Walter Reineke
Michael Schlömann

Environmental Microbiology

Springer Spektrum

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Walter Reineke
Bergische Universität Wuppertal
Wuppertal, Germany

Michael Schlömann
TU Bergakademie Freiberg
Freiberg, Germany

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Preface to the Third Edition

"Textbooks are outdated. Don't waste your time writing a textbook. Nowadays, teachers, students and practitioners download everything they need to know from the internet. They don't need to rely on textbooks anymore.

These and other similarly "encouraging" statements were made by some dear colleagues (…) when we announced our plan to write a 3rd edition. But (…) numerous others motivated us to sit down and think again about the (…) field (…) that has guided us throughout our academic careers. And you won. Here it is, the third edition. This is why we believe that textbooks are needed more than ever: at a time when the number of scientific publications continues to explode, from time to time there is a need to evaluate and (…) summarize more holistically the state of the art in a particular field, (…). In other words, between two physical or imaginary book covers, to attempt to define a particular field and give an account of where that field stands. Of course, such an attempt represents only the biased, personal view of the book's authors, but that is perhaps better than having no view at all."

(Translation of Preface, Schwarzenbach, R. P., Gschwend, P. M., Imboden, D. M. 2016. Environmental Organic Chemistry. 3rd ed. Wiley).

We can't describe the situation any better. We are also repeat offenders. What all has been changed:

Now an online version is to be available to the reader. According to the request of readers, colour illustrations were created when useful.

The chapter on exhaust air purification has been revised from scratch and presented more comprehensively following a suggestion by Christoph Sager (VDI). Many thanks for the suggestions.

When new data or research of interest emerged, it was incorporated.

Without the support and expertise of the publisher, especially Kent Muller and his team, in the revision and editorial work, the new edition would not have been produced.

We remember with gratitude the late founder of this book, Wolfgang Fritsche. We also remember David T. Gibson, the "father" of aerobic aromatic degradation and an exemplary scientific motivator.

Walter Reineke
Wuppertal, Germany

Michael Schlömann
Freiberg, Germany
January 2019

Preface to the Second Edition

Many years have passed since we set out on the journey to write the textbook "Environmental Microbiology". We have now worked through more topics from the "old" Fritsche and incorporated them into the second edition.

First of all, we would like to thank our readers for critical comments, advice on errors, wishes and suggestions for changes and additions.

What has been changed and added: An attempt has been made to update the chapter "Global Environment. Climate and microorganisms" with data from the IPCC. Nevertheless, it remains generally difficult to be always up to date with figures. However, old information is also important to be presented, such as the Haber-Bosch process. "Microorganisms, Players in the Environment" has been rewritten to include information on mycorrhizae and marine symbioses. In Microbial Metabolism, phototrophy and also autotrophy types are covered. Heavy metals and other toxic inorganic ions are discussed more broadly. Very topical are culture-independent techniques for the description of microbial communities: metagenomics, DNA-MicroArray. However, the discussion of artificial organisms is also stimulated. Biocorrosion and its effects are addressed. Our own experiences with organisms that degrade foreign substances are used to stimulate thoughts on the isolability of microorganisms. The specialists, which we both are, are interested in the biochemistry of aromatics and methane in the border area of oxic and anoxic. Current discussions such as biofuel and sustainability and their assessment were taken up. A fascinating topic is the ocean with its habitats, which has only become accessible in recent years.

Thus, for this edition, numerous chapters have been rewritten and expanded. It should become clear that the interaction of organisms in the balance of nature cannot be understood without a thorough knowledge of the physiology of bacteria. Bacteria are important, irreplaceable links in the cycles of substances on our planet.

In the preface to the first edition of "Stryer Biochemistry" was the following apt saying: "Were I to strive for perfection, my book would never be finished" (by the Chinese scholar Tai T'ung, who lived in the thirteenth century).

The fact that we now have a "finished" book is because of the commitment and expertise of the publisher. Without their support in the revision and editorial work, the new edition would not have come into being.

We hope that the book as it stands will serve its purpose as a reliable aid to the study of environmental science and that it will be a pleasure to work with.

We hope that readers will see environmental microbiology not just as a subject to be studied, but as a fascinating science that influences all areas of biology, including agriculture, right into our daily lives.

Walter Reineke
Wuppertal, Germany

Michael Schlömann
Freiberg, Germany
January 2020

Preface to the First Edition

About 20 years ago, a small-format book *Environmental Microbiology. Microbiology of Environmental Protection and Design* by Wolfgang Fritsche came into my hands at a time when I was beginning to develop the first lecture on environmental microbiology. It was a great help at the time when environmental issues were beginning to register with the public.

Then in 1998, the second edition in a larger format was given to students and also to teachers under the title *Environmental Microbiology. Fundamentals and Applications* was put into the hand.

Now the task of refreshing was placed in our hands. Each stone was touched, lifted, turned and then, depending on the assessment, put back in its old place or sorted out and replaced by another stone or perhaps just installed in a different place. One will therefore recognize some things, certainly not some things. People with a different environment, different orientations, different interests have worked on the change. The proven *Environmental Microbiology* by Fritsche has been completely revised. In particular, methodological aspects for the investigation of microbial communities were taken into account. Among other things, an attempt was made to make the principles behind modern molecular genetic investigation methods comprehensible to non-biologists. Greater consideration was also given to the physiological adaptations of microorganisms to different habitats. As before, the prominent role of microorganisms in various material cycles is presented. Global and local processes are addressed as well as the microbial influence on them. In addition to biochemical principles for the degradation of environmental chemicals, the use of microorganisms in environmental biological processes for the maintenance or purification of air, water and soil is discussed. Finally, as before, the use of microorganisms or their enzymes in product-oriented environmental protection is considered.

The book is intended not only for biologists with an interest in environmental microbiological issues, but also for students of chemical engineering or environmental process engineering, geoecology or geology, as well as students of other disciplines who do not *consider* environmental microbiology merely as a black *box* but want to gain an insight into the interrelationships and interdependencies.

Some of our own lectures have unmistakably been incorporated into the book here. The lecture "Environmental Microbiology" was intended to introduce biological, biochemical aspects to chemists in the context of "Environmental Chemistry", so the chemistry of environmental chemicals is an important part of the "New Fritsche".

Fruitful, but also critical discussions took place in the seminars and lectures to develop a realistic assessment of environmental problems. Microbiological solutions to environmental problems were critically analysed for their advantages and disadvantages compared to other concepts.

Our own roots in the field of environmental microbiology are certainly not to be overlooked either. Much of our knowledge of the environmental behaviour of chlorinated aromatics has therefore been incorporated.

Today, there are methodological possibilities that were not yet available or had not yet been thought of in our own initial research: the DNA of entire organisms has

now been sequenced, and biodiversity and evolution can be discussed in a more informed manner. It is also now possible to analyse the whole range of different biological and chemical levels associated with environmental chemicals: the organisms, the chemistry of degradation pathways, the biochemistry of the degradation pathways, the underlying gene structure in terms of structural genes and regulation, and possible origins for the degradation sequences.

But have we made any progress in describing/explaining ecosystems? Schleifer and Ziegler (2002) write:

"Since the use of new methods, especially molecular methods, has made it possible to identify microorganisms without prior cultivation and to gain insight into their function, our knowledge of the occurrence and ecological significance of these microorganisms has expanded enormously. Nevertheless, even today the majority of microorganisms have not been studied and their diverse functions in the ecosystem are far from being elucidated".

The biochemical services of microorganisms are unique and of global importance. In this book, a variety of these microbial functions have been presented. With this book we want to help to better understand some environmental phenomena, we want to draw attention, to enable a factual discussion.

Who do we need to, want to thank?

- Mr. Fritsche for entrusting the continuation of his work to us.
- The Spektrum Verlag, Martina Mechler and Dr. Ulrich Moltmann for their extraordinary patience and the firm belief that we will eventually deliver something meaningful.
- Mr. Christian Mandt for patient and very fruitful discussions. Always when the biologist needed a chemist for clarification. In general for his great never tired interest, his openness.
- To the students, who have shown great interest in the topics of environmental microbiology presented to them, and who have helped me to reach new horizons with their critical questions. Almost every event was the start for further searching and clarification.
- Last but not least, our heartfelt thanks go to our families, without whose constant help the book would not have come into being.

A book cannot be without mistakes. So we appreciate any comments from the users of this book. Mail us at:

reineke@uni-wuppertal.de or michael.schloemann@ioez.tu-freiberg.de

Schleifer, K.-H., Ziegler, H. 2002. foreword. *In:* Significance of microorganisms for the environment: round table of the Commission for Ecology, Bavarian Academy of Sciences. Verlag Dr. Friedrich Pfeil. Vol. 23, p. 9.

Walter Reineke
Wuppertal, Germany

Michael Schlömann
Freiberg, Germany
December 2006

Spellings and Abbreviations

For the sake of the simplest and clearest possible representation of the metabolic processes, conventional incorrectness have been adopted on purpose. Thus, charge ratios of organic compounds have not been taken into account. Organic acids have been designated as salts, for example, pyruvate instead of pyruvic acid, succinate instead of succinic acid or benzoate instead of benzoic acid, but have been consistently depicted as undissociated acids, although the dissociated compound is present in the cell in which the pH is around 7.

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Global Environment: Climate and Microorganisms

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W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_1

1

Life on Earth has created for itself the atmosphere necessary for its survival. The world climate is not only a function of atmospheric physics, but also of atmospheric chemistry. This is very dynamic and to a large extent the result of the biosphere and thus of microbial processes.

Most atmospheric gases are subject to cycles that are more or less dominated by the biosphere. Thus, the performance of microorganisms in global material cycles contributes decisively to the equilibrium that has emerged in the history of the Earth. The preservation of this equilibrium is the prerequisite for safeguarding the conditions of life on Earth. To ensure this, environmental problems caused by human activities must be identified and remedial action taken.

In addition to their important role in global material cycles, microorganisms play an indispensable role in the management or elimination of localised environmental problems.

► Examples of Global Environmental Problems

- Deterioration of air quality: Global pollution due to industrial combustion and biomass burning
- Increase in the occurrence of tropospheric oxidants including ozone and related impacts on the biosphere and human health
- Changes in the self-cleaning capacity of the atmosphere and in the residence time of anthropogenic trace gases
- Climatic and environmental changes in land use such as deforestation of tropical rainforest, draining of swamps
- Disruption of the biogeochemical cycles of carbon, nitrogen, phosphorus and sulfur
- Acid rain
- Climate change (global warming) resulting from increasing emissions of CO_2 and other greenhouse gases and consequences thereof
- Climatic effect (regional cooling) by sulphate aerosols due to anthropogenic SO_2 emission
- Decrease in stratospheric ozone (ozone hole) associated with an increase in UV-B radiation on the Earth's surface with its effects on the biosphere and human health ◄

► Examples of Regional (Localised) Environmental Problems in Water and Soil (Marine and Terrestrial Biospheres)

- Factory farming and the resulting contamination of groundwater with nitrates
- Eutrophication and runoff into water bodies
- Use of biocides and contamination of groundwater, for example with triazines
- Waste water and pollution of running waters, lakes and the sea with phosphates, for example eutrophication—oxygen depletion, disturbance of the self-purification capacity of water bodies
- Wastewater and the effect of heavy metals on human health: for example Minamata disease (mercury), Itai-Itai disease (cadmium)
- Accidents in industry: contamination of the Rhine by chemical fire in Basel
- Soil contamination and pollution of water bodies due to accidents during the transport of chemicals and oil
- Soil and groundwater contamination due to runoff from landfills and contaminated sites (pollutants) ◄

1.1 Climate System

1.1.1 Components of the Climate System

The climate system is an interactive system consisting of five main components (◘ Fig. 1.1): the atmosphere, the hydrosphere, the cryosphere, the land surface and the biosphere, driven or influenced by vari-

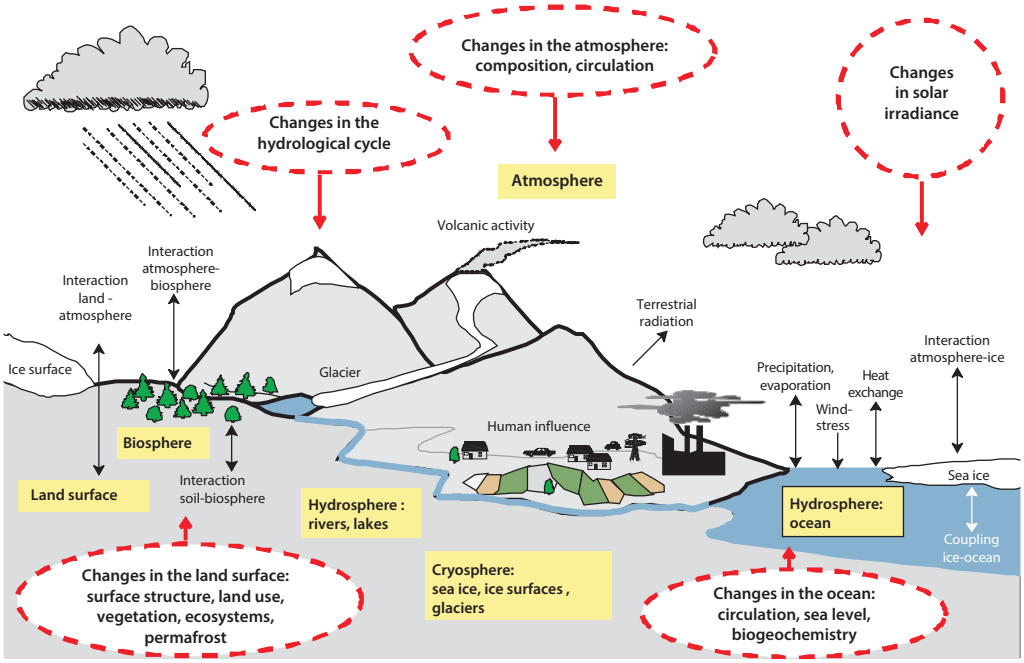


Fig. 1.1 The climate-determining components and their interactions. (Modified according to IPCC, 2001, 2007)

ous external forcing mechanisms, the sun being the most important. However, the direct effect of human activities on the climate system is also considered an external forcing.

The **atmosphere** is the most unstable and rapidly changing part of the system. Its composition, which has changed during the evolution of the Earth, is central to the problem of climate (for the structure and orientation in the different levels, see Fig. 1.2).

Earth's dry atmosphere is composed mainly of nitrogen (N_2), oxygen (O_2) and argon (Ar). These gases have little interaction with incoming solar radiation and they do not interact with the infrared radiation emitted by the Earth. However, there are a number of trace gases, such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O, laughing gas) and ozone (O_3), which absorb and emit infrared radiation. These so-called greenhouse gases, which however account for less than 0.1% of the total volume in dry air, play an essential role in the Earth's energy balance. The atmosphere also contains water vapour, which is also a greenhouse gas. Its contribution to the volume of air is highly variable, but it is of the order of 1%. Since these greenhouse gases absorb the infrared radiation emitted by the earth and emit it up and down, they cause the temperature to rise near the earth's surface. Water vapour, CO_2 and O_3 also absorb short-wave radiation (Table 1.1).

The distribution of ozone in the atmosphere and its role in the Earth's energy balance are unique. Ozone acts as a greenhouse gas in the lower part of the atmosphere, the troposphere and the lower stratosphere. Higher in the stratosphere, there is a natural layer of high ozone concentration that absorbs ultraviolet solar radiation. Thus, the so-called ozone layer plays an essential role in the radiation balance of the stratosphere, at the same time filtering out the harmful form of radiation.

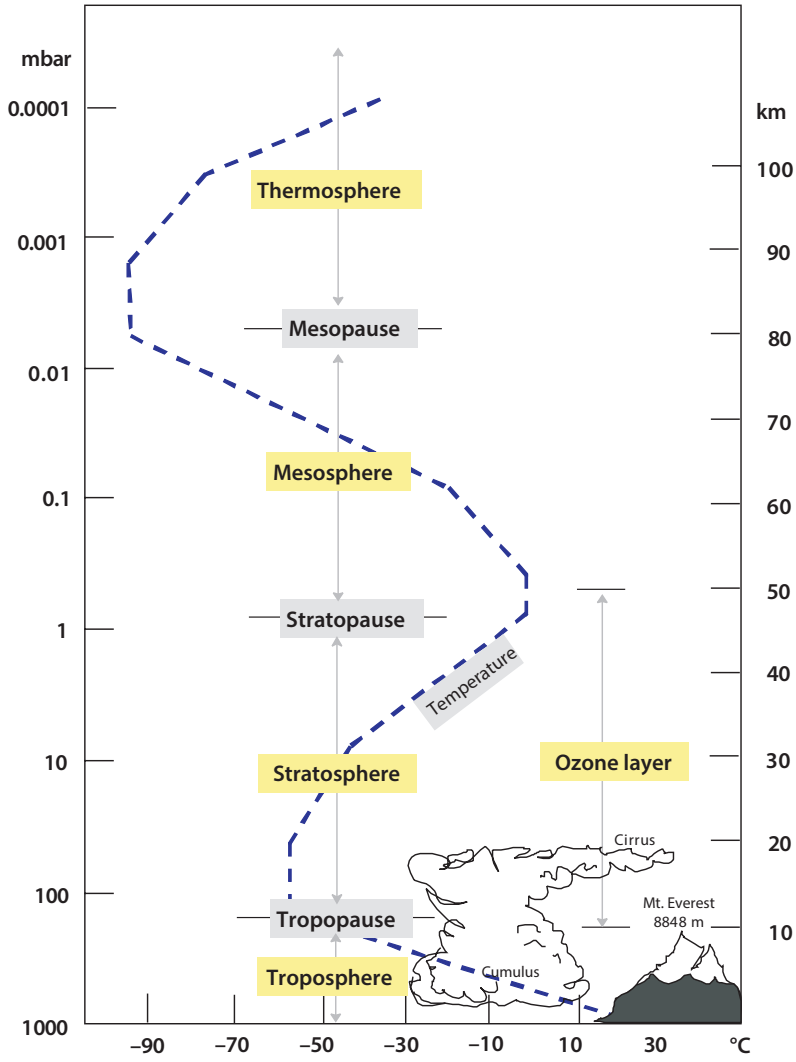


Fig. 1.2 Structure of the atmosphere with the temperature profile

In addition to these gases, the atmosphere contains solid and liquid particles (aerosols) and clouds, which interact with incoming and outgoing radiation in a complex and sometimes changing manner. The most variable component of the atmosphere is water in its various forms as vapor, cloud droplets, and ice crystals. Water vapor is the most potent greenhouse gas. The transfer between the different forms (evaporation/condensation/solidification) absorbs or releases a lot of energy. For these reasons, water vapour is important for the climate and its variability and change.

The **hydrosphere** is the component that includes subsurface water and all liquid surface, both freshwater of rivers, lakes, and aquifers and saltwater of oceans and seas. The runoff of freshwater from the land to the oceans by rivers affects the composition and circulation. The oceans cover about 70% of the Earth's surface. They store and transport a large amount of energy and dissolve and store large amounts of CO_2.

Table 1.1 Chemical composition of the atmosphere

Substance	Formula	Proportion in dry air	Primary source	Influence on atmosphere	Half-life
Nitrogen	N_2	78.084%	Biological	No	
Oxygen	O_2	20.948%	Biological		
Argon	Ar	0.934%		Inert	
Carbon dioxide	CO_2	360 ppmv	Incineration, ocean, biosphere	Greenhouse effect	
Neon	Ne	18.18 ppmv		Inert	
Helium	He	5.24 ppmv		Inert	
Methane	CH_4	1.7 ppmv	Biological and anthropogenic	Greenhouse effect, tropospheric and stratospheric chemistry	9 years
Hydrogen	H_2	0.55 ppmv	Biological, anthropogenic, photochemical	Insignificant	3 years
Nitrous oxide	N_2O	0.31 ppmv	Biological and anthropogenic	Greenhouse effect, stratospheric chemistry	120 years
Carbon monoxide	CO	50–200 ppbv	Anthropogenic and photochemical	Tropospheric chemistry	60 days
Ozone (troposphere)	O_3	10–500 ppbv	Photochemical	Greenhouse effect	
Ozone (stratosphere)	O_3	0.5–10 ppbv	Photochemical		
Non-methane hydrocarbons	For example isoprene	5–20 ppbv	Biological and anthropogenic		<1 day
Halocarbons		3.8 ppbv	85% anthropogenic	Partly ozone hole	
Nitrogen species	NO_x	10 ppt–1 ppm	Soils, lightning, anthropogenic		1 day
Ammonia	NH_3	10 ppt–1 ppb	Biological		5 days
Particulate nitrates	NO_3^-	1 ppt–10 ppb	Photochemical, anthropogenic		
Particulate ammonium	NH_4^+	10 ppt–10 ppb	Photochemical, anthropogenic		
OH radical	$OH^\bullet$	0.1–10 ppt	Photochemical	Purification of the atmosphere	

(continued)

Table 1.1 (continued)

Substance	Formula	Proportion in dry air	Primary source	Influence on atmosphere	Half-life
Peroxy radical	$HO_2^{\bullet}$	0.1–10 ppt	Photochemical	Purification of the atmosphere	
Hydrogen peroxide	H_2O_2	0.1–10 ppb	Photochemical		
Formaldehyde	CH_2O	0.1–1 ppb	Photochemical		
Sulfur dioxide	SO_2	10 ppt–1 ppb	Photochemical, volcanic, anthropogenic		3 days
Dimethyl sulfide	CH_3SCH_3	10–100 ppt	Biological	Cloud formation	1 day
Carbon disulfide	CS_2	1–300 ppt	Biological, anthropogenic		
Carbon oxide sulfide	COS	500 pptv	Biological, volcanic, anthropogenic	Aerosol formation	5 years
Hydrogen sulfide	H_2S	5–500 ppt	Biological, volcanic		4 days
Particulate sulfate	SO_4^{2-}	10 ppt–10 ppb	Photochemical, anthropogenic		

After Brasseur et al. (1999), Conrad (1996), Schlesinger (1997)

The circulation driven by the wind and density differences due to salinity and temperature gradients (*thermohaline circulation*) is much slower than the circulation in the atmosphere. Mainly due to the strong thermal inertia of the oceans, they dampen strong and rapid temperature changes and act as a regulator of the Earth's climate and a source of natural climate variability over a wide time horizon.

The **cryosphere**, which includes the ice sheets of Greenland and Antarctica, continental glaciers and snowfields, sea ice and permafrost, derives its importance for the climate system from its high reflectivity (albedo) for solar radiation, its low thermal conductivity, its high thermal inertia. It plays a particularly critical role in driving the circulation of deep ocean waters. Because the ice layers store large amounts of water, changes in volume are a potential source of sea level variance.

The vegetation and soils of the **land surface** affect how much of the energy received from the sun is returned to the atmosphere. Some returns as infrared radiation and warms the atmosphere as the land surface warms. Some of the energy causes water to evaporate from either soil or plant leaves, returning water to the atmosphere. Because evaporation of soil moisture requires energy, soil moisture has a strong effect on surface temperature. The texture of the land surface (its roughness) dynamically affects the atmosphere through wind over the surface. Roughness is influenced by topography and

vegetation. Wind also blows dust from the surface into the atmosphere, which in turn interacts with atmospheric radiation.

The marine and terrestrial **biospheres** have a significant influence on the composition of the atmosphere. Living organisms influence the uptake and release of greenhouse gases. Through photosynthetic processes, marine and terrestrial plants (especially forests) store significant amounts of carbon. Therefore, the biosphere also plays a central role in the carbon cycle as well as in the budget of many other gases such as methane and N_2O. Other emissions from the biosphere are the so-called volatile organic compounds (VOXs), which have significant effects on atmospheric chemistry, aerosol formation and therefore climate.

Since carbon storage and trace gas exchange are influenced by climate, feedbacks can occur between climate change and trace gas concentrations in the atmosphere. The influence of climate on the biosphere is preserved in fossils, tree rings, pollen and other records, so knowledge of past climate comes from such bioindicators.

1.1.2 Interactions Between the Components

Many physical, chemical and biological interaction processes occur between the different components of the climate system in a large space and over long periods of time. They make the system extremely complex. Although the components of the climate system differ greatly in their composition, physical and chemical properties, structures and behaviour, they are coupled by mass fluxes, heat and motion: all subsystems are open and interconnected.

As an example of this, the atmosphere and oceans are strictly coupled and exchange water vapor and heat through evaporation. This is part of the hydrological cycle and leads to condensation, cloud formation, precipitation and runoff, and feeds energy into the weather system. On the other hand, precipitation affects salinity, its distribution and *thermohaline circulation.* The atmosphere and oceans exchange CO_2 among other gases, maintaining a balance between dissolving in cold polar water, sinking to the ocean depths, and outgassing from relatively warm, rising water near the equator.

Some other examples: The ice of the sea prevents the exchange between the atmosphere and the oceans. The biosphere influences the concentration of CO_2 through photosynthesis and respiration, which is inversely influenced by climate. The input of water to the atmosphere is determined by evapotranspiration of the biosphere. The radiative balance is influenced by the amount of solar radiation that is reflected back, the albedo.

In conclusion, any change in the components of the climate system and their interactions or external forcings, whether natural or anthropogenic, can lead to climatic changes.

1.1.3 Energy Balance of the Earth

The fundamental reason for the existence of the "**greenhouse effect**" is that temperature decreases with altitude in the troposphere. Radiatively active gases (CO_2, H_2O) as well as clouds absorb radiation emitted by the warmer surface, while emission of radiation to space occurs due to colder atmospheric temperature. The interception of IR radiation by radiatively active molecules produces an increase in surface temperature of about 33 °C (assuming no change in albedo when the atmosphere is removed). Without the "greenhouse effect", the mean surface temperature would be only −18 °C and life would not be possible on Earth. At higher altitudes, radiative emission towards space through the 15 μm band of CO_2 contributes to cooling in the stratosphere and mesosphere.

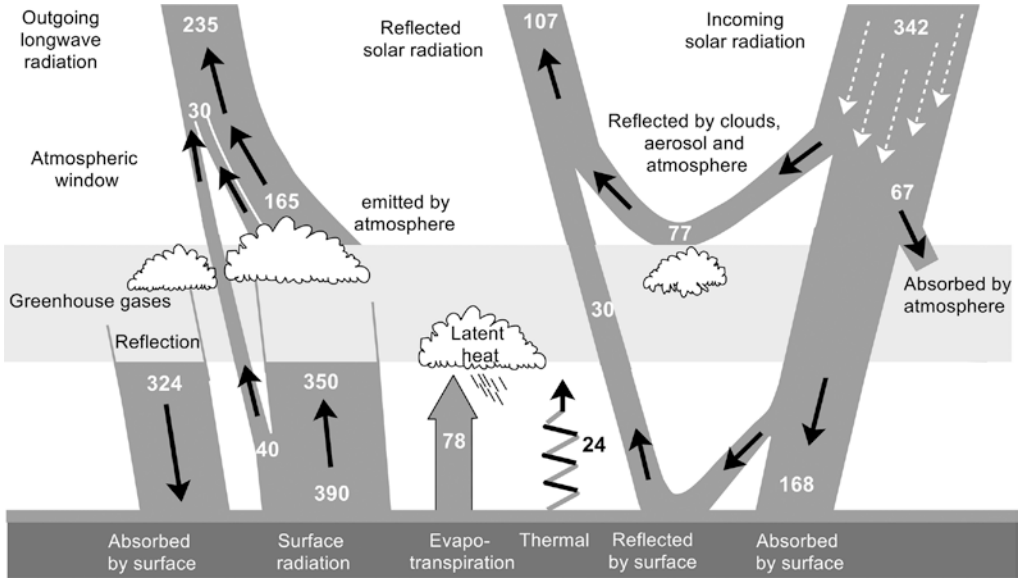


Fig. 1.3 Energy balance of the Earth. (Modified after Kiehl & Trenberth, 1997). Figures in W/m^2/year

The surface climate is directly influenced by the radiation balance between the incoming solar radiation and the outgoing radiation (reflected solar radiation and infrared radiation). The global energy balance of the Earth can be roughly represented as follows (Fig. 1.3): Solar energy striking the Earth accounts for about 342 W/m^2, of which about 107 W/m^2 (or 31%) is reflected into space (22% due to reflection by clouds, air molecules, and particles, and 9% due to reflection by the Earth's surface). 67 W/m^2 (or 20%) is absorbed in the atmosphere, by ozone in the stratosphere, and by clouds and water in the troposphere.

The remaining 168 W/m^2 (or 49%) is absorbed by the Earth's surface. Of the terrestrial energy radiated from the surface (390 W/m^2 or 114%), only 40 W/m^2 (12% of the incoming solar radiation) passes through the atmospheric window (cloudless sky) directly into space. The remaining 350 W/m^2 (or 102%) is absorbed in the troposphere by water vapor, CO_2, O_3, and other greenhouse gases, as well as clouds and aerosols. Finally, energy of the order of 324 W/m^2 (or 95%) is returned to the surface, while 195 W/m^2 (or 57%) is emitted to space. The excess energy received by the surface is compensated by non-radiative processes such as evaporation (latent heat flux of 78 W/m^2 or 23%) and turbulence (sensible heat flux of 24 W/m^2 or 7%).

Note the difference between the radiation emission from the Earth's surface (390 W/m^2) and the total infrared emission to space (40 + 195 = 235 W/m^2). The energy intercepted by the atmosphere represents the greenhouse effect.

Among the gases present in the atmosphere, the largest contribution to the greenhouse effect comes from water vapor, followed by CO_2 and other trace gases such as CH_4, N_2O, O_3 and chlorofluorocarbons (CFCs).

Clouds also absorb and emit infrared radiation. In addition, they increase the global albedo. The overall effect on climate is very complex. High altitude cirrus clouds are believed to contribute to warm-

ing, while lower altitude stratus clouds contribute to cooling. Overall, the presence of clouds causes the Earth to cool. However, the complicated feedback effects can lead to warming as well as cooling, depending on the change in clouds due to the changing climate.

As the concentration of some radiatively active gases has increased due to human activities, there is great anxiety about the possible increase in greenhouse forces. When the concentration of a radiatively active gas increases, the long-wave radiation into space is lowered first. As a consequence, the energy balance at the top of the atmosphere becomes unbalanced. At the same time, if the gas does not affect the absorption of solar radiation, the total radiant energy in the lower atmosphere as well as the Earth's surface will increase. The energy balance is restored by the warming of the surface troposphere system.

Changes in the Climate System Are Reported to the Public by the IPCC

The *Intergovernmental Panel on Climate Change* (IPCC) was established in 1988 by the *World Meteorological Organisation* (WMO) and the *United Nations Environment Programme* (UNEP). The IPCC's task is to determine at regular intervals the state of the climate system and its impact on human societal systems, and to identify options for political countermeasures.

The IPCC does not conduct its own research, but uses the scientific literature. Its reports are essentially produced by scientists working in the *World Climate Research Programme* (WCRP).

Five progress reports were produced between 1990 and 2013.

Information on the IPCC can be found at ► http://www.ipcc.ch and on the WCRP at ► http://wcrp.wmo.int.

1.1.4 Climate Change and Its Effects

The changes in the radiation balance responsible for the climate system are caused predominantly by carbon dioxide (66%), and to a lesser extent by other greenhouse gases (methane and nitrous oxide, 33%). Changes in solar irradiance, on the other hand, have only a minor influence.

The observed consequences in the climate system are an increase in temperature and, as a result, a rise in sea level, a decrease in ice cover and changes in the frequency and intensity of extreme weather events (storms, precipitation and droughts).

1. Heating

   There is no doubt about the warming of the climate system for the period 1906–2005. The global mean surface temperature has increased by 0.74 °C. Of the last 12 years, 11 have been the warmest since records began. The temperature increase over the last 50 years is twice that of the last 100 years. Satellite measurements since 1978 and weather balloon measurements show that the temperature of the lower 8 km of the atmosphere has increased by +0.05 ± 0.10 °C/decade.

   Extreme low temperatures were less common after 1950, with a weak increase in extreme high temperatures.

   On average, between 1950 and 1993, the nocturnal daily minimum over the country increased by about 0.2 °C/decade.

   Reconstructed data from observations and other sources, such as tree-ring data, indicate that temperatures over the past 50 years have very likely been higher than at any time in the past 500 years and probably higher than in the past 1300 years.

   The Arctic has warmed twice as much as the global average.

   The increase in ocean temperature over the last 100 years has been about half that of the mean air temperature over land (◘ Fig. 1.4).

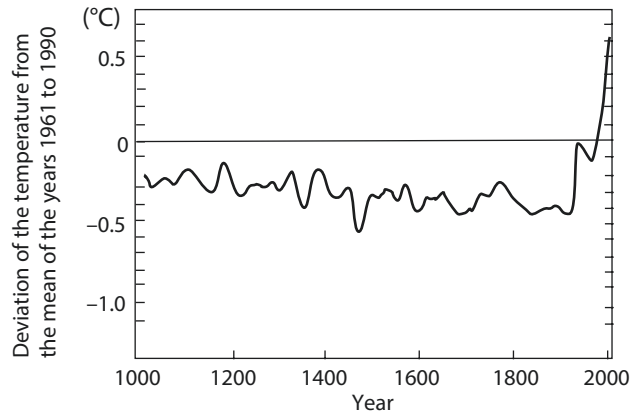


Fig. 1.4 Warming over the last 1000 years in the northern hemisphere. (After IPCC, 2001). Data obtained from thermometer measurements from 1861 onwards, earlier data from measurements of tree growth rings, coral growth, ice core probe studies and historical reports

2. Sea level

   The oceans have become warmer to depths of 3000 m on global average. This warming has contributed to sea level rise. Since 1993, sea level has risen by an average of about 3 mm/year, and by a total of 17 cm in the twentieth century. Of this, slightly more than half has been caused by thermal expansion of the warmer ocean, about 25% by melting of mountain glaciers (0.8 mm/year), and about 15% by melting of the ice sheets on Greenland and Antarctica (0.4 mm/year).

   One indicator of the changes in precipitation and evaporation and of the enhanced transport of water vapor in the atmosphere from low to higher latitudes is the observed change in ocean salinity.

3. Glaciers, ice sheets and permafrost

   The snow-covered area has decreased by about 5% since 1980. Glaciers are shrinking worldwide. Sea ice in the Arctic has declined by an annual average of 8% and by 22% in summer since 1978. The thickness of Arctic sea ice during late summer and autumn has decreased by 40% in the last decade.

   The ice sheets on Greenland and Antarctica are currently losing mass due to melting and glacier collapse.

   Temperatures in the upper layers of permafrost have increased by 3 °C since 1980, and the extent of seasonally frozen ground has decreased by 7% since 1900, and by as much as 15% in spring.

   The annual glaciation of rivers and lakes in the middle and higher latitudes of the northern hemisphere decreased by about two weeks in the twentieth century.

4. Precipitation

   The frequency of heavy precipitation has increased.

   Precipitation increased over the mid- and higher latitudes of the northern hemisphere by 0.5–1% per decade during the twentieth century. Rainfall has been more frequent over tropical countries by 0.2–0.3% per decade. However, it appears that rainfall over subtropical areas of the Northern Hemisphere has decreased by 0.3% per decade.

   In the middle and higher latitudes of the northern hemisphere, cases of severe precipitation have increased by 2–4% in the latter part of the twentieth century. This can be attributed to changes in atmospheric water content, thunderstorms and large-scale storms.

   Over mid- and high-latitude land, a 2% increase in cloud cover is thought to have occurred during the twentieth century.

UN Experts Warn of Danger from Thawing Permafrost Soils

Permafrost soils make up about 24% of the land surface in the Northern Hemisphere. In Canada, Russia, the USA and China in particular, large areas are permanently frozen. So far, they have only thawed temporarily at the surface in summer.

According to a UN report, the Arctic soils contain around 1700 Gt (billion tonnes) of carbon—twice as much as is currently circulating in the atmosphere. This carbon is gradually and irreversibly released during thawing as carbon dioxide (CO_2) and in the form of the even more potent greenhouse gas methane.

According to the report, the thawing of the soils will significantly increase global climate change. The rise in air temperature in the Arctic and the Alps will be about twice as fast as the global average.

Climate projections indicate a substantial loss of permafrost soils. Warming permafrost may release greenhouse gases equivalent to the climate effect of 45–135 Gt of carbon dioxide by 2100. By 2200, it may be 246–415 Gt.

Permafrost may eventually account for 39% of total emissions.

Compared to the 100 years before, warm periods of the El Niño phenomenon (El Niño-Southern Oscillation phenomenon, ENSO) have occurred more frequently, permanently and intensively since the mid-1970s.

El Niño

The same phenomenon, known as "El Niño" or "Southern Oscillation", has its origin in exceptional meteorological conditions occurring in regions several thousand kilometres apart. Usually, abundant rains pour down over Indonesia and northern Australia, while the coastal regions of Ecuador and Peru receive very little rain as a result of the circulation that occurs between the western and eastern Pacific oceans. The air rises over Indonesia while it sinks over the regions in the southeast Pacific, between Easter Island and Peru. As it rises, the air, laden with moisture at the ocean surface, cools and condenses. This releases a large amount of energy that sustains convection. In contrast, the cold air that sinks over Peru brings almost only dryness.

This cycle is disrupted on a large scale during the El Niño phenomenon. Convective activity, usually over Indonesia, shifts to the middle of the Pacific. As a result, areas in the western Pacific receive less rain, while the central and eastern Pacific receive more. A shift of convection over the western Pacific toward the center of the Pacific occurs on average only every 3–4 years. The time interval between two successive events varies. It may recur in 2 or 10 years.

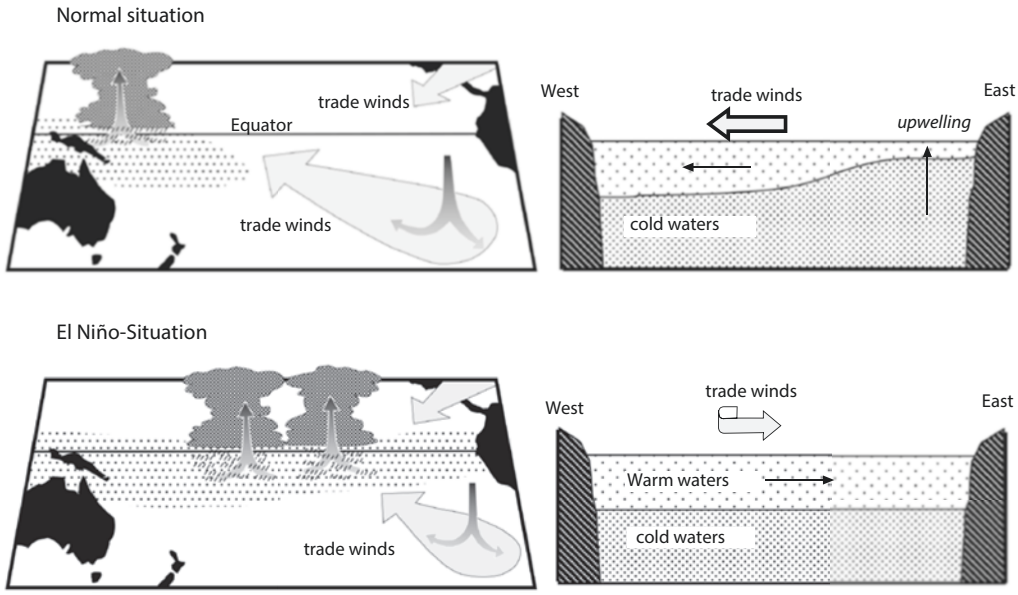
The Southern Oscillation is accompanied by a significant increase in the temperature of the surface waters of the Eastern Pacific. In a vast area around the equator, the water temperature, normally between 20 and 25 °C, increases by a few degrees, which is accompanied by an increase in rainfall. This event generally occurs at Christmas and is called El Niño in South American, after the Spanish name for the baby Jesus. The El Niño phenomenon is not limited to the Christmas period, however, and between 12 and 18 months can pass between the appearance and disappearance of the warm water in the eastern Pacific.

In normal times, cold coastal waters are formed by the upwelling of water masses from great depths. This event is known as *upwelling.* The circulation

1

brings numerous nutrients such as phosphates and sulphates to the surface, which ensure the proliferation of phytoplankton. During an El Niño event, this upwelling of deep water to the surface is blocked and the entire food chain is disrupted. The phytoplankton declines, so that the zooplankton, which feeds on the phytoplankton, disappears, resulting in the death or migration of fish to more fertile waters (◘ Fig. 1.5).

El Niño is a phenomenon resulting from the interaction between the atmosphere and the tropical ocean. In a so-called "normal" period, the water temperature in the tropical western Pacific is close to 28–29 °C, while in the east it reaches no more than 20–25 °C. This strong asymmetry of temperature controls the atmospheric circulation, which in turn maintains the temperature gradient. Indeed, the warmest waters provide the necessary heat and moisture to develop strong convective activity over the western Pacific, which drives the trade winds. The trade winds, in turn, maintain the east-west gradient in temperature. In the eastern Pacific, they induce a rightward deflected surface current in the northern hemisphere. In the southern hemisphere, this current is deflected to the left due to the Earth's rotation. It transports surface water on both sides of the equator, causing cold water to rise to compensate. In addition, the surface water is transported to the west, where it stops the *upwelling process.* Heated by the sun, these waters reach the highest oceanic temperatures, favouring intense convection. During the course of an El Niño event, oceanic and atmospheric circulation alternate in a process of mutual action and reaction. In particular, the warming of the central Pacific generates 28–29 °C warm water, shifting the strong convective activity to the east. This results in a weakening of the trade winds in the western Pacific, and even a reversal of



◘ **Fig. 1.5** Comparative representation of the normal and El Niño situation *over* the Pacific (left) and *in* the Pacific (right)

their direction. When trade winds are weak, the energy of the surface current decreases, and the warm waters of the western Pacific flow eastward. This, in turn, has the effect of warming the Central Pacific and disrupting the upwelling of cold waters off the South American coast.

El Niño years add to the already increasing amount of greenhouse gases such as carbon dioxide (CO_2) in the atmosphere. The powerful El Niño event of 2015–2016—the third strongest since the 1950s—has had a major impact on the Earth's natural climate system.

The 2015/2016 El Niño released an additional 8.8 billion tonnes of CO_2 into the atmosphere. This corresponds to about a quarter of the amount released worldwide each year by human activities. By comparison, around 0.8 billion t CO_2 were emitted from fossil fuels in Germany in 2015.

The new figure of 8.8 billion tonnes of additional carbon dioxide suggests that fire-related emissions have only a secondary effect on the carbon content of the atmosphere. The fact that the vegetation in the southern hemisphere, which was suffering from drought as a result of El Niño, was unable to absorb as much CO_2 as usual, is probably having a much greater impact.

Thus, the excess CO_2 flux results mainly from reductions in vegetation uptake due to drought and, to a lesser extent, from increased biomass burning (Patra et al., 2017).

Globally, there was no increase in the number of severe droughts and wetnesses on land masses between 1900 and 1995. In some regions of Asia and Africa, however, drought has become more frequent and severe in recent decades.

Contrary to earlier assumptions, however, some aspects important for climate did **not** occur. There has been no warming over a few areas of the Earth, such as over the oceans of the Southern Hemisphere and parts of Antarctica.

There was no clear trend in the global frequency and intensity of tropical and extra-tropical storms during the twentieth century, apart from some local variations. Systematic changes in the frequency of tornadoes, thunderstorm days and hail precipitation were not detectable.

2017 Was One of the Three Warmest Years on Record

According to the 28th State of the Climate report, 2017 was the third warmest year on record for the planet after 2016 and 2015. The planet also experienced record high concentrations of greenhouse gases as well as sea level rise.

Notable findings from the *American Meteorological Society*'s international report include:

- **Concentrations of greenhouse gases were the highest since records began.** Concentrations of greenhouse gases in the atmosphere—including carbon dioxide (CO_2), methane and nitrous oxide—reached new highs. The average global CO_2 concentration in 2017 was 405 ppm. This is the highest value measured in the modern 38-year global climate datasets and records from ice core samples.
- **Sea level rise reached a new high—about 7.7 cm higher than the 1993 average, and** global sea levels are rising at an average rate of 3.1 cm/decade.
- **Global land and ocean surface temperatures reached near-record levels.** Depending on the dataset, average global surface temperatures were 0.38–

0.48 °C above the 1981–2010 average, marking 2017 as the one with the second or third warmest annual global temperature since records began in the mid-to-late nineteenth century.

- **Sea surface temperatures reached near-record highs.** Although the global average sea surface temperature in 2017 was slightly lower than in 2016, the long-term trend remained upward.
- **In the uppermost 700 m of the world's oceans,** the **temperature reached a record high,** reflecting the continued accumulation of thermal energy.
- **Drought levels fell and recovered.** The global zone of drought fell sharply in early 2017, only to rise to above-average levels later in the year.
- **The Arctic's maximum ice cover fell to a record low.** The maximum extent of Arctic sea ice in 2017 was the lowest in the 38-year record. The September 2017 sea ice minimum was the eighth lowest in 25 years and 25% below the long-term average.
- **Antarctica recorded a record-low sea ice cover**, well below the 1981–2010 average. On March 1, 2017, sea ice extent fell to 2.1 million km^2, the lowest observed value in the continuous satellite record that began in 1978.
- **Unprecedented coral bleaching in several years.** Global coral bleaching lasted from June 2014 to May 2017, leading to unprecedented impacts on reefs. More than 95% of corals in some reef areas died.
- **The total number of tropical cyclones was slightly above average overall.** There were 85 named tropical cyclones in 2017, slightly more than the 1981–2010 average of 82 storms.

Bulletin of the American Meteorological Society. *State of the Climate in 2017 report*. Vol. 99. No. 8, August 2018.

Report is based on the participation of more than 500 scientists from 65 countries.

► http://www.noaa.gov/news/2017-was-one-of-three-warmest-years-on-record-international-report-confirms.

1.1.5 Which Substances Have Which Effect on the Climate?

The influence of the various components of the atmosphere on the climate is shown in ◘ Fig. 1.6. It becomes clear that some components lead to a warming, others contribute to the cooling of the earth. It is also noticeable that relatively large ranges of variation are still given for most climate-relevant parameters. Here, the effect of aerosols is to be addressed in particular.

The concentration of greenhouse gases is systematically increasing (◘ Fig. 1.7). The carbon dioxide content of the air has increased by 35% from 280 to 379 ppm between 1750 and 2005. The present value is the largest in the last 650,000 years. The rate of increase over the last 10 years is the highest in 50 years. Fossil fuel use is the main cause of the increase.

Concentrations of methane and nitrous oxide, long-lived greenhouse gases, have increased by 148% and 18% respectively since 1750. This is primarily caused by agriculture.

