to examine P sorption potential. One ditch had an organic sediment and was vegetated with *Pontederia cordata*, while the second ditch had a mineral sediment and was vegetated with a mixture of *Eichhornia crassipes* and *L. minor*. Results indicated P uptake was greater and was also subsequently released more rapidly in the vegetated ditch. It was determined that vegetated ditch residence time (0.46 and 0.11 days) was insufficient to promote mitigation of P, and an extension of residence time would benefit P mitigation (Collins et al. 2016).

Taylor et al. (2015) utilized mesocosms to explore the VADD concept and attempt to differentiate mechanisms which may be affecting N mitigation. Using three treatments (unvegetated control), rice cutgrass (*L. oryzoides*), and common cattail (*T. latifolia*), they examined the N mitigation capability of treatment systems, while also quantifying denitrification rates in each system. Systems with *L. oryzoides* retained 68% of nitrate loads in simulated runoff exposures, while *T. latifolia* and unvegetated controls retained 60% and 61%, respectively. Sediment cores removed from mesocosms planted in *L. oryzoides* had significantly higher mean denitrification rates (5.93 mg m^{-2} h^{-1}) than either *T. latifolia* or unvegetated controls (0.2 mg m^{-2} h^{-1} and −0.19 mg m^{-2} h^{-1}, respectively), indicating the strong potential for permanent removal of excess N through microbially mediated denitrification (Taylor et al. 2015).

Soana et al. (2017) utilized reach scale methods and laboratory incubations to estimate plant nutrient uptake in a study within the Po River plain of northern Italy.

N removal, primarily via denitrification, was greater within vegetated ditch reaches (38–84 mmol N m^{-2} d^{-1}) than in unvegetated reaches (12–45 mmol N m^{-2} d^{-1}), as reported by Soana et al. (2017). Castaldelli et al. (2018) utilized mesocosms to evaluate denitrification in systems vegetated with *P. australis* and unvegetated systems under a range of runoff flow velocities (0–6 cm s^{-1}). Results indicated that vegetated sediments had more denitrification activity (27–233 mmol N m^{-2} d^{-1}) than did unvegetated sediments (18–33 mmol N m^{-2} d^{-1}), as reported by Castaldelli et al. (2018). Likewise, nitrate removal and denitrification rates increased by an order of magnitude when the water velocity increased from 0 to 6 cm s^{-1} in the vegetated systems. Zhang et al. (2016) conducted a field-scale experiment examining ammonium removal and reduction of nitrous oxide emissions in ditches vegetated with *P. cordata* and *Myriophyllum elatinoides* versus unvegetated ditches. Results indicated that vegetated ditches increased ammonium removal while simultaneously decreasing nitrous oxide emissions (Zhang et al. 2016). Dominant ammonium removal pathways differed between the two plant species, with *M. elantinoides* vegetated ditches achieving removal primarily by plant uptake and by nitrification–denitrification processes mediated by microbes. Alternately, unvegetated ditches and those vegetated with *P. cordata* removed ammonium via sediment sorption (Zhang et al. 2016).

Soana et al. (2018) conducted mesocosm experiments to elucidate nitrate mitigation via denitrification within microbial biofilms colonizing dead *P. australis* stems during winter. Using chlorophyll *a* content as a proxy for the proportion of the autotrophic community on the biofilm, Soana et al. (2018) reported *P. australis* vegetated sediments were more efficient in conversion of nitrate through denitrification (7–17 mmol N m^{-2} d^{-1}) than were unvegetated sediments (3–5 mmol N m^{-2} d^{-1}). Results of this study indicated the best practice for ditch maintenance was to postpone mowing until the end of winter to promote nitrate removal throughout the year (Soana et al. 2018). Soana et al. (2019) conducted watershed modeling within the lowlands of the Po River Basin in Italy to determine nitrate mitigation through denitrification and the effects of ditch maintenance (mowing) within the watershed. Based on the current maintenance techniques, 11% of excess N was removed from the system (3300–4900 t N yr^{-1}). However, this could be improved to 4000–33,600 t N yr^{-1} if 90% of vegetated ditches were maintained (Soana et al. 2019). Additional gains in denitrification could be made by delaying ditch mowing at the end of the growing season, as pointed out previously by Soana et al. (2018).

Kumwimba et al. (2016) examined the nutrient mitigation capacity of six plant species, *Canna indica*, *Cyperus alternifolius*, *Colocasia gigantea*, *Acorus calamus*, *I. sibirica*, and *M. verticillatum*, and reported removal efficiencies ranging from 97–99%, 98–100%, and 90–98% for total N, ammonium, and total P, respectively. They also noted an 85–95% increase in aboveground biomass as plants sequestered nutrients, but a rapid nutrient loss occurred after 70 d during the senescent period (Kumwimba et al. 2016). Harvesting of plant biomass prior to senescence was suggested as a possible remedy for preventing nutrient release back into the vegetated ditch system. Kumwimba et al. (2020) examined VADD ability to retain nutrients during periods of low temperatures in China. Overwintering plants in the VADD

included *A. gramineus*, *M. aquaticum*, and *I. sibirica.* Approximately 43–46% of N species were retained, with 46–52% of P species being retained by the VADD in low temperatures. It was estimated that 5.37×10^3 kg N y^{-1} and 0.65×10^3 kg P y^{-1} were removed by the VADD. Further plant senescence did result in release of nutrients during the experiment, so caution in design was noted (Kumwimba et al. 2020). Other studies have also demonstrated the potential for nutrient release during plant senescence but relied on experiments designed to represent worst-case scenario by using chambers with plant material and water only to estimate nutrient release rates (Peverly 1985; Kröger et al. 2007b; Menon and Holland 2014; Wang et al. 2018). In contrast, Taylor et al. (2020) measured P release from senescent plant material in mesocosms during rain events throughout winter. Mesocosms representing intact ecosystems with sediment, root systems, senescent plant biomass, and associated microorganisms demonstrated balanced retention; that is, import during the growing season and export during the senescent period when exposed to low P loads. However, mesocosms receiving high P inputs had high retention (80–90%), which could be partially explained by excess P being translocated to extensive root systems in mesocosms (Taylor et al. 2020).

7.3.3 Complex Mixtures

Early studies of VADDs by the USDA-ARS focused on a particular contaminant class, such as pesticides or nutrients. However, ditches receive a variety of contaminants, many of them simultaneously. Several studies have examined overall VADD efficiency regarding complex mixtures of contaminants. In a mesocosm experiment, Moore and Locke (2020) examined the capacity of typical plant species in VADDs, *M. aquaticum*, *P. amphibium*, and *T. latifolia* to remove nutrients (orthophosphate, nitrate, and ammonium) as well as three pesticides, clomazone, propanil, and cyfluthrin during a simulated runoff event. The target inflow concentration for each nutrient species was 10 mg L^{-1}, while pesticide inflow concentrations were 20 μg L^{-1} for both propanil and clomazone and 10 μg L^{-1} for cyfluthrin. The simulated storm event was applied to individual mesocosms for 6 h (representing the HRT), followed by 48 h with no flow, a then a 6 h flush with unamended (no nutrients/pesticides) water. Samples were collected temporally throughout the experiment and load reductions were calculated. In the vegetated mesocosms, mean percent load reductions for orthophosphate, ammonium, and nitrate ranged from 39–52%, 47–62%, and 50–59%, respectively, while in the unvegetated mesocosms, mean percent load reductions were 42%, 52%, and 54% for orthophosphate, ammonium, and nitrate, respectively (Moore and Locke 2020). Cyfluthrin retention varied only slightly between the unvegetated (76%) and vegetated systems (ranging from 79–86%). Similar results were noted for propanil, with 69% retention in unvegetated systems, while retention in vegetated systems ranged from 63–71%. Mesocosms vegetated with *P. amphibium*

were most efficient at retaining clomazone (63%), followed by mesocosms vegetated with *T. latifolia* (44%), *M. aquaticum* (8%), compared to 5% retention in the unvegetated system (Moore and Locke 2020).

Kumwimba et al. (2021) evaluated the effectiveness of VADDs in rural China to mitigate nutrients and metals from rural wastewater, as well as identifying the standing stock concentrations in associated vegetation (*M. verticillatum*, *Acorus gramineus*, *Thalia dealbata*, *C. alternifolius*, *Hydrocotyle vulgaris*, *I. pseudacorus*, *C. gigantea*, *P. australis*, *A. calamus*, and *C. indica*) N and P species were reduced by 48–63% and 51–58%, respectively. Additionally, Ni, Cu, Cr, Zn, Cd, Pb, As, Fe, Al, and Mn were reduced by 50%, 56%, 63%, 79%, 67%, 80%, 60%, 52%, 19%, and 24%, respectively (Kumwimba et al. 2021). The primary location of metals in the plants was in either in the stems or roots, with Al, Fe, and Mn having the highest recorded concentrations. Based on their data, Kumwimba et al. (2021) determined plant biomass harvesting in either August or early September was optimal for effective metal removal in the VADD.