Global Warming Potential

To characterize the effects of various climate-influencing factors, the following measures are primarily used: **greenhouse potential, radiative forcing** and **global warming potential.**

- **Greenhouse potential** is the extent to which different greenhouse gases can absorb additional radiant energy when their concentration is increased,

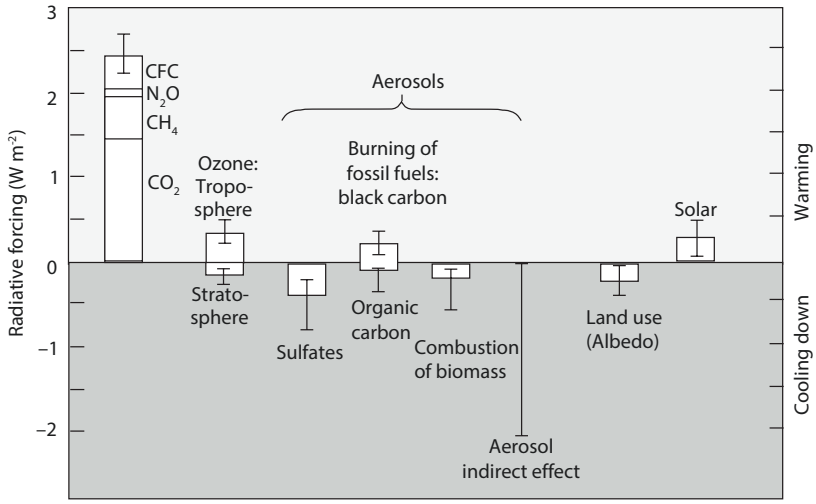


Fig. 1.6 Influence of various components on the Earth's radiation balance. (Modified according to IPCC, 2001)

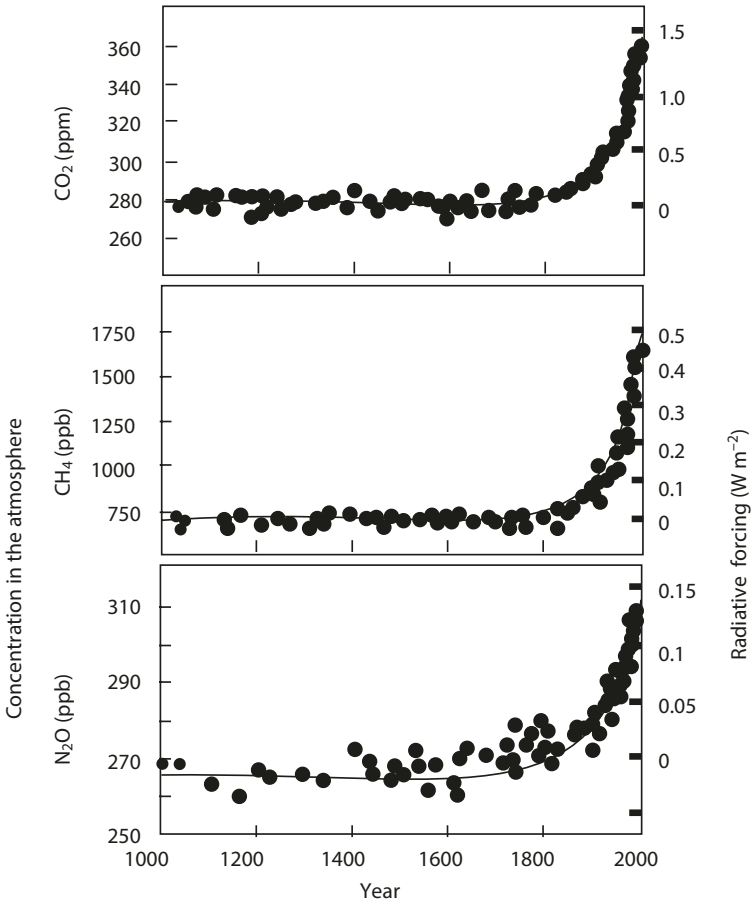


Fig. 1.7 Global concentration in the atmosphere of three well-mixed greenhouse gases CO_2, CH_4, N_2O. (Modified according to IPCC, 2001)

depending on their absorption, emission and scattering properties.

- While the global warming potential is thus limited exclusively to the radiation factor, the concept of **radiative forcing** was developed for the comparison of different influencing factors. This refers to the change in the global mean of the radiation balance at the stratopause and is thus a measure of the disturbance of the equilibrium between incoming solar energy and long-wave radiation emitted to space.
- The concept of **Global Warming Potential (GWP)** builds on that of radiative forcing and comprises the sum of all radiative forcing contributions of a gas up to a chosen time horizon, which are caused by the single release of a unit of measurement at the beginning of the period. Thus, it is possible to extrapolate the climate impact of greenhouse gases for different time horizons into the future. In most cases, the Global Warming Potential is given in relation to 100 years: CO_2: 1; CH_4: 30; N_2O: 300; SF_6 (sulfur hexafluoride): 23,900; HFCs (partly fluorinated hydrocarbons): 2530 (mean); PFCs (fully fluorinated hydrocarbons): 7614 (mean).

1.1.6 Projections

Climate models predict a further rise in temperature and sea level over the next 100 years, depending on energy use.

1. Heating

   For the last decade of the twenty-first century, the most likely value of global warming is 1.1–2.9 °C for the lowest scenario and 2.4–6.4 °C for the highest scenario. The greatest warming occurs in high northern latitudes. For the next 2–3 decades, the projected warming depends little on assumptions about future emissions, and even if all emissions were to end immediately, the inertia of the climate system would result in a further temperature increase of up to about 0.6 °C.
2. Sea level

   For sea level rise, the projections for 2090–2100 are: 18–38 cm for the lowest scenario and 26–59 cm for the highest scenario.

   Even after emissions have ceased completely, sea levels will continue to rise for many centuries due to further warming of the deep ocean.
3. Glaciers and ice sheets

   There is considerable uncertainty regarding the further development of the Greenland and Antarctic ice sheets. Sustained warming significantly above 3 °C over millennia would lead to a complete melting of the Greenland ice sheet, corresponding to a sea level rise of 7 m.
4. Gulf stream

   It is very likely that the meridional overturning motion in the Atlantic will decrease by an average of 25% in the twenty-first century. Temperatures in the Atlantic region will nevertheless increase because the influence of global warming will predominate. However, it is very unlikely that there will be an abrupt collapse in the twenty-first century.
5. Precipitation

   Precipitation is very likely to increase at higher latitudes, while the tropics and subtropics (including the Mediterranean region) are likely to experience a reduction in precipitation.

1.2 Global Cycles with Reservoirs and Material Flows

Biological cycles are important parts of global material flows, which in turn are relevant for the climate. For example, microorganisms play a central role in the mineralization of substances. Biological

cycles such as the nitrogen and sulfur cycles or metal reductions and oxidations depend on the activity of prokaryotes. In addition, prokaryotes represent significant pools. Approximately 500 billion t of carbon are sequestered in bacteria and archaea, accounting for about half of the total carbon found in biomass. In terms of nitrogen and phosphorus, almost 90% is bound in prokaryotes.

1.2.1 Global Carbon Cycle

Globally, carbon migrates through all of Earth's major carbon reservoirs: the atmosphere, the land (organic carbon in humus), the oceans and other aquatic environments (carbonate, bicarbonate, and dissolved CO_2), sediments and rocks (carbonates; organic carbon of biological origin fixed as fossil reduced carbon reserves in coal, natural gas, petroleum, and sediments), and biomass (living and dead).

Figure 1.8 shows the reservoirs and the material fluxes. The largest carbon reservoir is in the sediments and rocks of the Earth's crust, but the turnover time here is so long that the flux from this area is relatively insignificant by human standards. From the point of view of living things, a large amount of organic carbon occurs in land plants, which make up the bulk of the biomass of the land surface. This is the carbon of forests and steppes, which are the main sites for photosynthetic CO_2 fixation (► Sect. 3.2). However, there is more carbon in dead organic matter, humus, than in living organisms. Humus is a complex mixture of organic matter. It comes partly from the components of soil microorganisms that have resisted decomposition and partly from non-degradable plant matter. Some humus substances are quite stable, with a global turnover rate of about 40 years, while other humus components degrade much faster.

The fastest mode of global carbon transfer is via atmospheric CO_2. Carbon dioxide is removed from the atmosphere mainly by photosynthesis by terrestrial plants and released back into the atmosphere by respiration by animals and chemoorganotrophic microorganisms (► Sect. 3.2). Analysis of the different processes suggests that microbial decomposition of dead organic matter, including humus, is the most significant contributor to the CO_2 content of the atmosphere.

The oceans and seas have a primary production that is difficult to quantify. It is estimated to be 45×10^9 t C. Since the biomass of phytoplankton is much lower than that of land plants, at about 3×10^9 t C, this means that there is a higher turnover in the oceans than on the continents.

The oceans act as a CO_2 sink. They have an internal CO_2 or bicarbonate cycle. CO_2 is exchanged via the sea surface. The exchange between surface and deep water is very slow. Sedimentation of C compounds is of the order of 0.2×10^9 t C/year.

The increase in the CO_2 content of the atmosphere is caused by the increasing combustion of fossil fuels such as coal and oil and by deforestation. This results in an additional CO_2 formation of 8×10^9 t C.

The dimension of the natural conversion of substances is about two orders of magnitude higher than that of anthropogenic production.

- **Carbon Monoxide (CO)**

Carbon monoxide is a relatively minor component of the atmosphere compared to CH_4 and CO_2 and has a low impact in terms of climate gas (GWP = 1.4). It is addressed as an indirect climate gas because of its central role in tropospheric chemistry. CO is significantly involved in the control of $OH^{\bullet}$ radicals and the production of ozone. A significant annual increase of ~6% in global mean tropospheric concentration was observed in the mid-1980s. Natural sources of CO to the atmosphere are from the $OH^{\bullet}$-radical pathway as well as from hydrocarbons emitted by plants such as terpenes, but also bushfires and oceanic sources.

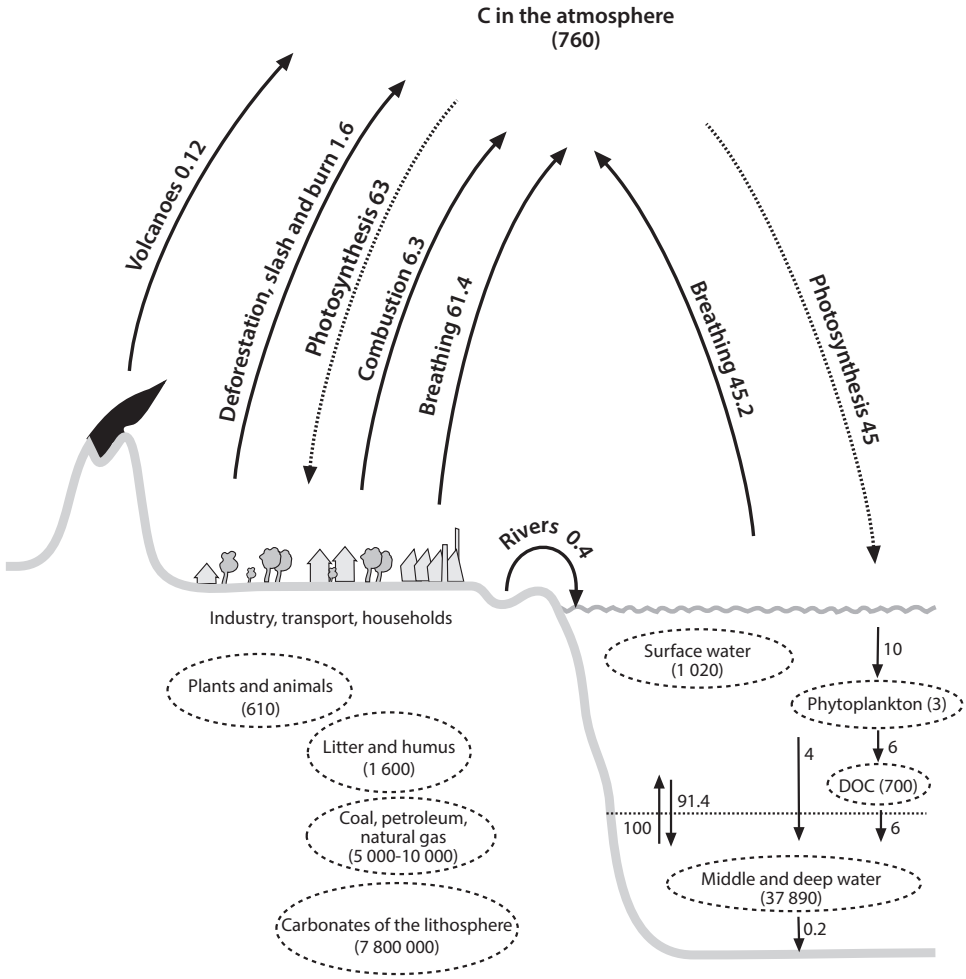


Fig. 1.8 The global carbon cycle. The figures at the arrows indicate the flux rates in 10^9 t C/year, the figures in brackets the C content of the various reservoirs in 10^9 t C. (Adapted from Mackenzie, 1997; ► http://www.pmel.noaa.gov/co2/)

Natural sources account for about 45% of the global CO budget, while the rest comes from anthropogenic sources (fossil fuel combustion, anthropogenic CH_4, slash-and-burn and savannah fires to produce agricultural land).

Tropospheric sinks involve the further oxidation of CO by $OH^{\bullet}$ radicals. In addition, a number of bacteria and fungi can metabolize CO, and soils can consume CO from the atmosphere. Overall, the global importance of soils as sinks is not known.

▪ Methane (CH_4)

Atmospheric CH_4 has experienced puzzling dynamics over the past 15 years. After a period of relative stagnation in the early 2000s (an increase of +0.5 ± 3.1 ppb/year on average over the period 2000–2006), atmospheric methane concentrations have increased more than tenfold since 2007 (+6.9 ± 2.7 ppb/year for the period 2007–2015, Dlugokencky, 2016). The growth rate of methane in the atmosphere accelerated to +12.5 ppb in 2014 and +9.9 ppb in 2015,

reaching an annual average concentration of 1834 ppb in 2015 (Dlugokencky, 2016). This emerging momentum underscores the growing contribution of methane to global warming versus the observed slower growth rates of CO_2 over the past three years (Jackson et al., 2016; Le Quéré et al., 2016) and the relatively constant growth rate of N_2O (Hartmann et al., 2013).

Based on recent atmospheric studies, global emissions are estimated at 559 [540–568] Tg CH_4/year for the period 2003–2012 (Saunois et al., 2016a, b). Tropical sources, including natural and anthropogenic sources, represent two-thirds of total global emissions and are dominated by emissions from wetlands. Approximately two-thirds of global emissions are also due to anthropogenic activities, including those from mid-latitudes and the tropics.

The main end products of the anaerobic mineralisation of organic compounds are methane and CO_2. Methanogenesis (► Sect. 4.5) mineralises about 10% of the organic matter produced. Despite the obligate anaerobiosis and specialised metabolism of methanogenic bacteria, they are quite widespread on Earth. Although methanogenesis is only strong in anoxic environments, such as swamps or the rumen of ruminants, the process also occurs in biotopes that might normally be considered oxic, such as forest and steppe soils. In such biotopes, methanogenesis occurs in anaerobic microenvironments, for example in the middle of soil crumbs. An overview of the rate of methanogenesis in different biotopes is given in ◘ Table 1.2. It is noteworthy that the biogenic production of methane by methanogenic archaea exceeds the production rate of gas sources and other abiogenic sources. About 1×10^9 t CH_4 escapes into the atmosphere annually. Of this, about 75% comes from microbial processes, about 25% is of industrial origin (combustion processes, leaking natural gas storage facilities and pipelines).

◘ **Table 1.2** Sources and sinks of methane in the atmosphere

Sources and sinks	10^6 t CH_4/year
Natural sources	
Wetlands	
Tropes	65
Northern latitudes	40
Other	10
Termites	20
Ocean	10
Freshwater	5
Geological	10
Total natural sources	160
Anthropogenic sources	
Fossil energy sources	
Coal mining	30
Natural gas	40
Oil industry	15
Coal combustion	15
Waste disposal systems	
Landfill sites	40
Animal waste	25
Domestic waste	25
Fermentation ruminant	85
Burning of biomass (slash and burn)	40
Rice fields	60
Total anthropogenic sources	375
Total sources[a]	535
Sink	
Reaction with OH	490
Distance	40

(continued)

Table 1.2 (continued)

Sources and sinks	10^6 t CH_4/year
Distance	30
Total sinks[a]	560
Atmospheric increase[b]	37

According to IPCC (1994, 1996)
[a] Calculated data
[b] Measured data (explanation for the discrepancy!)

Microbial methane formation occurs in ruminant rumen, rice paddies, and natural wetlands such as swamps, one third each. In addition, the formation of methane in termites is a noteworthy variable. The increase in rice cultivation and cattle breeding is an important cause of the increase in methane formation.

Methane hydrates on the seabed and in permafrost soils represent an exceptionally large methane deposit (► Sect. 4.5). The methane hydrates stored mainly at the continental margins of the oceans exceed the C quantities of the known oil, natural gas and coal deposits. The deposits have formed over millions of years under certain combinations of elevated pressure and low temperatures when sufficient methane was available.

Methane Is One of the Climate-Relevant Gases, Which Contributes About 15% to the Greenhouse Effect

- It absorbs long-wave radiation and is therefore a greenhouse gas.
- On a molecular basis, methane has a global warming potential about 30 times higher than CO_2.
- It is quite reactive and therefore plays an important role in tropospheric and stratospheric chemistry.
- The oxidation of methane by $OH^{\bullet}$ in the troposphere leads to the formation of formaldehyde, CO and in the environment of sufficient NO_x to ozone.
- In the stratosphere, methane acts as a sink for chlorine atoms and is therefore important for stratospheric ozone chemistry.
- Methane oxidation by $OH^{\bullet}$ is a major source of water vapor in the stratosphere.
- The half-life of methane in the atmosphere is 8–10 years.

1.2.2 Global Nitrogen Cycle

The main reactions of the global nitrogen cycle are summarized in Fig. 1.9.

For primary plant production, animal and human consumption, nitrogen is necessary in bound form. As nitrogen sources, plants use nitrate and ammonium, animals and humans use proteins and amino acids, respectively. Terrestrial plant production of about 180×10^9 t dry matter annually requires about 1.8×10^9 t N. Although the biomass of marine phytoplankton is much smaller, it also has a much greater turnover than terrestrial plants. Nitrogen consumption is likely to be of the same order of magnitude as for terrestrial vegetation. From the dimensions of the annual consumption, it can be deduced that most of the nitrogen is recycled again through the mineralization processes in the soil and in the waters.

Transfer of nitrogen to and from the atmosphere occurs largely as N_2 (biological fixation of elemental nitrogen [N_2]) (► Sect. 7.1) and the release of fixed nitrogen from soils and waters through denitrification (► Sect. 7.5.1) and smaller transfers of N_2O and NO and gaseous ammonia.

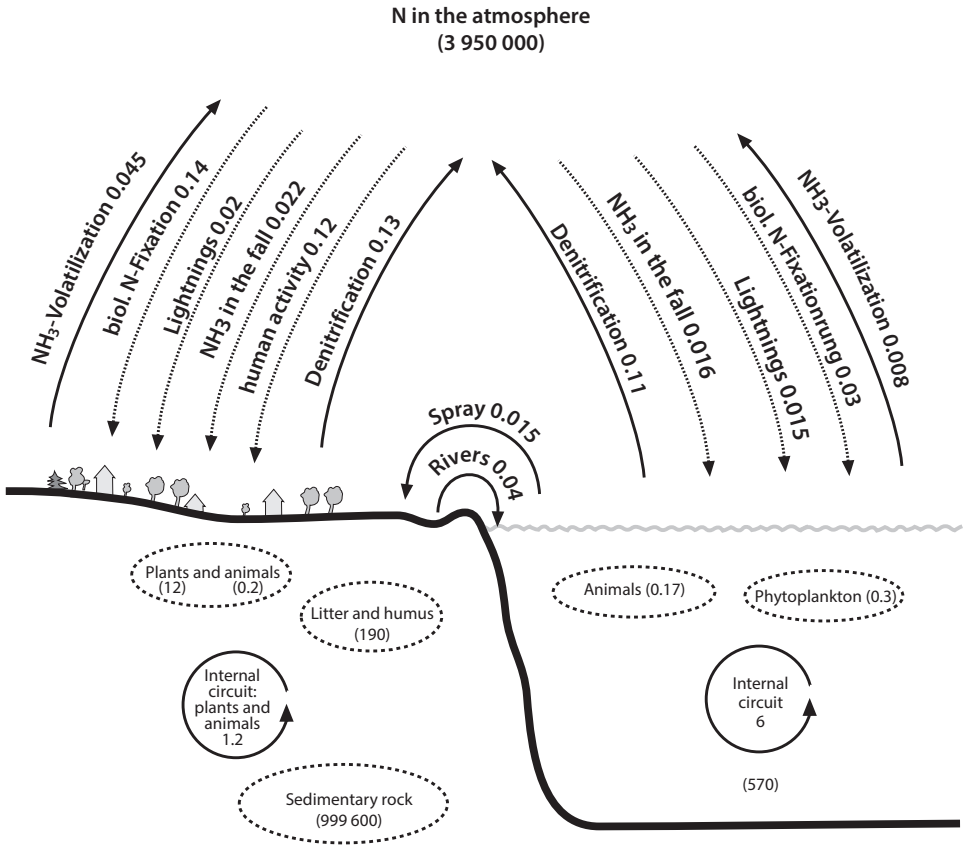


Fig. 1.9 The global nitrogen cycle. The data at the arrows indicate the material flows in 10^9 t N/year, the data in brackets indicate the N content of the various reservoirs in 10^9 t N. (Adapted from Mackenzie, 1997; Dentener & Crutzen, 1994; ► http://ic.ucsc.edu/~envs23/lecture14N-cycle_prt.htm; Mosier et al., 1998; Kätterer, 2001)

Compared to biological processes, the turnover rates of anthropogenic material flows are lower.

In both terrestrial and marine areas, bacterial atmospheric nitrogen fixation is the main source of fixed nitrogen. In terrestrial areas, it is comparable to chemical nitrogen fertilizer production.

Fertilization with mineral and organic fertilizers is used to increase agricultural productivity. About 9×10^7 t of mineral N fertilizer is produced annually. The usual method is to combine hydrogen and nitrogen in a 3:1 ratio at high temperature (300–500 °C) and pressure (400–1000 atm) in the presence of a catalyst (usually reduced iron) (see Box ► Haber-Bosch Process).

$$N_2 + 3H_2 \rightarrow 2NH_3$$

To produce nitrate fertilizer, ammonia is oxidized to nitrate. Since CO_2 and N_2O are emitted during this process, the production of nitrate is involved in the emission of greenhouse gases.

In addition to biological nitrogen fixation, fixation by atmospheric processes (thunderstorms, UV) is a non-negligible factor in the recycling of nitrogen. About 10% of the fixed nitrogen is supplied to the earth's surface from the atmosphere in this way.

Another important nitrogen flux from terrestrial systems is the volatilization of ammonium, some of which is returned to the earth's surface from the atmosphere by precipitation. Ammonium release is due to microbial degradation of organic compounds.

A significant factor in the global nitrogen cycle is the input of organically bound nitrogen compounds as well as ammonium and nitrate ions by rivers into the oceans (about 3.6×10^7 t/year).

Haber-Bosch Process

The industrial synthesis of ammonia from nitrogen and hydrogen had a greater fundamental significance for the modern world than the invention of the airplane, nuclear energy, space travel or the telephone. The expansion of the world population from 1.6 billion in 1900 to 7 billion today would not have been possible without the synthesis of ammonia through the Haber-Bosch process.

During the twentieth century, catalytic ammonia synthesis has grown into one of the most important industrial processes. Ammonia is now the most important bulk chemical along with sulfuric acid. Hundreds of mostly large plants are operated to ultimately produce fertilizer.

Today's ammonia synthesis has been greatly optimized in many details and functions with better energy efficiency than in the beginning. Today, more than 1% of total global energy use is still used for ammonia production. Consequently, further optimization could have no small impact on fossil fuel consumption. Despite continuous efforts to optimize the Haber-Bosch process, an iron-based catalyst similar to the one developed by Mittasch and co-workers is still used today.

Global ammonia emissions are 130 million tonnes, with 4/5 going into fertilisers, with urea being the most significant fertiliser.

Without the above synthesis, about 2/5 of the current world population would not exist. This dependency will increase further if there is an increase from 7 to 9 or 10 billion people.

An adult contains about 1 kg of nitrogen in the body.

In some countries, fertilizer use has risen to more than 50 kg of nitrogen/capita (the global average is about 13 kg).

The amounts of nitrogen fixed in fertilizer are comparable to half of that fixed by bacteria in all natural terrestrial ecosystems.

▪ Nitrous Oxide (N_2O)

In the global atmosphere, the accumulation of N_2O (nitrous oxide, laughing gas) leads to the destruction of the ozone layer in the stratosphere and to the greenhouse effect.

In addition to the natural formation of dinitrogen, this gaseous compound is formed primarily during high nitrogen fertilizer applications. N_2O is formed by side reactions of denitrification and nitrification. Oxygen limitation and a high N:C ratio promote its formation.

In terrestrial systems, nitrogen compounds cause the over-fertilisation of forests (recent forest damage due to the territorial accumulation of ammonia released by industrial animal production), and in aquatic systems, the eutrophication of water bodies and the nitrate pollution of groundwater.

As already mentioned, N_2O contributes to the destruction of the ozone layer in the stratosphere and to the greenhouse effect. Even though the amounts released are very small, N_2O is a very effective climate gas, as it is both very stable (half-life 150 years) and causes thermal absorption about 300 times higher than CO_2. Of the current global N_2

O emissions, about 25% are due to fertilization, 3% to combustion processes, with the majority coming from natural biogenic processes. Nevertheless, the additional anthropogenic N_2O release leads to environmental pollution.

Nitrous Oxide (N_2O) Is One of the Gases Relevant to Climate Change

- N_2O is predominantly released from soil and water into the atmosphere.
- Tropical soils are the most important natural sources of N_2O.
- Globally, temperate soils leak about half as much N_2O as tropical soils.
- Emissions of N_2O from fertilized agricultural land are the largest single anthropogenic source to the global amount of N_2O. Other sources (industrial activities, biomass burning, degassing of groundwater used for irrigation) are estimated to be smaller, although no quantification is available.
- The removal of N_2O from the atmosphere occurs primarily by photolysis in the stratosphere.
- Consumption by soils is another unquantified sink of N_2O.
- N_2O is a source of reactive nitrogen oxides in the stratosphere.
- It plays a significant role in the destruction of the stratospheric ozone layer.
- It is involved in global warming.
- Global half-life of N_2O in the atmosphere is 130–150 years.
- As a greenhouse gas, it is 300 times more effective than CO_2 (◘ Table 1.3).

◘ **Table 1.3** Sources and sinks for N_2O

Sources and sinks	10^6 t N_2O/year
Natural sources	
Tropical soils	
Wet forests	3
Dry savannah	1
Latitudinal soils	
Forests	1
Grassland	1
Ocean	3
Total natural sources	9
Anthropogenic sources	
Cultivated soils	3.5
Burning of biomass (slash and burn)	0.5
Industrial sources	1.3
Cattle herds	0.4
Total anthropogenic sources	5.7
Total sources[a]	14.7
Sink	
Distance in the stratosphere photochemistry	12.3
Distance in the ground	?
Total sinks[a]	12.3
Atmospheric increase[b]	3.9

After IPCC (1994)
[a] Calculated data
[b] Measured data

1

Animal Husbandry and Climate Change

The livestock sector plays an important role in carbon and nitrogen cycles from a global perspective. Regarding livestock, C and N cycles are closely related to the role of livestock in land use and land use change. Land use for livestock production includes pasture and agricultural land used for livestock feed production. When emissions across the entire production chain are considered, livestock production currently contributes about 18% of global warming. Livestock contributes about 9% of total CO_2 emissions. But 37% of methane and 65% of N_2O come from livestock production. The latter emission will increase considerably in the coming decades, as natural grazing has currently reached the maximum possible area in most regions of the world. However, the growth of the livestock sector in the future can only be guaranteed by the cultivation of forage crops.

▪ Ammonium (NH_4^+)

The majority of organic fertilizers come from livestock excreta and sewage sludge. In this way, some of the nitrogen from the faeces and urine is reused in agriculture. However, ammonia losses from excreta are very high when nitrogen-rich livestock wastes are reused. Ammonia emissions from the excreta of grazing animals are also high. Recent estimates suggest that in Europe up to 90% of total ammonia emissions to the atmosphere come from agriculture, with livestock waste being the main source.

Cattle farming, especially dairy farming, is considered the largest livestock source of ammonia release (Bussink & Oenema, 1998). Losses of NH_3 occur during sludge use, sludge storage, grazing, manure use and from crops. Total losses range from 17 to 46 kg N/year/cow, depending on the amounts and composition of excreta, sludge treatment, and soil and environmental conditions (Bussink & Oenema, 1998). High temperatures, high pH and good aeration promote ammonia evaporation. Consequently, rapid transport of livestock manure to anaerobic storage and rapid incorporation into the soil after application is a way to reduce ammonia release. A reduction in release losses can also be achieved by a well-balanced animal diet in which a combination of amino acids is optimised for efficient high nitrogen consumption by animals and consequently lower nitrogen concentration in livestock excreta (◘ Table 1.4).

◘ **Table 1.4** Sources and sinks for NH_3

Sources and sinks	10^6 t N/year
Sources	
Pets	21.3
Human excreta	32.6
Ground emission	16
Slash-and-burn	5.7
Wildlife	10.1
Industry	10.2
Fertilizer loss	9
Combustion of fossil fuels	0.1
Ocean	8.2
Sink	
Wet precipitation (land)	11
Wet precipitation (ocean)	10
Dry deposition (land)	11
Dry deposition (ocean)	5
Reaction with OH	3

After Dentener and Crutzen (1994)

Ammonium (NH_4^+)

- NH_3 is the third most abundant nitrogen gas in the atmosphere (after N_2 and N_2O).
- Ammonia has a relatively short half-life of about 10 days.
- NH_3 is primarily a product of biological activity as well as a byproduct of agricultural activity and waste production and processing.
- Atmospheric concentrations are higher over the continents than over the oceans.
- There is little transport of NH_3 or NH_4^+ from the ocean to the land masses.
- The transport from the land masses to the ocean is considerable.
- Ammonia is the only alkaline gas in the atmosphere and therefore an important neutralizer of anthropogenic acids through reactions such as

$$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2 SO_4$$

- The main sink of atmospheric NH_3 is the transformation to ammonium-containing aerosols, which sink by dry deposition or precipitation.
- $(NH_4)_2SO_4$ aerosols have a different radiation property than H_2SO_4 aerosols.

NO

- NO is a reactive gas that plays a major role in tropospheric photochemistry and thus contributes to acid rain.
- The photochemistry of NO is important for the formation of tropospheric O_3 and it thus regulates the oxidizing capacity of the troposphere.
- Upland soils are involved as a major source of tropospheric NO.

▪ Nitric Oxide (NO)

The combustion of fuels leads to the emission of NO. In the atmosphere, NO is oxidized to nitrogen dioxide gas (NO_2), which dissolves in water and can react to form HNO_3, one of the main components of acid rain. NO also plays a significant role in atmospheric chemistry as it participates in the balance of oxidants in the atmosphere.

1.2.3 Global Sulfur Cycle

The global transport cycle of sulfur is shown in ◘ Fig. 1.10.

The main reservoirs are marine sulphate (the most important sulfur reservoir for the biosphere) and sedimentary rock (in the form of sulfate minerals, mainly gypsum, $CaSO_4$, and sulfide minerals, mainly pyrite, FeS_2).

Sulfur is a chemical element that is essential for life on earth. Sulfur makes up about 0.2% of biomass. Living organisms, including plants, assimilate sulfur while at the same time sulfur is released in various forms by living organisms as end products of metabolism.

The main sulfur gases are dimethyl sulfide (CH_3SCH_3 or DMS), carbonyl sulphide (OCS), hydrogen sulfide (H_2S), dimethyl disulfide (DMDS), carbon disulfide (CS_2) and sulfur dioxide (SO_2) Over the last century, the sulfur cycle has been increasingly influenced by human activity. Today, globally, anthropogenic emissions account for about 75% of total sulfur emissions, 90% of which are in the northern hemisphere. If biomass burning is excluded, natural emission sources (marine, terrestrial and volca-

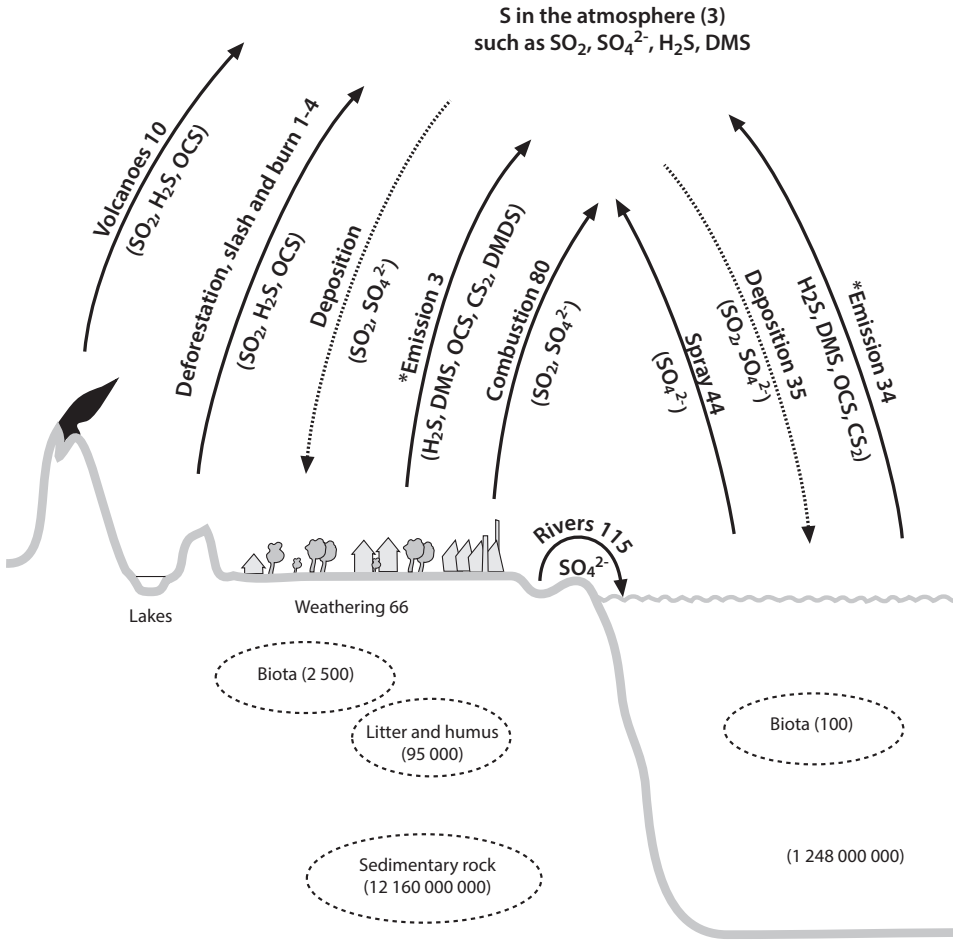


Fig. 1.10 The global sulfur cycle. The figures at the arrows indicate the material flows in 10^6 t S/year, the figures in brackets indicate the sulfur content of the various reservoirs in 10^6 t S. *Process that is partly or exclusively due to microbial activity. (Adapted from Madigan et al., 2009; Bates et al., 1992; Mackenzie, 1997)

nic) account for 24% of total emissions, with 13% in the northern hemisphere and 11% in the southern hemisphere. Although anthropogenic emissions (mainly SO_2) clearly dominate in certain regions, particularly the northeastern United States and areas of Europe and Asia, both natural and anthropogenic sources influence the global distribution of sulfur in the atmosphere.

The reduced sulfur compounds produced in the biosphere and hydrosphere, DMS, H_2S, CS_2 and OCS, are volatile and readily exchanged with the atmosphere.

As the atmosphere acts as an oxidation medium, reduced sulfur is converted to SO_2 and further to sulfuric acid (H_2SO_4). Sulfate particles formed by homogeneous and heterogeneous nucleation of H_2SO_4 are believed to be the main source of cloud condensation nuclei. Removal of sulfur from the atmosphere occurs by dry and wet deposition at the surface with momentous consequences for the environment. Acid precipitation and other similar negative impacts on living organisms are observed in some regions of the Northern Hemisphere.

If DMS, H_2S, CS_2, and OCS have entered the atmosphere, they are primarily converted to SO_2 by oxidation with $OH^•$, possibly also to H_2SO_4 or CH_3SO_3H (methanesulfonic acid). Sulfates return to the surface by dry and wet deposition. The reaction of OCS with $OH^•$ is very slow and therefore transport to the stratosphere takes place. There in the stratosphere OCS is photolyzed, SO_2 and finally H_2SO_4 aerosols are formed.

Source and Biogeochemical Significance of Dimethylsulfonium Propionate and Dimethylsulfide

- Dimethylsulfonium propionate (DMSP) is a sulfonium compound formed in high concentration in marine micro- and macroalgae and halophytic plants to control internal osmotic pressure.
- DMSP is ubiquitous in marine surface waters with concentrations ranging from less than 1 nM in the open ocean to several micromolar in phytoplankton blooms.
- Its release into ocean waters occurs through spill, death, or grazing and is one of the most significant sources of sulfur and carbon for marine bacteria.
- One of the degradation products of DMSP is volatile dimethyl sulfide (DMS), which escapes to the atmosphere at a significant rate.
- DMS is poorly soluble in water, but ocean water is supersaturated.
- Dimethyl sulfide, as a gas, is the primary cause of non-sea salt sulfate pollution in the atmosphere.
- The emission of DMS from the oceans causes atmospheric DMS aerosols or droplets. Their oxidation produces sulfuric acid, which acts as a condensation nucleus in cloud formation, leading to the re-radiation of solar energy. Model calculations have shown that reducing the amount of sunlight reaching the Earth's surface due to changes in the concentration of DMS would theoretically cause a decrease in global mean temperature. DMS is thus a "climate-cooling" gas.
- The total annual flux of biogenic DMS from the ocean has been estimated to range from 13 to 37 Tg (T = Tera = 10^{12}). This is an order of magnitude higher than the total annual flux from all other sources, such as soil, plants, salt marshes and freshwater marshes. The amount is particularly noteworthy because between 50% and 85% of the DSMP released is demethylated and a fraction of DMS is oxidized by bacteria in the water column before reaching the atmosphere.
- Although the total flux of DMS is only half that of anthropogenic sulfur dioxide emissions, the longer residence time of DMS oxidation products in the atmosphere and the global spread of DMS release result in a larger fraction of atmospheric sulfur pollution coming from DMS.
- DMS was identified as the missing gas component needed to understand the steady-state flux of sulfur between terrestrial and marine environments. This makes DMS emissions a key step in the global sulfur cycle and a transporter between sea and land.
- DMS is thus the most important organic sulfur compound with global ecological significance.
- A link between solar radiation and DMS concentration is known as the CLAW hypothesis. This claims that increasing levels of solar radiation and the associated higher temperatures promote the growth of DMSP-producing marine phytoplankton, thereby increasing overall DMSP production.

1

Carbonyl Sulphide (OCS)

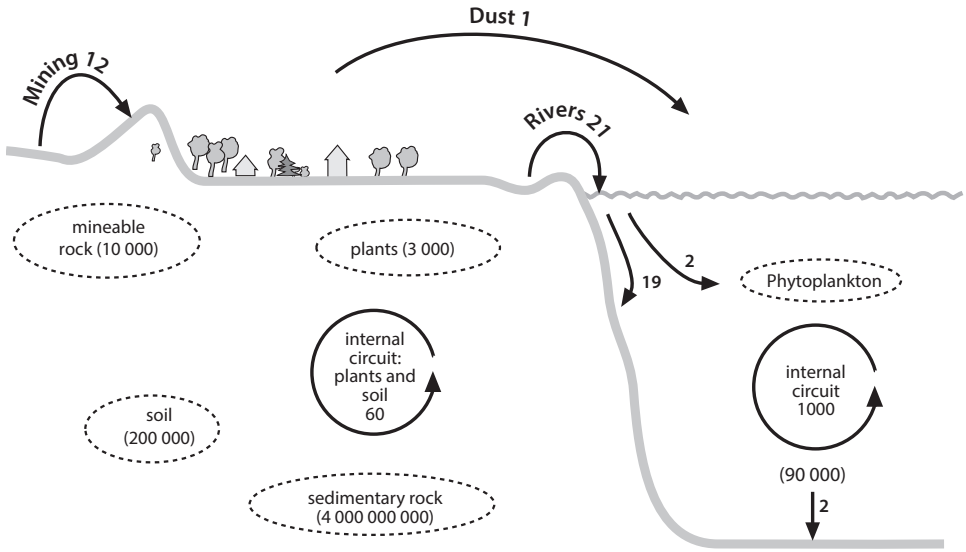
- Due to its low chemical reactivity, OCS is the most abundant sulfur gas in the atmosphere.
- The main tropospheric sinks are uptake by soils and hydrolysis by natural waters.
- The global lifetime of OCS is about 1.5 years.
- It can be transported to the stratosphere where it is photolyzed by solar UV radiation and acts as a source of SO_2 and sulfate particles.
- The sum of known OCS sources is about 50% greater than that of known sinks.

1.2.4 Global Phosphorus Cycle

The global cycle of phosphorus is unique among the cycles of major biogeochemical elements in that it has no significant gaseous component. The transfer of phosphorus via the atmosphere by dust and sea spray (1×10^6 t P/year) is much smaller than that of other transfers in the global phosphorus cycle. Nevertheless, it is known to make a significant contribution to the supply of available phosphorus when precipitation falls in some tropical forests and in the open ocean (◘ Fig. 1.11).

Unlike transfers in the global nitrogen cycle, the major source of reactive phosphorus is not fed by microbial reactions. Almost all phosphorus in terrestrial ecosystems is originally derived from the weathering of calcium phosphate minerals, especially apatite [$Ca_5(PO_4)_3OH$]. There is no process equivalent to nitrogen fixation that causes a significant increase in the availability of phosphorus to plants in phosphorus-limited habitats. The phosphate content of most rocks is not very high and in most soils there is only a small fraction of total phosphorus available to living things. Therefore, living things on land and at sea exist only because of a well-developed "recycling" of phosphorus in the organic form.

The main flow of phosphorus in the global cycle is carried out by rivers, which



◘ **Fig. 1.11** The global phosphorus cycle. The data at the arrows indicate the material flows in 10^6 t P/year, the data in brackets indicate the phosphorus content of the various reservoirs in 10^6 t P. (According to Schlesinger, 1997)

transport about 21×10^6 t P/year to the sea. About 10% of this is accessible to marine organisms, the rest is tightly bound to bottom particles and sediments rapidly on continental shelves. The concentration of PO_4^{3-} in ocean surface waters is low, but the large volume of deep water contains a substantial reservoir of phosphorus. The residence time for reactive, unbound phosphorus in the ocean is about 25,000 years. Turnover of phosphorus from the organic reservoir in ocean surface water occurs in a few days and the remainder is mineralized in deep water. Phosphorus sediments on the ocean floor, which is the largest reservoir of phosphorus near the Earth's surface. About 2×10^6 t P/year is added to open ocean sediments-roughly the amount that enters the oceans via rivers. On a time scale of hundreds of millions of years, "uplift" of sediments and rock weathering takes place, closing the global cycle.

Humans have increased the availability of phosphorus in many areas by mining phosphate rock for fertilizer. Most of the economically useful deposits of phosphate are sedimentary rocks of marine origin, so mining activities have directly increased turnover in the global phosphorus cycle. The flow (flux) of phosphorus across rivers as a result of erosion, pollution and fertilizer runoff is much higher than that in prehistoric times.

1.2.5 Summary of Global Cycles

Small biogeochemical cycles of nitrogen and phosphorus with relatively rapid turnover are coupled to large reservoirs with relatively slow turnover. The large reservoir for phosphorus is formed by unweathered rocks and soil, while the atmosphere is the main reservoir for nitrogen. The biogeochemical cycle for nitrogen begins with the fixation of atmospheric nitrogen, which transfers a small amount of inert N_2 to the biosphere. This transfer is balanced by denitrification, which returns N_2 to the atmosphere. The balance of these processes maintains a steady-state concentration of N_2 in the atmosphere with a turnover time of 10^7 years. In the absence of denitrification, most of the nitrogen stock on Earth would eventually have disappeared into the ocean and organic sediments. Denitrification thus closes the global nitrogen cycle and it causes nitrogen to be converted faster than phosphorus, which has no gas phase. The average residence time of phosphorus in sedimentary rocks is 10^8 years and the phosphorus cycle is only complete as a result of tectonic movements of the Earth's crust.

Only in the biosphere are the transformations of nitrogen and phosphorus much faster than in their global cycles. Thus, there are turnover rates in the range of hours (for soluble phosphorus in soil) to hundreds of years (for nitrogen in biomass).

Due to nutrient limitations, biological recycling in terrestrial and marine habitats allows much higher rates of net primary production than rates of nitrogen fixation and rock weathering alone would allow. The high efficiency of nutrient recycling may also explain why, despite widespread nitrogen limitation, only about 2.5% of global net primary production is via nitrogen fixation.

Human impacts on global nitrogen and phosphorus cycles are diverse and dramatic. Through fertilizer production, humans have doubled the rate at which nitrogen enters the biogeochemical cycle on land. It is not clear how quickly denitrification will respond to this global increase in nitrogen availability, but the increasing concentration of atmospheric N_2O is certainly indicative of the biological response taking place. Increasing nitrogen availability has led to local extinctions of species in polluted ecosystems and has shifted the limitation of "net primary production" in these systems from nitrogen to phosphorus. Increased transport of nitrogen and phosphorus by rivers has altered many estuarine and coastal ecosystems.

The main reservoir of sulfur in the global cycle are the minerals of the earth's crust gypsum and pyrite. In addition, there is sulfur dissolved in the ocean as sulfate. In terms of reservoir, the global sulfur cycle is similar to the global cycle of phosphorus, while in contrast, the largest reservoir of nitrogen is found in the atmosphere. In another relation, however, there is strong similarity of global nitrogen and sulfur cycles. In both cases, the main turnover of elements occurs through the atmosphere. Under natural conditions, the major part of the turnover is the formation of reduced gases of nitrogen and sulfur by living organisms. These gases return nitrogen and sulfur to the atmosphere, causing a closed loop with relatively rapid turnover. In contrast, the final pathway of phosphorus is storage in marine sediments, from where the cycling of phosphorus is complete only due to upwelling of sediments over very long periods of time. Biogeochemistry exerts a major influence on the global sulfur cycle. The largest reservoir of sulfur at the Earth's surface is found as a result of sulfate reduction as pyrite of sediments. Sedimentary data show that the relative extent of sulfate reduction has varied over geologic time. In the absence of sulfate-reducing bacteria, the concentration of SO_4^{2-} in lake water would be higher and that of O_2 in the atmosphere would be lower than present-day levels.