In Mexico, Moeder et al. (2017) studied a 3.6 km section of a VADD in Sinaloa State receiving both agricultural runoff and discharges of domestic wastewater from a nearby community. Five different points along the ditch were monitored on a monthly basis for 38 different pollutants, including pesticides, polycyclic aromatic hydrocarbons, artificial sweeteners, and pharmaceuticals. Sediment and plant samples were collected three times during the year and also measured for concentrations of pollutants. Results indicated that cattails (*T. domingensis*) absorbed 10 of the 38 measured pollutants and sediment sorption was of minimal influence. It was hypothesized that microbial activity and the subtropical climate contributed to effective pollutant mitigation within the VADD.

Vymazal and Březinová (2018) monitored a 200 m VADD for two years in the Czech Republic to examine its ability to mitigate levels of N and P, suspended solids, biochemical oxygen demand (BOD), and chemical oxygen demand (COD). Ditch vegetation was dominated by *P. australis*, *T. latifolia*, and *Glyceria maxima.* N and total P removal were estimated at 1,070 kg ha^{-1} y^{-1} and 804 kg ha^{-1} y^{-1}, respectively. Fourteen percent of removed P load was attributed to plant uptake (Vymazal and Březinová 2018). Removal of suspended solids, BOD, and COD were 2,0437 kg ha^{-1} y^{-1}, 1,500 kg ha^{-1} y^{-1}, and 7,000 kg ha^{-1} y^{-1}, respectively. Nitrogen and organic removal were influenced by temperature, whereas P and suspended solids removal were not affected by temperature (Vymazal and Březinová 2018).

7.3.4 New Technologies in VADDs

A common misconception is that by utilizing an individual conservation practice, one can solve all the environmental issues for a particular location. While individual practices can certainly have a significant impact, suites of various conservation practices typically provide improved mitigation efforts needed to meet environmental goals.

The capacity of VADDs to mitigate contamination from agricultural runoff is well-documented in the literature and is summarized in this chapter, but VADDs cannot to be considered a "silver bullet" for mitigating all agricultural runoff. Instead, it should be viewed as a valuable tool in a larger toolbox. Goeller et al. (2020) provides an excellent resource for "tool stacking" for N mitigation. They describe various N mitigation tools that can be implemented at multiple locations within a watershed for better nutrient attenuation. Locations of these mitigation measures include in-stream, within channel margins, riparian buffer and floodplains, and edge-of-field tools. Below is an overview of both old and new technologies that have been incorporated within VADDs that demonstrate the same "tool stacking" concept.

Perhaps the best example of technologies incorporated within VADD is research recently published by Phillips et al. (2021) describing an integrated vegetated treatment system (VTS) which included a sediment trap, vegetated ditch, compost swales, and a granulated activated carbon (GAC) or biochar polishing filter. Each component of the VTS was designed with a specific purpose in mind: coarse particulates would be removed by the sediment trap, while suspended sediment and insecticides would be removed by the vegetated ditch and compost swales. Any residual pesticides remaining would be treated by sorption using either GAC or biochar. Additionally, irrigation water for these experiments was treated with polyacrylamide (PAM), a long-chain polymer commonly used for erosion control, to minimize concentrations of suspended sediments. Both simulated and actual runoff events were examined for the ability of VTS to reduce concentrations and loads of the neonicotinoid insecticide, imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine] and the pyrethroid insecticide, permethrin. In the series of simulated runoff events, the VTS reduced the suspended sediment, imidacloprid, and permethrin concentrations by 30–82%, 88–92%, and 98–100%, respectively, in tests with the GAC polishing treatment. With a biochar polishing treatment, concentrations of suspended sediment, imidacloprid, and permethrin were reduced 42–85%, 89–94%, and 98–99%, respectively (Phillips et al. 2021). Following VTS treatment and GAC polishing, loads of suspended sediment, imidacloprid, and permethrin were reduced by 63–95%, 94–98%, and 98–100%, respectively. When biochar was substituted for the polishing step, load reductions were 82–96%, 98–99%, and 99–100%, respectively, for suspended sediment, imidacloprid, and permethrin. When actual runoff events from a lettuce field were routed through the VTS, concentrations of suspended sediment, imidacloprid, and permethrin were reduced by 78–84%, 74–80%, and 48–100%, respectively. Load reductions for these same events were approximately 98%, 99%, and 97%, for suspended sediment, imidacloprid, and permethrin, respectively (Phillips et al. 2021).

Addition of physical structures in VADDs have also been suggested to improve mitigation of agricultural runoff. Flora and Kröger (2014) examined in a mesocosm experiment the value of constructing low-grade weirs to VADDs containing *L. oryzoides* and *T. latifolia* that receive aquaculture pond effluent. In systems without weirs, there was a decrease in TP, total ammonia nitrogen (TAN), and nitrate loads of 47%, 43%, and 63%, respectively, but there was also a 154% increase in the loads of soluble reactive phosphorus (SRP). In VADDs with low-grade weirs, decreases of

SRP, TP, TAN, and nitrate loads were 80%, 86%, 89%, and 89%, respectively (Flora and Kröger 2014). Low-grade weirs increased the HRT by 32% and decreased flow velocity, which likely contributed to decreases in nutrient loads exiting the system. Iseyemi et al. (2018) reported that VADDs with weirs had significantly lower bioavailable P (0.018 mg g^{-1}) than VADDs without weirs (0.021 mg g^{-1}). Mean P sorption maxima ranged from 139–671.8 mg kg^{-1} in the summer to 525–1288 mg kg^{-1} in winter, with P binding energy measurements ranging from 0.63–1.34 L mg^{-1} in the summer to 0.09–0.30 L mg^{-1} in the winter (Iseyemi et al. 2018). Kröger et al. (2011, 2012) found the use of low-grade weirs created zones upstream which allowed for sedimentation to occur and further improved denitrification and bound P removal.

Faust et al. (2018a) provided a review of enhanced mitigation methods utilized in VADDs, in addition to VADDs alone. Riser and slotted board pipes had some reported success in decreasing nitrate loads in tile drainages, typically in northern and eastern states of the USA, but they noted limited evidence of the ability of these structures to enhance removals of ammonia, TN, TP, or total suspended solids (Faust et al. 2018a). Another physical structure similar in concept to VADD is the two-stage ditch. These special ditches are often used in the Upper Mississippi River Basin in the USA, and the "benches" created in these systems may serve to increase denitrification levels (Mahl et al. 2015; Powell and Bouchard 2010; Roley et al. 2012; Speir et al. 2020).

Faust et al. (2018a, b) also discussed incorporation of organic carbon amendments (if limiting) in VADDs with physical structures, such as weirs, as an additional step toward increased denitrification in these systems. Organic carbon amendments may include various wood media, corn stover (leaves, stocks, and cobs left over after harvest), rice straw, or Bermuda grass hay (Faust et al. 2018a). Eighty-nine drainage ditch sediments were collected from 35 sites throughout the Lower Mississippi Valley, USA, to determine baseline organic carbon and N content, finding organic carbon content ranging from 0.253% to 6.04% (Faust et al. 2018b). These contents are well below sediment organic carbon expected in restored and natural wetlands, so organic carbon may be limiting denitrification in Lower Mississippi Valley drainage ditches. Nifong et al. (2019) used a flow through, intact sediment core experiment to assess the effects of gypsum or hardwood mulch overlying layers on ditch sediment denitrification. Sediment denitrification rates were 0.6, 1.3, 9.2, and 11.2 mg N_2-N m^{-2} h^{-1} for control, gypsum, mulch-gypsum, and mulch cores, respectively. Mulch and mulch-gypsum treatments were estimated to remove 65 and 69% of N loads, respectively (Nifong et al. 2019). Cai et al. (2021) examined amendments with rice straw and the mineral zeolite (independently and in combination) in bench-scale experiments designed to improve N removal in drainage ditches receiving either high ammonium or high nitrate concentrations with low carbon content. Ammonium removal rates were greatest in the rice straw-zeolite combination (48.9–77.7%), as were nitrate removal rates (67.6–82.7%). The presence of microbial denitrifying genes (*nirK*, *nirS*, *narG*, and *napA*) was significantly enhanced in the rice straw-zeolite mixture, contributing to the improved nitrate removals (Cai et al. 2021).

Studies have also evaluated various VADD amendments for improved removal of P compounds. He et al. (2021) studied the use of akadama clay (particle size

1–6 mm) barriers in mesocosm-scale experiments as a method of removing phosphate in VADDs. Akadama clay is a reddish-brown granulate derived from volcanic sources in Japan, although it is exported around the world. Highest P removals were in VADD mesocosms with akadama clay barriers that had the following characteristics: akadama particle size of 1 mm; 10 cm height; and 90 cm length. Removal efficiencies were 97.1% for TP, 96.9% for particulate P, and 97.4% for dissolved P (He et al. 2021). While not specifically examining VADDs, Penn et al. (2007) did address several P sorbing materials that could be used to mitigate drainage ditch water. Materials suggested included limestone, byproduct gypsum, quick lime, and alum. They noted several factors including material cost, availability (including transportation), potential contaminants, physical properties, and P sorption characteristics as issues of concern for choosing a P sorbing material. When examining the physical properties of sorbing materials, it was noted that an acidic pH is most effective for P sorption with Al and Fe complexes, while Ca and Mg are more effective at pH 6–7.5 for precipitation of P (Penn et al. 2007). How these sorbing materials are implemented was also addressed, including the potential of "flow dosing," broadcast application, or the use of flow through structures, such as filter socks (Penn et al. 2007).

7.3.5 Challenge: VADD Maintenance

Maintenance of VADDs is by far the most common challenge of concern to both researchers and landowners. Routine ditch maintenance, including dredging, mowing, chemical weeding, and burning is necessary for flood attenuation and to maintain proper flow. How often and to what degree maintenance must occur is a critical knowledge gap. This is not merely a simple engineering issue of hydraulics. If VADDs are utilized for drainage and contaminant mitigation, a balance must be maintained to allow sufficient water flow, while still providing vegetation and other organic carbon sites for binding of nutrients and pesticides. Dollinger et al. (2015) reviewed drainage ditch design and maintenance, noting the key parameters for maintaining ecosystem services in VADDs were the degree of vegetated cover, ditch morphology, reach connections, and slope orientation. They similarly noted that the geochemical, geophysical, and biological processes providing these ecosystem services varied widely with different ditch characteristics, but in general, there were low adverse effects on biodiversity conservation and water purification abilities if VADDs were mowed during the proper season (Dollinger et al. 2015). Iseyemi et al. (2019) examined carbon sequestration capabilities of mowed and unmowed VADDs (with and without weirs) in summer and winter experiments. Average carbon content in mowed and unmowed ditches was 16.54 ± 0.52 and 16.60 ± 0.44 g kg^{-1}, respectively in summer, while winter averages were 15.86 ± 0.71 g kg^{-1} and 14.89 ± 0.77 g kg^{-1}, respectively for mowed and unmowed ditches. These results indicate no difference in maintained and unmaintained ditches regarding average carbon content (Iseyemi et al. 2019).

Another issue with VADD maintenance deals with the question of plant senescence. If plants take up excess nutrients during the growing season, what happens when the plants die? Will those nutrients then be released back into the ditch, nullifying any potential mitigation to downstream aquatic resources? Kröger et al. (2007b) examined this question by studying microcosms filled with harvested *L. oryzoides* plants in decomposition bags soaked in a control water of known nutrient composition. Treatments examined included enriched *L. oryzoides* samples (those exposed to 3.8 g m^3 of N and P), *L. oryzoides* samples from a reference ditch with no enrichment, and control microcosms with only water and no *L. oryzoides* plant samples. Decomposition and leaching were monitored for a 12-week period from December until February. Senescence of the enriched *L. oryzoides* samples resulted in higher concentrations of P present in microcosm water (2.19 ± 0.84 mg P L^{-1}) (Kröger et al. (2007b). Taylor et al. (2020) further elucidated N and P dynamics in larger mesocosms observed during both the summer growing and winter decomposition seasons using *L. oryzoides* as the model plant. Throughout the experimental seasons, both measured N retention and modeled denitrification rates did not vary between treatments; however, retention of P increased significantly with P enrichment treatments. They also reported winter export of N was less than 10% of the observed summer N uptake, and denitrification was likely responsible for approximately 40% of retained N. In mesocosms that lacked P enrichment, there was only 25% retention in the winter, while net P retention increased from 77 to 88% as enrichment treatments increased (Taylor et al. 2020).

Furthermore, a recent study by Martin et al. (2021) illustrated that greater bed and bank vegetative coverage in VADDs provided improved water quality when comparing upstream and downstream sites, while showing that nutrient values were higher in the non-production season relative to the production season. This further illustrates the importance of vegetation density and the potential negative mitigative effect due to senescence, indicating that VADD seasonality requires consideration when utilizing this mitigation strategy.

7.3.6 Future Directions

Vegetated agricultural drainage ditches hold significant promise as effective edge-of-field contaminant mitigation sites, even though results may vary by field, region, and contaminant due to a myriad of factors. Kumwimba et al. (2018) published a review of VADD designs, various management strategies, and other mechanisms which affect retention of contaminants in agricultural runoff, as well as components of domestic sewage. These authors highlighted the importance of vegetation, ditch substrate, and microbial activity as three key parameters for individual VADD success. Suggestions for future VADD research included further investigation on the effect of size, length, and slope of VADDs, vegetative cover and type, ditch substrate, microbial biofilms, organic carbon amendments for denitrification, impacts of low-grade weirs, and various maintenance practices for vegetation and substrates (Kumwimba et al. 2018).

While several aspects of nutrient mitigation were discussed in this chapter, the future direction of VADD research with regard to nutrients will likely gravitate toward studies that integrate spatial and temporal patterns in processes. For example, using the principles developed for stream metabolism (Hall and Hotchkiss 2017), research focused on agriculture can begin to understand linkages between biological processes and nutrient mitigation in VADDs. Recent USDA-ARS research utilized sensor measurements (i.e., dissolved oxygen and temperature loggers) and hourly sampling in artificial streams vegetated with *L. oryzoides* to determine N and P uptake, gross primary productivity (GPP), ecosystem respiration (ER), and denitrification (Nifong et al. 2020a; Nifong and Taylor 2021). Vegetated ditches had significantly higher N uptake rates and removal (2 h: 63%, 4 h: 44%, 6 h: 32%) than unvegetated ditches (2 h: 32%, 4 h: 21%, and 6 h: 17%). In vegetated ditches, GPP and ER were significantly higher, and an increased HRT resulted in increased respiration rates (Nifong and Taylor 2021). Studies of this magnitude, while more complex than simple measurements of inflow versus outflow, provide key details in mechanisms and expectations for nutrient mitigation in VADDs. Similar approaches based on diel patterns in dissolved N_2 gas are being explored to integrate denitrification estimates across temporal and spatial scales within ditches (Nifong et al. 2020b).

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Chapter 8
The "Green Liver" Concept: Green Liver Systems as Low-Impact Systems for Bioremediation Using Aquatic Macrophytes

Maranda Esterhuizen and Stephan Pflugmacher

Abstract Systems with aquatic macrophytes have been developed as low-impact, low-energy, and low-cost solutions for the remediation of water pollutants. As plants metabolize toxicants in much the same way that an animal liver would, the name "*Green Liver Systems*" was chosen. This technology relies on the ability of aquatic macrophytes to take up, biotransform, and intracellularly store xenobiotics; thereby, in this way, not releasing metabolites with unknown toxicity into the environment. Based on the wastewater pollutant composition, macrophytes can be screened in the laboratory to evaluate their affinity for contaminant uptake and select the most appropriate species for the system. The results of laboratory screening experiments to determine the uptake potential and remediation efficiency of several macrophytes species are presented here. The combination of the macrophytes used in the system may also affect the remediation potential due to differing uptake and biotransformation rates and possibly allelopathic effects. Therefore, various combinations of macrophytes can be used as a toolbox for correcting water quality issues. The most commonly utilized macrophytes in large-scale Green Liver Systems to date include species from the genera *Ceratophyllum*, *Myriophyllum*, *Elodea*, *Egeria*, *Azolla*, and *Pontederia* (formerly *Eichhornia*). The practical and large-scale application of this

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technology is illustrated with two case studies in China and Brazil. Additionally, the benefits and limitations of Green Liver Systems are discussed. Methods for utilizing spent biomass as a byproduct of Green Liver Systems need to be developed to ensure its sustainable maintenance as a green, low-impact phytoremediation system.

8.1 Necessity of Sustainable Water Remediation

Even though, the majority of the earth's surface is covered by water, freshwater amounts to less than 3% thereof, with the majority sequestered as ice and groundwater (Hinrichsen and Tacio 2002). The quality of freshwater ecosystems, such as lakes, streams, rivers, swamps, reservoirs, and aquifers, is deteriorating due to eutrophication and increased pollution (e.g., microorganisms and their toxins, pesticides, pharmaceuticals, and heavy metals), disrupting the health of these water bodies and drastically influencing biodiversity (Albert et al. 2021). Other impacts of polluted aquatic ecosystems include economic, social, and environmental consequences (Carpenter et al. 1998; Kumaraswamy et al. 2020). Although these outcomes directly impact humans, the sources or origins of pollution can mostly be traced to anthropogenic activities (Akhtar et al. 2021). Nevertheless, access to clean water is necessary to sustain all life. Both the growing global population and demand for water are growing. Thus, access to freshwater as a critical resource is emerging as a global crisis, and reasonable use patterns and the development of sustainable water purification technologies are essential goals. Keen interest has been expressed in shifting toward green technologies, including bio-, myco-, phyco-, and phytoremediation for the purification of polluted waters due to their overwhelming benefits relative to other treatment options (Vidali 2001).