The current human impact on the sulfur cycle is roughly doubling the annual mobilization of sulfur from the Earth's crust. As a result of the burning of fossil fuels, landscapes on the downwind side of industrial areas receive large amounts of acid precipitation from the atmosphere. This excess acidity leads to changes in rock weathering, forest growth, and ocean productivity.

Test Your Knowledge

- Name global and local environmental damage.
- Which components of the atmosphere are directly related to biological activities?
- Which compound **not** mentioned in the list on pages 5 + 6 has a high global warming potential? Hint: It says "fraction in dry air" in the first line.
- Why did it take so long for CFCs to reach the stratospheric ozone layer? Why do substances disperse quickly or slowly?
- Name biological methods of determining temperature even without a thermometer.
- Compare CO_2, N_2O and methane as climate gases.
- What is behind El Niño?
- What do the environment and microorganisms have to do with each other?
- How do the "Dutch" fertilise the farmland in North Rhine-Westphalia and Lower Saxony?
- Name climate-relevant N and S compounds.
- Which sinks for CO_2 do you know?

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Microorganisms, Actors in the Environment

Contents

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2

Different groups of organisms settle in the environment at different stages of an ecosystem. Plants act as primary producers, animals and humans as consumers, and microorganisms—bacteria and fungi—as destructors. In this function, microorganisms also play a decisive role in overcoming environmental problems. They are essential components of material cycles (see ► Chaps. 4, 6 and 7).

Microorganisms are objects that are individually invisible to the naked eye because they are smaller than the resolution limit of the human eye of about 20 µm. You cannot see them yourself, but you can see or smell the result of their metabolic activity or see them when they occur en masse in nature. For example, mass proliferation of cyanobacteria is seen in summer as "water blooms" in eutrophic lakes. In extreme locations such as saline and alkaline lakes or anaerobic, sulfide-rich lakes, bacteria can grow into extremely large phototrophic or halophilic populations and become visible by the purple color of the water, such as Lake Cisó, in northeastern Spain, Lake Tyrrell in Australia, or Lake Magadi in Kenya. The conspicuous stratification of colour-striped mudflats in intertidal areas of the sea is another example of microbial populations becoming visible (see ► Sect. 10.4). The fight against plaque formation by microorganisms is carried out by everyone on a daily basis (see ► Sect. 10.2, Biofilm on teeth). Everyone has held a huge number of yeast cells in the form of a cube of compressed yeast to prepare a pizza dough.

Microorganisms include fundamentally different organisms:

1. **Prokaryotes** are the focus of general microbiology and also environmental microbiology. Prokaryotes have simply constructed cells without a demarcated nucleus. They include the *Eubacteria* and *Archaea.*
2. Single-cell organisms with a true cell nucleus (**eukaryotes**) can be classified as animals (protozoa) or plants (algae or fungi).

2.1 Microorganisms, Assignment to Groups

After the RNA of the ribosomes, the rRNA, of a large number of bacteria had been isolated and the nucleotide sequence determined, a phylogenetic tree of the prokaryotes was arrived at. From the degree of similarity between the sequences of different prokaryotes, it was possible to conclude that all living things share a common root and that prokaryotes split early into two major groups, the *Eubacteria* and the *Archaea.* These two **kingdoms** stand on equal footing with the kingdom of the eukaryotes (or *eukarya*) (◘ Fig. 2.1).

Eubacteria and *Archaea* have great similarity in microscopic cell structure. Overall, the prokaryotes are relatively little differentiated morphologically. In terms of shape, only a few forms can be distinguished, all of which can be traced back to the sphere and straight and curved cylinders as the basic form. This uniformity, however, contrasts with a metabolic-physiological versatility and flexibility that is unparalleled. There are also similarities between *Eubacteria* and *Archaea* in energy and building block metabolism (◘ Table 2.1).

With regard to the molecular biology of the cell, for example in DNA, RNA and protein synthesis, *Archaea* have similarities with eukaryotes.

In addition to the two large groups of plants and animals, the fungi belong to the *Eukarya.* The fact that the fungi actually occupy their own independent rank was confirmed by phylogenetic analyses using molecular biological methods.

The fungal cell corresponds in its structure to the typical eucyte with nucleus, intracellular membranes, vesicles, vacuoles and mitochondria. The cell is surrounded by a membrane, to which the cell wall is attached. In addition to proteins and other polysaccharides, the cell wall contains chitin as an important structural component.

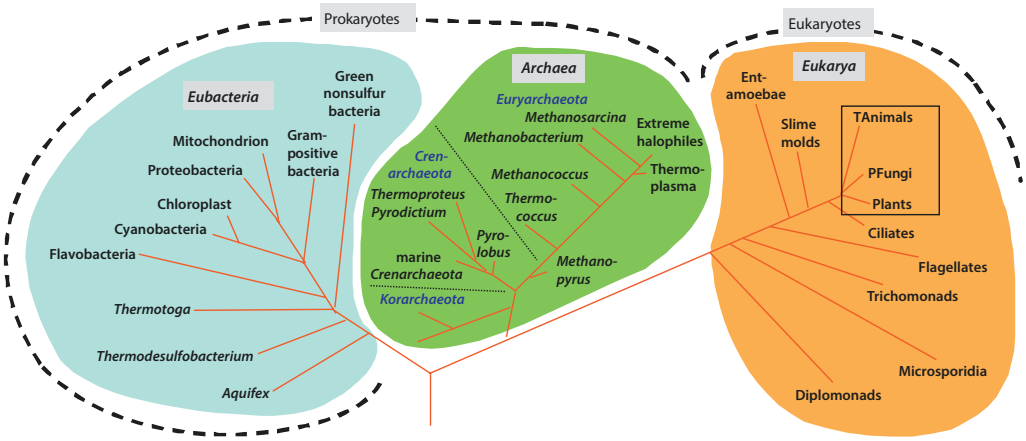

Fig. 2.1 Phylogenetic tree of organisms. The tree consists of the three domains: the *Eubacteria* and the *Archaea*, which have prokaryotic cells, and the eukaryotes. Only a few organisms are plotted in each domain. The groups highlighted in the box are macroorganisms. All other organisms in the phylogenetic tree are microorganisms. The length of the branches is a measure of the change in ribosomal DNA genes whose sequence comparison underlies the phylogenetic tree

Table 2.1 Differences between *Eubacteria*, *Archaea* and *Eukarya*

Characteristic	*Eubacteria*	*Archaea*	*Eukarya*
Organisational form	Single-cell	Single-cell	Single- or multicell
Organelles	No	No	Yes
Membrane enveloped nucleus	No	No	Yes
Membrane structure	Ester lipids	Ether lipids	Ester lipids
Cell wall contains murein	Yes	No	No
Cell wall contains pseudomurein	No	Yes	No
Cell wall contains chitin	No	No	Yes
Ribosomes	70S (30S + 50S)	70S (30S + 50S)	80S (40S + 60S) (70S: mitochondria, plastids)
RNA polymerase(s)	One (4 subunits)	Several (8–12 subunits each)	Three (12–14 subunits each)
Transcription factors	No	Yes	Yes
Sensitivity to chloramphenicol/streptomycin	Yes	No	No

Within the so-called *true fungi* there are organisms that live as single cells and reproduce by yeast-like sprouting or bifurcation, as well as filamentous growing forms that form a coherent mycelium containing septa for demarcation between the cells. Branching of the hyphae produces a mycelium. In some fungi, both growth forms occur side by side.

2

For the function of microorganisms in the environment, it is important to consider their number of species. Today, about 5000 species of bacteria and 100,000 species of fungi have been described. Many indications suggest that only a small percentage of the occurring species of microorganisms have been isolated and characterized, but that a much larger number (up to 99.9%, see ► Chap. 11) cannot be isolated at all (with today's methods).

Proteobacteria

The *Proteobacteria* form by far the largest group among the *Eubacteria.* This phylum is composed of six classes, *Alpha*, *Beta*, *Gamma*, *Delta*, *Epsilon*, and *Zetaproteobacteria*, each containing multiple genera with the exception of Zetaproteobacteria. Bergey's Manual of Systematic Bacteriology (2005) provides the description of more than 2000 species in 538 genera assigned to the phylum *Proteobacteria* (◘ Fig. 2.2).

Although 16S rRNA studies show that the *Proteobacteria* are phylogenetically related, they differ markedly in many respects. As a group, these organisms are all gram-negative. The morphology of these bacteria ranges from simple rods and cocci to genera of stalked, prostecate (appendaged), and coccygeal bacteria, and even those with fruiting bodies.

Members of the photosynthetic purple bacteria have been found within the *Alpha*, *Beta* and *Gammaproteobacteria.* This has led to the suggestion that the *Proteobacteria* evolved from a photosynthetic ancestor, presumably similar to the purple bacteria. Subsequently, photosynthesis was lost due to various events. New metabolic capabilities were acquired as the bacteria adapted to different ecological niches.

Physiologically, *Proteobacteria* can be either phototrophic, chemolithotrophic, or chemoorganotrophic, making them the most metabolically diverse group of all known bacteria. There is apparently no common pattern in the metabolism, morphology, or reproductive strategy of the *Proteobacteria.* They represent the majority of all known gram-negative bacteria of medical, industrial and ecological importance.

Alphaproteobacteria largely include two main phenotypes: Non-sulfur purple bacteria and aerobic bacteriochlorophyll-possessing bacteria. They are known for their symbiosis with plants.

Betaproteobacteria largely include chemoheterotrophs and chemoautotrophs, which obtain their nutrients by decomposing organic material. *Betaproteobacteria* play a role in nitrogen fixation in various types of plants, oxidizing ammonium to nitrite, an important chemical for plant function. Many of them are found in environmental samples such as wastewater or soil.

Gammaproteobacteria largely include facultative anaerobic and fermentative gram-negative bacteria. *Gammaproteobacteria* have several medically and scientifically important groups of bacteria. A number of important pathogens belong to this class, such as *Salmonella* spp., *Escherichia coli* and others. Some *Gammaproteobacteria* are methane oxidizers and many of them are found in symbiosis with animals inhabiting geothermal outcrop channels in the ocean.

Deltaproteobacteria largely represent morphologically diverse anaerobic sulfidogens. Some members of this group are bacterial predators that have bacteriolytic properties. *Deltaproteobacteria* have the ability of sulfur or sulfate reduction, other anaerobic bacteria have a different physiology such as iron reduction.

Epsilonproteobacteria largely include chemoorganotrophs. Most known species

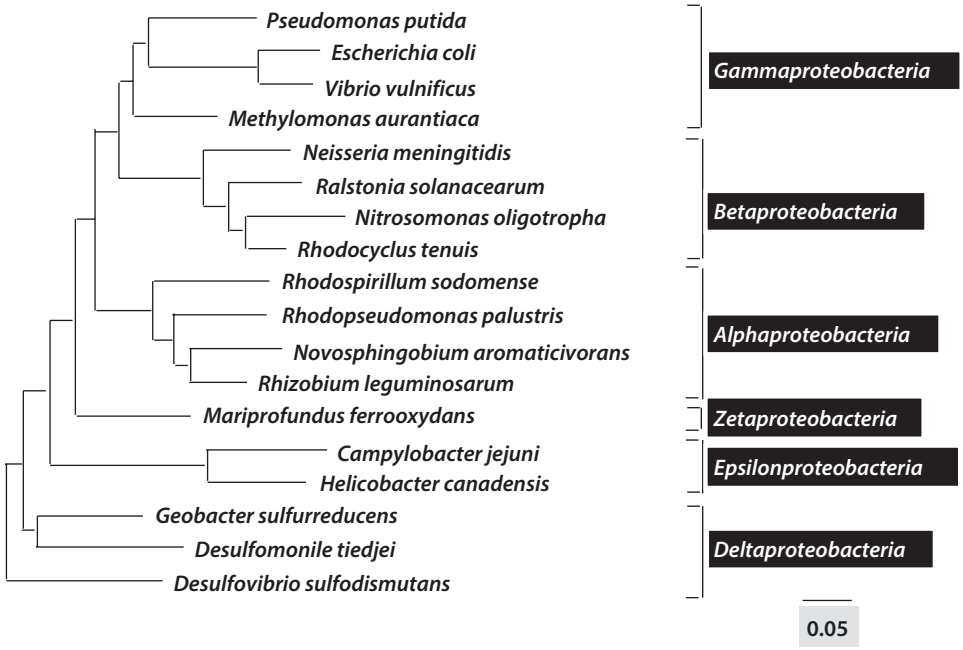

Fig. 2.2 The maximum-likelihood phylogenetic tree showing the evolutionary arrangement of representatives of the classes of *Proteobacteria.* The scale means 5 nucleotide substitutions per 100 positions

inhabit the intestinal tract of humans and animals and serve as symbionts. A vast amount of environmental sequences of *Epsilonproteobacteria* have been recovered from the deep sea from near habitats of hydrothermal vents and cold springs.

Zetaproteobacteria include chemolithotrophs and has only one member, *Mariprofundus ferrooxydans*, described in 2010, which is the only representative cultivated to date. This iron-oxidizing bacterium lives under microaerobic conditions and is widely distributed in deep-sea habitats.

An example of the taxonomic assignment of an environmentally relevant bacterium whose metabolism is frequently discussed in the book is *Pseudomonas putida*:

- Domain: *Bacteria*
- Phylum: *Proteobacteria*
- Class: *Gammaproteobacteria*
- Order: *Pseudomonadales*
- Family: *Pseudomonadaceae*
- Genus: *Pseudomonas*
- Species: *Pseudomonas putida*

2.2 Microorganisms, the Advantage of Small Size

The cell size of prokaryotes varies from 0.1–0.2 to 50 μm in diameter. Typical rod-shaped prokaryotes such as *Escherichia coli* have a size of 1 × 3 μm. In contrast, typical eukaryotic cells can range in diameter from 2 to 200 μm. Thus, the cells of prokaryotes are small compared to that of eukaryotes. The small size affects a number of the biological properties. For example, the rate at which nutrients and waste products enter and leave the cell, respectively, is generally inversely proportional to cell size. Metabolic and growth rates are strongly influenced by

Table 2.2 Relationship between surface area and volume using the example of spheres

Bullet[a]	Radius, r (µm)	Surface, $A = 4\pi r^2$ (µm²)	Volume, $V = 4/3\pi r^3$ (µm³)	Surface/volume
●	1	12.6	4.2	3
⬤	2	50.3	33.5	1.5

[a] Size comparison

transport speed. This, in turn, is to a certain extent a function of the available membrane surface, which is larger by volume in small cells. This can be illustrated using the example of a sphere (Table 2.2). The volume is a function of the third power of the radius, while the surface area is a function of the second power. Thus, a cell with a smaller radius has a greater surface area to volume ratio than a larger cell and can therefore exchange with its environment more effectively than a large cell. This advantage generally allows small prokaryotic cells to grow faster and form larger populations than eukaryotic cells in most microbial habitats. This in turn influences ecological conditions, as large numbers of cells with high metabolic activity can lead to strong physicochemical changes in an ecosystem in a relatively short time.

The large surface-to-volume ratio enables intensive interactions with the environment. Microorganisms have an "extroverted" way of life. In connection with the relatively small transport paths in the cell, this leads to high metabolic performance. Respiration is a measure of metabolic rate. In bacteria, the respiration rate is around 1000, in yeasts around 100, in animal and plant tissues around 1–10 (Table 2.3). There is an illustrative picture for bacterial metabolic rate. A lactose-fermenting bacterium converts 1000–10,000 times its own weight of substrate in one hour; a human being would need about 250,000 h for 1000 times the sugar conversion of its own weight. This corresponds to half of his life.

Another expression of the high microbial performance potential is growth. Bacteria such as *Escherichia coli* have a generation time of 20 min under favorable conditions, yeasts of 2 h. During this time, the biomass doubles. This continues in an exponential manner, as will be explained in ► Chap. 9. From calculations on microbial protein production comes the comparison that in a yeast factory with an initial biomass of 500 kg protein, 50,000 kg of protein can be produced within 24 h. In contrast, a bovine of 500 kg produces 50,000 kg of protein within 24 h. In contrast, a 500 kg cow produces only 0.5 kg of protein in one day. The biomass of young cattle therefore doubles in 1–2 months (about 2000 h).

In summary, it can be stated that microorganisms, in terms of biomass, can perform about 100–1000 times better than plants and animals.

While higher organisms have achieved a high degree of morphological-anatomical differentiation in the course of evolution, microorganisms, especially bacteria, possess a pronounced metabolic-physiological versatility and flexibility. This is the basis of their great importance in metabolic cycles. The biochemical diversity is expressed in the different types of energy production and carbon assimilation (see later ► Chap. 3).

For the bacteria, a high adaptive capacity is also a necessity, which can be attributed to their small dimensions. A *Micrococcus* cell only offers space for a few 100,000 protein molecules. Enzymes that are not required can therefore not be kept in stock. Cellular

Table 2.3 Differences between prokaryotic and eukaryotic cells. The figures are average numbers intended to illustrate orders of magnitude

Cell type	Diameter (µm)	Volume (μm^3)	Respiration rate (QO_2)	Generation time (h)
Bacteria	1	1	1000	0.3–1
Yeasts	10	1000	100	2–10
Plant and animal cells	100	>10,000	10	About 20

QO_2 = µL O_2/mg dry substance—h

regulatory mechanisms thus play a considerably greater role in microorganisms than in other living organisms.

2.3 Microorganisms, Small But Numerous

The high number of individuals in environmental media indicates the great importance of microorganisms for the environment. For the number and biomass of microorganisms in soils, an order of magnitude for forest soils may be given. In 1 g soil there are 10^6–10^9 bacterial cells and 10–100 m fungal mycelium. The ratio of the biomass of bacteria to that of fungi is made clear by the following values for the dry cell mass per ha: bacteria 40 kg, fungi 400 kg. Forest soils are richer in fungi than arable soils, but the large mycelial mass shows that the importance of fungi is often neglected. Since the metabolic activity of fungi, in relation to cell biomass, is about one power of ten less than that of bacteria (Table 2.3), the two groups of organisms are of approximately equal importance in substance transformations in soils. However, the other soil organisms (protozoa, earthworms and others) must not be ignored. The microfauna, which contributes significantly to the comminution of plant biomass in soils, corresponds to the biomass of bacteria with about 40 kg/ha. In conclusion, 10^9 bacterial cells have about the dry weight of 1 mg. A single cell of *Escherichia coli* has a weight of about 9.5×10^{-13} g when wet. A billion bacterial cells is the order of magnitude we find in 1 g of nutrient-rich soil and also in 1 mL of wastewater. Other numbers are: There are about 1×10^{10} cells in 1 g of baker's yeast. A cell paste of *Escherichia coli* of 1 cm^3 consists of about 1×10^{12} cells.

Microorganisms are small, but their biomass on Earth is enormous, even when compared to the biomass of higher organisms. Estimates of the total number of microbial cells on Earth, and especially the number of prokaryotes, show that this number is in the range of 5×10^{30} cells. The total carbon present in this very large number of very small cells is equal to that of all plants on Earth (and plant carbon far exceeds animal carbon). Furthermore, the nitrogen and phosphorus content of all prokaryotic cells is ten times that of all plant biomass.

So, as small as prokaryotic cells may be, they represent a major fraction of the biomass on Earth and are the most important reservoir of vital nutrients.

Microorganisms Can Become Huge

Usually, fungi and trees have a fruitful relationship. The mycelium entwines around the tree and supplies it with water and amino acids. In return, the fungus receives carbohydrates. The situation is

different in the case of the mycelium of the white rot fungus *Armillaria ostoyae* in the Malheur National Forest (Oregon, USA), which was discovered in 2000 as a result of a mysterious forest dieback. Because of its extent of over 880 ha, its calculated age of at least 2400 years and its mass of about 600 t, it is considered the largest known living organism on earth. Genetic analyses prove that the fungal plexus belongs to one and the same fungus. The mushroom produces only a few fruiting bodies and this is probably why it has not produced any offshoots. Without competition from other fungi, it spreads freely. Oregon's dry climate seems to favor its growth. It penetrates the soil and infested trees with its millimeter-thick filaments, thereby depriving the trees of food. Several spruce and Douglas spruce have already fallen victim to it.

Europe's largest Hallimasch clone (also *Armillaria ostoyae*) was discovered in 2004 in Switzerland near the Ofen Pass. It covers an area of about 500 m width and 800 m length—this corresponds to an area of about 35 ha. Its age is estimated to be more than 1000 years.

2.4 Microorganisms, Do Not Live Alone

In nature, microorganisms are not encountered as pure cultures, but each individual organism cooperates with others or competes with them. Microorganisms interact with each other and with the environment around them. In doing so, they fulfil important functions in the metabolism of substances and make a significant contribution to shaping the physical, chemical and biological conditions.

Plants and animals have evolved in an environment where almost all prokaryotic metabolic types were already present. It is therefore understandable that numerous partnership relationships have also developed between microorganisms and the higher life forms.

With regard to the relative benefits that partners derive from living together, a distinction can be made between several categories (◘ Table 2.4). However, the definitions must not lead to a superficial benefit-harm thinking; the respective cohabitation is of a complex nature. A togetherness and a counter-togetherness are only apparently of a fundamental nature, they involve interactions of various degrees.

In general, starvation is the typical living situation for microorganisms.

Below are some examples to illustrate the presence of interactions:

In many cases, partners can coexist without interacting with each other (**neutralism**). However, this can only occur when population densities are low, as in marine habitats or oligotrophic lake habitats. At high cell densities, competition for food or light occurs. There can also be no interference if the different populations have extremely different metabolic properties, i.e. they do not compete with each other for substances.

If a cohabitation has a favourable or positive effect on both partners, it is called **symbiosis** in the narrower sense or mutualistic symbiosis (**mutualism**). Such cohabitation can reach different degrees of spatial connectedness: If one partner lives outside the cells of the other partner, it is called **ectosymbiosis**. The intracellular settlement of the partner is called **endosymbiosis**.

Lichens are examples of highly developed ectosymbioses between microorganisms. In a **lichen**, a fungus and an alga are associated with each other in such a way that they form a single uniform body of vegetation. Both partners benefit from the symbiosis. As a rule, the fungal component of

Table 2.4 Categories of biological interactions between microorganisms and between microorganisms and other organisms

Categories/types	Characteristics
(a) **Neutralism**	The presence of two populations of different microorganisms that do not show any discernible interactions
(b) **Mutualism**	An interactive association between two populations of different microorganisms from which both derive a small advantage
(c) **Synergism**	A non-mandatory interactive association between members of two populations or one population that benefits both populations or all members of one population. In synergism, mutualistic coexistence leads to effects that qualitatively and quantitatively exceed the outputs of the partners
(d) **Syntrophism**	Two organisms complement each other with nutrients or with catabolic enzymes required for the utilization of a substrate (see ► Sect. 10.1.5)
(e) **Symbiosis**	An obligatory interactive association between members of two populations. Both partners are promoted by living together This is a stable state in which both organisms live in close proximity (ectosymbiosis) or in direct contact (endosymbiosis) to their mutual advantage
(f) **Protocooperation**	A symbiosis, but not obligatory
(g) **Commensalism**	One species uses the metabolites of another species without affecting it An interactive association between two populations of different microorganisms in which one population benefits from the association while the other is neither positively nor negatively affected
(h) **Kompetition**	An interactive association (a struggle) between two populations of different microorganisms that both require a limiting abiotic factor (for example, nutrients, light, oxygen, or space/surface area). If both share this factor, both grow at a suboptimal rate; if the factor is only used/utilized by one population, only that population grows
(i) **Amensalism**	An interactive association between two populations of different microorganisms by which one is harmed while the other is neither positively nor negatively affected
(j) **Antagonism**	An interactive association between two populations of different microorganisms in which one exerts a negative effect (inhibition, damage, killing) on the other
(k) **Parasitism**	An interactive association between two organisms from which the smaller (parasite) benefits and by which the larger (host) is harmed

Extended after Alexander (1977)
While an interaction listed in (b) to (f) is beneficial for both partners or at least one of them, at least one partner suffers a disadvantage in the case of the interaction characterized in (g) to (k)

the lichen, the mycobiont, is formative. The fungus obtains organic nutrients from the algae, the CO_2 fixation products. In return, it supplies the algae with minerals and protects them from unfavourable environmental influences, especially from desiccation. Green algae and cyanobacteria may occur as phycobionts in the lichens. The lichens colonize extreme ecosystems in which none of the partners could exist on their own.

In Some Lichens There Is a Third in the Group

In the case of the widespread bright yellow lichen of the genus *Vulpicida canadensis*, which grows on tree bark in North America, a yeast joins the symbiotic community of algae and fungus.

Thanks to new methods of genetic analysis, the yeast was identified as a partner of the lichen. The systematic study of lichens from around the world revealed that a yeast occurred in common species of lichens from Antarctica to Japan, from South America to the Ethiopian highlands.

"The discovery shakes up our fundamental knowledge of lichens. We need to investigate anew how these creatures develop and who assumes which functions in the community," says the evolutionary biologist, explaining the fundamental significance of the discovery (Spribille et al., 2016).

In the course of their evolutionary history, certain fungi have learned to form an extremely successful symbiosis in the soil with plants, the **mycorrhiza** (see Box ► Mycorrhiza). Mycorrhizal fungi help plants to obtain sufficient supplies of water, nutrients and trace elements in nutrient-poor soils. They decisively promote diversity and productivity of plant communities. Mycorrhizal plants show increased resistance to pathogen attack. In return, the fungus receives carbohydrates in the form of simple sugars from the plant.

Mycorrhiza

Mycorrhiza refers to a form of symbiosis between fungi and plants in which a fungus is in contact with the fine root system of a plant. About 80% of the vascular plants in the world form so-called arbuscular mycorrhiza or **endomycorrhiza**. **Ectomycorrhiza** is found in the large number of remaining plants, while only a minority of plants have no mycorrhiza. **Endomycorrhiza** dominates in grasslands and species-rich forests, while **ectomycorrhiza** dominates in forests where a single or few species predominate. Many plants can form more than one type of mycorrhizal community (◘ Fig. 2.3).

Ectomycorrhiza

Ectomycorrhiza (**EM**), which mostly occurs in forest trees, is only one of the known mycorrhizal types. Here, the fungi remain restricted in their growth to the apoplast of the outer root bark tissue of short roots (ectotrophic mycorrhiza) and sometimes form powerful hyphal coats enveloping the roots.

Ectomycorrhizal fungi can live independently of plant roots, as has been shown by growth in petri dishes.

These fungi often form above-ground fruiting bodies, which are known to us as poisonous and edible mushrooms. Fungal partners of the ectomycorrhiza are most of the cap fungi (basidiomycetes) of the forest, such as tube mushrooms, russulae and milter mushrooms. Some of these mycorrhizal fungi can only live in symbiosis with a few tree species, such as the golden reed fungus with larch and pine. Other fungal species are less specialized, such as the porcini and fly agaric, both of which can form a mycorrhiza with birch, beech, oak, spruce, pine, larch, and fir. The host specificity of individual mycorrhizal fungi can also be seen in their names, for example in the case of the birch fungus (*Leccinum scabrum*) or the larch boletus (*Suillus viscidus*). While the fungi are often specialized on a few tree species, trees show no preference for certain fungi.

The ectomycorrhizal fungi form an extensive mycelium in the forest soil and

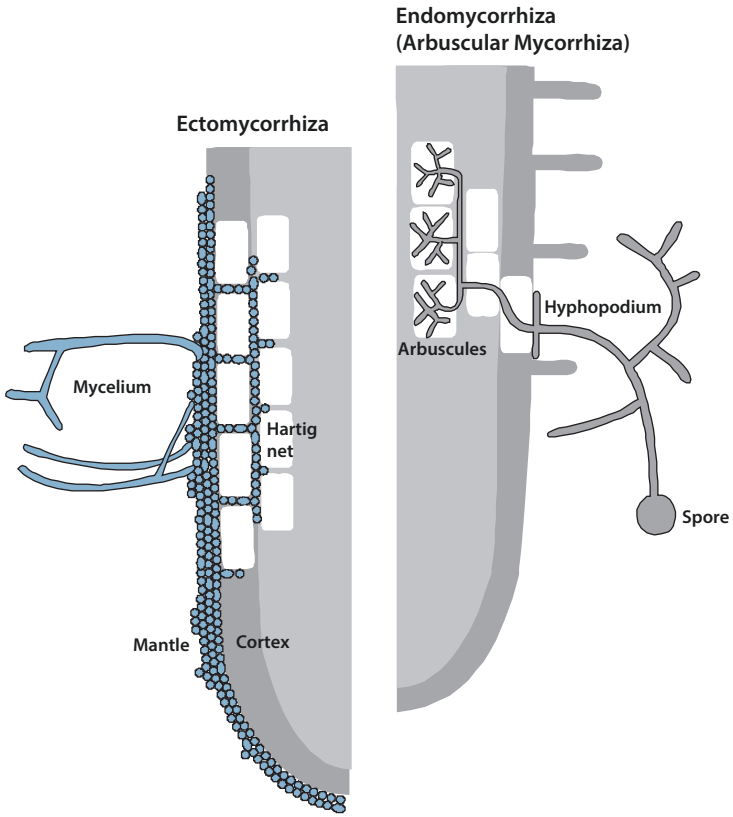

Fig. 2.3 Structures of root colonization in ectomycorrhiza and arbuscular mycorrhiza (endomycorrhiza). In the case of **ectomycorrhiza**, the fungus surrounds the root tip with a mantle of closely pressed hyphae, while the network of intercellular hyphae (Hartig's network) develops around the epidermal cells. In the case of **arbuscular mycorrhiza**, the root tip is usually not colonized. The hyphae develop from a spore and form a hyphopodium on the root epidermis. Colonization within the root occurs intracellularly and intercellularly, eventually leading to the formation of arbuscules, small fungal saplings within the inner cortex cells. During this process, the branching hyphe pushes the plasma membrane into the protoplast, leaving it and the tonoplast of the central vacuole intact. The formation of the arbuscules thus increases the protoplast surface of the host cells many times over, in maize for example from an average of 20,000–80,000 μm^2

are thus particularly important for the ecosystem. Since a tree can form mycorrhiza with several fungi at the same time and each fungus can connect several tree partners through the mycelium in the soil, many trees in a forest form a large network that contributes to the relative stability of the forest ecosystem. If the mycorrhiza is damaged, usually not only a single tree dies, but entire stands show tree damage, as was observed with the "tree death" as a result of acid rain and high pollution levels.

Many forest trees (beech, oak, spruce, pine, fir) are not viable without an ectomycorrhiza completely surrounding the roots, as this not only absorbs nutrients and water for the host, but also protects against soil-borne pathogens.

Endomycorrhiza

The arbuscular mycorrhiza (**AM**), which is widespread today, is particu-

larly beneficial to the plant under adverse conditions (nutrient deficiency, drought, salt or heavy metal stress, and pathogen attack). Arbuscular mycorrhiza occurs in most representatives of angiosperms, but is also found in some gymnosperms and ferns, less so in mosses and club-moss families. Today it is found in more than 80% of predominantly herbaceous plants.

Other endomycorrhizae are the **ericoid mycorrhiza**, which is of crucial importance for the phosphate nutrition of ericaceous plants in moor and heath landscapes, and the **mycorrhiza of orchids**.

Although AM host plants can survive if the fungal symbiont is removed, this condition is not known in nature. The AM fungus acts as a true helper microorganism and thus enhances overall plant fitness. Plant growth experiments under artificial non-symbiont conditions have shown that AM fungi are significantly involved in the uptake of soil mineral nutrients. They increase plant biomass and help the plant to resist stress and pathogens.

In contrast, AM fungi have been shown to be non-culturable without the host. Since the fungi are unable to take up carbohydrates—with exceptions within the plant cell—they are strictly dependent on their green hosts for growth and reproduction, which gives them the status of obligate biotrophs/symbionts. A saprophytic lifestyle without a host is not possible for AM fungi. AM fungi belong to the phylum *Glomeromycota*, a monophyletic group descended from the same common ancestors as *Ascomycota* and *Basidiomycota*.

From an evolutionary perspective, the ecological success of AM fungi demonstrates that the advantage of strict companionship with the plant eliminates the risks associated with loss of saprophytic properties.

Nutrient Exchange of the Symbiosis Partners

The fungi supply the plant with nutrient salts and water and in turn receive a portion of the assimilates produced by the plants' photosynthesis. This supply of carbohydrates to the fungus comes at a considerable "cost" to the plant. Estimates suggest that the proportion of primary production that is passed on to the fungus can be as high as 25%.

In contrast to other soil fungi, many mycorrhizal fungi lack the enzymes that are necessary to break down complex carbohydrates. Therefore, they depend on the supply from the plant.

The fungal hyphae radiating from the mycorrhiza increase the contact area with the soil. Fungal hyphae with their small diameter (2–12 μm) can absorb nutrients and water from a pore space that is inaccessible to roots. Mycorrhizal fungi thus have a much greater capacity to dissolve minerals and water from the soil compared to plants. The water, nitrogen and phosphate supply of the "infected" plants is thus frequently improved (◘ Fig. 2.4).

The different view of harm-benefit can be illustrated here as follows: Thus, in endomycorrhiza, mutualism is initiated by the parasitic invasion of the plant cell by the fungal partner. In later stages of development, the plant "digests" the fungus. Overall, however, both partners "profit" from the symbiosis.

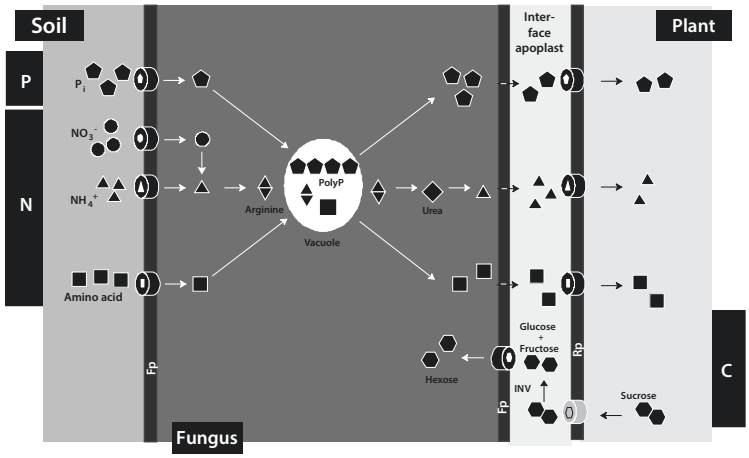

Fig. 2.4 Schematic representation of the main exchange processes of nutrients in EM and AM symbiosis. The focus is on the transport of phosphorus (P), nitrogen (N) and carbon (C) at the soil-fungus and fungus-plant interface. Inorganic P_i and mineral or organic forms of N, such as NH_4^+, NO_3^- and amino acids are carried into the mycelium outside the root by specialized transporter proteins localized in the fungal membrane. NH_3/NH_4^+ and P_i (the latter originating in AM fungi from the hydrolysis of polyphosphate) are brought from the symbiotic contact surface into the plant cell by selective transporters. Hexose transporters import the plant-fixed carbon into the fungus. It is debatable whether the processes are truly the result of active protein-mediated transport or involve passive export mechanisms. *INV* plant invertase, *Fp* fungiplasma membrane, *Rp* root plasma membrane

The situation is similar for another symbiotic community of microorganisms and plants that is of considerable importance for agriculture. For example, nitrogen-fixing bacteria form root nodules with certain agriculturally important butterfly plants (legumes), through which the plant obtains its nitrogen supply, but which also contribute significantly to the natural nitrogen supply of the soil. Many trees also meet their nitrogen needs by living together with nitrogen-fixing bacteria (see ► Sect. 7.1).

It is now recognized that a variety of important symbioses are also present in the ocean. The low availability of nutrients and energy has led to the evolution of numerous strategies to overcome these limitations. Symbiotic communities embody a key mechanism. Most notable are communities between chemosynthetic bacteria and marine animals that live well and successfully in nutrient-poor environments such as the deep sea, as the symbionts allow their hosts to grow with inorganic energy and carbon sources such as sulfide and CO_2. Chemosynthetic symbioses between bacteria and marine animals were discovered about 30 years ago on hydrothermal vents of the Galapagos Reef. Today, chemosynthetic symbioses are known to exist in a wide range of habitats worldwide (◘ Fig. 2.5).

A variety of groups of animals have formed communities with chemosynthetic bacteria. Currently, seven phyla of animals are known to harbour such symbionts (◘ Table 2.5). Some host groups are found in only one habitat, while others occur in many different ones (see also ► Sect. 10.4 with marine habitats).

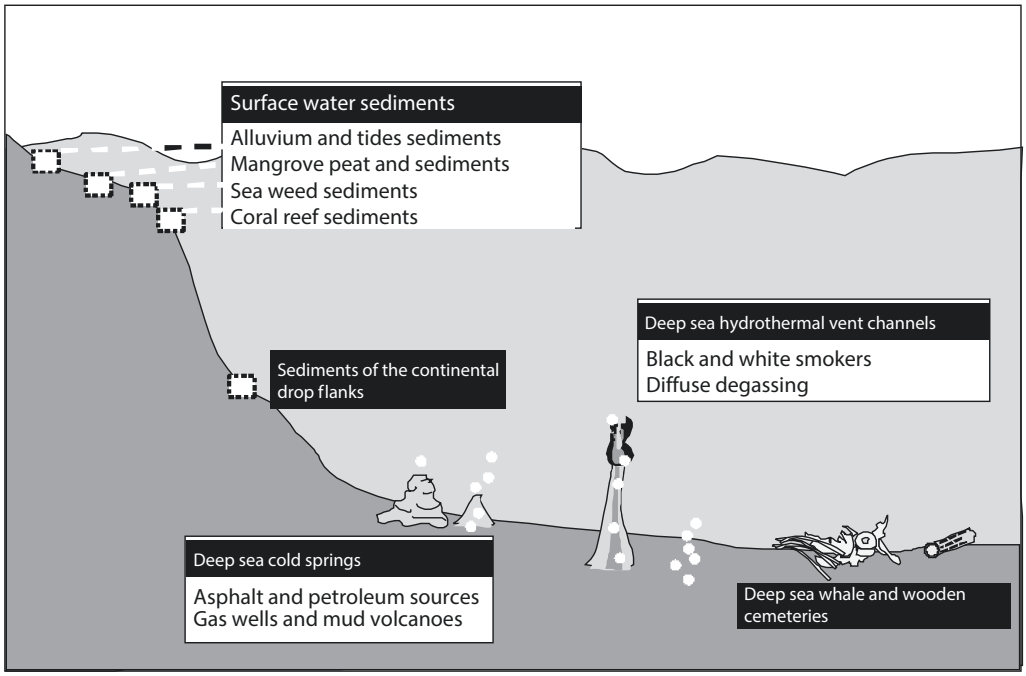

Fig. 2.5 Marine habitats in which chemosynthetic symbioses occur

The Elba Worm *Olavius algarvensis*, a Well-Studied Example of Marine Symbiosis

More than 100 gutless oligochaete species are known in marine sediments worldwide.

Gutless oligochaetes are small marine worms that possess neither mouth nor intestine nor nephridia, i.e. excretory organs. Instead, they harbor bacterial endosymbionts that have taken over the function of the digestive and excretory system.

Off the coast of Elba, the gutless oligochaete *Olavius algarvensis* is particularly abundant near seagrass beds.

The inconspicuous worm acquires its characteristic white coloration by the refraction of light on sulfur particles in the bacteria, which lie as symbionts extracellularly in a layer under the worm cuticle between extensions of the epidermal cells. This clearly distinguishes these oligochaetes from other marine ones and makes identification easy.

The symbionts provide food for the worm by using reduced inorganic sulfur compounds as an energy source to fix carbon dioxide into organic matter.

All gutless oligochaetes studied to date live in symbiosis with a *Gammaproteobacterium* related to free-living sulfur-oxidizing bacteria. These γ1-symbionts from different host species are phylogenetically closely related.

The thioautotrophic character (sulfur-oxidizing, CO_2-fixing metabolism) of the γ1-symbionts was clarified. In addition to the γ1-symbionts, some host species harbor a second *Gammaproteobacterium* and up to three different *Deltaproteobacteria*, while others are associated with *Alphaproteobacteria.*

In *Olavius algarvensis*, the deltaproteobacterial symbionts are sulfate reducers

Table 2.5 Phyla with chemosynthetic symbioses in the ocean

Phylum	Example		Occurrence in the ocean	Location of microorganisms in the host	Metabolic type of microorganisms
Ciliates (Ciliophora)		Colony-forming ciliates *Zoothamnium niveum*	Surface water	Ectosymbionts completely cover the surface of the colony	Sulfur-oxidizing symbionts
Sponges (Porifera)		Demospongiae, *Cladorhiza*	Deep sea cold springs	Intracellular and extracellular	Methane oxidizing symbionts
Flatworms (Platyhelminthes)		Retronectidae, *Paracatenula*	Surface water	Intracellular in trophosomes	Sulfur-oxidizing symbionts
Nematodes		*Astomonema*	Surface water	Bacteria inhabit the entire intestine	Sulfur-oxidizing symbionts
Annelids (Annelida)		*Alvinella* (Pompeii worm)	Deep-sea hydrothermal vents	Ectosymbionts were found on the dorsal surface (epibiont)	Chemoautotrophic symbionts
		Tubeworms of the family Siboglinidae	Surface water, deep sea: hydrothermal outcrop channels, cold springs, wood cemeteries	Endosymbionts colonize the trophosomes	Sulfur- and methane-oxidizing symbionts
		Oligochaete, *Olavius*	Surface water	Bacteria are endo-symbionts, occurring just below the cuticle in the extracellular space above the epidermal cells (but not intracellularly)	Sulfur-oxidizing and sulfate-reducing symbionts

(continued)

Table 2.5 (continued)

Phylum	Example		Occurrence in the ocean	Location of microorganisms in the host	Metabolic type of microorganisms
Arthropoda		Outbreak Channel Crab, *Rimicaris*	Deep-sea hydrothermal vents	Ectosymbionts were found in the mouth area and in gill chambers (epibiont)	Chemoautotrophic symbionts
Molluscs (Mollusca)		Gastropod, Snail	Deep sea hydrothermal vents	Endosymbionts occur intracellularly in the gill tissue	Sulfur- and methane-oxidizing symbionts
		Mussels	Deep sea: hydrothermal vents, cold springs, whale and lumber graveyards	Endosymbionts colonize the gill tissue intracellularly and extracellularly	Sulfur- and methane-oxidizing symbionts
		Bivalve molluscs of the family Vesicomyidae	Deep sea: hydro-thermal vents, cold springs, whale cemeteries	Endosymbionts colonize the gills intracellularly	Sulfur-oxidizing symbionts
		Mussels of the family Solemyidae	Surface water	Symbionts are intracellular in the gills	Sulfur-oxidizing symbionts
		Mussels of the family Lucinidae	Surface water, deep sea: hydrothermal vents, cold springs	Symbionts are intracellular in the gills	Sulfur- and sulfide-oxidizing symbionts
		Mussels of the family Thyasiridae	Surface water, deep sea: hydrothermal outcrop channels, cold springs, forest cemeteries	Extracellular symbionts occurring between the microvilli of the epithelial cells, in or on the gill tissue	Sulfur-oxidizing symbionts

Trophosome. Adult worms have neither a mouth nor a continuous intestine or anus. They feed through the trophosome, an organ developed from the intestine, which is created as a special tissue already in an early larval stage**Thiotroph**. An organism that uses reduced sulfur compounds such as sulfide as electron donors is called a thiotroph or sulfur oxidizer**Epibiont**. A symbiont that lives on the surface of its host**Endobiont**. A symbiont that lives within its host

that respire oxidized sulfur compounds such as sulfate to sulfide. The sulfide produced can be used by the sulfide-oxidizing symbionts for autotrophic fixation of CO_2.

For the growth of the worm and its symbionts, energy sources, such as organic matter dissolved in the pore water, must be taken up from the environment.

These gutless oligochaetes are a good example of mutualistic association with multiple symbionts:

1. Hosts that take up sulfate-reducing bacteria as additional symbionts can adapt to changes in the environment-such as the availability of sulfide, which can vary greatly but is essential for the γ1-symbiont.
2. The sulfide-oxidizing and sulfate-reducing symbionts do not appear to compete for resources, but instead benefit from each other in their conversion.

Each symbiont occupies a microniche through its substrate specificity and affinity, thus benefiting the mobile multisymbiosis. This is because during its migration through the sediment, the worm passes zones with different substrate offers as well as electron donors and acceptors on which the individual symbionts have specialized.

This may explain why, despite apparent functional redundancy with two sulfur-oxidizing γ-symbionts, there is a selective advantage in *O. algarvensis*. The only physiological features used by both symbionts are the use of reduced sulfur and carbon fixation via the Calvin cycle. On the other hand, they show great differences in the use of additional energy and carbon sources as well as electron acceptors. The γ3-symbionts can use CO and also the host-produced osmolyte betaine (*N*,*N*,*N*-trimethylglycine) as additional energy and carbon sources, whereas the γ1-symbionts can use fermentative waste products from their hosts as an additional carbon source. Furthermore, the γ1-symbionts appear to rely heavily on storage substances such as sulfur and polyhydroxyalkanoates, whereas storage substances do not appear to play a major role in the metabolism of the γ3-symbionts.

Resource partitioning is also apparent in the differences in electron acceptors used by the two symbionts. The γ1-symbionts are primarily dependent on oxygen for their respiration, whereas the γ3-symbionts probably cannot use this electron acceptor, but instead use the less energy-efficient nitrate.

Metaproteomic analyses also show that despite the genetic similarities, functional differences exist in the key metabolic pathways for chemosynthesis of the two symbionts.

All symbionts in *O. algarvensis* appear to use a remarkably similar metabolic strategy: All express proteins that function in highly efficient metabolic pathways for the uptake, recycling, and maintenance of energy and carbon sources.

These paths include

1. Several strategies of recycling waste products from the host.
2. The possible use of inorganic energy sources in addition to reduced sulfur compounds, such as hydrogen and CO.
3. The extremely strong expression of high-affinity transporters, which allow the uptake of a large number of substrates at very low concentrations.
4. Previously undescribed energy-efficient steps in the sulfate reduction and CO_2 fixation pathways (◘ Fig. 2.6).