Phytoremediation, which utilizes the ecosystem services of plants, has proven promising as a broad spectrum, low-energy, eco-friendly technology appropriate to manage pollution. These treatment options can be classified according to their mechanism of action. For example, phytoextraction and phytodegradation involve bioaccumulation, biotransformation, storage, and/or metabolism. Rhizofiltration is when microbial biodegradation is facilitated by the rhizosphere, and phytostabilization is when plant-produced chemicals immobilize contaminants at the root/soil interface. Lastly, phytovolatilization is when xenobiotics are taken up and released via transpiration (Pilon-Smits 2005). The selected plants are usually species that are native and noninvasive to where the phytoremediation system is used and are non-noxious, with minimal environmental adverse effects. Considering the materials needed for construction and operation, and the low maintenance requirements and negligible environmental impact of these systems, phytoremediation has been considered an economical, sustainable, and ecologically sound solution to various pollution issues. Concerning the phytoremediation of polluted waters, the efficacy of various plants

has been studied, including other wetland plants (Wang et al. 2002), trees (Luqman et al. 2013), and aquatic macrophytes (Pflugmacher et al. 2015). However, for water remediation, the use of aquatic macrophytes in their natural habitat is a more natural approach.

Aquatic macrophytes play an essential role in maintaining the health of aquatic ecosystems, as they increase habitat complexity by giving physical structure, availability of breeding habitat for other species, shelter, surface area for colonization by microorganisms (periphyton), and contribute to an overall increase in species richness and biodiversity (Esteves 1998; Thomaz and Cunha 2010). They act as a food source for aquatic herbivores and detritivores in the aquatic system (Bakker et al. 2016) and contribute to sediments as an organic matter source (Kennedy et al. 2004). Aquatic macrophytes significantly influence the nutrient cycle by retaining solids and nutrients and reducing nutrient release from sediments (Pott and Pott 2003).

Macrophytes are classified based on their growth in water. Emergent plants are rooted in sediment underwater with protruding stems and leaves, such as *Typha latifolia* (cattail). Floating macrophytes float on the water surface with their unanchored roots underwater, such as *Lemna* spp. (duckweed). In contrast, submerged macrophytes grow completely underwater, such as *Ceratophyllum demersum* (coontail) or *Myriophyllum* spp. (milfoil). All types of macrophytes can be used in the phytoremediation of wastewater (Mustafa and Hayder 2021); however, turbidity can inhibit the growth of submerged plants as photosynthesis may be hindered (Goldsborough and Kemp 1988). Within the context of phytoremediation, macrophytes play a pivotal role given their fast growth, high tolerance to and capacity for uptake of contaminants, as well as easy management and control. Additionally, the spent biomass could be used for biofuel production (Arefin et al. 2021), as well as other circular economy initiatives (Kurniawan et al. 2021).

8.2 Biotransformation of Pollutants

One of the properties allowing macrophytes to cope with and remediate pollutants in their environment is their ability to take up, chemically transform, and bioaccumulate harmful substances through biotransformation. This three-phase process utilizes the action of several enzymes to convert hydrophobic substances into hydrophilic metabolites, which can then be stored in cellular structures by plants (Fig. 8.1).

Biotransformation is a relatively universal biochemical function in living organisms, with similar enzymes involved in Phase I and Phase II biotransformations in both animals and plants (Sandermann 1992). Phase I, the transformation phase, involves the modification of the primary structure of the xenobiotics taken up by adding or unmasking functional groups, making the molecule more polar. This occurs through hydrolysis or redox reactions mainly catalyzed by cytochrome P450 monooxygenases. Phase II, or the conjugation phase, is the next step in modifying xenobiotics, where endogenous hydrophilic molecules are added to the intermediate formed in Phase I or directly to the xenobiotic. These primary

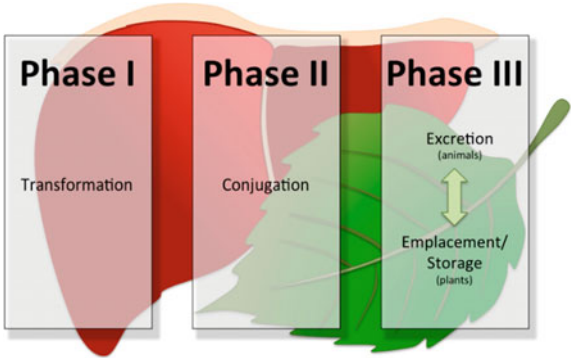

Fig. 8.1 Schematic of the three phases of biotransformation, starting with Phase I, during which xenobiotics are transformed by adding or unmasking functional groups. In Phase II, biomolecules are conjugated with the xenobiotic or the functional groups added in Phase I. Both phases aim to make the molecule more polar and Phase III involves the active transport of metabolites that are stored by plants

reactions include sulfation, acetylation, methylation, glutathione conjugation, and glucuronidation, which are catalyzed by enzymes such as glutathione S-transferases and UDP-glucosyltransferases (Sandermann 1992, 1994).

The core difference between plant and animal xenobiotic metabolism occurs in Phase III, or the sequestration phase, where animals excrete the formed metabolites via urine and feces. In contrast, plants will sequester the formed metabolites by storage in cell vacuoles or the apoplast or by covalent binding to cell wall fractions such as celluloses and hemicelluloses (Fig. 8.2). The formed metabolites reach the vacuole or the apoplast by active transport, moving them through plasma membranes or the tonoplast (Colemann et al. 1997). In the case of aquatic macrophytes, xenobiotics are eliminated from the water column without releasing potentially harmful metabolites, making them ideal candidates for the phytoremediation of contaminated waters.

8.3 Phytoremediation Using Aquatic Macrophytes

The use of macrophytes in phytoremediation has previously been reviewed, and several species were reported to remove organic and inorganic pollutants with high efficiency (Ansari et al. 2020; Mustafa and Hayder 2021). Due to their high prevalence and impacts as aquatic pollutants, a range of studies has focused on the phytoremediation of cyanobacterial toxins (Esterhuizen et al. 2011; Pflugmacher et al. 2015; Flores-Rojas et al. 2019; Flores-Rojas and Esterhuizen 2020), pharmaceuticals (de Oliveira et al. 2019), heavy metals (Mishra and Tripathi 2008), and mixtures of contaminants (Loise de Morais Calado et al. 2019). Among these studies, species from the genera *Ceratophyllum*, *Pontederia* (formerly *Eichhornia*), *Lemna*, *Egeria*,

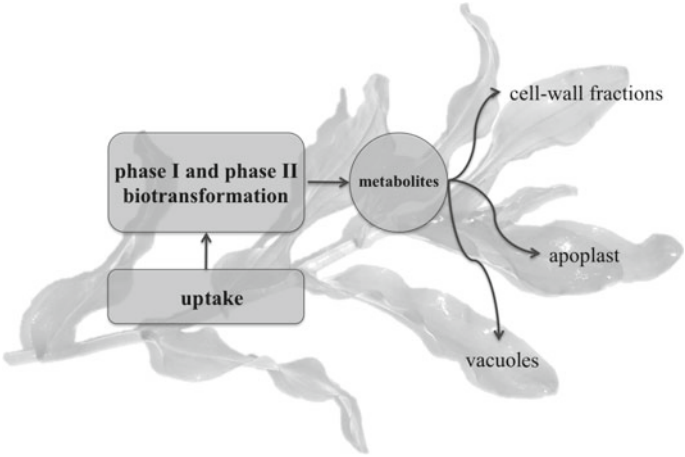

Fig. 8.2 Dynamics of pollutants in plants. Xenobiotics are taken up and biotransformed via Phase I and Phase II reactions before being distributed and stored in the cell wall, apoplast, and vacuoles

and *Myriophyllum* have been reported to efficiently bioaccumulate these contaminants with no or only minor adverse effects on the plants (Esterhuizen-Londt et al. 2011; Flores-Rojas et al. 2015; Vilvert et al. 2017).

Considering the phytoremediation of pharmaceuticals, there have been variable results reported in the literature. In shallow model constructed wetlands, macrophytes were found not to affect the removals of nutrients or pharmaceutical concentrations from artificial municipal wastewater containing carbamazepine, clofibric acid, fluoxetine, naproxen, sulfamethoxazole, and sulfapyridine. The macrophytes used in this study included *Typha* spp. (cattails), *Myriophyllum sibiricum* (northern watermilfoil), and *Utricularia vulgaris* (bladderwort), as reported by Cardinal et al. (2014). However, De Oliveira et al. (2019) reported efficient remediation of ibuprofen in vertical flow and free-floating macrophyte constructed wetlands containing *Heliconia rostrata* and *Pontederia crassipes* (formerly *Eichhornia crassipes*). However, a screening study for diclofenac (DCF) uptake by 42 different macrophytes revealed that not all aquatic plants have the same affinity for internalizing this anti-inflammatory pharmaceutical, as shown in Fig. 8.3.