If one takes the oligotrophic, nutrient-poor character of the environment of the Elba worm, in which organic compounds

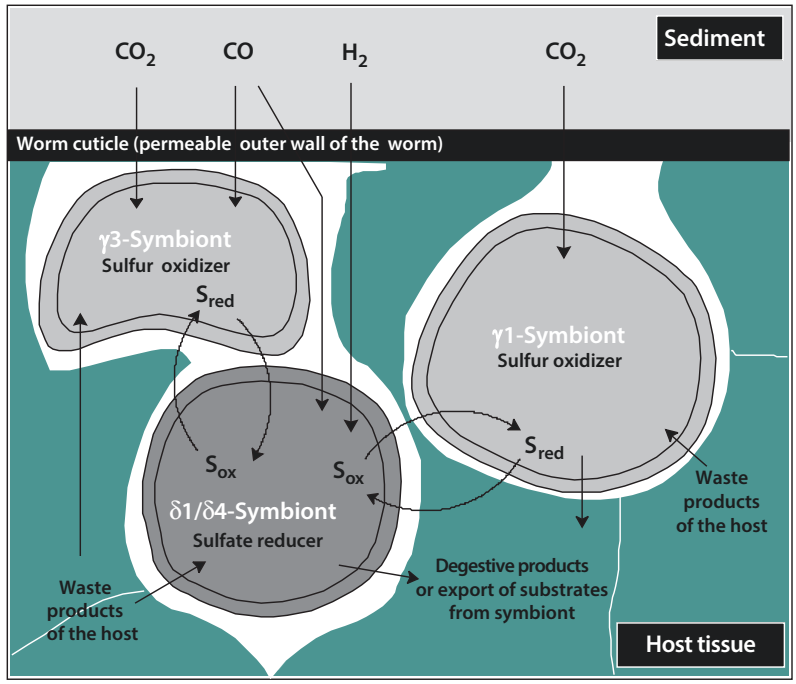

Fig. 2.6 Schematic diagram of possible sources of energy and carbon during symbiosis in *Olavius algarvensis.* External sources of energy and carbon may include carbon monoxide, hydrogen, and carbon dioxide; internal sources may include reduced sulfur compounds S_{red} and host waste products such as acetate and glycine betaine (*N*,*N*,*N*-trimethylglycine). S_{ox} mean oxidized sulfur compounds. The δ1- and δ4-symbionts are shown as one cell

are present near detection limits and reduced sulfur compounds are just detectable, the selective pressure on metabolic pathways must have been very strong, leading to the formation of this symbiosis, so that the acquisition and maintenance of energy and carbon was maximized.

Cooperation between metabolically different microorganisms is particularly common among anaerobic bacteria (see later ► Sect. 4.5). An example is the formation and consumption of hydrogen in the digestion tower of a sewage treatment plant or in the rumen or termite gut and the resulting formation of methane. Such an anaerobic feeding chain in the rumen involves the participation of a wide variety of microorganisms: utilizers of cellulose, starch and hemicellulose, sugar fermenters, fatty acid utilizers, methanogens, proteolytic and lipolytic bacteria.

In the digestive system of animals, bacteria face their host organisms as cooperative partners. Herbivores in particular have evolved highly developed cooperative systems that are highly beneficial to both partners (microorganism and cow).

If only one partner derives a benefit from the symbiosis without the other suffering any harm, this is referred to as **commensalism.** A typical interaction of this type is the degradation of cellulose by cellulolytic fungi, which produce organic acids that then serve as substrates for non-cellulolytic bacterial and fungal species.

Commensalism can also provide the right conditions in a habitat for certain microorganisms. For example, when a population of facultative anaerobic bacteria consumes toxic oxygen, it creates a habitat for the growth of obligate anaerobes.

Another example is the production and excretion of vitamins and amino acids by microbial populations, which can then be used by other fastidious microorganisms for growth.

The removal/consumption of toxic factors such as H_2S by a population of *Beggiatoa* is an example of how H_2S-sensitive aerobic populations are enabled to grow. In contrast, photoautotrophic bacteria require H_2S for their metabolism. It is supplied by sulfate breathers, which produce it from sulfate (see later ► Sects. 7.1 and 8.1).

Another example that can be counted to commensalism is the substrate chain during nitrifying: *Nitrosomonas* oxidizes ammonium to nitrite, which can then be used by *Nitrobacter* for its metabolism and nitrate is formed (see later ► Sects. 6.3 and 7.3).

Inhibition of pathogenic fungi by fluorescent pseudomonads is an example of **amensalistic** interaction between microorganisms, in which the bacterial partner acts against the fungus by *in situ* production of a phenazine-type antibiotic.

Typical predatory and **parasitic** interactions between microorganisms are the attacks on soil bacteria by Protozoa on one side and *Bdellovibrios* on the other.

Test Your Knowledge

- Compare prokaryotes and eukaryotes.
- How does a small size of cells affect their growth rate?
- In what numbers are bacteria found in soil or wastewater? Compare the biomass of bacteria with that of fungi in the forest soil.
- Which forms of togetherness are positive for both partners of a cohabitation?

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Relationship Between Microbial Energy Production and Material Cycles

Contents

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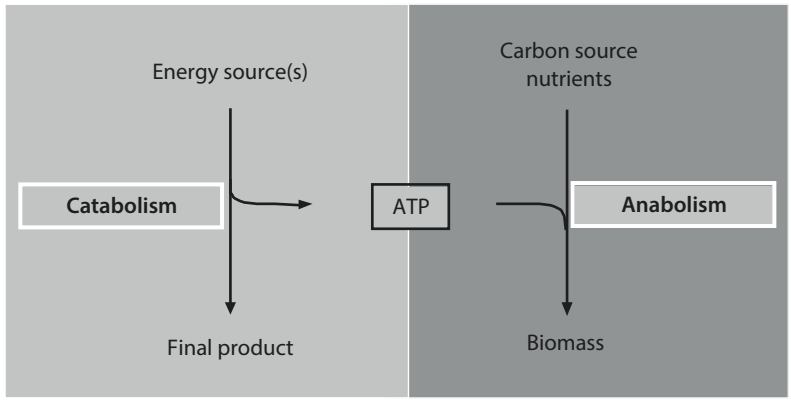


Fig. 3.1 Relationship between energy production and biomass formation. The carbon source is in many cases identical with one of the energy sources

The main reasons for the great importance of microorganisms in many metabolic cycles lie in the intensity of their metabolic processes and the diversity of their metabolic pathways. Whereas higher organisms, as discussed in ► Chap. 2, achieved a high degree of morphological-anatomical differentiation in the course of evolution, microorganisms, considered as a whole, possess a pronounced biochemical differentiation.

Every cell is a substance and energy converter. The aim of these substance and energy transformations is the maintenance and/or reproduction of the cell substance. Every organism, including every microorganism, in order to grow and maintain its cell structures, must first obtain biochemical energy. The cell functions as an irreversibly operating bioreactor that takes up energy in the form of chemical energy or light energy and usually converts it into the chemical energy of the energy-rich compound adenosine triphosphate, ATP (► Sect. 3.1). The aim is to achieve the highest possible yield of ATP. The processes that contribute to energy production, often degradation processes, are called **catabolism** (◘ Fig. 3.1). The organism then synthesizes biomass with the help of the energy gained and the available nutrients. The synthesis processes that take place for this purpose are summarized under the term **anabolism,** whereby the incorporation of carbon compounds, carbon assimilation, represents an important aspect.

The biochemical diversity of microorganisms is expressed in particular in the different types of energy production and carbon assimilation. In the following, some basic thoughts on biochemical energy production will be explained first. Subsequently, a first overview of the diverse, microbial metabolic types will be given.

3.1 Principles of Energy Production

Some microorganisms can obtain energy from light **(phototrophy).** However, even more microorganisms carry out chemical reactions to produce energy **(chemotrophy)**.

In most cases, chemotrophic energy metabolism can be described as a **redox process**. Macroscopically, there is an oxidative and a reductive part of the energy metabolism. In the oxidative part, a substrate, the **electron donor**, is oxidized and so-called reduction equivalents (hydrogen or electrons) become available and transferred to an intermediate electron carrier. From this, the reduction equivalents are then trans-

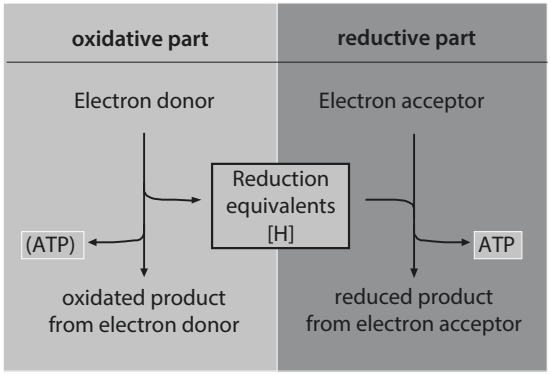


Fig. 3.2 Energy metabolism as a redox process. Energy in the form of ATP is usually obtained in the reductive part, in the case of organic electron donors also in the oxidation part (hence ATP in brackets)

ferred to another substrate, the **electron acceptor**, which is reduced (Fig. 3.2). Each reduction equivalent formed is also consumed again. The terms electron donor and (terminal) electron acceptor are of central importance for the characterization of microbial metabolic pathways (► Sect. 3.2).

In terms of the overall metabolism, the electron donor and the oxidized product from the electron donor (left side in Fig. 3.2) form a redox pair and the electron acceptor and the reduced product from the electron acceptor (right side in Fig. 3.2) form a second redox pair. Basically, the redox couple with the lower **reduction potential** E represents the electron donor, and the one with the higher reduction potential represents the electron acceptor. Then the difference of the reduction potentials ΔE of the reactants is positive. Based on the equation.

$$\Delta G = -n \times F \times \Delta E \quad (n, \text{number of electrons transferred; } F, \text{Faraday constant, } 96.485\,\text{J/V}\cdot\text{mol})$$

consequently, the change in **free enthalpy** (Gibbs free energy) ΔG is negative, and the reaction proceeds voluntarily.

For an initial estimate of the direction of a redox reaction, or how much energy it can supply, the **standard biological reduction potentials** $E^{0'}$ of various redox pairs are often compared. For some redox pairs that are particularly important with regard to microbial metabolism, the reduction potentials under standard biological conditions are given in Fig. 3.3.

In order to calculate the total driving force available for a metabolic reaction, the **difference between the reduction potentials is** determined by subtracting that of the oxidative partial reaction from the reduction potential of the reductive half-reaction. For example, under standard biological conditions, the data in Fig. 3.3 give the following for the oxidation of glucose to CO_2 using oxygen:

$$\text{Ox}: C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24e^- + 24H^+ \left(E^{0'} = -0.43\,\text{V}\right)$$

$$\text{Red}: 6O_2 + 24e^- + 24H^+ \rightarrow 12H_2O \left(E^{0'} = 0.82\,\text{V}\right)$$

$$\Delta E^{0'} = E^{0'}_{\text{red}} - E^{0'}_{\text{ox}} = 0.82\,\text{V} - (-0.43)\,\text{V} = 1.25\,\text{V}$$

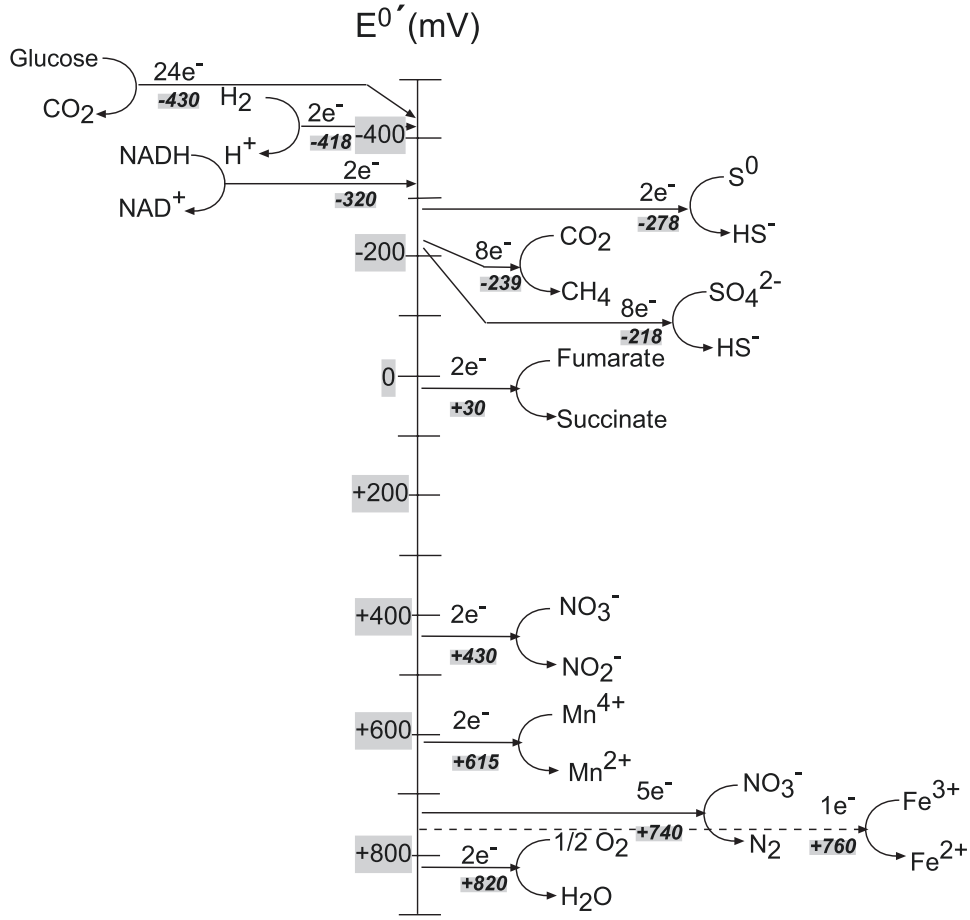


Fig. 3.3 Standard reduction potentials at pH 7 ($E^{0'}$) of some redox pairs important for microbial energy production at a temperature of 25 °C

The energy released per mole of glucose in such a reaction, the free enthalpy under standard biological conditions $\Delta G^{0'}$, is given as:

$$\Delta G^{0'} = -n \times F \times \Delta E^{0'} = -24 \times 96.485 \text{ J/V} \cdot \text{mol} \times 1.25 \text{ V} = -2890 \text{ kJ/mol}$$

Basics: Reduction Potential

The **reduction potential** E is used to characterize the tendency of a redox pair to accept electrons and thus react to the reduced form. It is given in comparison to the potential of the standard hydrogen electrode, which is defined as 0 V.

The reduction potential of redox pairs depends not only on the species involved as such, but also on the concentration or partial pressure (more precisely, the activity) in which substances are present. In order to eliminate this influence for the comparison of different reduction

potentials, the **standard reduction potential E0** has been defined in chemistry as the reduction potential at which the respective substance is present pure or in a solution with activity 1 at a pressure of 1 bar.

Standard chemical conditions would mean that, for reactions in which protons are transferred, the pH would have to be 0 (that is, the proton concentration would be 1 M). Since such a concentration does not occur in most biological systems, the **reduction potential under standard biological conditions** has been defined $E0'$. It refers to a half-reaction taking place in aqueous solution at pH 7 and a pressure of 1 bar, in which the remaining reactants are present in activity 1.

The **influence of the concentration of** the respective oxidized and reduced form on the reduction potential of a half reaction can be calculated using **Nernst's equation**:

$$E' = E^{0'} + \frac{R\ T}{n\ F}\ \ln\frac{[A_{ox}]}{[A_{red}]}$$

Here, E' is the reduction potential at pH 7 and $E^{0'}$ is the standard biological reduction potential for the half-reaction $A_{ox} + n\ e^- \rightarrow A_{red}$, n is the number of electrons transferred, F is Faraday's constant (96.485 J/V·mol), R is the general gas constant (8.3145 J/mol·K), T is the absolute temperature, $[A_{ox}]$ is the concentration of the oxidized form of the reactant, and $[A_{red}]$ is the concentration of the reduced form.

For a temperature of 25 °C (298.15 °K), the formula (with $\ln X = 2.303\ \lg X$) can also be simplified to:

$$E' = E^{0'} + \frac{0.0592\text{V}}{n}\ \lg\frac{[A_{ox}]}{[A_{red}]}$$

Since the concentrations of the reaction partners involved practically never correspond to the standard conditions, the respective **concentrations of** electron donor, electron acceptor and products formed from both must be taken into account for a more precise determination of the respective reduction potentials according to Nernst's equation. The potential differences determined by taking into account the actual concentrations of the reaction partners can deviate considerably from the $\Delta E^{0'}$ values determined from ◘ Fig. 3.3. Consequently, different values for the free enthalpy ΔG of the reaction result than under standard conditions. This fact plays a particularly important role under anoxic (i.e. oxygen-free) conditions. We will see in ► Sect. 4.5.1 that the interaction of different types of metabolism makes reactions possible that would be impossible under standard conditions.

The free enthalpy made available by the redox reactions is partly used for the **synthesis of ATP**. For many degradation processes, the energetic efficiency, i.e. the free enthalpy stored in ATP relative to the free enthalpy of the energy-producing reaction, is about 50%. ATP synthesis can occur in two fundamentally different ways (◘ Fig. 3.4):

- In the oxidative part of the energy metabolism of organic electron donors, ATP can be formed in part by forming phosphorylated metabolites from the organic substrate and transferring a phosphate group from these to ADP. This type of formation of ATP is called **substrate step phosphorylation.** It requires that phosphorylated metabolites occur that are sufficiently energetic to allow transfer of the phosphate group to ADP. This requires a $\Delta G^{0'}$ of hydrolysis of about –44 kJ/mol (Box ► Background:ATP).

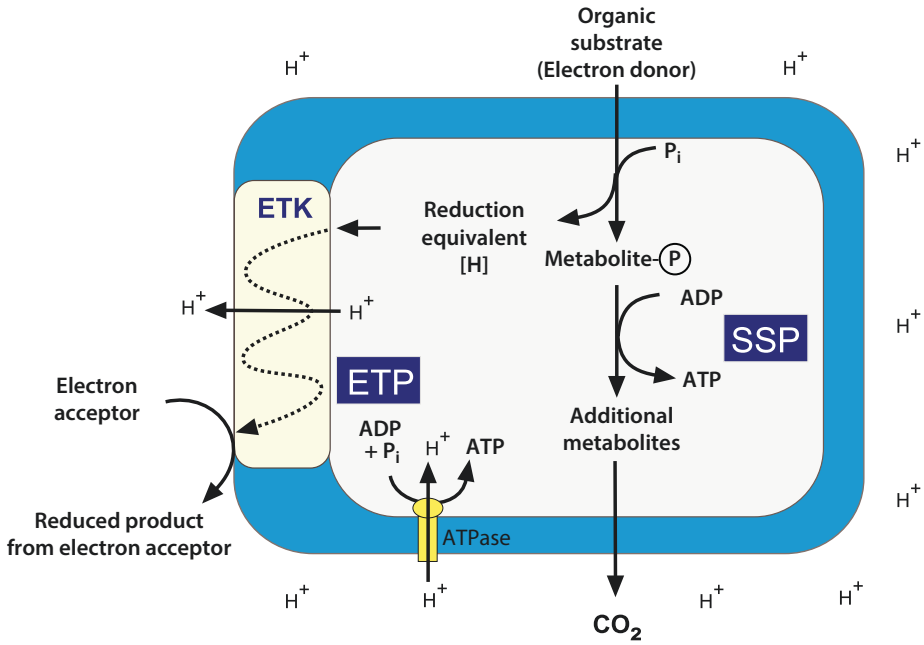


Fig. 3.4 Possibilities of ATP synthesis by substrate step phosphorylation (SSP) and electron transport coupled phosphorylation (ETP) using the example of energy production by oxidation of an organic electron donor. Metabolite-P indicates energy-rich phosphorylated metabolites. *ETK* Electron transport chain

- In the reductive part of energy metabolism, the reduction equivalents are transferred to the terminal electron acceptor following the reduction potential via a series of electron intermediates. Through this series of redox reactions, protons are carried out of the cell (or, in higher cells, out of the interior of the mitochondria). A **proton gradient** is created at the cytoplasmic membrane (or inner mitochondrial membrane, respectively). The electrochemical potential given by the proton gradient, the so-called proton motor force, can on the one hand serve directly as an energy source for various processes (membrane transport, movement). On the other hand, it can be used for ATP synthesis via so-called **electron transport-coupled phosphorylation.** In this process, an ATP-synthesizing membrane-bound enzyme, the ATP synthase (Box ► ATPase), couples the influx of protons to the phosphorylation of ADP to ATP (Fig. 3.4).

In contrast to substrate step phosphorylation, oxidation of one mole of substrate does not have to yield at least one mole of ATP in electron transport-coupled phosphorylation. Phosphorylation in exergonic reactions that have a $\Delta G^{0'}$ of smaller magnitude than −44 kJ/mol are still possible because the electrons that flow off from the redox reactions are initially used only for proton transport across the membrane. In a sense, the proton gradient integrates across the many small energy packets and is subsequently used for ATP production. This energetic flexibility is probably one reason for the widespread use of electron transport-coupled phosphorylation even in anaerobic bacteria, where the low $\Delta G^{0'}$ values do not allow substrate step phosphorylation in some cases.

Background:ATP

The most important compound in which energy can be kept available for syntheses and other metabolic activities is adenosine triphosphate, **ATP**. It is an excellent energy store because significant amounts of energy go into the formation of its phosphoric anhydride bonds, which are later made available again very easily either by hydrolysis or by transferring the phosphate moiety into new energy-rich bonds. Upon hydrolysis of the phosphoric anhydride bond found in ATP to form adenosine diphosphate, ADP, and inorganic phosphate, P_i , energy is released because (1) the repulsion of the negative charges in ATP is reduced, (2) the inorganic phosphate is resonance stabilized, (3) the neutral pH promotes ionization of the resulting ADP, and because (4) the charged reaction products are further stabilized by solvation (◘ Fig. 3.5).

The actual concentrations of ATP, ADP and P_i in the cell, which deviate from the standard concentrations, mean that the real $\Delta G'$ value is significantly greater than the specified $\Delta G^{0'}$ value. It can change over time and is affected by binding of ATP and ADP to Mg^{2+} and proteins. The magnitude of the $\Delta G^{0'}$-value is estimated to be about 44 kJ/mol (Lengeler et al., 1999). In the synthesis of ATP, at least the amount of energy released during hydrolysis must be available.

NADH and $FADH_2$ are the Most Important Electron Carriers

Nicotinamide adenine dinucleotide (NAD+) is the major electron acceptor in the oxidation of food substances (◘ Fig. 3.6). The reactive part of NAD^+ is its nicotinamide ring system, a pyridine derivative. When a substrate is oxidized, the nicotinamide ring accepts a proton and two electrons, which formally corresponds to a hydride ion. The reduced form of this carrier is called NADH.

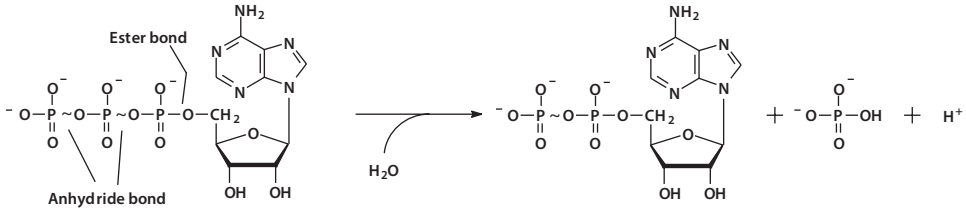
In the oxidized form, the nitrogen atom is formally four-bonded and positively charged, as indicated by NAD^+. In the reduced form, NADH, it is trivalent.

NAD^+ occurs as a mobile electron acceptor in many reactions of the following type:

In this dehydrogenation, one hydrogen atom of the substrate is transferred directly to NAD^+ while the other appears in solution. Both electrons from the substrate are transferred to the nicotinamide ring.

The other important electron carrier in the oxidation of food substances is the **flavin adenine dinucleotide** (◘ Fig. 3.6b). The symbols FAD and $FADH_2$ are used as abbreviations for the oxidized and reduced forms, respectively. FAD is the electron acceptor in reactions of the following type:

The reactive part of FAD is its isoalloxazine ring (◘ Fig. 3.6). Like NAD^+, FAD can accept two electrons. Unlike NAD^+, FAD formally accepts one proton and one hydride ion.



◘ **Fig. 3.5** Structures of ATP and the hydrolysis products ADP and P_i ($\Delta G^{0'}$ = –32 kJ/mol)

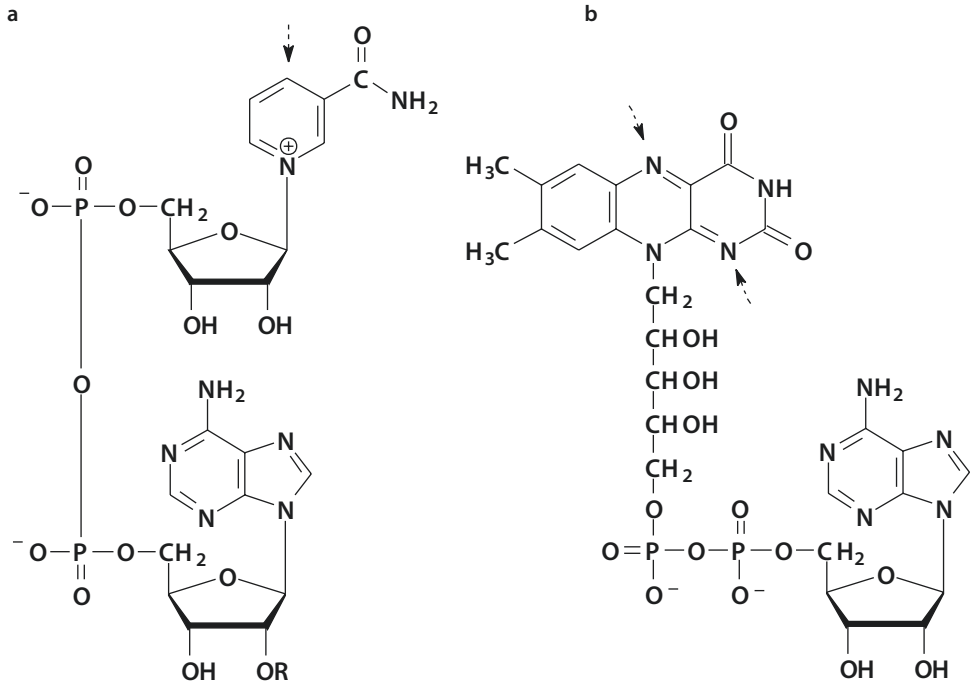


Fig. 3.6 Structures of the oxidized form of **a** nicotinamide adenine dinucleotide (NAD^+, R = H) and nicotinamide adenine dinucleotide phosphate ($NADP^+$, R = phosphate), respectively, and **b** flavin adenine dinucleotide (FAD). The arrows show the reaction sites on the nicotinamide and isoalloxazine rings

3.1.1 Respiratory Chains and ATP Synthase

Crucial for the energy production of aerobic organisms as well as of anaerobic organisms with anaerobic respiration is the point that the reduced hydrogen intermediates (reduction equivalents or better electron intermediates) NADH and $FADH_2$ produced during the oxidation of a substrate are used as "fuel" for the **respiratory chain,** where they can supply considerably higher amounts of energy via **electron transport phosphorylation** than is possible through substrate step phosphorylation. By transferring the reduction equivalents via a series of electron intermediates to the electron acceptor, a **proton gradient** is established. Thus, a charge separation occurs in which the exterior of the cell, or the periplasm, is positively charged and slightly acidic. This proton gradient can be used directly, for example, for movement processes or membrane transport or for ATP synthesis.

A proton gradient can inevitably only be established at a membrane that is normally not permeable to protons, in prokaryotes the cell membrane or invaginations thereof. This requires proteins in the membrane that transport the protons out of the cytoplasm or into the periplasm. These proteins must be correctly aligned and several of them must have access to both the environment and the interior of the cell (transmembrane proteins). The reduction equivalents delivered for the respiratory chain, i.e. hydrogen bound to NAD^+ or quinones, separate into the protons, which are transported to the outside, and the electrons, which are transferred to the various electron intermediates in turn. The driving force for the outward proton transport is the electron transport

from a donor compound with a low reduction potential, for example NADH/NAD^+ ($E^{0'}$ = –320 mV), to an acceptor compound with a higher reduction potential, for example O_2/H_2O ($E^{0'}$ = +820 mV).

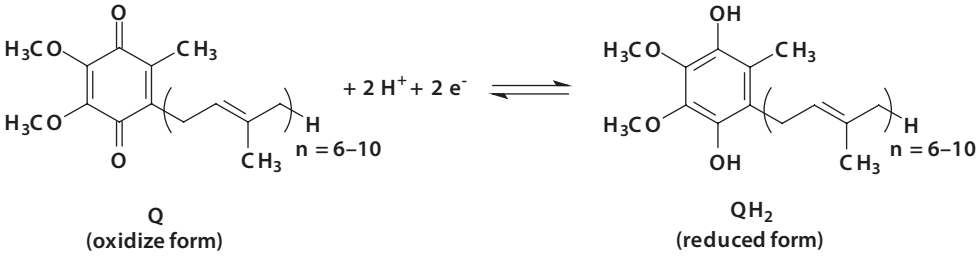
Different proteins are involved in the creation of the proton gradient in different bacteria and depending on the growth conditions. In most cases, however, a **quinone** in the membrane, an ubiquinone or a menaquinone, is first reduced to a **hydroquinone** (or quinol), abbreviated: Q → QH_2 (◘ Fig. 3.7).

This reaction is catalyzed by different dehydrogenases depending on the hydrogen donor, for example by an NADH dehydrogenase. Like the NAD^+ in the cytoplasm, the quinones in the membrane serve to collect reduction equivalents of different origin. The reduced quinones can then be directly re-oxidized by quinol oxidases with oxygen, which in this case would already complete the electron transport. Often, however, the electrons are first transferred to a **cytochrome c**, a small protein with a heme ring whose iron can be reduced. In such cases, it is the cytochrome oxidase that transfers the electrons to oxygen, forming water.

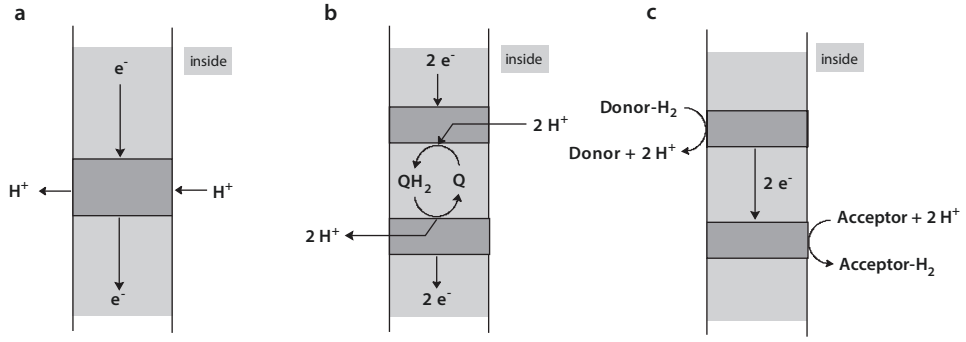
It should be noted that these processes do not occur without the terminal electron acceptor O_2.

Depending on the organism and growth conditions, different mechanisms in different combinations can lead to the formation of the proton gradient during electron transport across these steps (◘ Fig. 3.8).

For example, there are **proton pumps** that change their conformation during electron transport and release protons to the outside



◘ **Fig. 3.7** Structure of coenzyme Q. The C_5 unit in the side chain is an isoprenoid and in bacteria usually consists of n = 6 subunits



◘ **Fig. 3.8** Mechanism of the generation of a proton gradient at a membrane by electron transport. The electron transport enzymes are shown as grey boxes in the membrane. Q, ubiquinone or menaquinone, **a** proton pump, **b** Q cycle, **c** redox loop, scalar mechanism

(or into the periplasm). This is the case for some NADH dehydrogenases, quinol oxidases and cytochrome oxidases. A second important mechanism is the **Q-cycle.** Here, quinones are reduced on the inside of the membrane by uptake of protons from the cytoplasm and re-oxidized on the outside of the membrane by release of protons to the outside, this orientation being dictated by the enzymes involved. Finally, a proton gradient can be increased without proton transport across the membrane. This occurs when **proton release and consumption occur on different membrane sides**, for example, when protons are consumed on the inside of the membrane by transfer to the terminal electron acceptor oxygen, but are released from corresponding donors on the outside. It is called a **scalar mechanism,** in contrast to the **vectorial mechanism in** which protons are pumped from the inside to the outside.

In the presence of a respiratory chain with optimal use of the possibilities for the discharge of protons and with a good oxygen supply, a maximum of about 10 protons can be discharged per NADH molecule that is oxidized with oxygen. However, in many organisms and under less optimal conditions, the value can also be significantly lower.

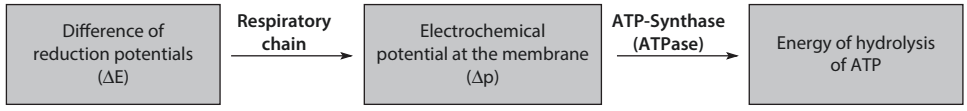
The energy from the difference in the reduction potentials of the electron donor and acceptor ΔE is partly used to form an electrochemical potential (Δp), the so-called proton motor force, at the membrane (◘ Fig. 3.9). The next step to be considered here is the use of this electrochemical potential for the production of ATP.

ATP synthesis from electron transport phosphorylation takes place at the **ATP synthases** in all organisms. These protein complexes, which are relatively similar in different organisms, are anchored in the membrane and extend into the cytoplasm of the cell. They exploit the proton gradient by coupling the influx of protons to conformational changes of specific subunits for the synthesis of ATP from ADP and inorganic phosphate. In this coupling, a rotational movement of some subunits relative to non-rotating other subunits plays a crucial role (Box ► ATPase).

Measurements of the stoichiometry between protons and ATP show that four protons are consumed per ATP formed. This means that about three (calculated 2.5) molecules of ATP can be formed from the oxidation of NADH with oxygen. The oxidation of reduced FAD with oxygen assumes a higher reduction potential and therefore yields only two molecules of ATP per $FADH_2$.

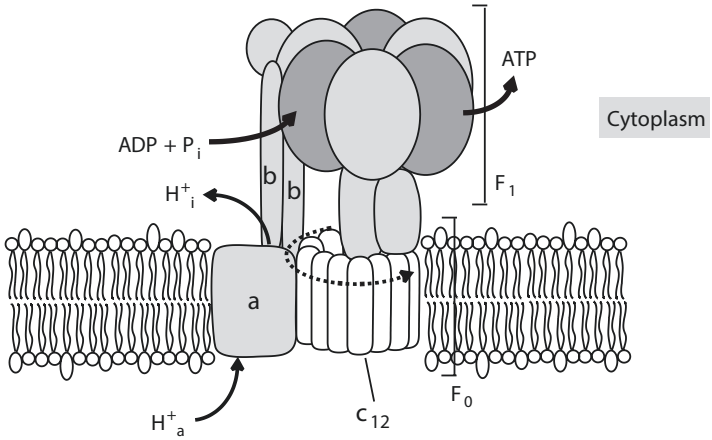
ATPase

The F_1/F_0-ATPase is the smallest known biological motor. The movement of protons through subunit **a** of **F0** drives the rotation of c proteins. The torque generated is transmitted through the γε subunits to **F1**. This leads to conformational changes in the β-subunits. Thus, it is a potential energy that can be used for ATP formation. This is possible because the conformational changes in the β-subunits allow sequential binding of ADP + P_i to

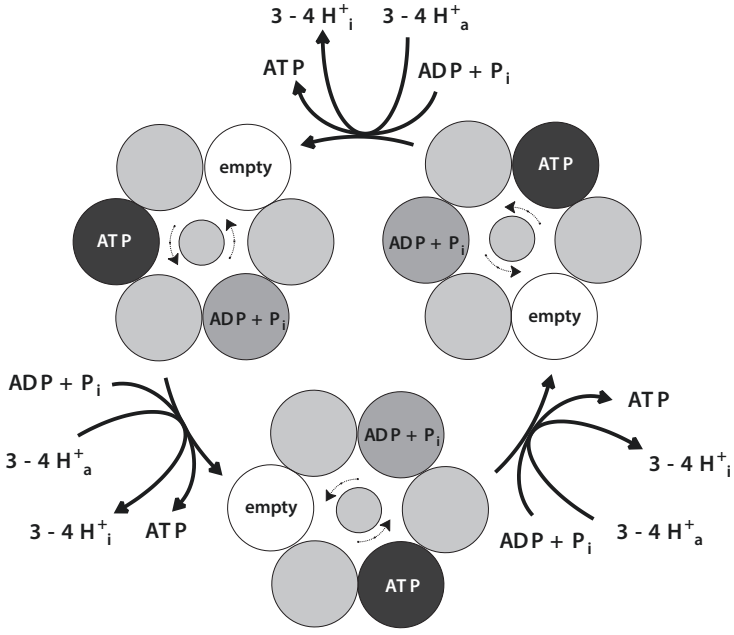


◘ **Fig. 3.9** Energy conversion during ATP synthesis by electron transport phosphorylation

each subunit. Conversion to ATP occurs when the β-subunits return to their original conformation. The **b2 δ-subunits** of **FI** act as uprights to prevent the α- and β-subunits from rotating with the γε-subunits and interfering with the β conformational changes. Thus, the rotation of the ATPase is used for ATP synthesis. Measurements of stoichiometry show that 3–4 protons per ATP formed are transported through the ATPase (■ Figs. 3.10 and 3.11).



■ **Fig. 3.10** Structure of ATPase with its subunits in F_1 and F_0. H_i^+proton inside, H_a^+, proton outside the cell. c_{12}: 12 subunits c



■ **Fig. 3.11** View from above of the schematically shown differently occupied conformational states of the β-subunits of the ATPase. The changes resulting from an overall rotation of the γε-subunits are shown

3.2 Main Types of Microbial Metabolism

Now that it has been clarified that electron donor and terminal electron acceptor are of decisive importance for most energy production processes and that the energy produced during metabolic processes is usually made available in the form of ATP (and/or in the form of a proton gradient), the various metabolic types will now be presented and classified in an overview.

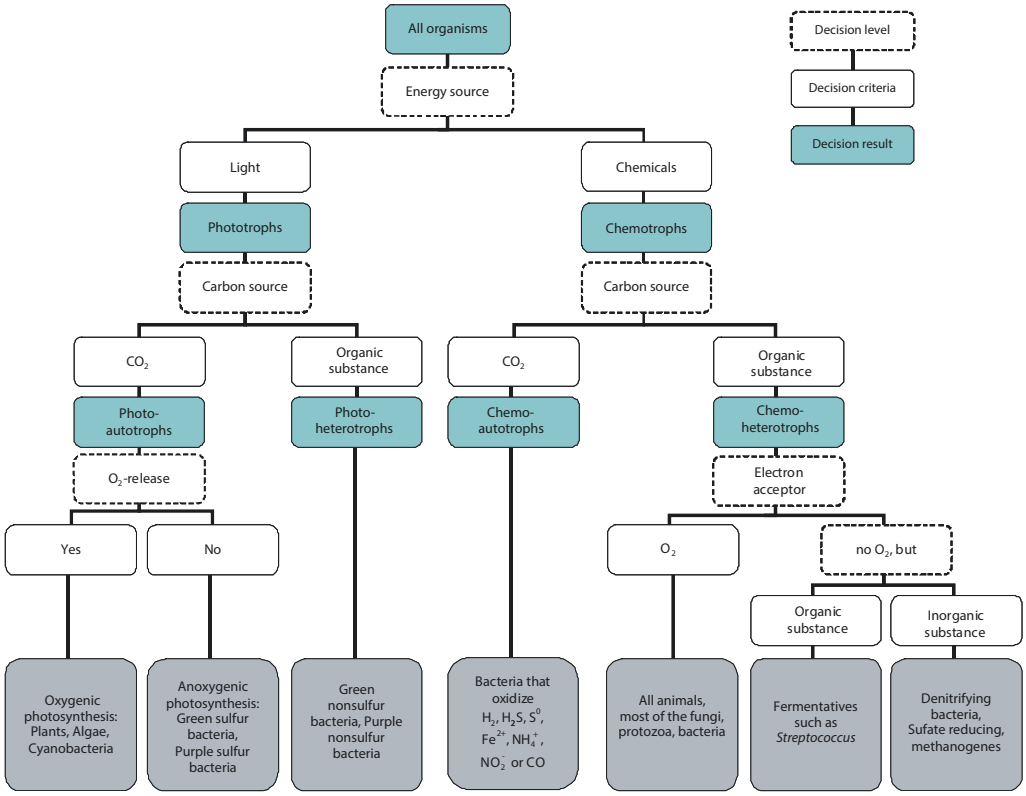
Metabolic types are mainly subdivided according to the type of **energy production**, but also according to the type of **carbon source** used to build up the cell substance (see ◘ Fig. 3.12).

With regard to energy production, the main division is first of all that already mentioned above between the microorganisms that use light and those that use chemical reactions for energy production. The first group is called **phototrophs**, the second **chemotrophs**.

3.2.1 Phototrophy

In the conversion of light energy into biochemical energy, one of the most important biological processes, two types of mechanisms can be distinguished. In both cases, a proton gradient is established, which is then used for ATP synthesis, as presented above (► Sect. 3.1.1).

Whereas in **"actual phototrophic organisms"** there is a light-driven redox process with photosynthetic pigments such as chlorophyll, various bacteriochlorophylls as well as carotenoids and phycobilins, the light-



◘ **Fig. 3.12** Flow chart for the subdivision of organisms into metabolic types

driven build-up of the gradient in **halobacteria** occurs at a purple membrane without a redox reaction.

The first-mentioned organisms carry out photosynthesis, i.e. energy production and simultaneous CO_2 fixation, in other words a light and a dark reaction. A subdivision of the "actual phototrophic organisms" is made according to whether they release oxygen during photosynthesis **(oxygenic photosynthesis)** or not **(anoxygenic photosynthesis). Water** is the electron donor in oxygenic photosynthesis, while in anoxygenic it is often—but not exclusively—**reduced inorganic sulfur compounds** such as H_2S. In both cases, these are lithotrophic processes, since inorganic electron donors are used.

The second mechanism takes place in the simple, chlorophyll-free photosystem of halobacteria or similarly in marine proteobacteria, with the best-known representative *Pelagibacter ubique.* These simple systems have **retinal** as their central molecule in the purple membrane, which carries out proton transport by means of a light-driven conformational change (◘ Table 3.1).

◘ **Table 3.1** Light utilization in pro- and eukaryotes

Organisms	Light-driven proton pump with redox process	(Bacterio) Chlorophylls	Proteorhodopsine	Electron donor	CO_2-fixation
All phototrophic eukaryotes (plants and algae)	+	+	–	H_2O	+
Cyanobacteria (for example *Prochlorococcus*, *Synechococcus*)	+	+	–	H_2O	+
Sulfur purple bacteria (for example *Chromatium*, *Thiocapsa*)	+	+	–	H_2S/organic compounds	+
Non-sulfur purple bacteria (for example *Rhodobacter*, *Rhodospirillum*)	+	+	–	Organic compounds	+
Green sulfur bacteria (for example *Chlorobium*, *Prosthecochloris*)	+	+	–	H_2S	+
Green non-sulfur bacteria (for example *Chloroflexus*)	+	+	–	H_2/organic compounds	±
Heliobacteria	+	+	–	Organic compounds	?
Halobacteria	–	–	+		–

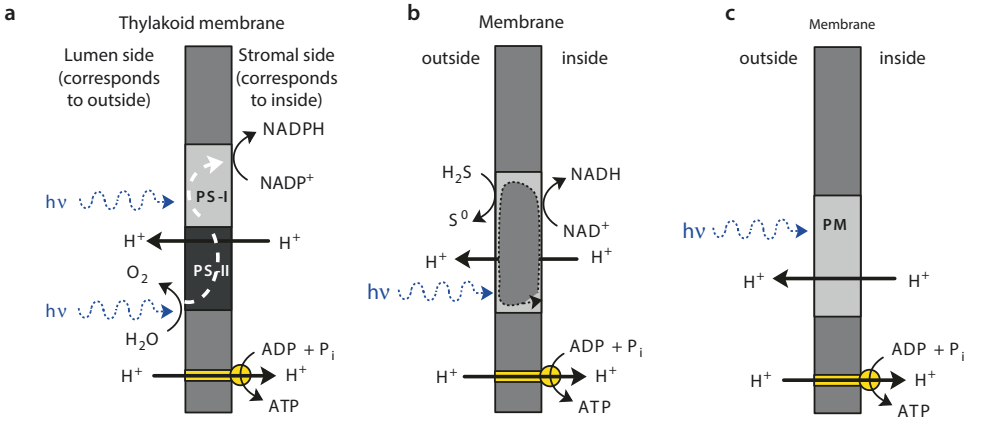
Principle of Photosynthesis

Photosynthesis is a light-driven redox process and uses light energy to establish a proton gradient at membranes. Chlorophyll excited by light energy splits off an electron in the reaction center, which is raised to an acceptor molecule with a more negative redox potential. After charge separation, the electron returns to the oxidized reaction center via an electron transport chain. In the process, a proton gradient is established. This is used for the synthesis of ATP. The photosynthetic apparatus consists of antennae, reaction center, electron transport components and H^+-ATP synthase. In many cases, it is localized on intracytoplasmic membranes (◘ Fig. 3.13).

Aerobic Anoxygenic Phototrophs

In addition to oxygenic phototrophs and organisms that possess proteorhodopsin, there are other prokaryotes that use light energy in marine waters. These **aerobic anoxygenic phototrophic bacteria** are a special case. Like anoxygenic phototrophic purple bacteria, they possess bacteriochlorophyll *a*. However, unlike anoxygenic purple bacteria, which photosynthesize only under anoxic conditions, aerobic anoxygenic phototrophs perform photophosphorylation **only under aerobic conditions**; they are therefore strictly aerobic.

They are mostly marine, organotrophic *Alphaproteobacteria* such as *Citromicrobium*, *Erythrobacter* or *Roseobacter* that live among algae.



◘ **Fig. 3.13** Systems of photosynthesis. **a** In cyanobacteria, as in green plants and algae, the photosynthetic apparatus is localized in the thylakoid membrane. Oxygenic photosynthesis uses two photosystems. In photosystem II (PS-II), the electron removed is replaced by oxidation of water with O_2 release. Water splitting and electron transport between photosystem II and I are used to generate a proton gradient. Photosystem I (PS-I) then raises the electron to an even more negative potential, allowing $NADP^+$ reduction. **b** Anoxygenic photosynthesis uses only one photosystem. It operates without water splitting and uses cyclic electron flow to generate the proton potential necessary for energy conservation. Inorganic or organic substrates (H_2S in this case) are oxidized to reduce $NAD(P)^+$. **c** A particularly simple, chlorophyll-free photosystem is represented by the bacteriorhodopsin of halobacteria. This simple system, the purple membrane (PM) acts as a light-driven proton pump. The generated proton gradient is then used for ATP synthesis

Aerobic anoxygenic phototrophs do not grow autotrophically and therefore rely on organic carbon as a carbon source. Thus, as in *Pelagibacter* and its proteorhodopsins, aerobic anoxygenic phototrophs probably use ATP formed via the photocomplex to supplement the chemotrophic metabolism used on the other hand. Proteorhodopsin has not yet been detected in *Roseobacter.*

A great diversity of aerobic anoxygenic phototrophs exists in marine waters. *Roseobacter* species account for up to 25% of the total microbial community in nearshore and polar oceans. Oligotrophic and highly oxic freshwater lakes are also habitats of these interesting phototrophic bacteria. Light, oxygen and organic excreta from the algae give them a selection advantage.