In this study, the highest DCF uptake rates were recorded among the *Myriophyllum* spp.; with 2.1 ng/g/h for *Myriophyllum roraima*, 1.9 ng/g/h for *Myriophyllum quitense*, 1.7 ng/g/h for *Myriophyllum aquaticum and Myriophyllum mattogrossense*, and 1.1 ng/g/h for *Myriophyllum tuberculatum*. The *Ceratophyllum* spp. also internalized DCF at a high rate, with an average uptake rate of 1.3 ± 0.1 ng/g/h. Other species with high uptake rates included *Aegagropila linnaei* (1.4 ng/g/h) and *Hydrilla verticillata* (1.0 ng/g/h). However, the remaining tested macrophytes took up DCF at a rate below 1 ng/g/h. Therefore, preliminary assessment tests like these beaker uptake experiments are essential to select the most suitable plants for inclusion in a phytoremediation system.

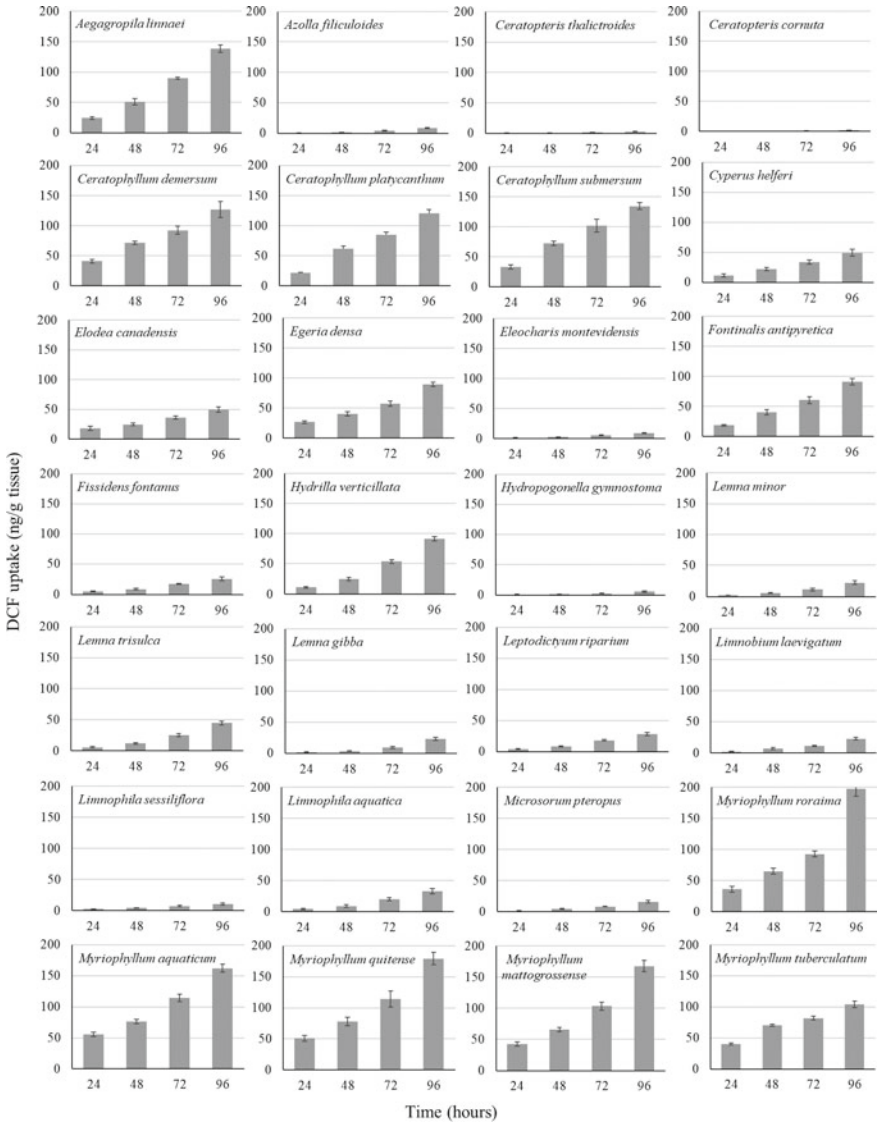

Fig. 8.3 Varying concentrations (ng/g) of diclofenac (DCF) accumulated among 42 species of aquatic macrophytes over four days. Assessing the diclofenac uptake with time was performed as static exposures in beakers where 5 g of plant material from each macrophyte was exposed to 250 μg/L diclofenac in DMSO. The exposures were maintained under a day-night cycle of 14 h light (100 mE/m^2.s) and 10 h moonlight blue light. Plant biomass samples were collected after 24, 48, 72, and 96 h and extracted with methanol, as described by Loise de Morais Calado et al. (2019) and analyzed via liquid chromatography-tandem mass spectroscopy (LC-MSMS), as reported by Esterhuizen-Londt et al. (2017b)

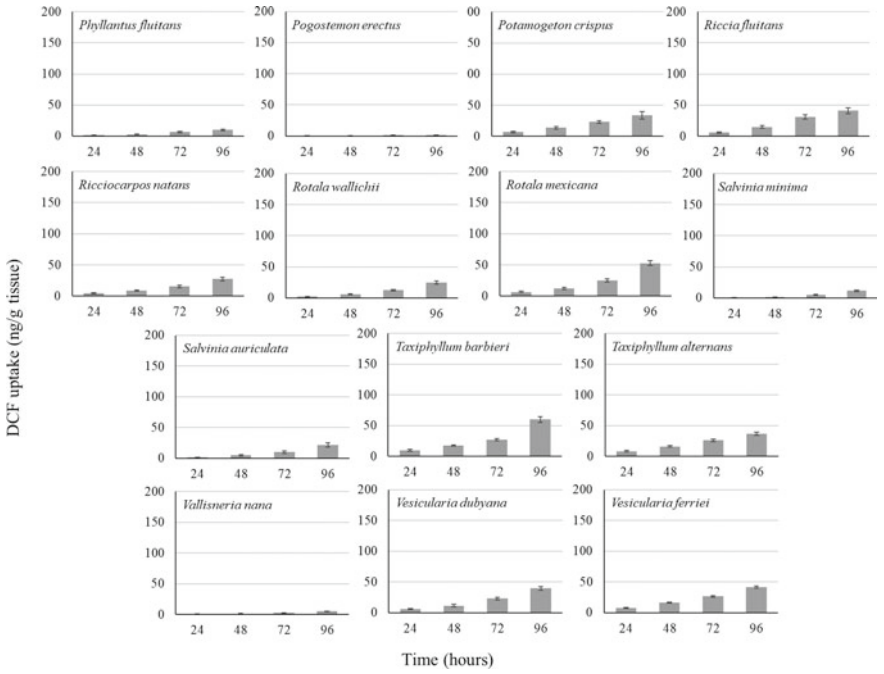

Fig. 8.3 (continued)

8.4 Green Liver System Development and Optimization

Constructed wetlands, which are phytoremediation systems consisting of plants, substrates, and associated microorganisms, rely on physical, chemical, and biological mechanisms to eliminate pollutants (Ingrao et al. 2020). However, the breakdown products produced by these microorganisms are largely unknown and may be equally toxic, as demonstrated for the metabolites of the cyanobacterial toxin microcystin-LR (Schmidt et al. 2014; Pflugmacher et al. 2015). Nevertheless, to make use of the ability of aquatic plants to effectively remove environmental pollutants from water bodies, low-impact systems using only floating and submerged aquatic plants with negligible roots to minimize associated microbial growth were developed. As plants metabolize toxicants as a liver would, the name "*Green Liver Systems*" was chosen. Green Liver Systems rely on the principle that plants take up contaminants, subsequently internally metabolize and store them in cellular components. Thus, the macrophytes do not release the products of biotransformation, and the contaminants are removed from the water phase. These simulated phytoremediation systems do not contain any natural substrates in order to minimize root anchoring and associated microbial growth (Pflugmacher et al. 2015).

As discussed above, not all plants have the same abilities to remove and metabolize contaminants. Therefore, the efficiency of using single aquatic plants versus

combinations of three plants was tested in a laboratory scale Green Liver System. The test system used, as illustrated in Fig. 8.4, was a glass tank divided into three linked compartments with a total volume of 60 L. Since the laboratory scale Green Liver System was compartmentalized and all three compartments in the experimental tanks had the same size and volume, the fresh weight of 150 g per macrophyte type was used per compartment. For the sediment, pure quartz sand was added to give the plants stability. Flow, using a commercially available aquarium pump, was adjusted to 4 mL/min, resulting in a complete circulation of the entire water volume in the system over 24 h. Light was provided using LED lights with a 14:10 light/dark cycle and an irradiance of 100 mE/m^2.s. The synthetic water used was prepared according to EN ISO 7346-3 (1996), with a pH of 7.1, conductivity of 300 μS/cm, and a dissolved oxygen content of 7 mg/L.

Three different aquatic macrophytes, *C. demersum*, *Myriophyllum spicatum*, and *Elodea canadensis*, were chosen for the Green Liver System optimization experiments based on established uptake efficiencies. *C. demersum*, commonly referred to as coontail or hornwort, is a submerged, free-floating aquatic plant. The distribution of this macrophyte is global, with the exception of Antarctica. *C. demersum* has no roots; therefore, uptake occurs entirely via the surface of the vegetative plants. Therefore, root uptake is not a confounding factor (Hak et al. 2020). *M. spicatum*, commonly called watermilfoil, is widely distributed geographically and a common species in freshwater lakes globally. This perennial submerged plant, which grows in

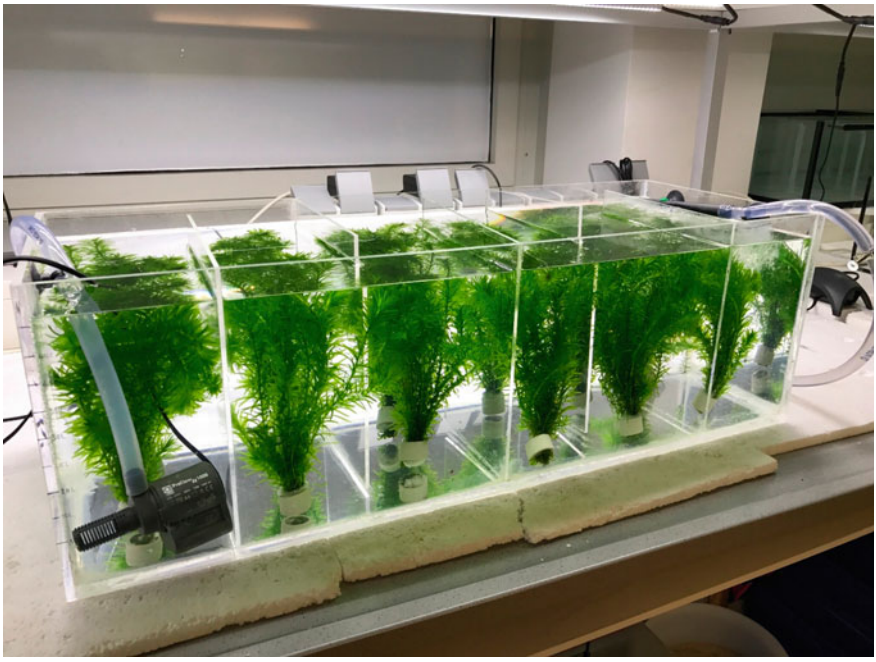

Fig. 8.4 Laboratory scale Green Liver test system

slow-moving waters, has two orders of lateral roots (Xie et al. 2007). The perennial *E. canadensis*, also called waterweed or pondweed, grows submerged in aquatic ecosystems globally. While *E. canadensis* produces roots, they can grow free-floating. When the roots are anchored in soil, they produce root hairs, while their water roots do not (Cormack 1937).

As test contaminants, the cyanobacterial toxin, microcystin-LR (MC-LR), the pharmaceutical DCF, and the insecticide cypermethrin (CYP) were used. MC-LR is a hepatotoxin produced by several cyanobacterial species that bloom in freshwater habitats, thus posing a risk to water quality in terms of potability, recreational use, agriculture, and aquaculture (Esterhuizen-Londt and Pflugmacher 2020). As a commonly detected pharmaceutical pollutant of freshwater ecosystems with known adverse effects on aquatic organisms (Lonappan et al. 2016), the anti-inflammatory drug DCF was included on the first Watch Lists established by the Commission Implementing Decision (EU) 2015/495, but has subsequently been removed (European Commission et al. 2020). CYP is a class II pyrethroid pesticide used domestically and agriculturally. The insecticide, which can have neurotoxic effects on terrestrial non-target organisms, has also been reported as toxic to some aquatic organisms (Saha and Kaviraj 2008).

The uptake of the three test contaminants (MC-LR, DCF, and CYP) in single contaminant experiments was tested in the laboratory scale Green Liver system containing only one species of each macrophyte at a time (Fig. 8.5). The exposure concentration for each contaminant was 10 μg/L in the single contaminant experiments. Water samples of 50 mL were taken at the outlet of the recirculating system after the initial application of the compound, as well as after one, three, and seven days. All experiments were repeated fivefold. The concentrations of the contaminants were analyzed using LC-MS/MS following the published methods for MC-LR (Esterhuizen-Londt et al. 2017a), DCF (Esterhuizen-Londt et al. 2017b), and CYP (Singh et al. 2016).

Several macrophytes species have been tested previously for their remediation efficiency of cyanobacterial toxins for inclusion in the Green Liver System (Pflugmacher et al. 2015, 2016; Contardo-Jara et al. 2015). In the experiments with MC-LR in the present study, *C. demersum* ($R^2 = 0.956$), *E. canadensis* ($R^2 = 0.992$), and *M. spicatum* ($R^2 = 0.980$) linearly decreased the concentration of the cyanotoxin over seven days (Fig. 8.5a). *C. demersum* had the highest removal rate of 56.5 μg/day for the 60 L system, amounting to a 69.0% removal of MC-LR (Table 8.1) after seven days ($p = 0.033$). A multifactorial repeated-measures analysis of variance (ANOVA) with multiple comparisons revealed that *E. canadensis* statistically had a similar remediation efficiency as *C. demersum* ($p = 1.000$), amounting to a removal percentage of 57.6% (Table 8.1). Though a previous study showed equal uptake rates for MC-LR among the two species (Cao et al. 2019), in the present study, *M. spicatum* had a significantly lower removal efficiency than *C. demersum* ($p = 0.033$), equaling 28.9% (Table 8.1). The capacity of several macrophytes to bioaccumulate MCs from their environment has previously been demonstrated (Pflugmacher 2004; Saqrane et al. 2007). Pflugmacher et al. (1998) reported that *C. demersum* took up

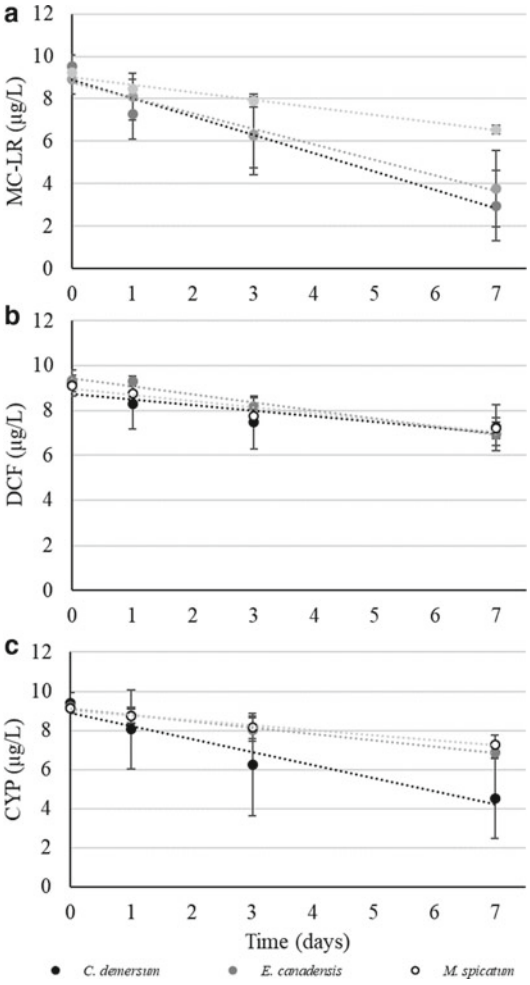

Fig. 8.5 Single species exposure to (**a**) MC-LR, (**b**) diclofenac, and (**c**) cypermethrin, each at a concentration of 10 µg/L in the laboratory scale Green Liver System for seven days. The concentrations of the three contaminants were monitored in the water on days one, three, and seven using LC-MS/MS. Data represent the mean contaminant concentration and standard deviation ($n = 5$) sampled at the system outlet

Table 8.1 Remediation percentage using a Green Liver System with a single species plant set-up after seven days of exposure in recirculated water

Plant species	Remediation %		
	MC-LR	Diclofenac	Cypermethrin
C. demersum	69.0	21.5	51.8
E. canadensis	57.6	25.6	25.3
M. spicatum	28.9	21.1	20.2

1.98 µg/g wet weight of MC-LR in seven days, indicating that 95% of the administrated radiolabeled MC-LR was internalized. From the available data, it appears that several factors, such as the exposure concentration, plant species, and tolerance to the contaminant, can influence the amount of MC-LR taken up by macrophytes.