3.2.2 Chemotrophy

3.2.2.1 Chemotrophy: Electron Donor

In chemotrophy, a distinction must be made between the use of organic and inorganic compounds as **electron donors:**

- **Chemoorganotrophic** organisms use organic compounds (such as carbohydrates, organic acids or amino acids) as electron donors.
- **Chemolithotrophic** organisms obtain energy from the oxidation of inorganic compounds such as hydrogen (H_2), carbon monoxide (CO), hydrogen sulfide (H_2S), sulfur (S^0), ammonium (NH_4^+), nitrite (NO_2^-), divalent iron (Fe^{2+}), or divalent manganese (Mn^{2+}), yielding the respective oxidation products H_2O, CO_2, SO_4^{2-}, NO_2^-, NO_3^-, Fe^{3+}, or Mn^{4+}.

3.2.2.2 Chemotrophy: Electron Acceptor

The labels mentioned so far do not include the **terminal electron acceptor.** As with electron donors, microorganisms also show great diversity in the use of electron acceptors.

- Often, as in most higher organisms, oxygen is the terminal electron acceptor. Metabolism is then normal, **aerobic respiration** with respect to the electron acceptor.
- Many microorganisms use nitrate (NO_3^-), trivalent iron (Fe^{3+}), tetravalent manganese (Mn^{4+}), sulfate (SO_4^{2-}), sulfur (S^0) or carbon dioxide (CO_2) as terminal electron acceptors for energy production. As in normal aerobic respiration with oxygen, ATP is also obtained in these cases primarily by electron transport-coupled phosphorylation. Therefore, the corresponding energy production processes are also referred to as **anaerobic respiration,** with nitrate respiration, iron respiration, manganese respiration, sulfate respiration, sulfur respiration or carbonate respiration, depending on the terminal electron acceptor.
- Even more common, however, are sometimes other names, depending on the product formed from the terminal electron acceptor. For example, many bacteria reduce nitrate to molecular nitrogen, and this process is usually referred to as **denitrification** instead of nitrate respiration. Instead of carbonate respiration, the formation of methane (CH_4) as a product is called **methanogenesis**, and the formation of acetic acid as a product is called **acetogenesis.**
- Even unusual inorganic ions such as arsenate (AsO_4^{3-}), selenate (SeO_4^{2-}), and hexavalent uranium (UO_2^{2+}) can apparently be used as terminal electron acceptors. And even organic compounds such

as fumarate and even perchloroethene or 3-chlorobenzoate can be terminal electron acceptors for energy production with electron transport-coupled phosphorylation. In these cases, we speak of fumarate respiration or, in the case of halogenated compounds, of dehalorespiration (► Sects. 6.2.1 and ► 6.2.4).

- Finally, in the absence of external terminal electron acceptors, various organic intermediates formed from the original substrate can serve as electron acceptors without electron transport-coupled phosphorylation. In these cases, the microorganisms obtain their energy by substrate step phosphorylation. In such cases one speaks of **fermentation.** Instead of a redox reaction between electron donor and terminal electron acceptor, a disproportionation of the substrate into an oxidized compound (CO_2) and relatively reduced compounds such as ethanol (C_2H_5OH), lactic acid ($CH_3CHOHCOOH$), butyric acid (C_3H_7COOH) or hydrogen (H_2) usually occurs.

Among the microorganisms that can grow with oxygen, i.e. aerobically, there are those that also do so anaerobically, i.e. without oxygen. These are called **facultative anaerobes.** These include, for example, many denitrifiers. Other microorganisms can only grow in the absence of oxygen and are called **obligate anaerobes.**

The above lists of possible electron donors and electron acceptors already show that chemotrophic organisms have a large number of different combinations and thus different metabolic types. Some examples are listed in ◘ Table 3.2.

◘ **Table 3.2** Examples of chemotrophic metabolic types

Electronics Donor	Terminal electron acceptor	Carbon source	Designation of the metabolic type according to	
			Electron donor and carbon source	Terminal electron acceptor (or reduced products)
Organic substance	O_2	Organic substance	Chemoorgano-heterotroph	Aerobic respiration
Organic substance	NO_3^-	Organic substance	Chemoorgano-heterotroph	Nitrate respiration (denitrification)
Organic substance	SO_4^{2-}	Organic substance	Chemoorgano-heterotroph	Sulfate respiration
NH_4^+	O_2	CO_2	Chemolithoautotroph (nitrification)	Aerobic respiration
H_2S	O_2	CO_2	Chemolithoautotroph (sulfide oxidation)	Aerobic respiration
H_2	SO_4^{2-}	CO_2	Chemolithoautotroph	Sulfate respiration
H_2	CO_2	CO_2	Chemolithoautotroph	Carbonate respiration (methanogenesis, acetogenesis)
CH_4, CH_3OH	O_2	CH_4, CH_3OH	Chemolithotroph (methylotrophy)	Aerobic respiration
Organic substance	Organic intermediate	Organic substance	Chemoheterotroph	Fermentation

3.2.3 Carbon Source: Heterotrophy and Autotrophy

The type of **carbon source** is often used in addition to identify the metabolic type:

- Organisms that use organic substances to build up their cell substance are called **heterotrophs**,
- those that assimilate CO_2 as **autotrophs**.

The aforementioned labels of energy production and carbon source are often combined. For example, many chemolithotrophic bacteria build up their cell substance from CO_2 and are therefore called **chemolithoautotrophs**.

Phototrophic bacteria are also autotrophic organisms, as indicated above, since CO_2 can be assimilated via different fixation pathways, the dark reaction (see later ► Sect. 4.3).

Some phototrophs use light as an energy source, but cannot convert CO_2 into sugars. These **photoheterotrophs** need organic substances such as alcohols, fatty acids, other organic acids and carbohydrates as a carbon source. They are anoxic. Green non-sulfur bacteria such as *Chloroflexus* and non-sulfur purple bacteria such as *Rhodopseudomonas* are photoheterotrophs.

Without light, there is facultative chemotrophy in cyanobacteria, purple sulfur bacteria, non-purple sulfur bacteria, and green non-sulfur bacteria.

The halobacteria and aerobic anoxygenic phototrophic bacteria cannot fix CO_2 and are therefore heterotrophic.

The above illustration shows, on the one hand, that microorganisms play a major role in the carbon cycle via the oxidation of organic compounds to CO_2 and the fixation of CO_2 in organic compounds. On the other hand, it shows that other elements such as nitrogen, sulfur or iron can also be oxidised by some microorganisms and reduced by other microorganisms, depending on the environmental conditions. This is the basis for the outstanding importance of microorganisms in the material cycles, which will be considered in more detail in the following chapters.

Test Your Knowledge

- Is a well-fed living being in thermodynamic equilibrium? Give reasons for your opinion!
- Name at least six different inorganic electron donors and the products formed from them in metabolic processes!
- Name various possible electron acceptors as well as the products resulting from them and rank these pairs according to the extent to which they enable energy production with organic compounds as electron donors!
- If oxygen does not occur at a site with high concentrations of organic carbon, but serious concentrations of sulpfate, Fe(III), nitrate and CO_2, what are the dominant metabolic processes to be expected? Why?
- Label and name the two fundamentally different ways of ATP synthesis!
- What distinguishes fermentation from other types of metabolism occurring under anaerobic conditions?
- Label the following metabolic types and indicate the probable metabolites. (a) Electron donor and C source acetate, electron acceptor NO_3^-. (b) Electron donor NO_2^-, C source CO_2, electron acceptor O_2. (c) Electron donor H_2, C source CO_2, electron acceptor NO_3^-. (d) Electron donor and C source fructose, electron acceptor SO_4^{2-}.

Further Reading

Animation ATPase (n.d.): www.sigmaaldrich.com/Area_of_Interest/Life_Science/Metabolomics/Key_Resources/Metabolic_Pathways/ATP_Synthase.html.

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Carbon Cycle

Contents

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4.1 Formation of the Earth's Atmosphere and Fossil Raw Materials

The composition of the Earth's atmosphere today can be traced back to biological processes in the course of Earth's history. Four billion years ago, the primordial atmosphere contained mainly water, CO_2 and reduced compounds. It is likely that CH_4, H_2 and H_2S were assimilated by the ancestors of the Archaea, which are thought to have formed about 3.8 billion years ago. From this anoxic early phase, first deposits of organic carbon have been detected in sediments. First stromatolites were found in 3.5 billion years old Precambrian sediments (Warrawoona Group, Australia). Stromatolites are biogenic sedimentary rocks that are probably formed from sediment-forming minerals by microbial activity, similar to present-day bacterial mats. The sediments have a lamellar structure.

Evidence for the evolution of oxygenic photosynthesis comes from 3.5 billion year old iron oxide deposits. Over billions of years, oxygen formed by biological photolysis has oxidized the divalent iron present through weathering to Fe_2O_3. The iron oxides are insoluble and have been deposited as banded iron formations (BIFs) in marine basins (see ► Sect. 8.2.1 for alternative hypothesis of BIFs). In addition to divalent iron, sulfides were also oxidized to sulfates. Another type of oxygen sequestration is the "red beds", redstone deposits on the primeval continents. Only after the reduced iron and sulfur compounds were oxidized was oxygen released to the atmosphere. This process began about 2 billion years ago and, as shown in ◘ Fig. 4.1, led to the gradual formation of our present atmosphere. Probably only 4% of the oxygen formed during evolution was released into the atmosphere, the majority being in mineral-bound form. The oxygen enrichment led to the formation of the ozone shield against UV radiation.

How from the sum equation of photosynthesis is evident

$$n\ CO_2 + n H_2O \rightarrow [CH_2O]_n + n O_2$$

oxygen formation is associated with CO_2 consumption and the formation of organic matter, simplified as $[CH_2O]_n$. These organic substances, formed in a stoichiometric ratio to oxygen, have accumulated in sediments and have been transformed in a variety of ways by geochemical processes. They are hydrocarbons, usually in dispersed form; they are called kerogens. These are the beginnings of petroleum formation. Already in archaeal deposits hydrocarbons with branched chains can be detected, which probably go back to the isoprenoid structure of the cell membrane of the archaea.

Higher organized and structured eukaryotic microorganisms evolved about two billion years ago. They developed an effective energy exchange in the form of respiration and at the same time mechanisms to detoxify the oxidative agent oxygen. According to the endosymbiont theory, the organelles of the eukaryotic cell trace back to phylogenetic precursors of prokaryotes, the chloroplasts to cyanobacteria and the mitochondria to heterotrophic, respiring bacteria. The eukaryotic cell was the "starting material" for the evolution of biodiversity, which began 700 million years ago, at the transition from the Precambrian to the Cambrian.

Earth history, organismal evolution and the formation of fossil resources are closely linked. The oil and gas reservoirs evolved from marine microorganisms, mainly bacteria and algae. The dead biomass settled as a kind of putrid sludge (sapropel) in which no mineralization of organic matter occurred due to lack of oxygen. The formation of the rock and lignite deposits from the terrestrial vegetation of the Carboniferous and Tertiary periods by processes that we can

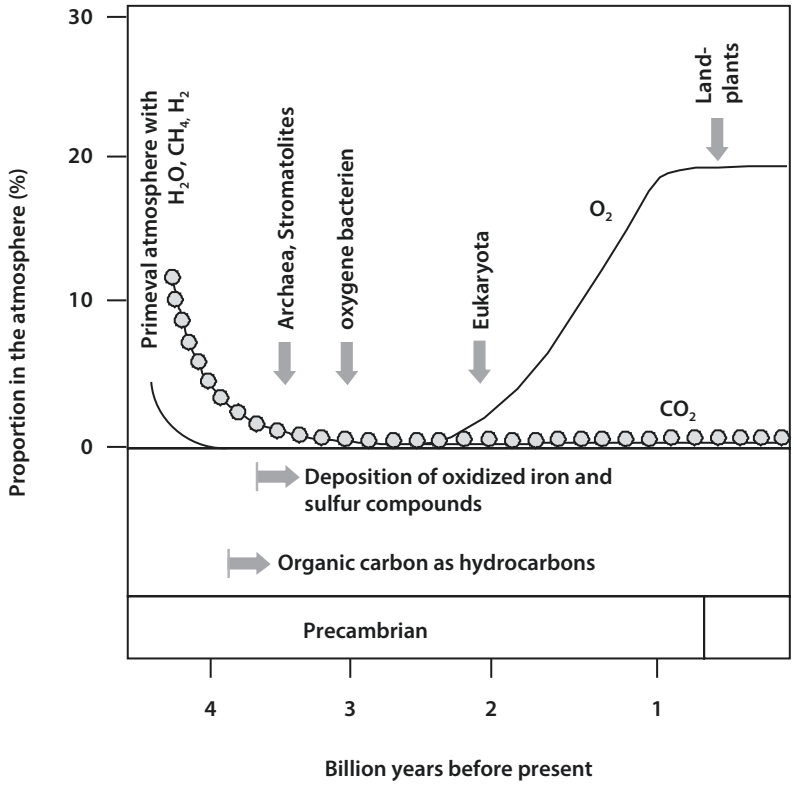


Fig. 4.1 Earth-historical periods of the formation of the Earth's atmosphere and fossil carbon deposits. Photosynthesis splits H_2O, and the resulting hydrogen is used to reduce CO_2 to organic matter. Oxygen gradually accumulated in the atmosphere after iron compounds were oxidized in the oceans and on land. The arrows indicate the beginning of the respective processes

observe today in the formation of peat is well known. All of these fossil resources are products of photosynthesis that began billions of years ago. This process created two values crucial to our existence, the oxygen-containing atmosphere and fossil fuels. The latter are solar energy stored over millions of years. Through photosynthesis, CO_2 was reduced to energy-rich compounds and fixed.

In the geological periods of biogeochemical formation of fossil raw materials, production far outweighed consumption, resulting in the high net gain of biomass. In the present we have a very balanced and delicate equilibrium between primary production and decomposition, photosynthesis and respiration as well as mineralization processes, which must be maintained.

4.2 Material Flows in the Carbon Cycle

Photosynthesis and chemosynthesis (CO_2 fixation by chemolithotrophs) are the only significant pathways to synthesize new organic carbon on Earth.

Phototrophic and **chemolithotrophic organisms** therefore form the basis of the carbon cycle. Phototrophic organisms occur in nature almost exclusively in biotopes where light is available. **Oxygenic phototrophic organisms** can be divided into two

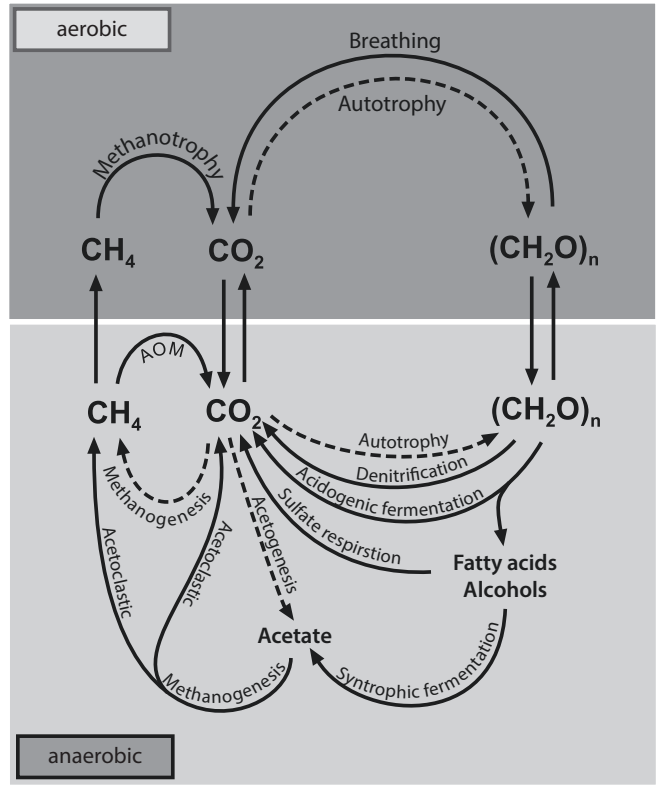
major groups: higher plants and microorganisms. Higher plants are the dominant phototrophic organisms in terrestrial biotopes, while phototrophic microorganisms are the dominant photosynthesizers in aquatic biotopes. **Anoxygenic phototrophic organisms** are other aquatic autotrophs. Chemolithoautotrophy is found under aerobic conditions for example in the **nitrifiers** or **sulfur oxidizers,** but also in the absence of oxygen for example in the **methanogens** and **acetogens.**

The redox cycle for carbon is shown in ◘ Fig. 4.2. The entire carbon cycle is based on a positive net balance of the rate of photosynthesis versus that of respiration, which in turn leads to the formation of CO_2 (◘ Table 4.1).

Photosynthetically fixed carbon is degraded over time by various microorganisms, essentially to two oxidation states: Carbon dioxide (CO_2) and methane (CH_4). These two gaseous products are formed by the activity of various chemoorganotrophs through aerobic respiration, anaerobic respiration (dissimilatory denitrification) or fermentation (CO_2) or methanogenesis (CH_4).

Methane produced in anoxic biotopes is nearly insoluble (35 mL in 1 L water at 17 °C) and is readily transported to oxic environments where it is oxidized by methanotrophs to CO_2. All organic carbon eventually returns to CO_2 from where autotrophic carbon cycle metabolism begins again.

The balance between the oxidative and reductive parts of the carbon cycle is very important; the metabolic products of some organisms form the substrates for others. Therefore, the cycle must remain in balance if it is to continue as it has for many billions of years. In terms of degradation, CO_2 release by microbial activities far exceeds that by eukaryotes, and especially in anoxic environments.



◘ **Fig. 4.2** The carbon cycle. $(CH_2O)_n$ stands for carbohydrates (cell material). Pathways leading to CO_2 are shown with solid arrows, pathways leading away from CO_2 such as fixation reactions are shown with broken arrows

Table 4.1 Processes in the carbon cycle

Process with function	Organisms
Autotrophic CO_2-fixation [$CO_2 \rightarrow (CH_2O)_n$] Aerobic: phototrophic Chemolithotroph Anaerobic: phototrophic Chemolithotroph [H_2 as electron donor]	Plants, algae, cyanobacteria Nitrifiers, *Thiobacillus* Sulfur bacteria: *Chromatiaceae, Chlorobiaceae* Acetogens, Methanogens, Sulfidogens
Respiration [$(CH_2O)_n \rightarrow CO_2$]	Heterotrophic organisms
Denitrifiers [$(CH_2O)_n \rightarrow CO_2$] nitrate respiration, dissimilatory nitrate reduction	*Pseudomonas, Paracoccus*
Acidogenic fermentation [$(CH_2O)_n \rightarrow$ fatty acids, CO_2]	*Enterobacteriaceae, Clostridium,* Propionibacteriaceae
Sulfidogens [fatty acids $\rightarrow CO_2$] complete oxidation	*Desulfobacter, Desulfobacterium, Desulfococcus, Desulfonema*
Syntrophic fermenters [fatty acids $\rightarrow$ acetate] Secondary fermenters	*Syntrophomonas wolfei, Syntrophobacter* sp.
Sulfidogens [fatty acids $\rightarrow$ acetate] incomplete oxidation	*Desulfovibrio, Desulfomicrobium, Desulfomaculum, Desulfobulus*
Carbonate respiration ($CO_2 \rightarrow$ acetate) CO_2 as terminal electron acceptor, homoacetogenic bacteria	*Clostridium thermoaceticum, Acetobacterium woodii*
Acetoclastic methanogens [acetate $\rightarrow CO_2 + CH_4$]	*Methanosarcina, Methanotrix*
Carbonate respiration [$CO_2 \rightarrow CH_4$]CO_2 as terminal electron acceptor	Methanogens: *Methanococcus, Methanomicrobium, Methanospirillum, Methanothermus*
Methanotrophs [$CH_4 \rightarrow CO_2$] Carbon and energy source: aerobic	*Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocystis,* some yeasts
Methanotrophs [$CH_4 \rightarrow CO_2$] anaerobic: AOM	Syntrophy between anaerobic methane oxidizers and sulfidogenic organisms

General Information on Autotrophic CO_2 Fixation

Assimilation of CO_2 (oxidation number of +4) to cellular carbon (average oxidation number of 0, as in carbohydrates) requires 4 reduction equivalents.

An energy supply is also required for the reductive conversion of CO_2 into cell carbon. This is supplied by the hydrolysis of ATP.

Both anaerobic and aerobic autotrophic fixation pathways have evolved.

While aerobes usually rely on NAD(P)H as a reducing agent, anaerobic organisms often use electrondonors with a low potential such as reduced ferredoxin (ferredoxin, $E^{0'} \approx -400$ mV; NADPH, $E^{0'} = -320$ mV) for CO_2-fixation. Because reduced ferredoxin contains more energy than NADPH, aerobic fixation pathways usually require more ATP equivalents than anaerobic ones.

Carboxylating enzymes attach either CO_2 or HCO_3^- to organic acceptor molecules,

which must then be provided again in subsequent steps of the assembly pathways.

These inorganic carbon species are related to each other in a pH-dependent equilibrium:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}, \text{with a pKa} \left[HCO_3^- / CO_2 \right] \text{of 6.3}$$

The concentration of CO_2 in water in equilibrium with air is 0.012 mM at pH 7.4 and 20 °C. In contrast, that of bicarbonate is 0.26 mM in seawater, which has a pH between 7.5 and 8.4. Consequently, the concentration of bicarbonate under slightly alkaline conditions in marine water is much higher than the concentration of dissolved CO_2. Thus, the use of bicarbonate instead of CO_2 is an advantage.

The products of the autotrophic assembly pathways are key metabolites such as acetyl-CoA, succinyl-CoA, pyruvate, phosphoenolpyruvate, malate, oxaloacetate, from which carbohydrates, proteins, nucleic acids and lipids are assembled.

All anaerobic autotrophic fixation pathways lead to acetyl-CoA. Therefore, the biosynthesis of C_3 to C_6 compounds requires "lifting" starting from acetyl-CoA. All three anaerobic pathways have the identical section of reductive conversion of acetyl-CoA to succinyl-CoA.

4.3 Autotrophic CO_2-Fixation

Carbon dioxide is an abundant source of carbon that must be reduced to cellular material of the stage $(CH_2O)_n$. The process requires reduction power and energy.

Organisms that require CO_2 as a primary carbon source are **autotrophs** and they serve as primary producers in ecosystems. Autotrophic CO_2-fixation allows **phototrophic** and **chemolithotrophic organisms** to live independently of an externally supplied organic carbon source.

Autotrophic carbon dioxide fixation in eukaryotes occurs solely through the **Calvin-Benson-Bassham cycle (CBB cycle)**. It is also the main mechanism by which many prokaryotes fix CO_2 in biomass. Although the main global carbon fixation occurs via this cycle, five other pathways of carbon fixation are currently known. These are

1. the reductive or reverse citrate cycle or reductive tricarboxylic acid cycle (rTCA cycle)
2. the reductive acetyl-CoA pathway or acetogenesis or the Wood-Ljungdahl pathway (WL pathway)
3. the 3-hydroxypropionate/4-hydroxybutyrate cycle (3-HP-/4-HB cycle)
4. the dicarboxylate/4-hydroxybutyrate cycle (DC-/4-HB cycle) and
5. the 3-hydroxypropionate bi-cycle (3-HP bi-cycle).

The fact that five alternative autotrophic fixation pathways exist in prokaryotes is often ignored. This limited view can lead to significant misconceptions in models of the global carbon cycle, in hypotheses on the evolution of metabolisms and interpretations of geological data.

The rTCA cycle and the WL pathway were found to operate in very different groups of microorganisms (◘ Fig. 4.3). The rTCA cycle appears to be restricted to bacteria and functions in *Aquificales, Chlorobiales, Epsilonproteobacteria, Nitrospirae* and individual strains of other *Proteobacteria* groups.

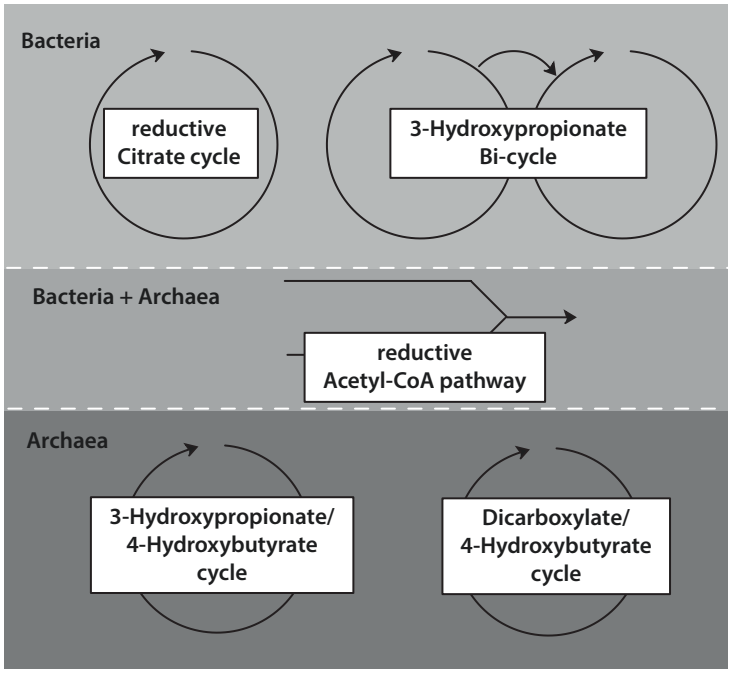


Fig. 4.3 Distribution of alternative pathways of autotrophic CO_2 fixation in archaea and bacteria

The WL pathway, which occurs in bacteria (*Deltaproteobacteria*, *Firmicutes, Planctomycetes*, *Spirochaeta*) as well as in *archaea* (*Euryarchaeota*), is probably the oldest of the fixation pathways.

The other pathways seem to occur less frequently. Thus, in *Chloroflexaceae* the 3-HP-Bi cycle operates, while in *Crenarchaeota* both the 3-HP/4-HB and DC/4-HB cycles are found.

The distribution of the six autotrophic pathways is summarized in Table 4.2.

The so-called alternative carbon fixation pathways make a relevant contribution to fixation in a number of oceanic environments. Of particular note are those at deep-sea hydrothermal vents, the meso- and bathypelagic ocean, and in oxygen deficient zones and redox layers as well as in euxinic water.

Of particular relevance are:

- the **rTCA cycle,** which appears to be the main carbon fixation pathway at deep-sea outcrop channels,
- the **3-HP/4-HB cycle,** which most likely occurs in nitrifying *Crenarchaea* in the dark pelagic regions, and
- the **WL pathway,** which operates in methanogenic *Archaea* and possibly in anammox-catalyzing *Planctomycetes.*

The rTCA cycle seems to be the predominant pathway in habitats characterized by temperatures between 20 and 90 °C. In contrast, the CBB cycle and other fixation pathways, such as the WL pathway or the DC/4-HB cycle, are the main pathways below 20 °C and above 90 °C. The CBB cycle proceeds in some thermophiles, but never in hyperthermophiles—the upper temperature limit here seems to be around 70–75 °C. The heat instability of some intermediates of the cycle, mainly of glyceraldehyde-3-phosphate, is an explanation for this upper limit.

At deep-sea hydrothermal vents, there is a clear niching of carbon fixation pathways with respect to temperature. This is also reflected in the presence of oxygen, as habitats

Table 4.2 The six pathways of autotrophic CO_2-fixation

Way	Group of organisms	Representative organisms
Calvin Cycle	Higher plants and algae	Plant chloroplasts *Anabaena cylindrica*
	Oxygene phototrophic bacteria	
	Anoxygenic phototrophic bacteria	*Chromatium vinosum* *Rhodospirillum rubrum* *Rhodobacter sphaeroides*
	chemolithoautotrophic bacteria	Nitrifiers, sulfur oxidizers, Hydrogen and carboxydobacteria Iron oxidizer *Thiobacillus ferrooxidans* *Curpriavidus necator*
Reductive citrate cycle	Green sulfur bacteria	*Chlorobium limicola* *Chlorobium thiosulfatophilum*
	Thermophilic hydrogen bacteria	*Hydrogenobacter thermophilus*
	Few sulfate-reducing bacteria	*Desulfobacter hydrogenophilus* *Aquifex pyrophilus*
Reductive acetyl-CoA pathway	Homoacetogenic fermenters	*Clostridium thermoaceticum* *Acetobacterium woodii* *Sporomusa* sp.
	Most sulfate-reducing bacteria	*Desulfobacterium autotrophicum* *Desulfovibrio baarsii* *Archeoglobus lithotrophicus*
	Methanogens	*Methanobacterium thermoautotrophicum* *Methanosarcina barkeri* *Methanococcus jannaschii*
	Denitrifier	*Ferroglobus placidus*
	Euryarchaeota, Planctomycetes, Spirochaetes	
3-Hydroxypropionate-/4-hydroxybutyrate cycle	*Sulfolobales,* marine *Crenarchaeota* Group I	
		Sulfolobus metallicus
Dicarboxylate-/4-hydroxybutyrate cycle	*Desulfurococcales, Thermoproteales*	*Pyrolobus fumarii, Thermoproteus neutrophilus*
3-Hydroxypropionate bi-cycle	Green non-sulfur bacteria	*aurantia chloroflexus*

with high temperature generally have a lower oxygen concentration than those with lower.

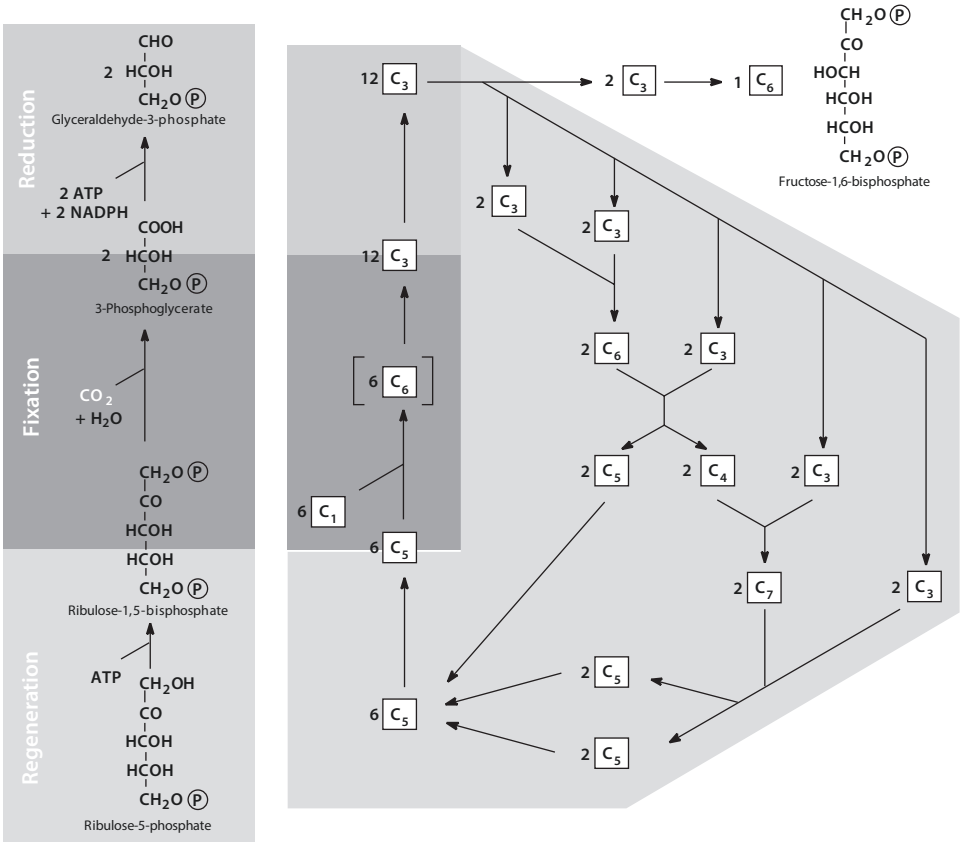
It should be noted that, to date, the reductive acetyl-CoA pathway is the only CO_2 fixation pathway found in both bacteria and archaea.

4.3.1 Calvin Cycle

The **Calvin cycle (=reductive pentose phosphate cycle)** (◘ Fig. 4.4), first discovered in green algae, is present in many photolithotrophic and chemolithotrophic bacteria, where it serves as the main mechanism of carbon assimilation. The CBB cycle operates in plants, algae, *cyanobacteria,* and many aerobic or facultative aerobic *Proteobacteria* which belong to the *alpha, beta*, and *gamma* subgroups. It has also been found in *Sulfobacillus* spp, iron- and sulfur-oxidizing members of the *Firmicutes,* some *Mycobacteria,* and green non-sulfur bacteria of the genus *Oscillochloris* (phylum *Chloroflexi*).

The success of the CBB cycle is probably explained by the enormous robustness of its enzymes to molecular oxygen.

The cycle can be divided into three phases, the fixation, reduction and regeneration phases. It requires NAD(P)H and ATP as well as the two key enzymes, **ribulose**



◘ **Fig. 4.4** The Calvin cycle. To make clear the formation of a C_6-body from 6 CO_2-molecules, 6 molecules of ribulose-1,5-bisphosphate are used in the scheme, which with 6 CO_2-molecules give 12 C_3-bodies. From this, one C_6-body, fructose-1,6-bisphosphate, is formed, while the remaining 10 C_3-bodies are converted back into 6 C-bodies$_5$

bisphosphate carboxylase (RubisCO for short) and **phosphoribulose kinase**.

RubisCO is the most abundant protein in the world and can account for up to 50% of the total soluble protein in chloroplasts of C_3 plants or in bacteria using this cycle. This fact is consequence of the known catalytic inefficiency of the RubisCO: low affinity to the CO_2, low catalytic turnover rate and a devastating oxygenase side reaction responsible for photorespiration, so that in a senseless cleavage of the substrate the toxic side product phosphoglycolate is formed.

Thus, the first step in the Calvin cycle is catalyzed by **ribulose bisphosphate carboxylase**. CO_2 is bound to ribulose-1,5-bisphosphate. The product of this reaction spontaneously decomposes to two molecules of 3-phosphoglycerate. In a reaction that runs in the opposite direction to that of glycolysis (see ► Sect. 4.4.1.2), 3-phosphoglycerate is reduced by the provided reducing equivalents, NAD(P)H, with water splitting and ATP consumption, producing glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is a key intermediate in glycolysis. From there, glucose can be formed by reversing the first steps of glycolysis by first converting two molecules of triose phosphate to fructose-1,6-bisphosphate.

CO_2 fixation is essentially completed with the formation of triose phosphate; what remains is the regeneration of the C_5 acceptor molecule for the fixation of CO_2. In a sequence of conversion reactions, in each of which phosphorylated sugars are involved or appear as intermediates (for example, also C_4 and C_7), 6 ribulose 5-phosphate (C_5) are synthesized from 10 triose phosphate (C_3). Finally, a final phosphorylation step yields the ribulose-1,5-bisphosphate again. Phosphoribulose kinase is another enzyme that occurs only in the Calvin cycle.

A total of 12 NADPH are required for the reduction of 6 CO_2 molecules. 18 ATP are consumed, 6 for the phosphorylation of the 6 ribulose-5-phosphate molecules and 12 during the reduction phase, the generation of the 12 glyceraldehyde-3-phosphates.

The balance for the formation of a sugar is:

$$6\,CO_2 + 18\,ATP + 12\,NAD(P)H \rightarrow \text{Hexose P} + 18\,ADP + 12\,NAD(P)^+ + 17\,P_i$$

P_i = inorganic phosphate.

Carbon dioxide fixation via the ribulose bisphosphate cycle is the most important reaction sequence in the biosphere today for the synthesis of organic matter from CO_2. However, it is not the only form. Autotrophic bacteria and archaea have other mechanisms of CO_2 assimilation.

4.3.2 Reductive Citrate Cycle

Alternative mechanisms for autotrophic CO_2 fixation are found in the green sulfur bacteria—*Chlorobiaceae*—and the green non-sulfur bacteria. In *Chlorobium limicola,* CO_2 fixation occurs by reversing steps of the citrate cycle (see ► Sect. 4.4.1.3), a pathway termed the **reductive** or **reverse citrate cycle** (◘ Fig. 4.5) *Chlorobium* contains two enzymes coupled to ferredoxin that catalyze reductive fixation of CO_2. These are the carboxylation of succinyl-CoA to 2-oxoglutarate and the carboxylation of acetyl-CoA to pyruvate. A further reductive carboxylation of 2-oxoglutarate takes place.

Most of the reactions of the reverse citrate cycle are catalyzed by enzymes that also work against the normal oxidative direction of the cycle. However, the

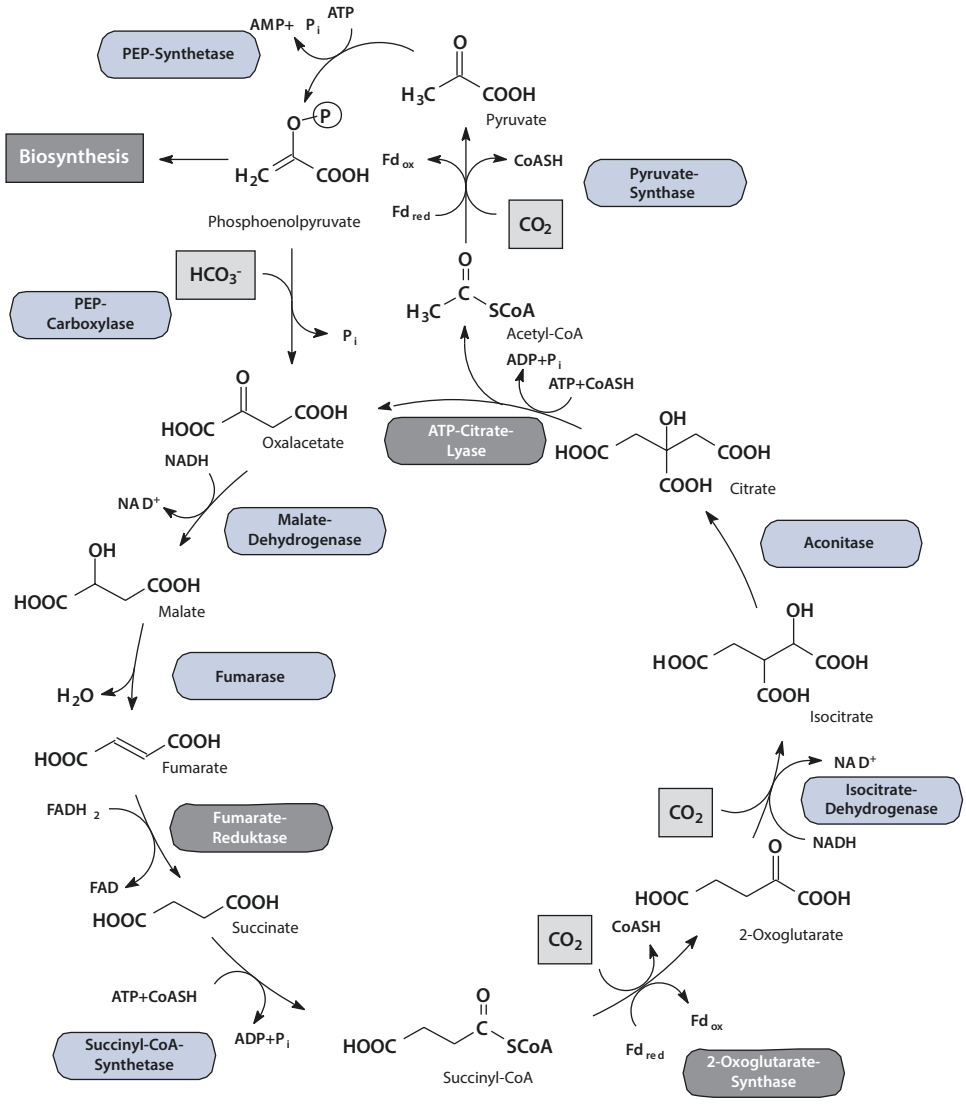


Fig. 4.5 The reductive or retrograde citrate cycle. The particular enzymes are indicated in dark grey They were studied in *Hydrogenobacter thermophilus*

Chlorobiaceae have replaced succinate dehydrogenase with a **fumarate reductase**, exchanged a **2-oxoglutarate dehydrogenase-ferredoxin oxidoreductase (2-oxoglutarate synthase)** for the normal 2-oxoglutarate dehydrogenase complex, and exchanged the irreversible citrate synthase with an **ATP-citrate lyase.**

Since its discovery in the *Chlorobiaceae,* this reductive tricarboxylic acid cycle has also been found in the *delta* subphylum of *Proteobacteria—Desulfobacter hydrogenophilus* -, in microaerophilic members *Aquifex* and *Hydrogenobacter thermophilus* of phylum *Aquificae,* an autotroph branching very early from the phylogenetic tree of bacteria, and in *anaerobic* members of the *Crenarchaeota—Sulfolobus.*

The cycle has been studied most intensively in the thermophilic aerobic, hydrogen-

oxidizing bacterium *Hydrogenobacter thermophilus (Aquificae)*. The actually anaerobic fixation cycle can take place there in the presence of oxygen, since the solubility of oxygen is strongly reduced at the optimal growth temperature of *Hydrogenobacter* (70 °C) (microaerophilic conditions).

4.3.3 Reductive Acetyl-CoA Pathway (Acetogenesis)

This fixation pathway is preferred by prokaryotes that live close to the thermodynamic limit such as acetogenic bacteria and methanogenic archaea (◘ Fig. 4.6).

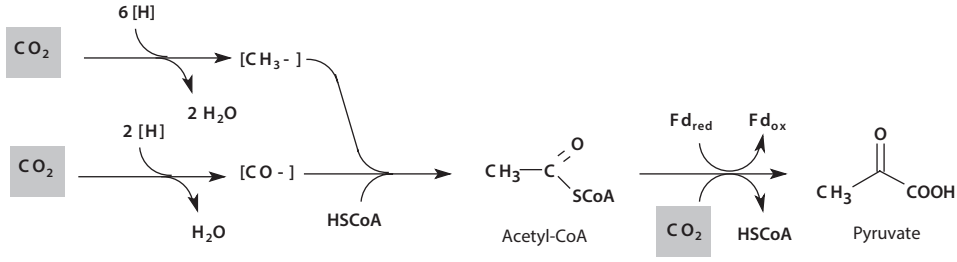
By chemolithotrophic methanogens, sulfidogens and homoacetogens using hydrogen or carbon monoxide as H donor, the reductive acetyl-CoA pathway is used as CO_2 fixation pathway under anaerobic conditions. In this process, 2 CO_2 are converted to acetyl-CoA. The metabolic pathway is largely identical to acetate synthesis from 2 CO_2, as catalyzed in the energy metabolism of homoacetogenic bacteria.

Unlike other autotrophic pathways such as the Calvin cycle or the reverse citrate cycle, the acetyl-CoA pathway of CO_2 fixation is not a cycle. Instead, in it, the reduction of CO_2 occurs in two linear pathways—one molecule of CO_2 is reduced to the carbonyl group, and the other CO_2 molecule is reduced to the methyl group of acetate—whereupon they are combined at the end to form acetyl-CoA. A key enzyme in the acetyl-CoA pathway is carbon monoxide (CO) dehydrogenase. The CO produced forms the CO group of the carboxyl function of acetate. The methyl group of acetate comes from the reduction of CO_2 by a series of reactions involving the coenzyme tetrahydrofolate. The methyl group that forms is transferred from the tetrahydrofolate to an enzyme that contains vitamin B12 as a cofactor. In the final step of the pathway, the CH_3 group is joined to CO in CO dehydrogenase to form acetate. The reaction mechanism requires the CH_3 group bonded to a nickel atom of the enzyme to combine with CO bonded to an Fe atom of the enzyme, along with coenzyme A, to form the final product acetyl-CoA.

The energy from acetyl-CoA can be conserved as ATP in catabolism or used for biosyntheses.

The acetyl-CoA is reductively carboxylated to pyruvate, a reaction that requires an electron donor with a negative redox potential (for example, ferredoxin ox/red, $E^{0'} = -390$ mV). Pyruvate cannot be directly converted to PEP for thermodynamic reasons. PEP synthesis costs 2 ATP. Starting from PEP, triose phosphate is then formed via a reversal of glycolysis.

The acetogenic pathway is responsible for much of the CO_2 fixation occurring in anoxic habitats.



◘ **Fig. 4.6** Schematic representation of the reductive acetyl-CoA pathway

4.3.4 CO_2-Fixation Cycle in *Crenarchaeota*

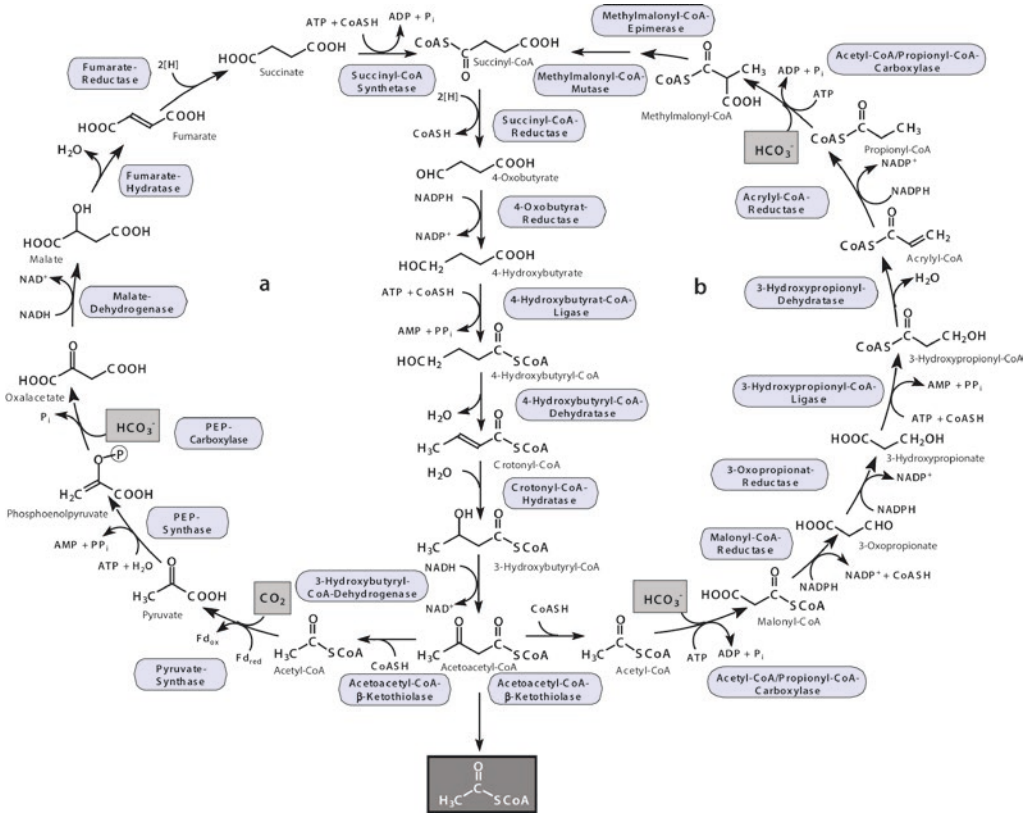
In *Crenarchaeota* there are two different autotrophic carbon fixation cycles. One is found in anaerobic organisms, the other in aerobic organisms. The two cycles differ in how they are coupled to central metabolism. However, they have the identical section of conversion of succinyl-CoA to acetyl-CoA.

4.3.4.1 Dicarboxylate/4-Hydroxybutyrate Cycle

The dicarboxylate/4-hydroxybutyrate cycle functions in the **anaerobic** autotrophic members of the *Desulfurococcales* and *Thermoproteales,* but also in *Pyrolobus fumarii (Desulfurococcales)*, which has adapted to facultative, aerobic energy metabolism with low oxygen partial pressure.

The cycle can be divided into two parts (◘ Fig. 4.7a). In the first part, acetyl-CoA, a CO_2 and a bicarbonate are converted to succinyl-CoA via C_4-dicarboxylic acids. In the second part, succinyl-CoA is transfected into two molecules of acetyl-CoA via 4-hydroxybutyrate. One acetyl-CoA can be used for biosynthesis, while the other serves as a CO_2 acceptor in the next round of the cycle.

The cycle begins with the reductive carboxylation of acetyl-CoA to pyruvate and is catalyzed by pyruvate synthase, an oxygen-sensitive enzyme that requires ferredoxin as an electron donor. This explains why these



◘ **Fig. 4.7** **a** Dicarboxylate/4-hydroxybutyrate cycle. Anaerobic metabolism. The enzymes have been studied in *Thermoproteus neutrophilus*. **b** 3-Hydroxypropionate-/4-hydroxybutyrate cycle. Aerobic metabolism. The enzymes have been studied in *Metallosphaera sedula*

reactions are restricted to strict anaerobes or at most microaerobes.

Pyruvate is converted to phosphoenolpyruvate (PEP), followed by carboxylation of PEP to oxaloacetate catalyzed by an Archaea PEP carboxylase. The link to central carbon metabolism is established with these metabolites. Subsequent oxaloacetate reduction involves an incomplete reductive citrate cycle that stops at succinyl-CoA.

Succinyl-CoA is further reduced to 4-oxobutyrate and then to 4-hydroxybutyrate. The compound is ultimately cleaved into two acetyl-CoA molecules, which requires the key enzyme 4-hydroxybutyryl-CoA dehydratase. This is a [4Fe-4S] cluster- and FAD-containing enzyme that accomplishes the elimination of water from 4-hydroxybutyryl-CoA. The product, crotonyl-CoA, is converted into two molecules of acetyl-CoA via normal β-oxidation reactions.

4.3.4.2 3-Hydroxypropionate/4-Hydroxybutyrate Cycle

This cycle runs in aerobic autotrophic *Crenarchaeota of* the order *Sulfolobales—Metallosphaera sedula*—including the strictly anaerobic *Stygiolobus azoricus,* which has reverted to an anaerobic lifestyle. This group includes extreme thermoacidophiles from volcanic areas that grow best at pH ~2 and a temperature of 60–90 °C.

The enzymes of the cycle are oxygen tolerant, as is necessary for life with oxygen. One of the key enzymes, 4-hydroxybutyryl-CoA dehydratase is sufficiently insensitive to oxygen in *Sulfolobales* to function under microoxic or even oxic conditions. In the mesophilic marine Group I *Crenarchaeota,* such as *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus, as* well as related, very common marine *Archaea* as well as corresponding bottom species, a similar cycle is probably present.

In the cycle, one molecule of acetyl-CoA is formed from two molecules of bicarbonate (◘ Fig. 4.7b). The carboxylating enzyme is a bifunctional biotin-dependent acetyl-CoA/propionyl-CoA carboxylase.

The cycle can be divided into two sections. The first section converts acetyl-CoA and two bicarbonate molecules into succinyl-CoA via 3-hydroxypropionate, as will be described later for the 3-hydroxypropionate bi-cycle. However, the enzymes for the synthesis of propionyl-CoA from malonyl-CoA are not homologous, although the intermediates are identical. The second section is apparently common in autotrophic *Crenarchaeota,* since it also occurs in the two orders *Desulfurococcales* and *Thermoproteales,* which use the dicarboxylate/4-hydroxybutyrate cycle for autotrophic carbon fixation. It in turn converts succinyl-CoA to two molecules of acetyl-CoA via 4-oxobutyrate, 4-hydroxybutyrate, 4-hydroxybutyryl-CoA, crotonyl-CoA, 3-hydroxyacetyl-CoA, and acetoacetyl-CoA. Thus, rotation of the cycle leads to regeneration of the CO_2 acceptor and forms an additional molecule of acetyl-CoA from two molecules of bicarbonate.

Acetyl-CoA and succinyl-CoA are possible intermediates for central carbon metabolism. Succinyl-CoA has been shown to serve as the main precursor for cellular carbon. Acetyl-CoA plus another two bicarbonate molecules are therefore converted to succinyl-CoA by another half turn of the cycle. Succinyl-CoA is then transformed to pyruvate via oxidative decarboxylation of malate by Malic enzyme: That is, a total of **one and a half** turns of the cycle are required to form this succinyl-CoA from four molecules of bicarbonate.

Thus, this strategy of pyruvate formation differs from that of the anaerobic pathways, in which acetyl-CoA is reductively carboxylated with ferredoxin to pyruvate.

4.3.5 3-Hydroxypropionate Bi-cycle

The photrophic green non-sulfur bacterium *Chloroflexus aurantiacus* and other related *Chloroflexi* use the 3-hydroxypropionate biocycle for autotrophic CO_2 fixation. The cycle starts with acetyl-CoA. Normal ATP- and biotin-dependent acetyl-CoA and propionyl-CoA carboxylases function as carboxylation enzymes.

Each circulation of the first cycle yields one molecule of glyoxylate with a fixation of two molecules of bicarbonate (◘ Fig. 4.8). Acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase. The reduction of malonyl-CoA to propionyl-CoA is catalyzed solely by two enzymes, bifunctional malonyl-CoA reductase and trifunctional propionyl-CoA synthase. Propionyl-CoA is carboxylated to methylmalonyl-CoA, followed by isomerization to succinyl-CoA. These reactions are used for propionate assimilation in many organisms.

Note that these steps are identical to those from the first part of the 3-hydroxypropionate/4-hydroxybutyrate cycle. Succinyl-CoA is needed for the activation of *(S)*-malate by CoA transfer, which generates succinate and *(S)*-malyl-CoA. The succinate, on the other hand, is oxidized to *(S)*-malate. In the final step, *(S)*-malyl-CoA is cleaved into acetyl-CoA and glyoxylate. Acetyl-CoA then serves again as a starter molecule for another round. Since glyoxylate is not a direct precursor for the formation of cellular building blocks, it must first be metabolized in round 2 (hence the term bi-cycle). This is done by combining it with propionyl-CoA, the intermediate of round 1, and forming β-methylmalyl-CoA. This condensation is followed by a series of C_5 -conversion reactions to give *(S)*-citramalyl-CoA. *(S)*-Citramalyl-CoA is then cleaved to acetyl-CoA and pyruvate by a trifunctional lyase, which previously cleaved *(S)*-Malyl-CoA and formed β-methylmalyl-CoA.

The confusing disproportionation of glyoxylate and propionyl-CoA to acetyl-CoA and pyruvate is solved without any redox reaction in an elegant and economical way and only three additional enzymes are needed. The entire bi-cyclic cycle results in the formation of pyruvate from three molecules of bicarbonate. 19 reaction steps are carried out by only 13 enzymes.

4.3.6 Comparison of the CO_2-Fixation Processes

Thermodynamically, more energy is required for the reduction of CO_2 to organic carbon in aerobic, oxidized environments compared to those in anaerobic, reduced habitats.

In addition to thermodynamic reasons, there are also biochemical ones that explain why aerobic chemolithoautotrophs mainly use the oxygen-tolerant but energy-consuming CBB cycle. In anaerobic, usually energy-limited chemolithoautotrophs, the more energy-efficient but oxygen-sensitive carbon fixation pathways are in use.

The anaerobic processes operate considerably more economically than the aerobic ones: for example, the CO_2 -fixation pathways used by the anaerobic or microaerophilic microorganisms (reductive acetyl-CoA pathway, rTCA cycle, DC/4-HB cycle) require 1–5 ATP for the synthesis of 1 mol of pyruvate from 3 mol of CO_2. The pathways that function under completely aerobic conditions (CBB cycle, 3-HP Bi cycle, 3-HP/4-HB cycle) require 7–9 ATP. This also makes sense, since anaerobes have much less energy available due to the absence of the respiratory chain. So aerobic fixation pathways usually require more ATP equivalents than anaerobic ones. However, this

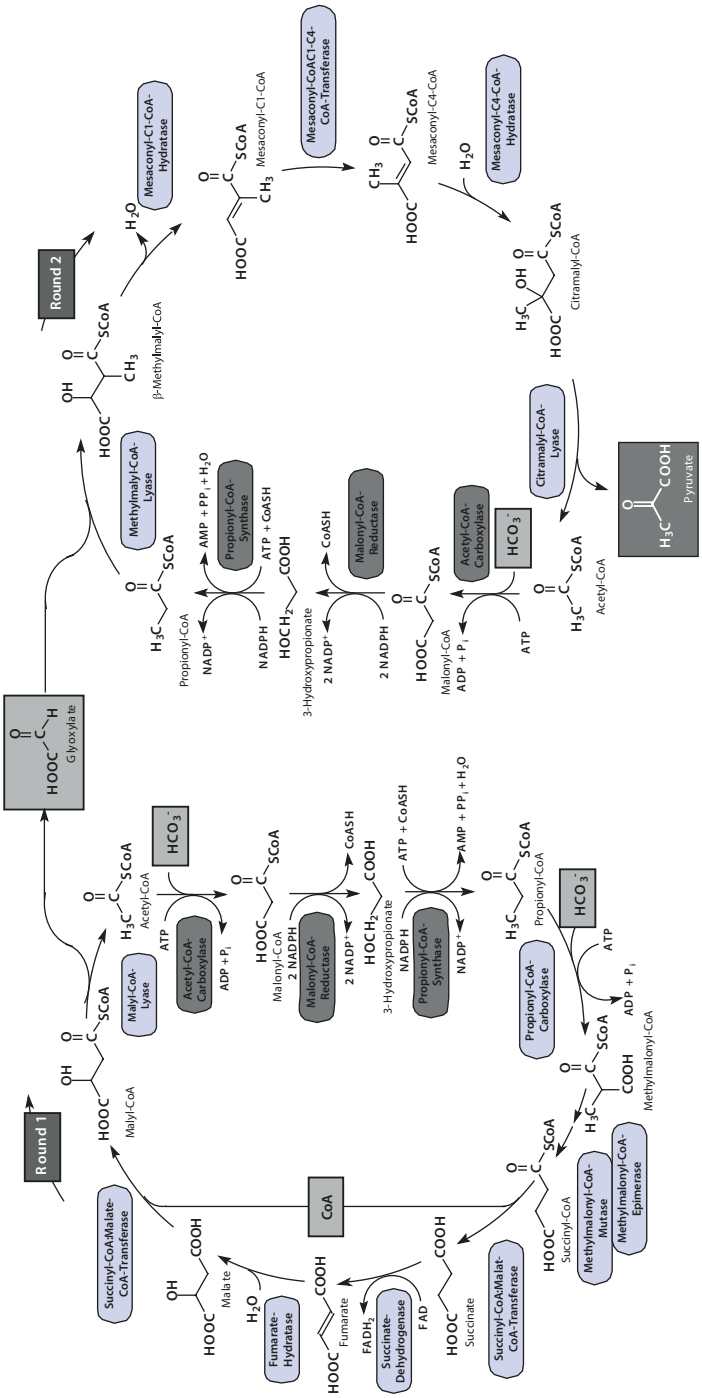


Fig. 4.8 3-Hydroxypropionate bi-cycle in *Chloroflexus aurantiacus*

Table 4.3 Comparison of energy input for autotrophic CO_2 -ixation

Path of CO_2-fixation	Amount of ATP	Reducing agent quantity	Behaviour towards oxygen
	For the synthesis of 1 pyruvate		
Calvin cycle	7	5 NAD(P)H	Aerobic
Reductive citrate cycle	2–3	2 NAD(P)H, 2 ferredoxin, 1 NADH or unknown electron donor for fumarate reductase	Anaerobic/ microaerobic
Reductive acetyl-CoA pathway	~1	NAD(P)H, Ferredoxin, $F_{420}H_2$	Strictly anaerobic
Dicarboxylate-/4-hydroxybutyrate cycle	5	1–2 NAD(P)H, 2–3 ferredoxin, unknown electron donor for fumarate reductase	Anaerobic/ microaerobic
3-Hydroxypropionate-/4-hydroxybutyrate cycle	9	6 NAD(P)H	Aerobic
3-Hydroxypropionate Bi-cycle	7	6 NAD(P)H	Aerobic

requires ferredoxin as a reducing agent in anaerobes. Reduced ferredoxin contains more energy than NADPH (Table 4.3).

4.4 Degradation of Natural Substances

Natural substances occur in nature largely as polymers. In this form, they are too large to be easily absorbed through the cell membrane. In addition, prokaryotes are not capable of phagocytosis.

The macromolecules are broken down into smaller building blocks outside the cell by **exoenzymes** so that the latter can then be taken up into the cell (Fig. 4.9). In bacteria, these are usually hydrolytic enzymes; in fungi, they are sometimes oxidases.

After uptake, the monomers formed enter various areas of the intermediary metabolism and are mineralized. The central position of pyruvate and especially acetyl-CoA as well as the citrate cycle for the metabolism becomes clear in Fig. 4.9.

4.4.1 Degradation of Carbohydrates

In the last chapter it was made clear that in microbial metabolism energy from reduction-oxidation reactions is conserved in the form of ATP or a proton gradient for various cell functions. The reactions that take place here will first be discussed in more detail using the example of aerobic carbohydrate metabolism.

Firstly, carbohydrate metabolism is of outstanding importance in purely quantitative terms, since cellulose in the cell walls of green plants and other polymeric carbohydrates (hemicelluloses, chitin, starch) represent the largest supply of organically bound carbon. Second, the pathways involved in carbohydrate metabolism are consequently widely distributed from many bacteria to humans. Thirdly, in many organisms, carbohydrate metabolism represents, so to speak, the centre of metabolism, into which many other degradation pathways flow and from which the building blocks of biomass, such

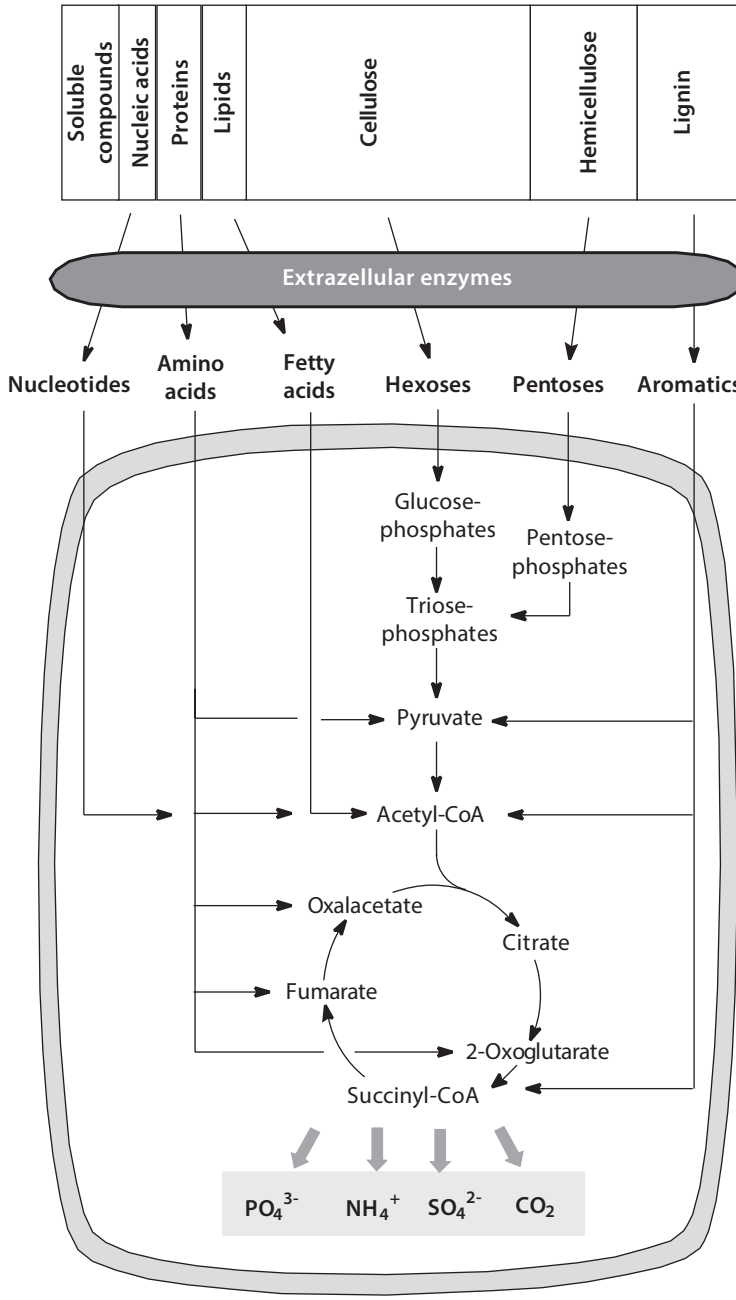


Fig. 4.9 Degradation of plant biomass to minerals

as amino acids, lipids or DNA building blocks, can be synthesized (Fig. 4.9).

4.4.1.1 Glycolysis

The glucose already present or formed from starch or cellulose is converted in several steps, the first of which is the formation of C_3 bodies from the C_6 body. A biochemical pathway for the degradation of glucose that is particularly widespread in both aerobic and anaerobic organisms is **glycolysis**, also known as the **Embden-Meyerhof pathway** after the most important discoverers, or the **fruc-**

tose-1,6-bisphosphate pathway after the characteristic intermediate.

Glycolysis includes those reactions that convert glucose into two molecules of pyruvate (pyruvic acid). The reduction equivalents resulting from this oxidation are transferred to NAD^+ so that 2 molecules of NADH are formed per glucose molecule. For anaerobic microorganisms in particular, it is of crucial importance that part of the energy released during this oxidation process can be stored in the form of 2 molecules of ATP per molecule of glucose. For glycolysis, the sum reaction equation is:

$$C_6H_{12}O_6 + 2\,NAD^+ + 2\,ADP + 2\,P_i \rightarrow 2\,C_3H_4O_3 + 2\,NADH + 2\,ATP + 2\,H_2O$$

The ATP formed during glycolysis is the result of **substrate step phosphorylation**. How it is possible to conserve energy in the form of ATP in the course of chemical transformations was discussed in ▶ Chap. 3. In order to understand the reactions within glycolysis, it is useful to divide them into two major sections (◘ Fig. 4.10).

In **section I, activation,** biochemical energy is first used, i.e. ATP is consumed, and no oxidation takes place yet (◘ Fig. 4.10). Glucose is phosphorylated with ATP to form glucose-6-phosphate, which is converted to an isomeric form, fructose-6-phosphate. A second phosphorylation leads to the formation of fructose-1,6-bisphosphate, a major intermediate of glycolysis characterized by two repulsive, negatively charged phosphate groups. An aldolase catalyzes the cleavage of fructose-1,6-bisphosphate into two C_3 molecules: Dihydroxyacetone phosphate and its isomer glyceraldehyde-3-phosphate. Since both can be converted into each other and only the latter is further converted in glycolysis, two molecules of glyceraldehyde-3-phosphate can be regarded as the result of section I of glycolysis.

In **section II** of glycolysis, an **oxidation reaction** occurs and is accompanied by **energy production through substrate step phosphorylation.** The oxidation reaction occurs during the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Glyceraldehyde-3-phosphate is oxidized from the aldehyde step to the acid step with the formation of NADH, and inorganic phosphate is incorporated into the molecule to form 1,3-bisphosphoglycerate. Due to the **mixed acid-anhydride bond,** this intermediate is sufficiently energetic that the phosphate group can be transferred to ADP in the next step to form ATP. Isomerization of the formed 3-phosphoglycerate to 2-phosphoglycerate and elimination of water gives phosphoenolpyruvate (phosphoric ester of the enol form of pyruvate). Since phosphoenolpyruvate contains an energy-rich bond (phosphoric acid ester) to phosphate, this phosphate residue can also be transferred to ADP to form ATP and pyruvate.

Group Translocation

In eubacteria, glucose, fructose, mannose and other carbohydrates are taken up into the cells by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). A cascade of protein kinases is involved in group translocation, a chemical modification during transport (◘ Fig. 4.11).

The phosphate group is not transferred directly by PEP, but a sequential phosphate transfer takes place. In this process, the proteins of the phosphotransferase system are alternately phosphorylated and dephosphorylated in a cascade before the phosphate group reaches enzyme IIc. Enzyme IIc is an integral membrane protein that forms the channel

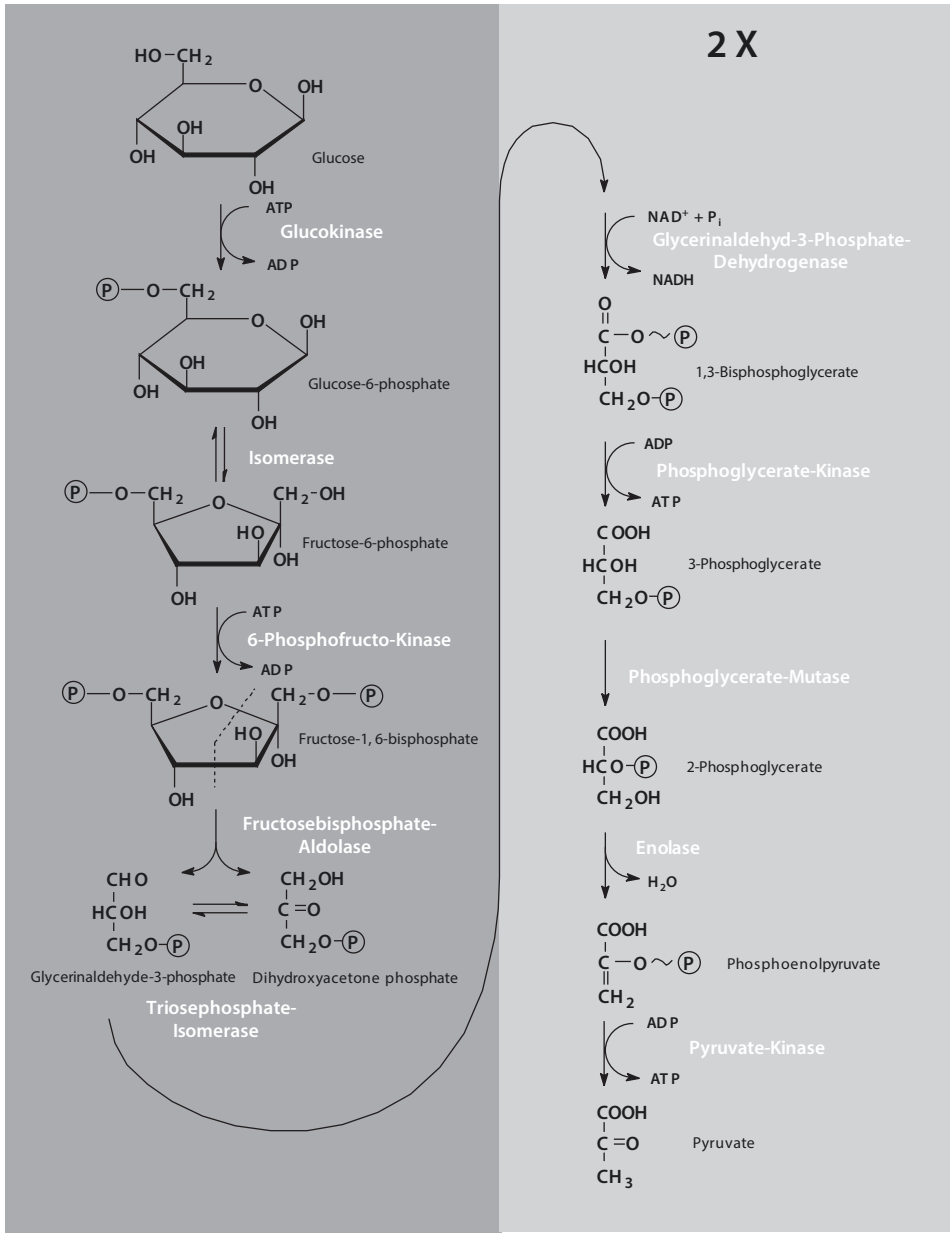


Fig. 4.10 Glycolysis

and catalyzes the phosphorylation of the sugar.

Enzymes II are specific for each sugar, whereas the cytoplasmic enzymes enzyme I (E_I) and histidine protein (HPr) are involved in the transport of all sugars entering the cell through the PTS.

Thus, group translocation prepares glucose for entry into central metabolism.

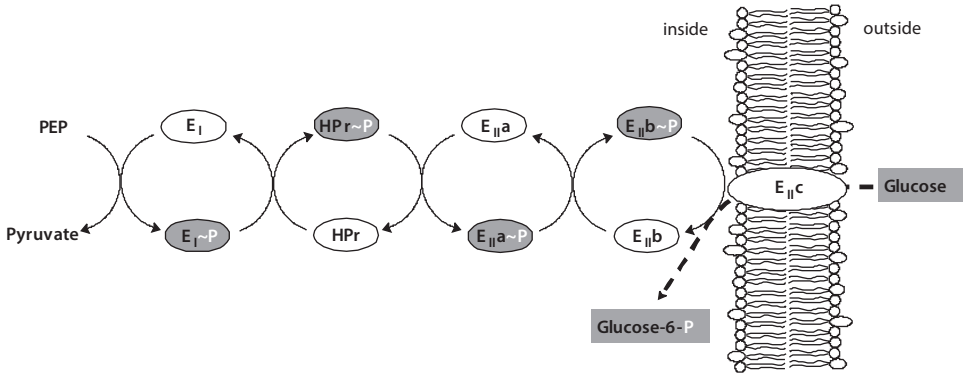


Fig. 4.11 Transport of glucose by phosphoenolpyruvate: glucose phosphotransferase system (PTS). HPr = histidine protein, PEP = phosphoenolpyruvate

Since, as mentioned above, two molecules of glyceraldehyde-3-phosphate are formed from glucose, the reaction chain from glyceraldehyde-3-phosphate to pyruvate occurs twice in terms of the balance. In this reaction chain, ATP is synthesized at two points, in the reaction of 1,3-bisphosphoglycerate to 3-phosphoglycerate and in the reaction of phosphoenolpyruvate to pyruvate. Thus, in total, four ATP molecules are gained and two are consumed per molecule of glucose in glycolysis. Thus, the organism gains a total of two molecules of ATP per glucose molecule.

4.4.1.2 Oxidative Pyruvate Decarboxylation and Tricarboxylic Acid Cycle

The pyruvate formed by glycolysis can be fed to very different reactions in different organisms depending on their physiological state. With regard to the energy production of aerobic organisms, the most important reaction of pyruvate is the oxidative decarboxylation by the pyruvate dehydrogenase complex. In this process, the resulting acetyl residue is transferred to a thiol group of coenzyme A (abbreviated HSCoA):

$$C_3H_4O_3 + HSCoA + NAD^+ \rightarrow CH_3 - COSCoA + CO_2 + NADH$$

Several enzymes and coenzymes are involved in the pyruvate dehydrogenase complex. As a result, one molecule of CO_2 is formed per molecule of pyruvate, a reduction equivalent is provided in the form of NADH for the respiratory chain and the remaining C_2 body is activated by a thioester bond so that it can be introduced into the tricarboxylic acid cycle or used elsewhere in the cell.

The acetyl-CoA formed by the pyruvate dehydrogenase complex is then completely oxidized to CO_2 in a cyclic metabolic pathway, the **tricarboxylic acid cycle** (also known as the **citrate cycle**) (Fig. 4.12). The reduction equivalents produced in this process are also transferred to electron intermediates, the greater part to NAD^+ and the smaller part to FAD, producing the respective reduced forms (NADH and $FADH_2$).

The tricarboxylic acid cycle is initiated by the addition of the acetyl residue (C_2-body) of acetyl-CoA to the C_4-body oxaloacetate, forming the C_6-body citrate. After

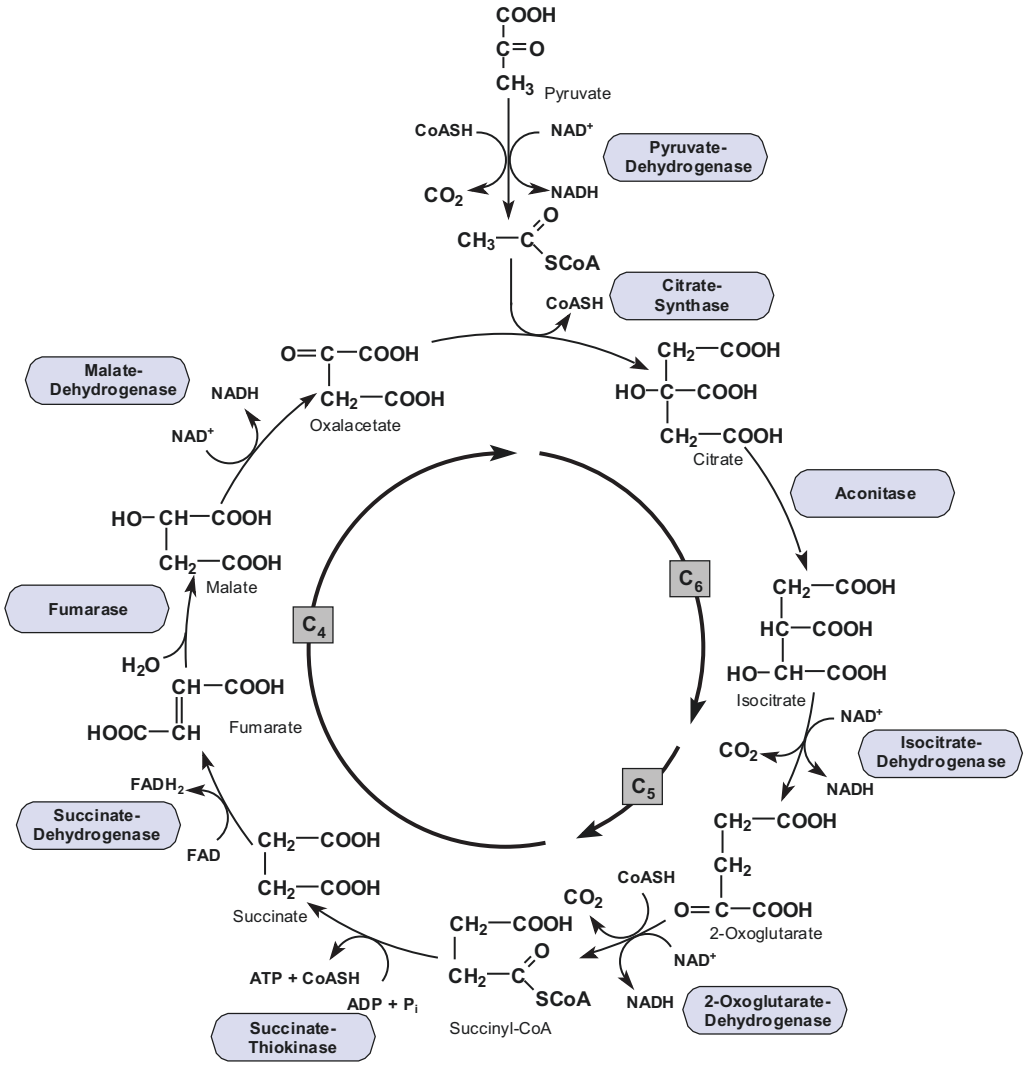


Fig. 4.12 Tricarboxylic acid cycle (also citrate cycle)

isomerization of citrate to isocitrate, oxidation yields a β-keto acid , which decarboxylates to form the C_5-body 2-oxoglutarate. This too undergoes oxidative decarboxylation in a reaction analogous to oxidative pyruvate decarboxylation, yielding succinyl-CoA in addition to CO_2. This (like acetyl-CoA) has an energy-rich thioester bond, which in this case is used to obtain ATP or GTP via substrate step phosphorylation. In the further course of the tricarboxylic acid cycle, the resulting, still relatively reduced C_4-body succinate is oxidized again to oxaloacetate via fumarate and malate. Note that because of the reduction potential of the fumarate/succinate pair, electrons from succinate cannot be transferred to NAD^+, but can be transferred directly to an FAD, which has a more positive reduction potential. If we assume for simplicity that the substrate step phosphorylation of the tricarboxylic acid cycle yields ATP, the balance of the tricarboxylic acid cycle can be summarized by the following reaction equation:

$$CH_3 - COSCoA + 3\,NAD^+ + FAD + ADP + P_i + 2\,H_2O \rightarrow 2\,CO_2 + HSCoA + 3\,NADH + FADH_2 + ATP$$

Glycolysis, oxidative pyruvate decarboxylation and the tricarboxylic acid cycle are not only important for the aerobic carbohydrate metabolism described here. Rather, degradation pathways of many natural products, such as proteins, lipids, and aromatics, feed into these pathways. At the same time, the metabolites of the metabolic pathways considered here are starting points for many reactions for the synthesis of microbial biomass.

Why Are Cyanobacteria Strictly Autotrophic?

According to an accepted hypothesis, cyanobacteria are strictly autotrophic because they do not have a complete citrate cycle. They lack the enzyme 2-oxoglutarate dehydrogenase. Therefore, it was previously believed that the complete oxidation of the storage substance glycogen to carbon dioxide is not possible in cyanobacteria.

As oxygenic phototrophs, cyanobacteria use light energy for ATP synthesis and NADPH formation with the help of two photosystems. Additional electron or energy sources are not necessary. CO_2 is the carbon source. Only limited organic compounds can be oxidized and assimilated. Glycogen, for example, is oxidized during the night.

Today, it has been demonstrated that most cyanobacteria do possess a closed but modified citrate cycle: 2-oxoglutarate decarboxylase and succinate semialdehyde dehydrogenase catalyze the conversion of 2-oxoglutarate to succinate.

Mutants lacking these enzymes are significantly slower than wild-type cells in their growth under strictly phototrophic conditions, which do not require a complete citrate cycle, under both continuous light and light-dark cycles.

So the question remains: why do cyanobacteria need the cycle in the light?

4.4.1.3 Balance of Aerobic Respiration and Energy Storage

If we summarize the balance equations for the partial steps of glucose degradation and take into account that the oxidative pyruvate decarboxylation and the tricarboxylic acid cycle must occur twice per glucose molecule, we obtain the following reaction equation:

$$C_6H_{12}O_6 + 10\,NAD^+ + 2\,FAD + 4\,ADP + 4\,P_i + 2\,H_2O \rightarrow 6\,CO_2 + 10\,NADH + 2\,FADH_2 + 4\,ATP$$

Glucose was thus completely oxidized to CO_2 and the reduction equivalents were transferred to intermediate hydrogen carriers. At the same time, 4 molecules of ATP were obtained by substrate step phosphorylation (2 each in glycolysis and in the tricarboxylic acid cycle).

Considering the ATP yield from the respiratory chain, oxidation of NADH under aerobic conditions adds up to 30 ATP (aerobically up to 3 ATP per NADH) and oxidation of 2 reduced quinone molecules contributes up to 4 ATP (up to 2 ATP per reduced quinone). In total, the aerobic deg-

radation of one molecule of glucose can provide up to 38 molecules of ATP.

4.4.1.4 Anaerobic Degradation of Carbohydrates

How much ATP can a microorganism make when utilizing glucose that lacks the respiratory chain or does not have the electron final acceptor oxygen or another available? Fermenters produce 2–3 ATPs per mole of glucose when glycolysis is used. This is about the maximum amount of ATP made by fermentation; many other substrates yield less energy. The energy potentially released in a given fermentation can be calculated from the balanced reaction and from the free energy values: For example, fermentation of glucose to ethanol and CO_2 theoretically yields –235 kJ/mol, enough to produce approximately 7 ATPs (–31.8 kJ/mol ATP),. However, only 2 ATPs are actually produced. This makes it clear that a microorganism operates at significantly less than 100% efficiency and that some of the energy is lost as heat.

- **Oxidation-Reduction Equilibrium/ Formation of Fermentation Products**

Why does a fermenter produce a product like ethanol? In the oxidative part of glycolysis, during the formation of two molecules of 1,3-bisphosphoglycerate, two NAD^+ are reduced to NADH. However, a cell contains only a small amount of NAD^+. If all of this were converted to NADH, the oxidation of glucose would come to a halt. The oxidation of glyceraldehyde-3-phosphate can only continue if NAD^+ is present to accept electrons. In fermentation, oxidation of NADH to NAD^+ avoids blockage by reducing pyruvate to a variety of **fermentation products**. In the case of yeast, pyruvate is reduced to ethanol with the release of CO_2. In lactic acid bacteria, pyruvate is converted to lactate. Other means of pyruvate reduction in fermenting prokaryotes are known, but the result is ultimately always the same: **NADH must be converted to the oxidized form NAD^+** in **order for the energy-producing reactions of glucose degradation to continue.** NADH diffuses from glyceraldehyde-3-phosphate dehydrogenase to lactate dehydrogenase, pyruvate is reduced to lactate, to diffuse away after conversion to NAD^+ so that the cycle can start again.

In any energy-producing process, oxidation must be balanced by reduction, and for every electron removed there must be an electron acceptor. In this case, the **reduction of** NAD^+ in one enzymatic step of glycolysis is balanced by its **oxidation** in another step. The end products must also be in oxidation-reduction equilibrium with the starting substrate, glucose. Therefore, the end products explained here, ethanol and CO_2 and lactate and protons, respectively, are in equilibrium with the starting molecule glucose with respect to charge and atomic sums.

In any fermentation reaction, there must be a **balance between oxidation and reduction.** The total number of electrons in the products on the right side of the equation must equal the number of electrons in the substrates on the left side of the equation.

In some fermentation reactions, electron balance is maintained by the production of molecular hydrogen, H_2. In H_2 production, protons derived from water (H^+) serve as electron acceptors. The production of H_2 is generally associated with the presence of an iron sulfur protein, ferredoxin a very electronegative electron transfer agent, in the organism. The transfer of electrons from ferredoxin to H^+ is catalyzed by hydrogenase. The energetics of hydrogen production are rather unfavorable, which is why most fermenting microorganisms produce only a relatively small amount of hydrogen among other fermentation products. Hydrogen production therefore serves mainly to maintain redox equilibrium. For example, if hydrogen production is prevented, the oxidation-reduction balance of the other fermentation products is shifted toward more reduced products. Therefore, many fermenting microorganisms that produce H_2

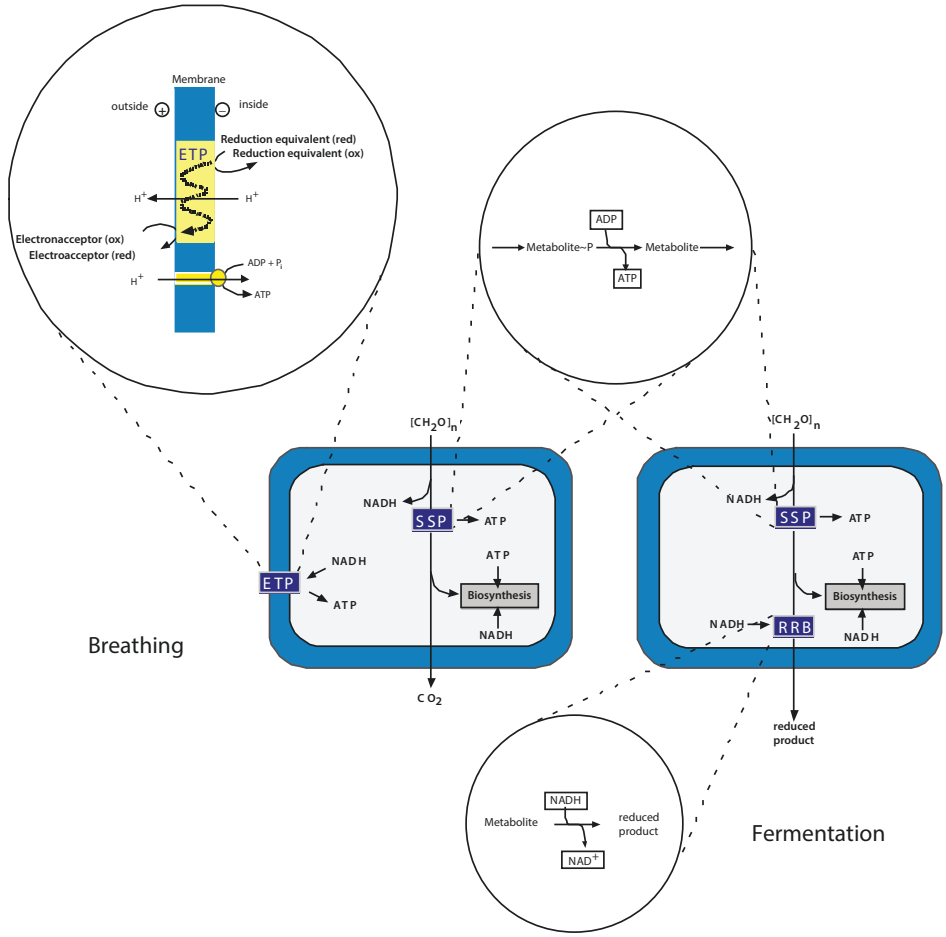


Fig. 4.13 Comparison of respiration and fermentation. *ETK* Electron transport chain, *SSP* Substrate step phosphorylation, *ETP* Electron transport phosphorylation, *RRB* Reduction equivalent regeneration box

produce both ethanol and acetate. Since ethanol is more reduced than acetate, its formation is favored when hydrogen production is inhibited. The production of acetate or certain other fatty acids is energetically advantageous because it allows the microorganism to produce ATP by substrate step phosphorylation. The major intermediate in acetate formation is acetyl-CoA as an energy-rich intermediate. Acetyl-CoA can be converted to acetyl phosphate and the high-energy phosphate group of acetyl phosphate can subsequently be transferred to ADP by acetate kinase to produce ATP (Fig. 4.13).

4.4.2 Degradation of Proteins

Like other high-molecular substances, proteins are initially broken down into permeable fragments outside the cell by exoenzymes. Proteolytic enzymes (exo- and endoproteinases: at the end or within a protein chain) hydrolyse peptide bonds in the protein. The cleavage pieces consisting of polypeptides and oligopeptides are taken up by the cell and degraded to amino acids by peptidases. These are either used directly by the cell for protein synthesis or deaminated, i.e. NH_4^+ is released and the deaminated compound is introduced into the intermediary metabo-

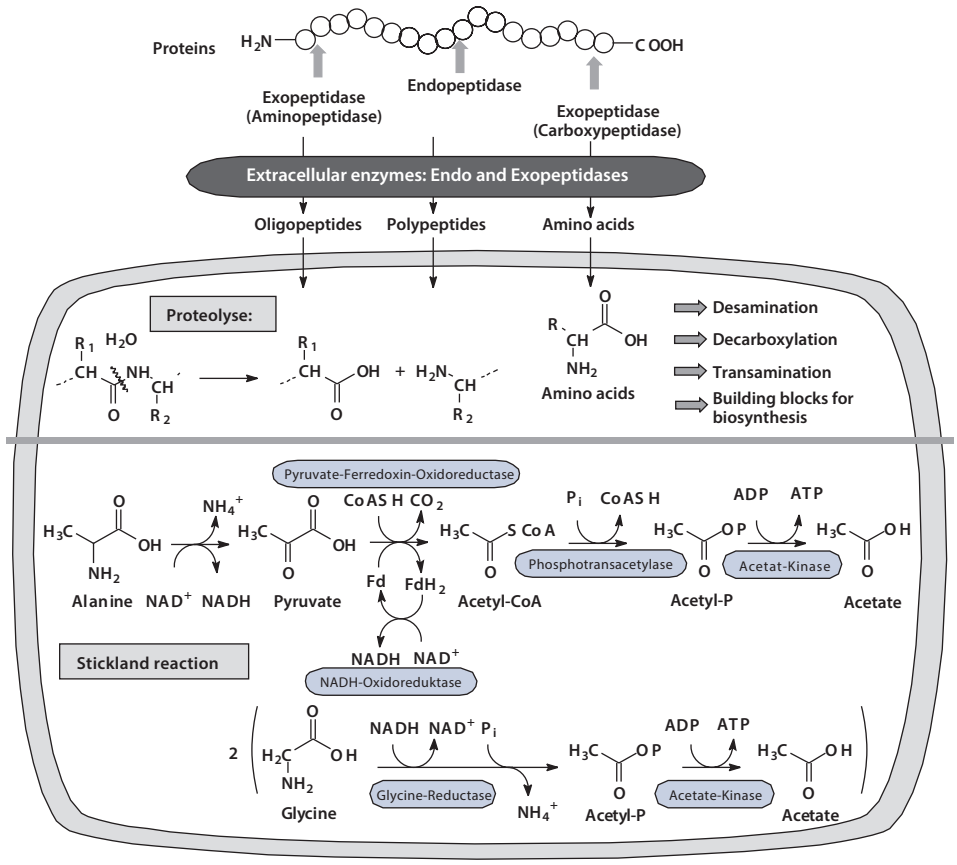
lism. A large number of fungi and bacteria are involved in protein degradation.

Ammonia formation accompanies protein breakdown in the soil, ammonification.

Another degradation step is decarboxylation, which leads to biogenic amines. The primary amines occur during normal intestinal putrefaction and other anaerobic decomposition processes of proteinaceous substances.

Oxidative deamination is the most common type of amino acid degradation. Glutamate is oxidatively deaminated by glutamate dehydrogenase to 2-oxoglutarate, the intermediate of the citrate cycle, and thus fed to the intermediate metabolism.

The proteolytic clostridia hydrolyse proteins and ferment amino acids. However, some amino acids are not converted on their own. Stickland found, however, that a mixture of, for example, alanine and glycine is rapidly fermented by *Clostridium sporogenes,* while the individual amino acids were not converted. Alanine was found to act as an H-donor during fermentation, while glycine fulfilled the role of H-acceptor. In this complex process, energy is obtained by a coupled oxidation-reduction reaction. Alanine, leucine, isoleucine, valine, serine, methionine can act as H-donors, while glycine, proline, arginine, tryptophan can serve as H-acceptors. The donor amino acid is deaminated to a keto acid, which is then oxidized to a fatty acid by oxidative decarboxylation. ◘ Fig. 4.14 shows the so-called Stickland



◘ **Fig. 4.14** Protein degradation outside and inside the cell. top: outside the cell; middle: proteolysis and formation of amino acids as well as the subsequent reactions; bottom: Stickland reaction

fermentation using the example of alanine and glycine.