Figure 8.5b shows that equal remediation efficiencies were achieved for DCF among the three macrophyte species after seven days, averaging 17.99 ± 2.16 μg/day or 22.7 ± 2.5% ($p = 0.396$). Previous studies proved the low biodegradation but high rates of photodegradation of DCF in aquatic systems (Matamoros et al. 2009; Matamoros and Salvadó 2012). Thus, light may be a key factor resulting in similar remediation efficiencies in these uptake experiments.

Menone et al. (2005) reported that CYP does not cause major adverse effects in *C. demersum*. Nonetheless, in the present study, the remediation efficiency of *C. demersum* was only 51.8%. Statistically, the differences between remediation efficiencies among the three macrophytes exposed to CYP were insignificant ($p = 0.134$; Fig. 8.5c). An average removal rate of 25.82 ± 13.94 μg/day was achieved for CYP by the three macrophyte species, amounting to a removal percentage of 32.4 ± 17.0% after seven days. Although macrophytes have been shown to reduce the levels of pesticides (Moore et al. 2001; Cooper et al. 2004), CYP removal has only been demonstrated with *Lemna* sp. in laboratory microcosmos (Mugni et al. 2011). None of the plants alone could completely remove the tested contaminants (Table 8.1).

The Green Liver System's remediation efficiency was also tested with all three plants, one per compartment, in various combinations, to test whether possible interactive effects could be observed in the downstream plants. This phenomenon, called allelopathy, includes all chemically mediated interactions between plants or microorganisms. The chemical substances or allelochemicals, which are released into a surrounding environment, can elicit either positive or harmful responses in target organisms (Rice 1984). Some macrophytes produce allelochemicals that induce photosynthesis, which decreases carbon dioxide levels and cause the pH to increase, which would influence the growth of other biota, including other macrophytes (Lundholm et al. 2005).

After three days, a significant split could be observed among the combinations for their ability to remove MC-LR ($p < 0.05$; Fig. 8.6a). However, after seven days, all tested combinations completely remediated the 10 μg/L of MC-LR administered to the Green Liver System (Fig. 8.6a). The average MC-LR removal rate was 76.69 ± 1.13 μg/day. For DCF, the combinations of *E. canadensis* + *C. demersum* + *M. spicatum*, *E. canadensis* + *M. spicatum* + *C. demersum*, and *M. spicatum* + *E. canadensis* + *C. demersum* were not statistically different ($p < 0.05$; Fig. 8.6b) and yielded the lowest average remediation percentage of 57.4 ± 6.2% (Table 8.2). The macrophyte combination consisting of *C. demersum* + *E. canadensis* + *M. spicatum* removed 75.9% (Table 8.2) of DCF from the system after seven days at a rate of 57.9 μg DCF/day. The other combination with *C. demersum* as the species in the first compartment (*C. demersum* + *M. spicatum* + *E. canadensis*) did not differ statistically in remediation percentage ($p = 0.621$). Differences in CYP remediation among the combinations were not statistically significant ($p = 1$; Fig. 8.6c). After seven days, an average of 43.4 ± 9.7% was removed at an average rate of 31.1 ± 8.5 μg CYP/day (Table 8.2).

In a study by Loise de Morais Calado et al. (2019), a combination of *Egeria densa*, *C. demersum*, and *M. aquaticum* was used in a laboratory scale Green Liver System to evaluate removals of a mixture of paracetamol (0.7 μg/L), DCF (12 μg/L), and

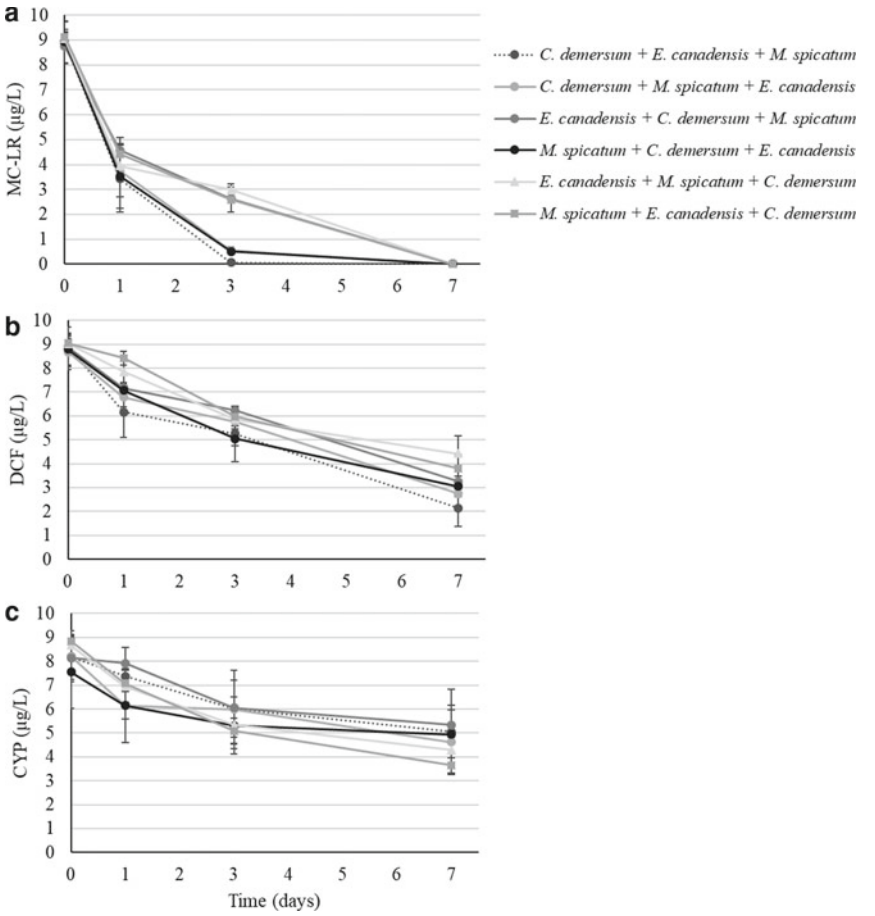

Fig. 8.6 Evaluation of the remediation efficiencies of the combinations of the three species (*Ceratophyllum demersum*, *Elodea canadensis*, and *Myriophyllum spicatum*) in tests with exposure to (**a**) MC-LR, (**b**) diclofenac, and (**c**) cypermethrin each at a concentration of 10 µg/L using the Green Liver System set-up with a 60 L glass tank for seven days. Data represent the mean and standard deviation ($n = 5$)

MC-LR (2 µg/L). After seven days, the DCF concentration in the system remained unaffected. However, after 14 days, 93% was removed. For MC-LR, 69% was degraded after 24 h and 100% after three days (Loise de Morais Calado et al. 2019). Comparing the data from the two studies highlights the importance of the macrophyte combination to achieve optimal remediation.

From the screening data for DCF uptake (Fig. 8.3), it is understood that various macrophytes have various uptake affinities for components. Therefore, the Green Liver system can be customized, like a tool kit, and the combinations of macrophytes can be selected and adjusted based on the contaminants that need to be remediated.

Table 8.2 Remediation percentage using a Green Liver System with a three species plant set-up after seven days of circulation

Macrophyte combination	Remediation %		
	MC-LR	Diclofenac	Cypermethrin
C. demersum + *E. canadensis* + *M. spicatum*	100	75.9	38.3
C. demersum + *M. spicatum* + *E. canadensis*	100	68.3	43.8
E. canadensis + *C. demersum* + *M. spicatum*	100	63.3	34.5
M. spicatum + *C. demersum* + *E. canadensis*	100	65.3	34.6
E. canadensis + *M. spicatum* + *C. demersum*	100	50.9	50.5
M. spicatum + *E. canadensis* + *C. demersum*	100	58.0	58.7

Based on the success of the laboratory scale Green Liver systems, large-scale systems have been globally constructed.

8.5 Large-Scale Green Liver Systems

Due to eutrophication, Lake Chao in Hefei, Anhui in the Peoples Republic of China, experiences toxic cyanobacterial bloom year-round. However, water from this lake is intended as a source for drinking water production. Therefore, the first pilot plant Green Liver System® (dimensions: 25 m × 10 m × 1.5 m, volume: 375 m^3) was built at the water treatment plant located at Lake Chao to remediate eutrophication as well as the cyanotoxins produced by the harmful algal blooms. The first two compartments of the system were planted with *Lemna* sp., followed by *Hydrilla* sp. in compartments two and three, *Myriophyllum* sp. in compartment four, and *Phragmites* sp. in compartments five and six. Prior to the construction of the system, the hepatotoxin congeners MC-LR (59.0 μg/L), MC-YR (1.7 μg/L), and MC-RR (42.6 μg/L) were detected. After installation and sustained operation and maintenance, the Green Liver System® removed 80 ± 5% of the toxins (Nimptsch et al. 2008).