4.4.3 Degradation of Fats

Lipids are very frequent in nature. As triacylglycerides they are storage substances of fatty seeds and fruits but also of microorganisms. The cytoplasmic membranes of all cells contain phospholipids. Both substances are biodegradable and, being energy-rich, are excellent substrates for microbial energy metabolism.

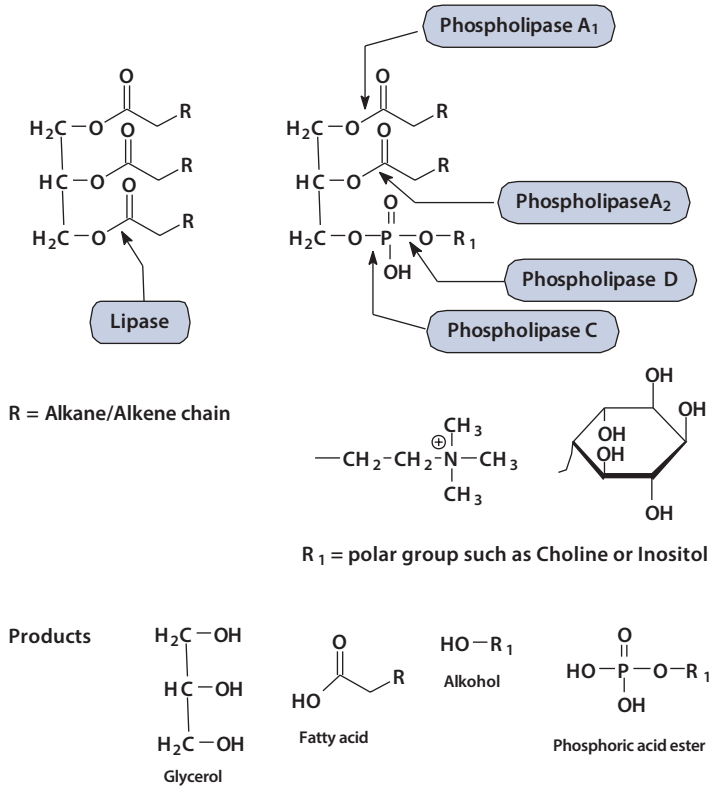
Fats are esters of glycerol and fatty acids. **Lipases**, extracellular enzymes, are responsible for the hydrolysis of the ester bond. Lipases are very non-specific and attack fats containing fatty acids with different chain lengths.

Phospholipids are hydrolyzed by specific **phospholipases** that cleave different ester bonds. Phospholipases A_1 and A_2 cleave fatty acid esters and are thus similar to lipases. Phospholipases C and D cleave phosphate ester bonds and are therefore completely different types of enzymes.

Lipase activity thus leads to the release of fatty acids and glycerol, substances that can be attacked both anaerobically and aerobically by various chemoorganotrophic microorganisms. The degradation of fatty acids by β-oxidation is discussed in ► Chap. 5 under alkane degradation (◘ Fig. 4.15).

4.4.4 Degradation of Plant Substances/Lignin and Other Natural Substances/Humus Formation

The plant dry matter consists of about 80% **lignocellulose**. In plant cell walls, the **cellu-**



◘ **Fig. 4.15** Sites of action of lipase and various phospholipases on lipids and phospholipids

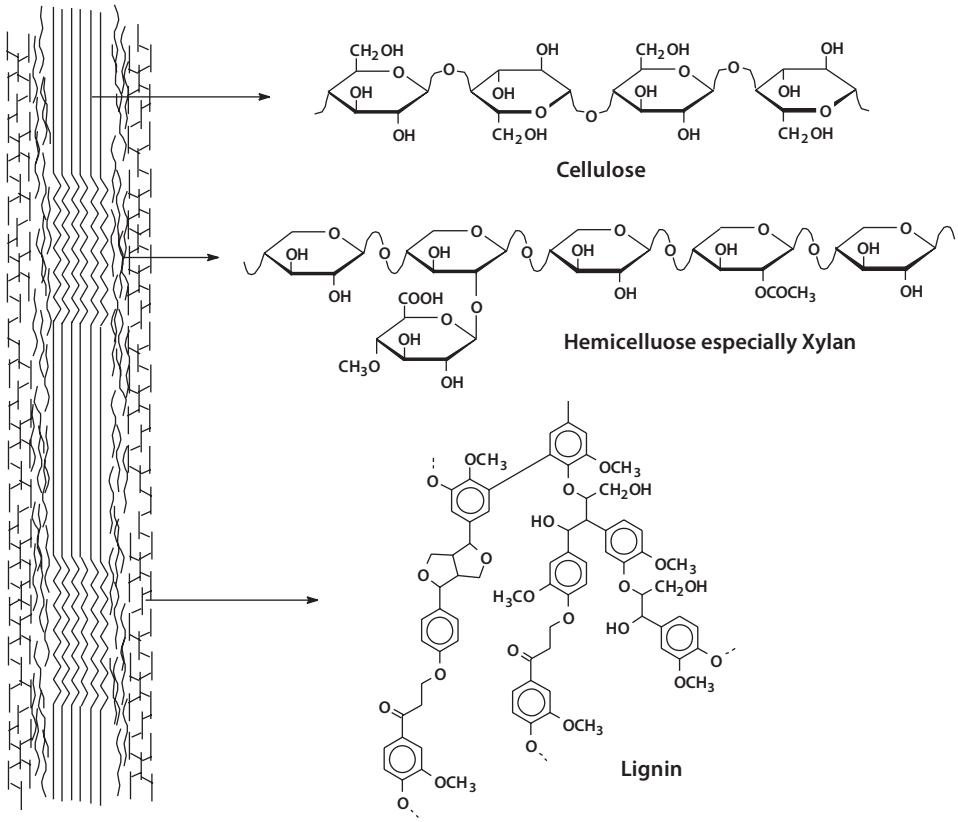


Fig. 4.16 Main components of the plant cell wall. The arrangement of the components is shown schematically

lose microfibrils are embedded in an amorphous matrix of **hemicelluloses** and **lignin** (Fig. 4.16). The three polymers are linked by hydrogen bonds and covalently. Their proportions vary according to plant species and age. On average, lignocellulose consists of 45% cellulose, 30% hemicelluloses, and 25% lignin. Grasses and straw are richer in cellulose and hemicelluloses than wood. The basic chemical structures are shown in Fig. 4.16.

4.4.4.1 Degradation of Starch

Starch is the main storage substance of plants. The polysaccharide is composed of *D*-glucose units that are linked to each other by α-1,4-glycosidic bonds. This results in a helical structure (see Fig. 4.17). In addition to amylose (up to 6000 glucose units), the linear polysaccharide that makes up to 35% of starch depending on the plant, amylopectin (molecular weight $10^7 - 2 \times 10^8$ daltons) is a component of starch as a 1,6-branched polymer. The branching is found on about every 25th glucose.

Microbial starch degraders are widespread in nature. Among aerobic bacteria, many *Bacillus* and *Streptomyces* species possess high amylase activity, and among anaerobes, Clostridia. *Aspergillus niger* and *A. oryzae* are known fungal amylase producers.

Four different groups of hydrolytic enzymes alone may be involved in the degradation of starch to glucose (Fig. 4.17): (1) α-amylases cleave the α-1,4-glycosidic bond of starch inside the starch molecule, leading

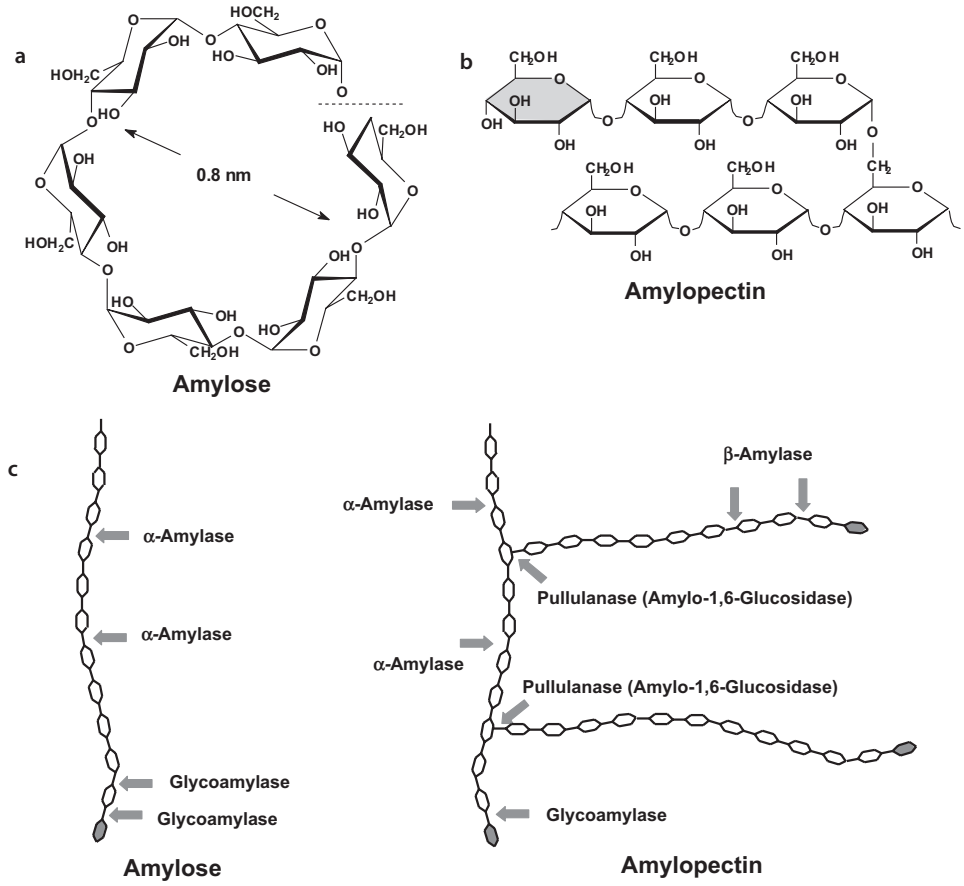


Fig. 4.17 Strength. **a** Structure shown in armchair helix form, **b** Structure of amylopectin shown in Haworth projection, **c** Degradation with sites of attack by the various hydrolytic enzymes. Non-reducing ends are marked

to a breakdown of starch into smaller units. (2) β-Amylases hydrolyze the α-1,4-glycosidic bond from the non-reducing ends to form maltose. (3) Pullulanases cleave the α-1,6-glycosidic bonds. (4) Glucoamylases directly cleave glucose from the non-reducing ends of the starch molecule. Maltose formed by β-amylase is cleaved into glucose by maltase.

4.4.4.2 Degradation of Cellulose

Cellulose is a linear polymer consisting of thousands (up to 14,000) of glucose building blocks linked by β-1,4-glycosidic bonds. 60–70 cellulose chains are linked by hydrogen bonds to form elementary fibrils. These are assembled into microfibrils, which in turn are united into bundles. Highly ordered crystalline regions alternate with less ordered amorphous regions. The complex structure is shown in Fig. 4.18.

Fungi and bacteria degrade cellulose. If the cellulose is more lignified, only fungi can attack the complex. Aerobic bacteria that degrade cellulose are, for example, *Cellulomonas, Cytophaga* and *Streptomyces* species. Important representatives among anaerobes are *Bacteroides, Clostridium* and *Ruminococcus* species. Among fungi, the ability is present in three ecological groups, the molds of the soil (for example, *Aspergillus* and *Trichoderma*), some phytopathogenic

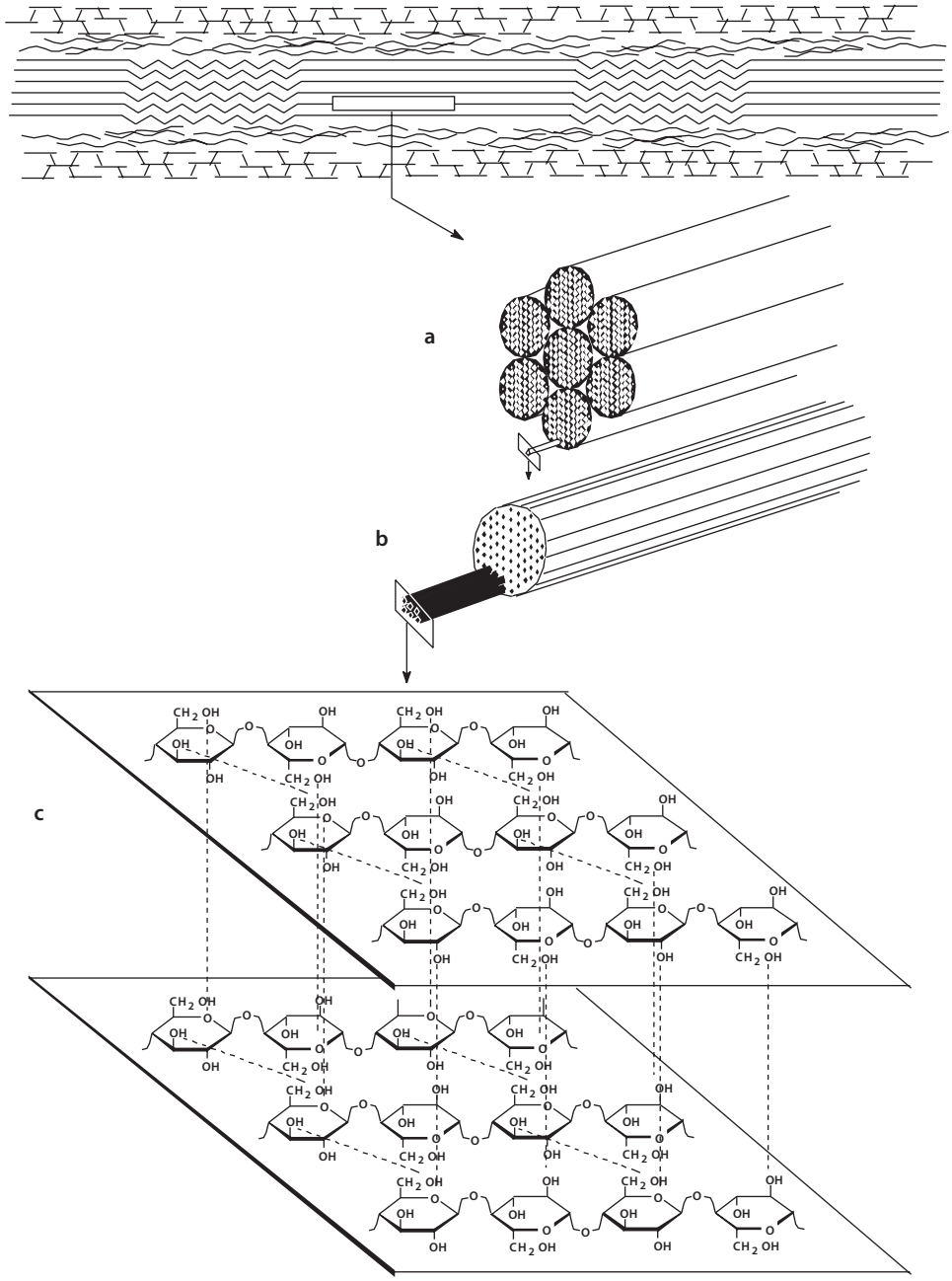


Fig. 4.18 Complex structure of cellulose. **a** Bundle of several microfibrils showing the structure of elementary fibrils in the section. **b** Structure of an elementary fibril in which the cellulose molecules are arranged in parallel. Six cellulose chains are pulled out in the section. **c** Crystalline arrangement of six cellulose chains in the elementary fibril. Some hydrogen bonds between the cellulose chains are shown in dashed lines

fungi (*Fusarium, Rhizoctonia*), and the wood-destroying basidiomycetes and ascomycetes. Among the wood-degrading fungi, it is again primarily the brown-rot pathogens that preferentially attack the cellulose, leaving the lignin skeleton as a brown-colored substance. Representatives of the **brown-rot-fungi** are the dry rot *Serpula lacrymans* and the birch-porling *Piptocarpus betulinus.* White-rot-fungi also break down cellulose beside lignin. Cellulose, but not lignin, can be used as the only carbon source.

Macromolecules such as cellulose cannot be taken up by microorganisms. The degradation of cellulose is made more difficult by the fact that the elongated cellulose molecules are connected to each other by a large number of hydrogen bonds. This results in microcrystalline areas where the cellulose molecules are very close together. This makes it difficult for microorganisms and also exoenzymes to access the bonds to be hydrolysed. Therefore, cellulose is preferably attacked in amorphous areas. The degradation is carried out by extracellular enzymes, which are excreted. They are often localized on the cell surface and there is close contact between the organisms and the cellulose fibers.

With regard to enzymatics, there is again an analogy to starch. Here, too, there are hydrolases, so-called endocellulases, which cut the β-1,4-glycosidic bond of cellulose rather inside the molecule, resulting in shortened cellulose chains. Other enzymes, so-called exocellulases, cleave at the resulting free, non-reducing ends.

A gradual depolymerization of the cellulose takes place: Fungi such as *Trichoderma viride* start the degradation in the macromolecule at the amorphous regions by an endocellulase. The resulting free ends are broken down by an exocellulase to di- and oligosaccharides, which are finally hydrolyzed to glucose by a β-glucosidase. Some bacteria preferentially attack the crystalline cellulose regions. In the anaerobic bacterium *Clostridium thermocellum,* which lives in the rumen, the enzymes are present at the cell surface as a multienzyme complex, the cellulosome. It consists of 14–18 polypeptides, which include glucanases as well as xylanases. Glucose as well as xylose enter the intermediary metabolism (◘ Fig. 4.7). The extracellular enzymes lead to degradation products that are also used by microorganisms that do not possess these enzymes (◘ Fig. 4.19).

4.4.4.3 Degradation of Xylan (Hemicellulose)

Hemicellulose is a collective term for a heterogeneous group of matrix polysaccharides that surround the cellulose microfibrils and link them by hydrogen bonds to form a network. Hemicelluloses are the alkali-soluble polysaccharides of the cell wall other than cellulose and pectin. Characteristic sugar building blocks are pentoses (*D*-xylose, *L*-arabinose) modified by acetylations (◘ Fig. 4.20). They are linked by β-1,4-glycosidic bonds to form units of about 30–500 pentose building blocks. Xylans, composed primarily of pentoses, are the most common hemicelluloses; hardwoods are 20–25% xylans, and softwoods are 7–12% xylans. Xylans contain further sugars, arabinose, galactose, glucose and glucuronic acids (for example arabinoglucuronoxylan) in side chains.

The ability to degrade xylan is more widespread than that to degrade cellulose. In addition to many cellulose-degrading species, xylanolytic microorganisms include the anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricum, Thermoanaerobacterium thermosaccharolyticum, Thermobacteroides* and *Thermoanaerobium* species, which ferment xylan to ethanol, acetate and lactate. Yeasts such as *Candida shehatae, Cryptococcus albidus* and *Pichia stipitis* and mycelial fungi such as *Fusarium oxysporium, Monilia* and *Neurospora crassa* degrade xylan to xylose and utilize pentoses.

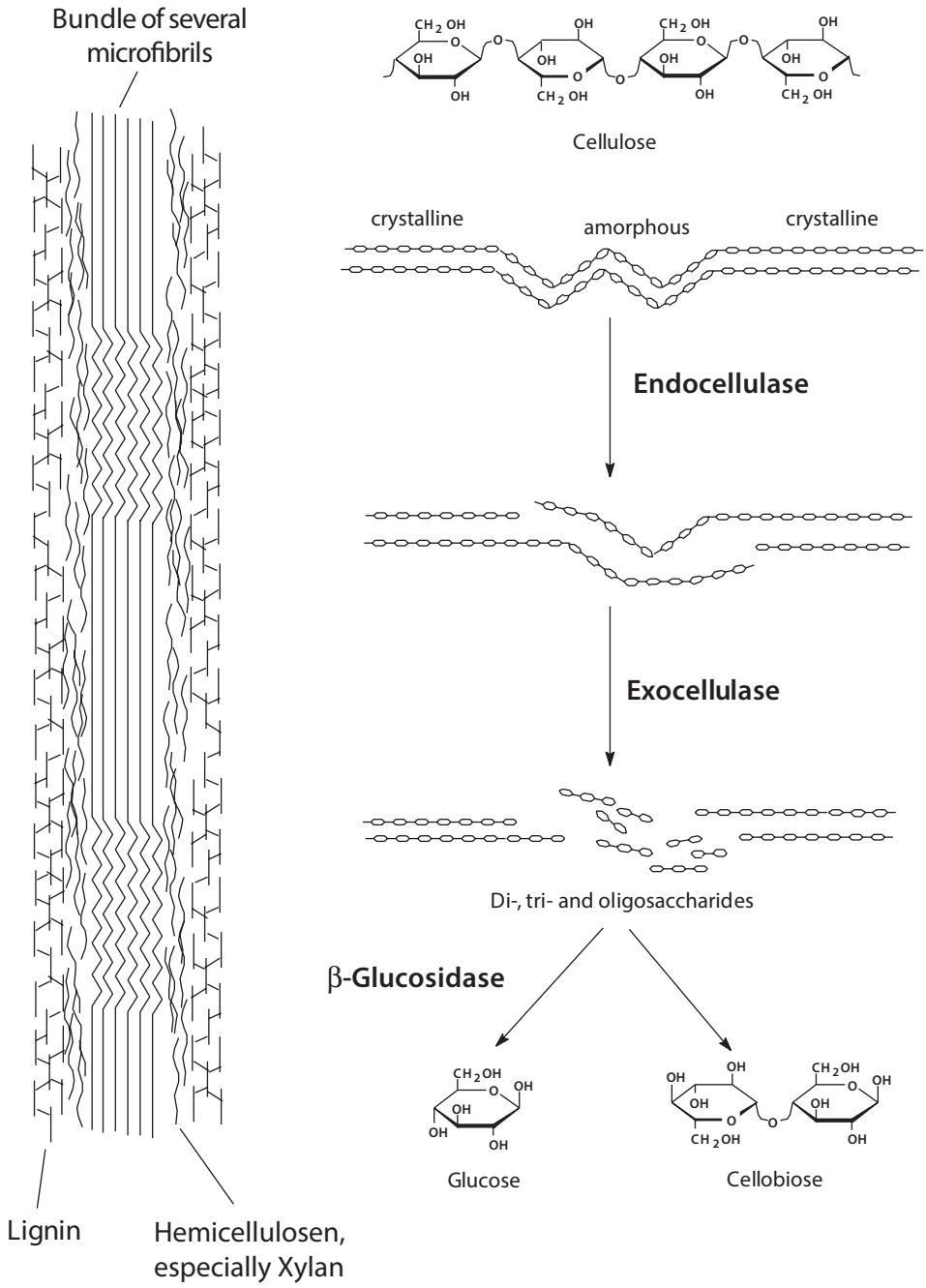


Fig. 4.19 Microbial cellulose degradation. Depolymerization of the macromolecule occurs stepwise by three extracellular enzymes to glucose

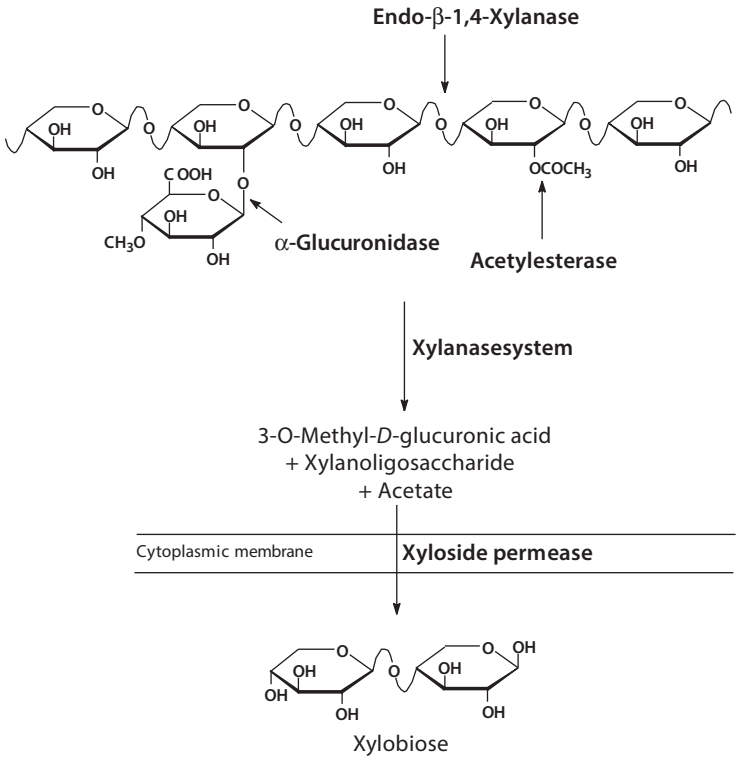


Fig. 4.20 Microbial xylan degradation

The extracellular xylanase system consists of several enzymes that hydrolyze the polysaccharide into xylobiose and cleave acetate. Xylobiose is degraded via *D*-xylose and *D*-xylulose in the pentose phosphate pathway after uptake into the cell.

4.4.4.4 Degradation of Pectin

Pectins are polygalacturonides composed of α-1,4-glycosidic linked *D*-galacturonic acids. Some carboxyl groups are esterified with methanol. Pectins are the building blocks of the middle lamina of plant tissues. Many soil bacteria degrade pectin under aerobic and anaerobic conditions. *Erwinia carotovora, Paenibacillus macerans* and *P. polymyxa* belong to the aerobic, Clostridia to the anaerobic pectin degraders.

The degradation of polygalacturonides to galacturonic acid is shown in ◘ Fig. 4.21. Depolymerization occurs by pectinases and subsequent hydrolysis to galacturonic acid by oligogalacturonidases. Methanol is cleaved by pectin methyl esterase. Pectins are the source of this C_1 compound in nature.

4.4.4.5 Degradation of Lignin

Lignin is not only a component of wood, but also of the cell walls of almost all land plants. It encrusts the cellulose and hemicellulose structures in the cell walls. This is what gives rise to the high stability and elasticity of plants. It is also the structure that protects plants from rapid microbial attack, as it is difficult to degrade. Lignin is a three-dimensional heterogeneous polymer composed of **phenylpropane building blocks** that are cross-linked in a variety of ways by ether and C-C bonds (◘ Fig. 4.16). These bonds and the irregular arrangement cause the difficult degradability.

Lignin is mainly degraded by **white rot fungi**, the remaining cellulose causes the light appearance of the affected wood. In addition to *Phanerochaete chrysosporium,* well-studied white rot fungi include the but-

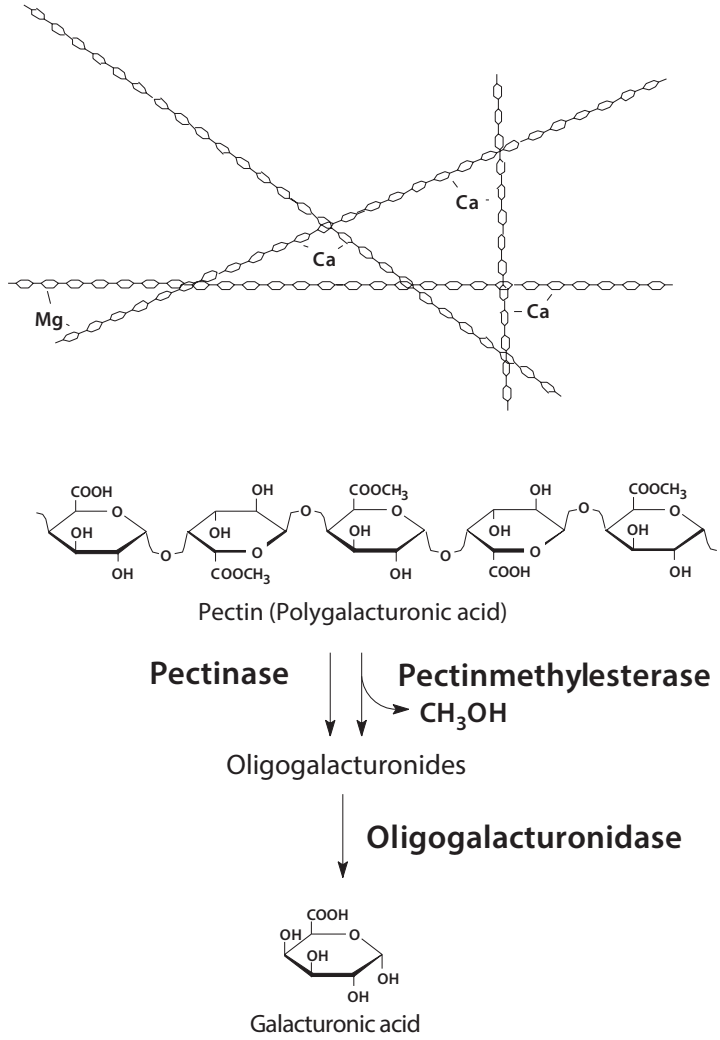


Fig. 4.21 Microbial pectin degradation

terfly spore *Trametes versicolor* , the comb fungus *Phlebia radiata* and the oyster mushroom *Pleurotus ostreatus*. But also litter degraders like the roundheads (for example *Stropharia rugosa-annulata*) possess enzymes for lignin degradation. Three enzymes play an essential role in wood degradation, **lignin peroxidase**, **manganese-dependent peroxidase** and **laccase**. In some fungi the above enzymes have been detected with several isoenzymes, in others only one of the peroxidases. Very common are the laccases, non-specific acting polyphenol oxidases. The interaction of peroxidases and laccases is insufficiently clarified.

The fungi cannot use lignin as their only source of carbon and energy, but need a growth substrate, usually cellulose. They eliminate the lignin encrustation and thus obtain the polysaccharides as substrate. The process is aptly called "enzymatic combustion". The extent to which degradation products are assimilated is unclear. Probably, the degradation leads to the release of nitrogen sources, which are a growth-limiting substrate in wood.

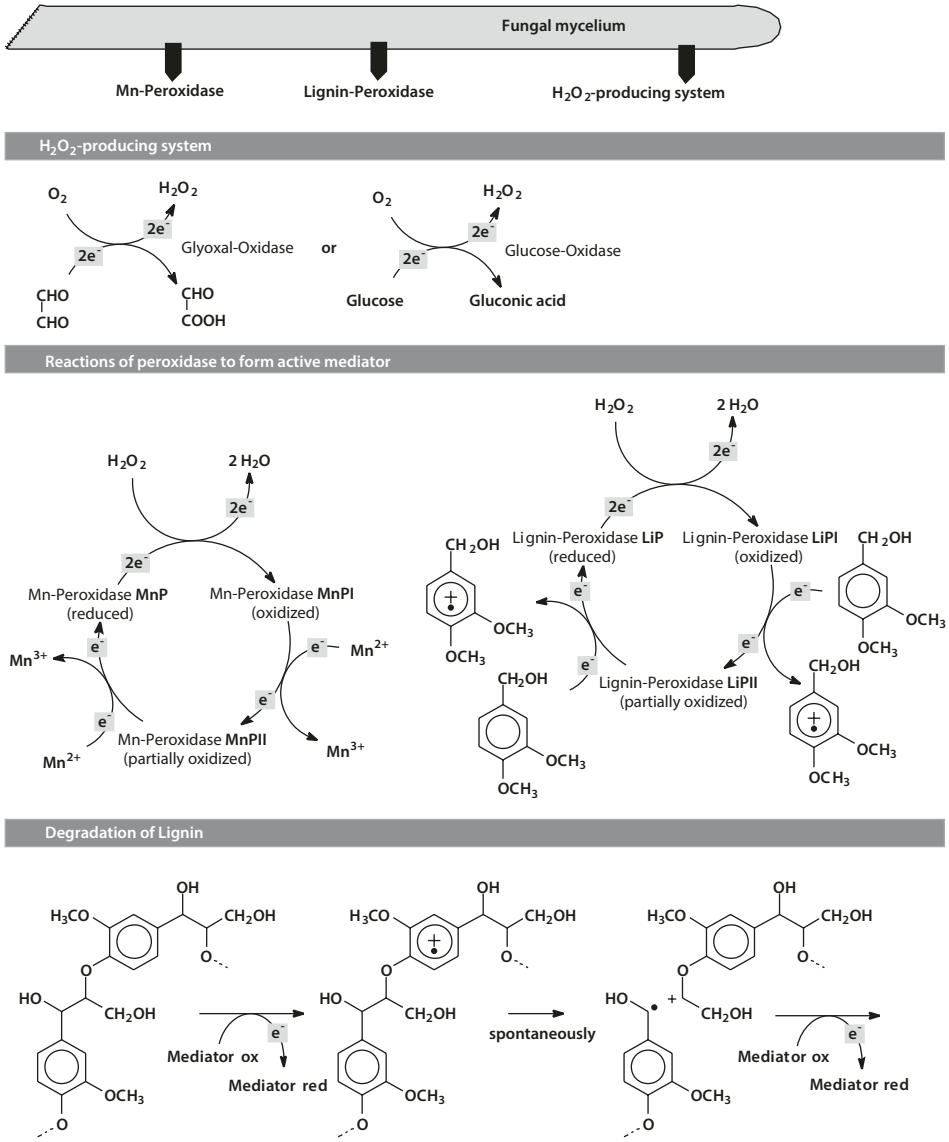


Fig. 4.22 Lignin degradation by the peroxidase system. *Upper part:* H_2O_2-generating system. *Middle part:* formation of a cation radical of the mediator or Mn^{3+}-formation by withdrawal of an electron. Lignin peroxidase oxidizes veratryl alcohol to the veratryl alcohol cation radical. *Lower part:* Mediator-mediated degradation of native lignin

The H_2O_2 necessary for the peroxidase reactions is supplied by various enzyme systems. In addition to the glyoxal oxidase mentioned in Fig. 4.22, glucose oxidase can also act as H_2O_2 donors.

Lignin peroxidases, whose mechanism of action is shown in Fig. 4.22, are extracellular enzymes with a pH optimum in the acidic range. They are potent oxidants and each removes an electron, producing a cation radical. To act as a potent oxidant, the prosthetic group of the protoporphyrin system of lignin peroxidase is first deprived of two electrons by the H_2O_2. The circle is com-

pleted by being replenished in conjunction with the change in valence of iron by twice withdrawing an electron from donor molecules of lignin or other substrates.

During the degradation of lignin, a mediator system is necessary because the large lignin peroxidase molecule cannot directly contact the lignin network. The peroxidase oxidizes **mediators** such as veratryl alcohol to the veratryl alcohol cation radical, which reacts with the lignin. Veratryl alcohol is a secondary metabolite of fungi, but it is also produced during lignin degradation. What other mediator molecules play a role in nature is insufficiently studied. The involvement of mediators explains the apparent non-specificity of lignin peroxidase, its actual substrate is H_2O_2, the nature of the substrates from which electrons are withdrawn is non-specific.

Manganese peroxidase (Mn-II peroxidase) is also a hemoprotein, its activity is dependent on H_2O_2 and manganese. The mechanism of action is similar to that of lignin peroxidase. During the change of valence of iron in protoporphyrin, divalent manganese acts as an agent to be oxidized. The resulting trivalent manganese forms chelate complexes with organic acids such as malonate or oxalate, which diffuse into the lignin framework and remove electrons from phenolic structures. In this case, therefore, the manganese complexes act as mediators of electron transfer. Manganese peroxidase mainly attacks phenolic structures, whereas lignin peroxidase also oxidizes nonphenolic methoxylated aromatic structures. The Mn-peroxidase is very common in fungi and is also able to oxidize a variety of xenobiotics.

The following reactions take place in the lignin: When an electron is removed from the aromatic nuclei of lignin, unstable radicals are formed. These lead to the cleavage of the aryl side chains between the α-β-atoms, but also to the cleavage of the ether bridges and the aromatic ring as well as to hydroxylation.

4.4.4.6 Humification

The process of **humification** requires advanced microbial degradation of the plant substances so that reactive degradation products such as monosaccharides from carbohydrates, peptides and amino acids from proteins and phenolic substances from cell wall components are present. In such a mixture of substances, polymerisation of the monosaccharides, cyclic amino acids and phenols then takes place to form high-polymer humic substances as mixed polymers. The principle of humic substance formation, which is still largely unknown today, is based on the linkage of cyclic basic substances already present in plants, such as lignins, dyes and tannins, or the ring compounds formed by the cyclization of linear cleavage products.

Different stages of biogenesis can be distinguished (◘ Fig. 4.23): In the metabolic phase, partial microbial degradation of high and higher molecular weight substances takes place. This is where the humifiable material is formed. An introductory phase of humification begins with the aromatic natural substances from plants. The genesis of humic acid precursors begins with the formation of radicals. An uptake of non-aromatic starting materials can be observed in the conformation phase. The non-aromatic starting materials come from carbohydrates, fats and proteins.

The phase of formation of a humic substance system can so far only be described on the basis of model reactions: These model humic syntheses include the autoxidation of various phenols to radicals and their reactions, as well as the **Maillard reaction** known from food chemistry. Using hydroquinone as an example, it was shown that this phenol undergoes autoxidation in alkaline solution in the presence of oxygen, leading to the formation of intensely brown-colored, non-uniform products with humic-like properties. The browning reaction, termed the Maillard reaction, which is

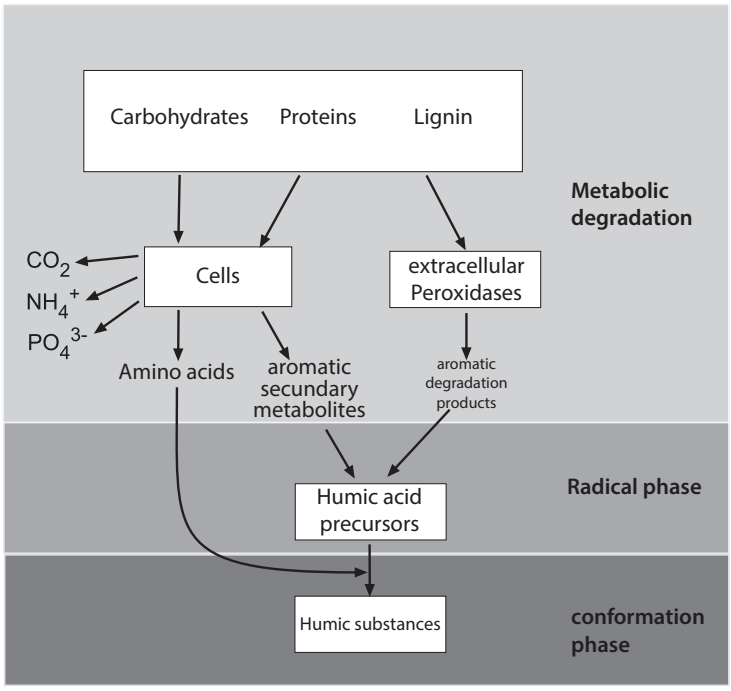


Fig. 4.23 Sections of humic substance synthesis

observed in the reaction of reducing sugars with amino acids, also yields humic-like products. In the first phase of formation of the humic system, numerous stable complexes of humic substances (or of humic precursors) with non-humic substances such as phenols, carbohydrates and amino acids, but also with polycyclic hydrocarbons, steroids and proteins are also observed. A conceptual differentiation of humic substances from non-humic substances is possible as follows: non-humic substances include all substances from dead plants or animals that occur in the stage of biological and abiological degradation and also transformation. Humic substances include those products which have been abiologically synthesized as transformation and build-up products.

Humic synthesis is characterized by an immense variety of reaction partners and no dominance of any reaction mechanism. Due to this fact, no defined structural formula and no uniform building principle can be given for humic substances. Nevertheless, there are many attempts to describe a structure for humic substances. Elemental analysis of humic substances reveals C, O, H and N with an average of 54/33/4.5/2.7% as the main elements, although nitrogen is not considered an obligatory element for humic substances. The most important functional groups of humic substances are carboxy, carbonyl, amino, imino, methoxy and hydroxyl groups. In addition, humic substances also contain larger hydrophobic (inner) regions. The relative molecular masses of humic substances vary between 1000 and in extreme cases 500,000 g/mol. Simplistically, they are high molecular weight hydroxy and polyhydroxycarboxylic acids that may be linked by various covalent bonds (for example, C-C, oxygen, or C-N bonds) or connected by hydrogen bonds, charge-transfer relationships, or VAN-DER-WAALS forces. The hypothetical structure includes aromatic nuclei, carboxy and hydroxyl groups, and peptide and carbohydrate side chains. Due to the constant build-up, transformation and degradation

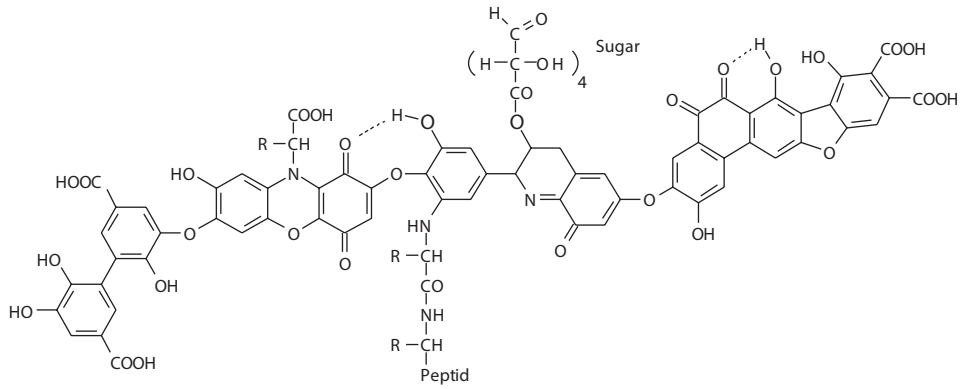


Fig. 4.24 Hypothetical humic acid molecule. The functional hydroxyl and carboxyl groups at the edges are decisive for the reactivity

in abiological and also biological processes, even a hypothetical structure can only approximate a temporary state. The most important structural elements of humic substances are shown in the hypothetical humic acid molecule (Fig. 4.24).

Humic substances always have an acidic character. The different solubility behaviour of individual humic fractions leads to the classification into the three groups of fulvic acids, humic acids and humins. The differences in solubility are correlated with different chemical properties of humic acids and fulvic acids. Thus, the yellow to reddish-brown fulvic acids have high acidity, while the brown to black humic acids are only moderately acidic. The black humic acids, which play only a minor role in terms of soil properties, are weakly acidic. In contrast to the humic acids, the fulvic acids also possess many carbonyl groups. On the other hand, the humic acids have a higher aromatic content.

Humification is important for the fertility of the soil, the formation of compost (► Sect. 17.2) and the fixing of pollutants such as TNT and PAHs (► Sect. 6.3).

Humic Substances as Electron Accep tors

Humic substances are normally resistant to microbial metabolism. Although an accurate $E^{0'}$ cannot be measured for natural humic substances because they are undefined complex mixtures, model low molecular weight humic compounds such as fulvic acid have an $E^{0'}$ of about +500 mV, indicating that humic substances should generally be good electron acceptors. Indeed, humic substances have been shown to be used as electron acceptors for the anaerobic oxidation of organic compounds and of H_2, providing energy and supporting growth. For example, the bacterium *Geobacter metallireducens* grows anaerobically with acetate or H_2 as electron donors and humins as electron acceptors.

The use of **humins as electron acceptors** indicates a previously unsuspected geochemical role for these substances. This is of particular importance as it is known that reduced humins can chemically react with metals such as Fe^{3+} and various organic compounds, mobilizing these substances for transport through waterlogged soils or into groundwater.

4.5 Methane Cycle/Methanogenic Food Chain/Methanotrophy

4.5.1 Methane Formation

Methane is of microbial or thermogenic origin and is the most abundant and chemically stable hydrocarbon. The microbial origin is usually evident from the low $^{13}C/^{12}C$ ratio and the absence of other gaseous hydrocarbons (ethane, propane, butane). Thermogenic methane (like other thermogenic hydrocarbons) is formed either by chemical transformations (catagenesis, metagenesis) of buried organic carbon or by reaction of water, iron(II)-containing rocks, and CO_2 at several hundred degrees Celsius. Large deposits of methane hydrates—ice-like solid solutions of the composition $(CH_4)_9$—$(H_2O)_{46}$ or $(CH_4)_{24}$—$(H_2O)_{136}$, composed of methane compacted into a hydrate structure, the dissociation of 1 m^3 of methane hydrate leading to 0.8 m^3 of water and about 170 m^3 of methane gas—with a mass exceeding conventional fossil fuel deposits by a factor of two, are buried in the deep, sulfate-free zones of marine sediments.

Methane formation is of particular importance for environmental microbiology from various aspects. Methane, which contributes about 15% to the greenhouse effect, originates predominantly from microbial processes. Secondly, methane is the main component of biogas as an energy source from waste (sewage sludge) and regenerable raw materials (see ► Chap. 14).

Much of the methane that escapes into the atmosphere (in the order of 535—10^6 t/year) comes from biogenic processes. The main sources are processes occurring in the rumen of ruminants (85 – 10^6 t), rice cultivation in temporarily flooded fields (60 – 10^6 t), and swamps, lakes and bogs (115 – 10^6 t). The anthropogenic increase in methane is due to the doubling of cattle farming and expanded rice cultivation in recent decades.

Methane formation occurs in a food chain involving fermenters and two other groups of microorganisms, the **acetogens** and the **methanogens.**

Methanogenesis is carried out by a group of archaea, the **methanogens,** which are strictly anaerobic. Most methanogens use **CO_2 as a terminal electron acceptor in anaerobic respiration (carbonate respiration)** and reduce it with H_2 to methane. Only a few other substrates, including mainly acetate, can be converted directly to methane by methanogens. Therefore, for the conversion of most other organic compounds to CH_4, methanogens must associate with partner organisms that provide them with the substrates they require. This is the task of **syntrophes**, which are of great importance for the entire anoxic carbon cycle. Substances with large molecular mass, such as polysaccharides, proteins and lipids, are converted to CH_4 and CO_2 by the cooperative interaction of several physiological prokaryote groups.

For the degradation of a typical polysaccharide, such as cellulose, the process begins with cellulolytic **bacteria** that cleave the high molecular weight cellulose molecule into cellobiose (glucose-glucose) and free glucose. Glucose is then converted by **primary fermenters**, the acidogens, into a variety of fermentation products, of which acetate, propionate, butyrate, succinate, alcohols, H_2 and CO_2 are the most important. All of the H_2 produced in the primary fermentation processes is immediately consumed by methanogens, homoacetogens, or sulfate-reducing bacteria (in environments containing sufficient amounts of sulfate).

Low hydrogen partial pressure ($<10^{-4}$ bar) allows electrons to be released at the redox potential of NADH (–320 mV) as molecular hydrogen and the fermentation pattern can change to higher production of acetate, CO_2 and hydrogen compared to ethanol or butyrate formation. This allows for additional ATP synthesis through substrate

step phosphorylation, compared to the production of reduced fermentation products (ethanol, lactate and butyrate). In this way, the fermenters benefit from the hydrogen oxidizing partners, but they are not dependent on this cooperation.