Similarly, wastewater from a tilapia fish farm near the city of Itacuruba in the state of Pernambuco, Brazil, released hormones and nutrients into the Luiz Gonzaga Dam, causing eutrophication and the development of cyanobacterial blooms. As the wastewater from aquacultural ponds needed to be cleaned before use for agricultural irrigation or released into this nearby reservoir, a second pilot plant of a Green Liver System was constructed with a size of 100 m × 25 m × 2 m and a final volume of 5000 m^3. The primary contaminants in the wastewater from the tilapia farm were oxytetracycline added in medicated feeds as a prophylactic antibiotic, methyltestosterone added for sex reversal of female fish since males grow faster, and cyanobacterial toxins due to the persistent blooms. Prior to construction, two cyanobacterial toxins were monitored and detected at concentrations of 22.4 μg/L for MC-LR and 31.2 μg/L for MC-RR. By stocking the Green Liver System with *P.*

crassipes (formerly *E. crassipes*) in two compartments, *E. densa* in three compartments, and *M. aquaticum* in one compartment, there were removals of 32% of MC-LR and 100% of MC-RR (Esterhuizen and Pflugmacher 2020).

8.6 Green Liver System Advantages and Limitations

Macrophytes used in the Green Liver Systems are customarily taken from water bodies in the vicinity, which also means that they are preconditioned for the climate conditions. One to two weeks are usually given for plants to establish themselves in the system before starting the water flow. As plants are native to the area and conditions, no specific care needs to be taken regarding testing the soil. Whether the selected macrophytes will flourish with the water quality of inflow must be tested at a laboratory scale before implementation.

To avoid plants from migrating through the system with water currents created by the flow, (a) surface plants such as *Pontederia (formerly Eichhornia)* were kept in place with fishnets, and (b) for submerged plants, bundles were formed and attached to stones using soft ropes. Each bundle was given approximately 50 to 60 cm of space to allow growth.

The primary benefit of the Green Liver System is the removal of pollutants from the water column without the addition of hazardous chemicals. By using combinations of macrophytes, including emergent and floating species, the entire water column can be covered. The contaminants are stored within the plants with no release of unknown metabolites. With the correct maintenance scheme, the whole plant can be removed from the system before expiry, thus safely removing harmful substances. The harvested biomass can be used to produce bioenergy (Wilkie and Evans 2010). However, the potential environmental impacts need to be comprehensively assessed. As native plants can be used, the system efficiently functions with little upkeep other than replacing macrophytes before expiration, therefore guaranteeing low cost and sustainable functioning. Additionally, macrophytes play a significant role in aquatic carbon cycling and carbon storage, supporting the removal of carbon dioxide as a greenhouse gas (Marba et al. 2015). Several macrophyte species are known to produce allelochemicals suppressing microalgal growth (Kurashov et al. 2021) and have antimicrobial properties (Juan et al. 2014). An added socio-environmental benefit of Green Liver Systems is its aesthetic appearance.

Reduced macrophyte health and fitness are significant risks to a Green Liver system's functioning and continual remediation efficiency. Dead and decaying plants will not actively take up and biotransform metabolites, meaning they would be useless biomass. Furthermore, continuous plant litter decomposition will increase dissolved and particulate organic carbon (Noller et al. 2003) and potentially release harmful chemicals. Therefore, how pollutants affect macrophytes on a morphological, physiological, and toxicological level needs to be assessed in the laboratory, as the decay of the plants could lead to the release of the contaminants. These data are also necessary to predict the rate at which the vegetation needs to be renewed. The second

threat is the release of the stored metabolites back into the environment by decaying plants. The fitness of the macrophytes is, therefore, directly correlated to the whole system's efficiency. Therefore, careful maintenance to ensure macrophyte vitality and the removal of spent biomass is critical for Green Liver Systems. Additionally, allelopathic interactions may lead to the suppression and death of particular species when grown in combination with others and, as previously highlighted, is a critical interaction to establish in the laboratory before applying in practice.

Environmental factors threatening the functioning of a large-scale Green Liver System include flooding and droughts, as well as animal and plant invasions. Water level fluctuation can significantly affect the survival and growth of aquatic vegetation (Sousa et al. 2010). A rapid increase in the system's water level would affect the availability of nutrients due to dilution and reduced available sunlight, thereby affecting biomass (Best et al. 2001). The quality of the inflow may directly impact the macrophyte. Highly turbid waters and rising water levels would decrease the available sunlight reaching submerged macrophytes, thus, affecting photosynthesis. Furthermore, pH changes may also affect some plants (Song et al. 2018). During droughts, contaminants and nutrients can be concentrated, dissolved oxygen may decrease, and water temperature may increase, threatening the plants' survival (Bond et al. 2008). As the macrophytes are selected based on the contaminants to be remediated, a rapid, sharp change in the types and concentrations of the contaminants may render the system less effective. Therefore, the waters should be tested occasionally for variations in the pollution types, with at least monthly testing recommended. Low-cost probes may be used for continual in-line monitoring, or commercial ELISA tests may be used (e.g., test for cyanobacterial toxins). Changing the macrophytes when drastic shifts in the inflow contaminant content are detected is suggested to ensure the system's efficiency in maintaining a high remediation performance.

Wild or domestic animals and birds may invade the system and forage on the contaminated plants, affecting the grazer's health and the system's functionality. For the Green Live System constructed in Itacuruba in Brazil (Esterhuizen and Pflugmacher 2020), this issue was overcome by constructing wooden fences around the system to exclude goats and chicken wire to restrict birds. Similarly, invading plants carried into the system by birds or wind could outcompete the planted macrophytes (Fleming and Dibble 2015). Suitable covering, such as surrounding the system with shade cloth or hard plastic, could lessen this threat. However, care should be taken in selecting the cover so as not to reduce illumination and hinder photosynthesis. Nevertheless, through good management and maintenance, invasive plants can be removed before they become a nuisance. The applicability of Green Liver Systems as a remediation technology may also be seasonally limited at certain altitudes and in certain climates, as winter temperatures may hamper macrophyte growth and survival (Yan and Xu 2014). For example, *C. demersum*'s optimal growth temperature is between 15 to 30 °C; however, it can survive at −2 °C. *Myriophyllum* spp., on the other hand, prefer warmer conditions with an optimal growth temperature between 26 and 32 °C and is only able to tolerate minimum water temperatures of 18 °C. Below this temperature, they are likely to produce turions (Aiken and Walz 1979). Therefore, it

is suggested to use native macrophytes that have likely evolved to acclimate to the prevailing environmental conditions (Hyldgaard and Brix 2012).

Green Liver Systems may adversely affect the environment as plants that are grazed upon, and decaying plants could emit methane as a greenhouse gas (Petruzzella et al. 2015). The system may function as a breeding ground for pests, such as mosquitoes (Greenway et al. 2003), especially since the system is artificial with few macroinvertebrates present to control pest numbers. However, pest breeding could be reduced by maintaining a sufficient flow rate. Typically, a flow rate of 8 L/h is maintained in large-scale Green Liver Systems (Esterhuizen and Pflugmacher 2020). With its curved separation walls, the system is designed not to have any "dead" zones with stagnant water, which will act as a breeding zone for mosquitoes.

Another hazardous byproduct of Green Liver Systems is the spent contaminated macrophytes that need to be harvested and replaced periodically for maintenance purposes. These plants cannot be utilized as animal feed or agricultural fertilizer because of pollutants, and their metabolites may be biotransferred (Pflugmacher et al. 2015). Micro-mining for heavy metals and combustion to produce bioenergy seem adequate approaches to deal with the used macrophytes, but the environmental implications of these processes need to be studied.

Proper system maintenance can reduce most of these environmental risks and disadvantages identified above. As Green Liver Systems® are entirely artificial systems containing a limited amount of different aquatic macrophytes, the management of these systems is straightforward and low cost. The flow in the system can be maintained with solar-powered pumps or gravitational flow and is thus a low-energy option. The primary maintenance requirements are to ensure constant flow through the system and to sporadically replace the macrophytes, which could be cultured onsite. Contaminants should constantly be monitored in the inflow to adapt the system to changing pollutant levels.

8.7 Conclusion

Green Liver Systems utilize the ecological services of macrophytes to remediate contaminants by uptake, biotransformation, and intracellular storage, with no extracellular release of metabolites with unknown environmental effects. The systems are fully customizable for mixtures of pollutants in wastewaters by selecting suitable macrophytes with high uptake affinities for the substances in question. The technology is green, low maintenance, low cost, and low energy, making it especially appealing to developing countries. However, the application of the system is limited by seasonality, climate, and altitude due to the growth requirements of the macrophytes. In the future, technologies should be developed to process used macrophytes containing toxins and pollutants to minimize their environmental impact.

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