As **secondary fermenters**, syntrophs oxidize fatty acids and produce H_2. *Syntrophomonas wolfei*, for example, oxidizes C_4 – to C_8-fatty acids, producing acetate, CO_2 if the fatty acid contained an odd number of carbon atoms, and H_2. Other species of *Syntrophomonas* use fatty acids up to C_8, including some unsaturated fatty acids. *Syntrophobacter wolinii* is specialized in propionate oxidation and produces acetate, CO_2 and H_2. However, these reactions allow growth of syntrophs only in co-culture with an H_2-consuming partner.

In a balanced anoxic sediment, in which the active hydrogen-consuming population maintains a low hydrogen partial pressure, the flux of carbon and electrons is almost exclusively through the "outer" pathways of the scheme (◘ Fig. 4.25) Therefore, the reduced fermentation products play a minor role. Despite all this, the "central" pathways will never go down to zero because long-chain and branched-chain fatty acids and other products always occur in the fermentation of lipids and amino acids. The reduced intermediates of the central pathways become more important when the hydrogen supply increases for any reason, such as increased supply of fermentable substrates or inhibition of the hydrogenotrophic methanogens due to a drop in pH (<6.0) or the presence of toxic substances. Under such conditions, the amount of fatty acids increases and may even cause the pH to drop further.

Acetate, which is an essential intermediate in the methanogenic food chain, is derived from primary and secondary fermentations, but also from acetogenesis. Acetogenic bacteria live strictly anaerobic and form a taxonomically very heterogeneous group in the Gram-positive phylum for example with the following genera: *Acetobacterium, Butyrobacterium, Clostridium* and *Eubacterium.*

The function of **homoacetogenic bacteria** in the overall process is altogether less well understood. They combine the supply of C_1 compounds and hydrogen with that of acetate. Acetogens synthesize acetate according to the overall reaction

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2\,H_2O \qquad \Delta G^{0'} = -95\,\text{kJ / mol Acetate}$$

The metabolic versatility of acetogens is evident in their ability to ferment sugars as well.

In certain habitats, for example at low pH or low temperature, they can successfully compete with the hydrogenotrophic methanogens and take over their function to a certain extent.

The formation of the acetate is shown in detail in ◘ Fig. 4.26. The formation of the methyl group from CO_2 requires three two-electron reductions, with formate and formaldehyde seen as intermediates. Because of its high reactivity, formaldehyde is toxic and is therefore normally handled in the cell as N^5, N^{10}-methylenetetrahydrofolate. Five enzymes are required for the conversion of CO_2 to N^5-methylenetetrahydrofolate. Further conversion is carried out by the highly oxygen-sensitive acetyl-CoA synthase, a complex enzyme with the following individual functions: (1) a cobalamin-dependent methyltransferase (methyl form of vitamin B12), which takes the methyl group

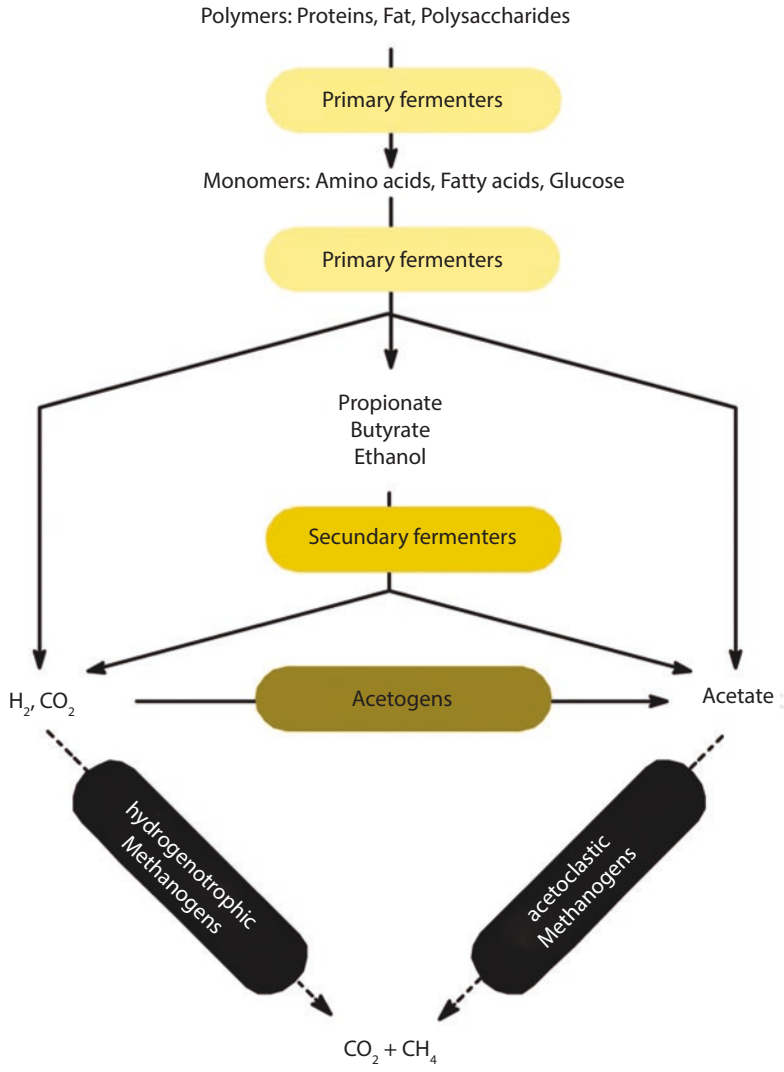


Fig. 4.25 Mass flow during methanogenic degradation of complex organic material. The groups of prokaryotes are: (1) primary fermenters, (2) hydrogen-oxidizing methanogens, (3) acetate-splitting methanogens, (4) secondary fermenters (syntrophes), and (5) homoacetogenic bacteria

from the tetrahydrofolate, (2) a nickel- and [4Fe-4S]-containing CO dehydrogenase, which provides CO by reducing CO_2, and (3) a nickel- and [4Fe-4S]-containing carbonylase. The energy of the acetyl-CoA formed can then be stored in the form of ATP, resulting in acetate.

Under standard conditions, the formation of acetate from CO_2 and H_2 provides enough energy to generate 1 mol ATP/mol acetate. However, in the environment, acetogens face a much lower H_2 concentration. Since 4 moles of H_2 are required for 1 mole of acetate, the change in Gibbs energy falls dramatically with decreasing H_2 concentration:

$$\Delta G' = \Delta G^{0'} + \mathrm{RT} \ln [\mathrm{Acetate}] / [\mathrm{CO_2}]^2 [\mathrm{H_2}]^4$$

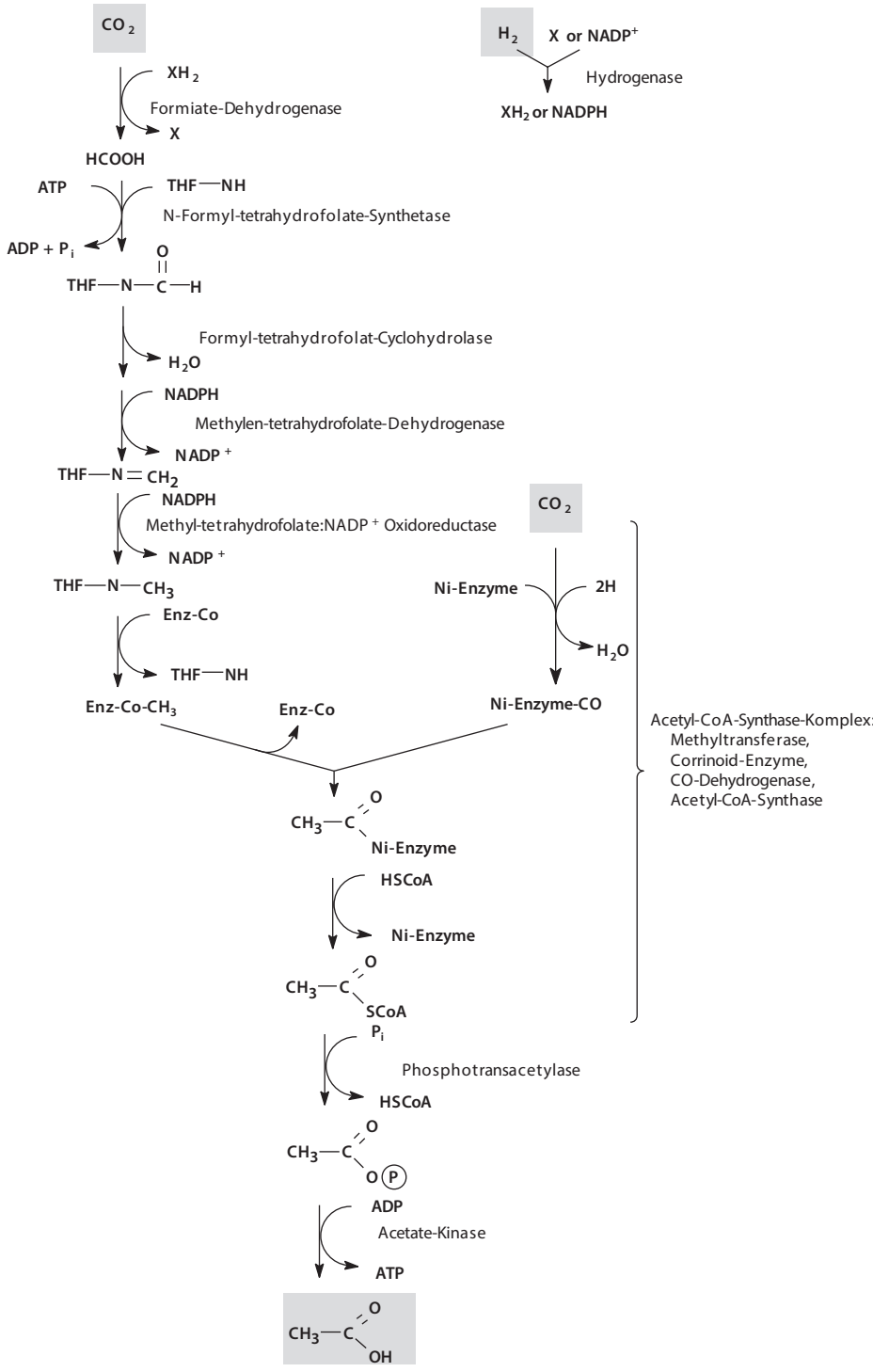


Fig. 4.26 Acetate formation by acetogens

Thus, under these conditions ($[H_2]$ = 100 Pa (equivalent to 1 mM), $[CO_2] = 10^5$ Pa (equivalent to 1 M), and [acetate] = 1 M), only

$$\Delta G' = -95 + RT\ln 10^{-12}\,kJ/mol = -27\,kJ/mol\,Acetate.$$

However, this energy yield is not sufficient to generate 1 mol ATP/mol acetate, for which at least −70 kJ are required. This consideration makes it clear that substrate step phosphorylation is impossible. Moreover, the ATP that can be produced from acetyl-CoA is already consumed during activation of the formate. Consequently, with CO_2 as substrate, electron transport phosphorylation (that is, anaerobic respiration) is the only way of energy storage. Thus, cytochrome b is also found in *Clostridium thermoaceticum* and H^+ has been shown to be the ion for electron transport phosphorylation. Na^+ is the corresponding ion in *Acetobacterium woodii,* in which a Na^+ -dependent ATP synthase was discovered.

Methanogens, the final members of the methanogenic food chain, form CH_4 under strictly anoxic conditions by cleavage of acetate and by reduction of C_1 compounds. The C_1 -reducing methanogens produce methane mainly from CO_2/H_2, but also from CO, formate, methanol, methylamines and methyl sulfides.

Methanogens have only been found within the *Euryarchaeota.* Representatives of the methanogens are identified by the prefix "Methano-": for example *Methanobacterium, Methanococcus, Methanomicrobium, Methanospirillum* and *Methanothermus.*

The methanogens are one of the groups of *Archaea* that have evolved independently and differ fundamentally from the eubacteria in the structure of the cell wall and cell membrane as well as important coenzymes.

The methane-forming reactions can be divided into the following groups, (1) reductions of C_1 compounds with molecular hydrogen as an electron donor, carried out by the **hydrogenotrophic methanogens**, and (2) the apparent decarboxylation of acetate, which is actually a disproportionation and is carried out by the acetoclastic **methanogens.**

Reduction of C_1 compounds:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^{0'} = -131\,kJ/mol\,CH_4$$

Disproportionation of acetate:

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \Delta G^{0'} = -36\,kJ/mol\,CH_4$$

The pathway of reduction of CO_2 to methane with molecular hydrogen has been elucidated in great detail. Coenzymes unique to Archaea function in it: methanofuran, tetrahydromethanopterin, coenzyme F_{420}, coenzyme M, coenzyme B, 5-hydroxybenzimidazolylhydroxycobamide and coenzyme F_{430}.

Only tetrahydromethanopterin (H_4MPT), coenzyme F_{420} and 5-hydroxybenzimidazolylhydroxycobamide resemble coenzymes of bacteria and eukaryota. Tetrahydromethanopterin is related to tetrahydrofolate (H_4F) and has the same role in methanogenic archaea as tetrahydrofolate in the bacteria and eukaryota. It carries C_1 compounds (from formyl to methyl residues) and protects the organism from the free, reactive formaldehyde.

Coenzyme F_{420} has comparable properties to NAD^+. However, the redox potential of coenzyme F_{420} (oxidized/reduced) is

40 mV more negative than that of the NAD^+/NADH pair (–320 mV).

5-Hydroxybenzimidazolylhydroxycobamide is very similar to hydroxycobalamin (hydroxy form of vitamin B_{12}), which is found in acetogenic bacteria.

The reduction of CO_2 (oxidation state +4) to CH_4 (–4) takes place in several stages (see ◘ Fig. 4.27). The C atom remains covalently bonded to special single-carbon carriers. First, CO_2 is reduced to the methanofuran-bound formyl stage (+2). The energy source for CO_2 activation is not yet known. The formyl group is then transferred to tetrahydromethanopterin (H_4-MPT). Removal of water provides the methenyl group, which is reduced to the methylene stage (0). Coenzyme-F_{420} provides electrons from the hydrogenase. Further reduction with coenzyme-F_{420} yields methyl-H_4-MPT (–2), from which the methyl group is transferred to coenzyme M. The resulting methyl-CoM is then reduced to methane, with nickel porphyrinoid coenzyme F_{430} serving as a cofactor. The actual reducing agent is coenzyme B, which is oxidized in the process and forms a heterodisulfide with coenzyme M. CoM and CoB are subsequently recycled by reduction of the disulfide by disulfide reductase. The electrons originate from the H_2.

Energy is stored during methane formation by chemiosmotic coupling. The methyl-CoM reductase (MCR) is coupled with a membrane-bound H^+-pump, which forms an electrochemical potential gradient. Furthermore, Na^+-electrochemical potentials are involved in energy storage. Substrate step phosphorylation does not occur in methanogens.

The formation of electrochemical ion gradients allows organisms to adapt their energy yields to varying substrate concentrations in nature. While standard conditions (10^5 Pa H_2; $\Delta G^{0'} = -131$ kJ/mol CH_4) allow the formation of more than 1 mol ATP/mol CH_4, in most methanogenic habitats H_2 concentrations are in the range of 1–10 Pa: this means that only $\Delta G'$ values of −22 to −45 kJ/mol result. The large dependence on H_2 concentrations stems from the consumption of 4 mol H_2/mol CH_4.

The vast majority of methane in nature is formed from acetate. It is converted to methane by the **acetoclastic methanogens.** Only the two genera *Methanosarcina* and *Methanotrix* excel in the utilization of acetate. Overall, the disproportionation of acetate to CO_2 and methane is an internal redox process. Initially, acetate is activated to acetyl-CoA by a joint activity of acetate kinase and phosphotransacetylase or it is directly activated by an acetyl-CoA synthetase.

$$\text{Acetate} + \text{ATP} + \text{CoASH} \rightarrow \text{Acetyl CoA} + \text{AMP} + \text{PP}_i$$

Acetyl-CoA is decarbonylated to methyl-H_4 MPT and CO, which is oxidized to CO_2. The reduction equivalents formed are used to generate methane in the subsequent pathway. CO dehydrogenase (or acetyl-CoA synthase), which performs the decarbonylation of acetyl-CoA to methyl-H_4-MPT, CoASH, and CO and the subsequent oxidation of CO, is a nickel-cobalt-iron enzyme similar to acetyl-CoA synthase of acetogenic bacteria. The further pathway corresponds to that shown for the hydrogenotrophic methanogens with methyltransferase, methyl-CoM reductase as well as heterodisulfide reductase (◘ Fig. 4.28).

The amount of energy conserved by the disproportionation of acetate is not very high because most of the ATP formed by the H^+-translocating ATP synthase is consumed for the activation of acetate. The

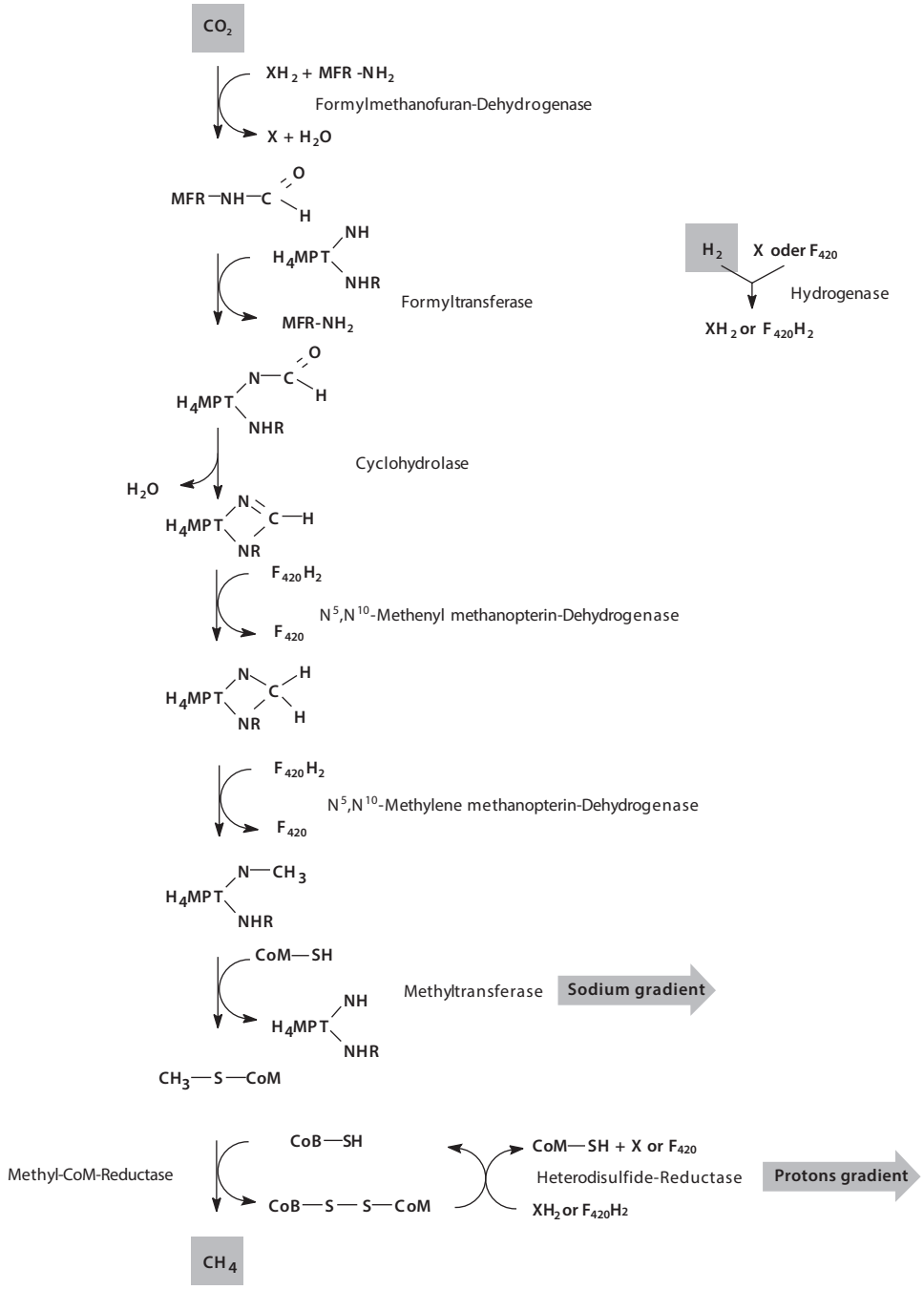


Fig. 4.27 Sequence of methane formation by hydrogenotrophic methanogens. (X = unknown)

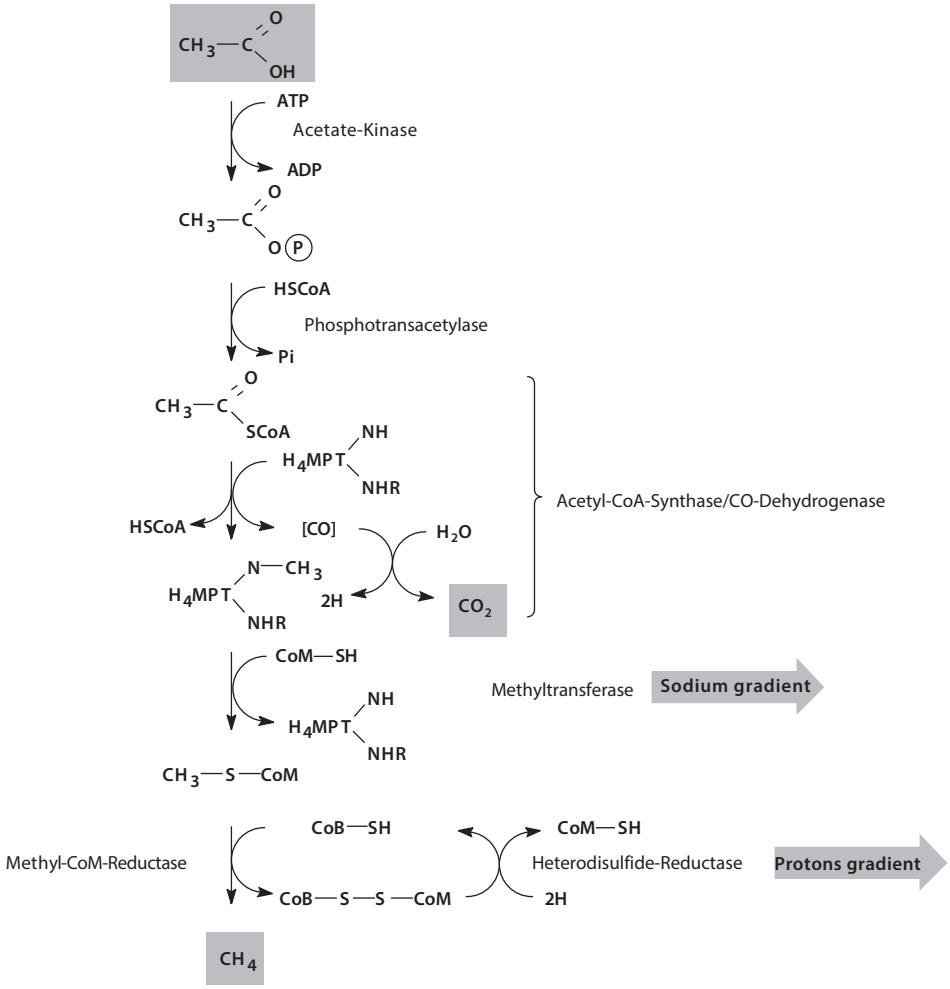


Fig. 4.28 Sequence of methane formation by acetoclastic methanogens

sodium ion gradient formed during the transfer of the methyl radical contributes to ATP synthesis through a Na^+/H^+-antiporter. Overall, approximately 1/3 mol of ATP can be conserved per mol of CH_4 formed. This agrees well with the calculated value of the change in Gibbs energy ($\Delta G^{0'} = -30$ kJ/mol).

Methanogenesis is the main pathway of anaerobic biomass degradation in freshwater sediments and swamps. In anaerobic marine biotopes rich in sulfate, sulfate-reducing bacteria successfully compete for the substrates H_2 and acetate of methanogens.

4.5.2 Methane Degradation

A considerable amount of the methane formed is released into the atmosphere, especially from water bodies and rice fields. In rice plants, the transport takes place through the vessels and tissues of the plants. The methane formed in anaerobic habitats rises to the surface in gaseous form and either escapes to the atmosphere or is partially utilized at the aerobic upper layers of soils and waters by the methanotrophic bacteria.

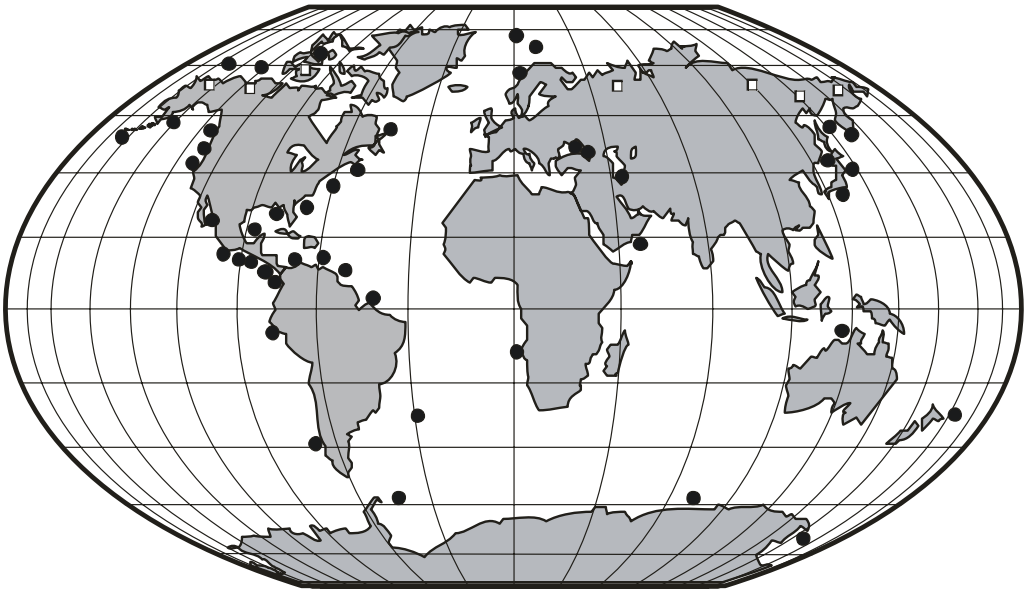


Fig. 4.29 Worldwide locations where samples of gas hydrate have been taken or where there is an encounter with seismic reports. The areas include both the occurrence at continental margins and permafrost regions

Methane also escapes into the atmosphere from the digestive tracts of ruminants and other animals (for example termites) (Fig. 4.29).

Large reservoirs of methane hydrates are buried in deep, sulfate-free zones of marine sediments. Despite permanent upwelling and new thermogenic or microbial production, only small amounts of methane (about 2% of the global methane budget) escape from marine gas hydrates and sources at the continental margins of the oceans into the hydrosphere. Are microbial mechanisms also responsible for the strong minimization of released methane, even though the habitats are anoxic?

4.5.2.1 Aerobic Degradation (Methylotrophy)

Methanotrophic bacteria represent a group of aerobic microorganisms specialized in the utilization of C_1 compounds. In addition to the small group of methane-using species present in soils and waters, there is a large group that can use oxidized C_1 compounds such as methanol, methylamines, formate and formamide. They are called methylotrophic species, and many representatives bear the prefix "methylo-". Methylotrophs are also some yeasts such as *Candida boidinii* and *Hansenula polymorpha.* The extended substrate spectrum results from the pathway of energy production shown in Fig. 4.30, in which some of the substrates mentioned occur as intermediates. The methanotrophs are distinguished from the methylotroph species by the possession of a methane monooxygenase. The oxidation of methane to methanol occurs by the incorporation of one atom of O_2, the second being reduced to water.

The methanotrophic and methylotrophic microorganisms use C_1 compounds as their sole C source. Assimilation occurs in two different pathways, the serine pathway and the ribulose monophosphate pathway. The serine pathway, simplified in Fig. 4.31, shows the basic reactions of formaldehyde and CO_2 assimilation and is used by *Methylocystis* and *Methylosinus.*

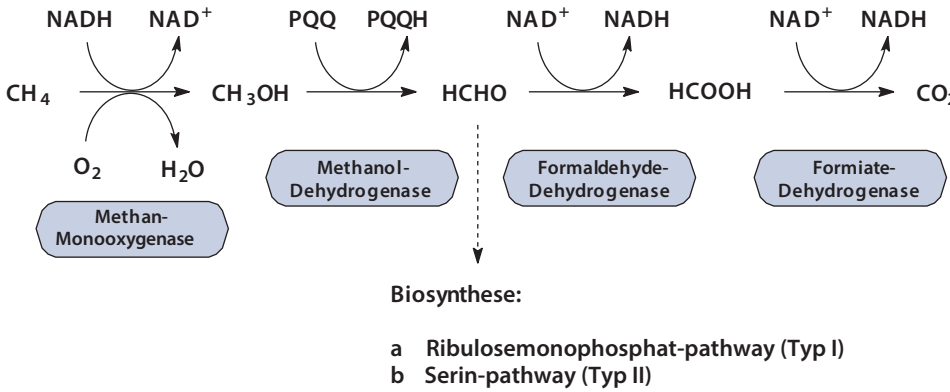


Fig. 4.30 Methane as a carbon and energy source, PQQ, methoxatin (2,7,9-tricarboxy-1H-pyrrolo(2,3-f) quinoline-4,5-dione) (2,3-f-)quinoline

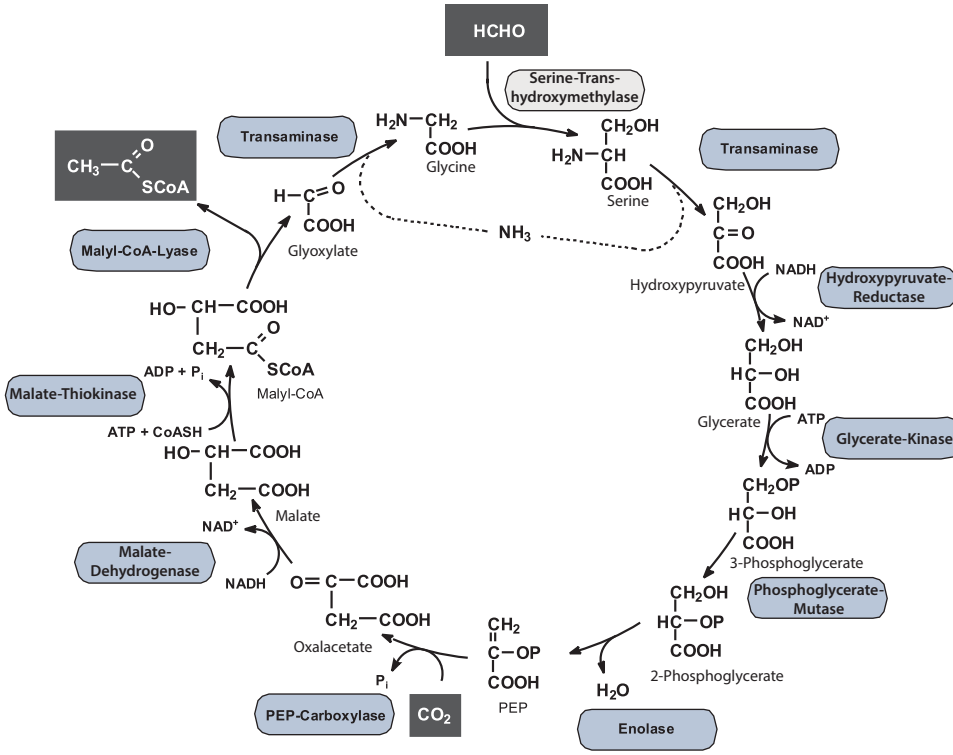


Fig. 4.31 Serine pathway of assimilation of formaldehyde

In the ribulose monophosphate pathway, a C_1 compound is attached to a C_5 compound, similar to the Calvin cycle. The C_1 compound is formaldehyde, and ribulose-5-phosphate acts as the C_5 compound, yielding fructose-6-phosphate. The summation equation is (Fig. 4.32):

$$3\,\text{Formaldehyde} + \text{ATP} \rightarrow \text{Glycerinaldehyde}\ 3\ \text{phosphate} + \text{ADP}$$

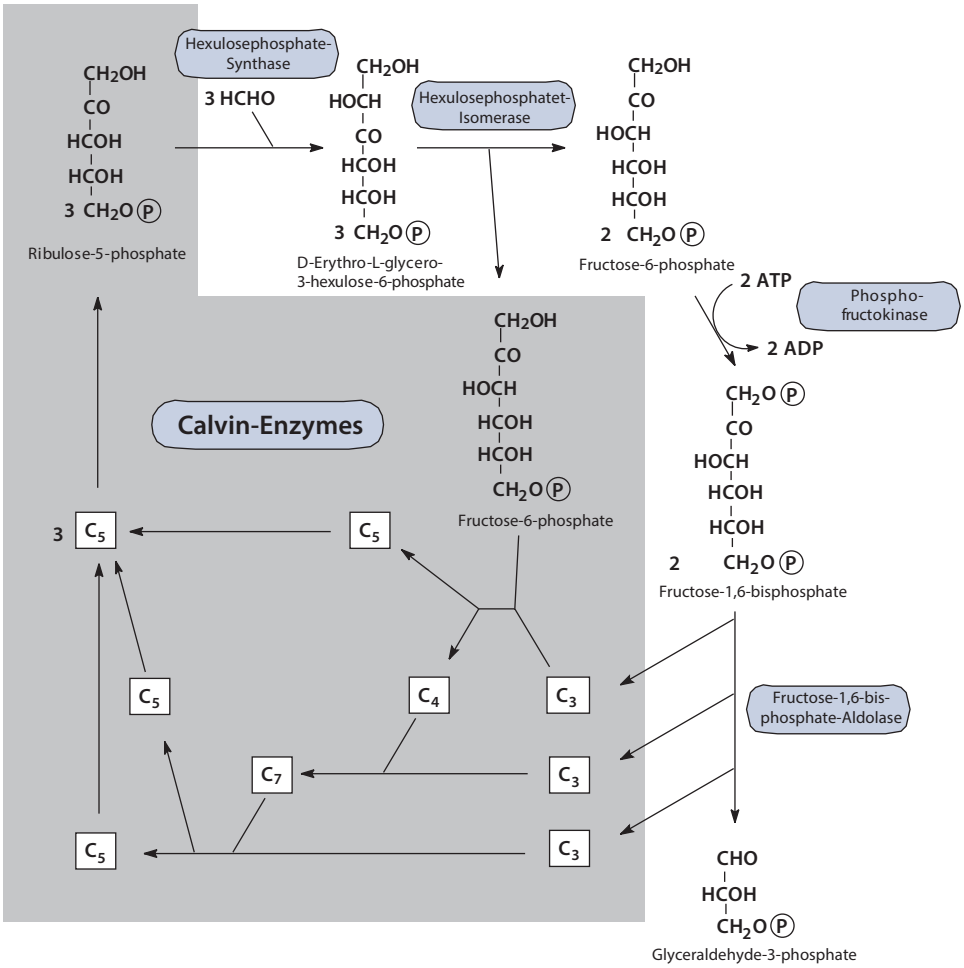


Fig. 4.32 Ribulose monophosphate pathway of assimilation of formaldehyde

Representatives that assimilate C_1 compounds via this pathway are *Methylobacter, Methylococcus* and *Methylomonas.* The above-mentioned methanol-utilizing yeasts possess a xylose monophosphate pathway very similar to the ribulose monophosphate pathway.

Euxinian Environment in the Black Sea

In the case of poor mixing and limited exchange of bottom water, as can occur in largely isolated sedimentary basins, an euxinic, i.e. oxygen-free or reducing, environment develops. Here, too, putrid mud forms, which, after diagenesis has taken place, is called black shale. Such conditions exist today at the bottom of the Black Sea. The dark colour is due to finely distributed carbon and iron sulphide compounds, especially pyrite.

4.5.2.2 Anaerobic Degradation of Methane

Until recently, it was believed that only aerobic bacteria using oxygen as an electron acceptor as well as a substrate for monooxygenase could form significant biomass from methane in natural habitats. However, a growing body of work now makes it clear that anaerobic

oxidation of methane (AOM) occurs in methane-rich sediments, especially in association with methane hydrate areas and methane sources as well as in euxinian water columns.

The net release of methane to the atmosphere is about 500 Tg per year and is projected to increase about 1% annually (Reeburgh, 2007; Cicerone & Oremland, 1988; Simpson et al., 2002).

A net rate of AOM in marine sediments of 70–300 Tg of CH_4 per year was estimated, corresponding to an average consumption of 2 nM per day. More than 99% of the CH_4 present there is consumed by AOM (Reeburgh, 1996, 2007; Cicerone & Oremland, 1988; Simpson et al., 2002).

The main niche for anaerobic methanotrophs on Earth is the so-called "sulfate-methane transition zone" (SMTZ) (Reeburgh, 2007). SMTZs are found at all anoxic aquatic systems where the transport of methane from below and that of sulfate from above creates a niche that allows minimal yield of energy for anaerobic methanotrophs. Methane is completely consumed in the SMTZ, which is found at thicknesses ranging from decimeters to tens of meters below the seafloor, depending on the rate of deposition of reactive organic matter, the depth of the methane production zone, and the rate of transport of methane and sulfate and rates of consumption. That is, AOM occurs at the base of the sulfate-reducing zone.

Table 4.4 The influence of methane partial pressures in the different environments on the free enthalpy

Habitats	CH_4 Partial pressure (atm)	$\Delta G'$ (kJ/mol)
Waters	0.007	–15
Typical sediment	0.35	–25
Submarine springs and eruption channels of volcanoes	20	–35

The influence of the methane concentration on the process of an AOM becomes clear when considering the Gibbs free energy (see Table 4.4).

$$CH_4 + SO_4^{2-}\,(10\text{mM}) \rightarrow HCO_3^-\,(20) + HS^-\,(2\text{mM}) + H_2O\,(\text{Temp.}4°\text{C}, \text{pH}\,7.2)$$

Valentine (2002)

It follows that in anoxic waters AOM is weak compared to that in typical sediments as well as submarine springs and eruption channels of volcanoes. Elevated concentrations of CH_4 in euxinic sedimentary basins compared to oxidized seawater (12 μM in the Black Sea versus nM of normal seawater), on the other hand, allow the higher rates of AOM.

Inputs of CH_4 from sediments, trapped methane sources on the continental margin, and methane-rich mud volcanoes on the deep-sea plain make the Black Sea the world's largest surface water reservoir of dissolved methane, causing high rates of AOM in the anoxic zones there.

AOM is carried out by a consortium of methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB). However, there is also evidence that some archaea oxidize CH_4 without tight coupling to syntrophic partners.

In such consortia, the archaea strain ANME-1 is found, which is distantly related to Methanomicrobiales and *Methanosarcinales*.

Other archaea in the ANME-2 group are closely related to the *Methanosarcinales,* a group of largely methylotrophic methanogens, including all known acetoclastic methanogens. The *Methanosarcinales* have the broadest substrate breadth among the known orders of methanogens, and some

species perform oxidative metabolism (from methyl to CO_2) by dismutation of methylated compounds. ANME-3 is closely related to *Methanococcoides*.

So far, all known ANME-related strains belong to the methanogenic *Euryarchaeota* (Knittel et al., 2005). No single member of these groups has yet been isolated as a pure culture.

It is likely that the organisms use many oxidative steps that are also used in methylotrophic methanogenesis. It is assumed that they produce reduced intermediates such as H_2, acetate or other methylated compounds.

The sulfate-reducing bacteria in the CH_4-oxidizing consortium belong to the *Desulfosarcina/Desulfococcus* a branch of the *Deltaproteobacteria*. These groups of sulfate-reducing bacteria are usually complete oxidizers of organic acids and are often involved in the degradation of hydrocarbons. Although little is yet known about these bacteria and the archaea, it seems reasonable to assume that the bacteria directly reduce sulfate and receive any reduced intermediates from the *archaea*. However, H_2, acetate or methanol are not freely transferable intermediates in the syntrophy of the AOM.

The biochemical pathway of the anaerobic oxidation of methane by the consortium of *Archaea* and SRB remains speculative for now. During AOM, methane is oxidized with equimolar amounts of sulfate, and carbonates and sulfides result accordingly. The generation of basic pH favors the precipitation of calcium ions (10 mM) abundant in seawater as calcium carbonate corresponding to the following net reaction:

$$CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO_3 + H_2S + H_2O$$

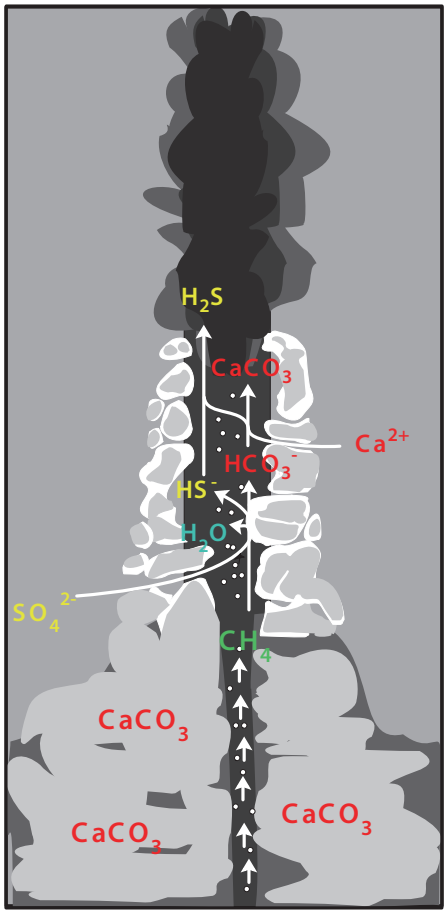
This explains the formation of the black smoker tubes as a common, geologically important subsequent reaction of the anaerobic oxidation of methane at high rates.

The stoichiometry of AOM shows that almost all methane is used for sulfate reduction and little for assimilation of carbon into the cell. This is in contrast to aerobic methanotrophs, which channel up to 60% of methane carbon into biosynthesis (Leak & Dalton, 1986).

Growth of AOM consortia is slow, with generation times of months to years, owed to the low energy yield of the reaction and the fact that only 1% of the total methane consumed is fed directly into biosynthesis.

Despite the restrictive thermodynamic limits, microbially mediated AOM reduces the release of methane from the marine environment (◘ Fig. 4.33).

Anaerobic oxidation of methane is also possible coupled to denitrification of nitrate (Raghoebarsing et al., 2006). Recently, Ettwig



◘ **Fig. 4.33** Chimney of a black smoker. The bacterial mat, consisting of methane oxidizers and sulfate reducers—responsible for AOM—is shown as a white layer on the precipitated calcium carbonate (grey)

et al. (2010), using metagenome analyses and biochemical assays of cultures highly enriched in *Candidatus* Methylomirabilis oxyfera, made it clear that metabolism lies in the grey zone between aerobic and anaerobic methane oxidation.

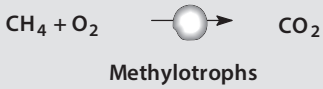
There is some evidence that anaerobic oxidation of methane also occurs coupled to the reduction of manganese (birnessite $(Na_{0.3}Ca_{0.1}K_{0.1})(Mn^{4+}, Mn^{3+})_2O_4 \cdot 1.5\ H_2O$) and iron (ferrihydrite, $(Fe^{3+})_2O_3 \cdot 8\ 0.5H_2O$).

4

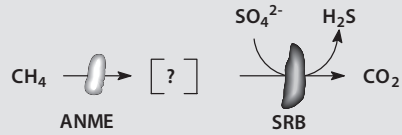
Comparison of the Four Types of Methane Metabolism by Microorganisms

(a) Methanogenesis by anaerobic digestion of organic matter to fermentation products and subsequent H_2 and CO_2 or acetate, which serve as substrates for the utilization of methanogenic Archaea.

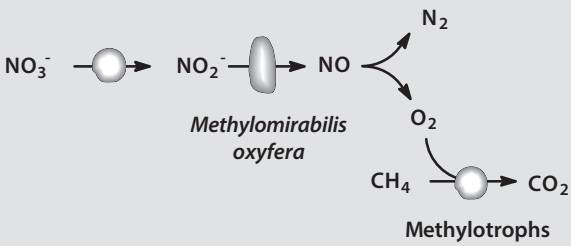
(b) Aerobic oxidation of methane, as carried out by bacteria such as *Methylococcus capsulatus*. They settle at the transition between the methane-rich anoxic and oxygen-rich aerobic phases, where both gases meet.



(c) Reverse methanogenesis: the anaerobic oxidation of methane (AOM) involves an archaeon (designated ANME, Anaerobic Methanotrophic Archaea) related to methanogens. The ANME organism oxidizes methane in a sequence that is the reverse of methane formation in (a). An unknown intermediate [?] is exchanged between the ANME and sulfate-reducing bacteria (SRB). The linkage eventually allows the sulfate reducers to oxidize the carbon of methane to CO_2. The electrons generated by the reaction thereby reduce sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S).



(d) Nitrite-coupled methane oxidation by '*Candidatus* Methylomirabilis oxyfera' as a freshwater methane sink. Nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by other microorganisms. M. oxyfera reduces nitrite to nitric oxide (NO), which is then converted to N_2 and O_2 in a dismutative reaction. The intracellular oxygen is then the oxidant for the normal 'aerobic' reaction pathway of methane oxidation (b), which is initiated by a methane monooxygenase.



Interaction Between the Biogeochemical Cycles of Iron and Methane

Carbon and nitrogen cycles have been dramatically altered by human activities. Methane-producing (methanogenic) and methane-consuming (methanotrophic) microorganisms control the emission of methane, one of the most potent greenhouse gases, into the atmosphere.

Methanotrophic microorganisms that use methane as an electron donor and oxygen as an electron acceptor have long been known. Anaerobic oxidation of methane (AOM) with nitrate and sulfate is also well documented. Nitrite-dependent methane oxidation has been reported recently. In contrast, AOM coupled to the reduction of oxidized metals has only been shown in environmental samples.

Archaea of the order *Methanosarcinales* related to "*Candidatus* Methanoperedens nitroreducens" have now been described that couple the reduction of environmentally relevant particulate forms of iron (Fe^{3+}) and manganese (Mn^{4+}) to methane oxidation.

An enrichment culture of these archaea from the Twentekanaal in the Netherlands was obtained under anaerobic nitrate-reducing conditions with a continuous supply of methane. Batch incubations with [^{13} C] methane demonstrated that soluble iron (Fe^{3+} as Fe citrate) and nanoparticulate forms of Fe^{3+} and Mn^{4+} supported methane oxidizing activity. CO_2 and iron (Fe^{2+}) were prepared in stoichiometric amounts.

Thus, the link between previous findings of iron-dependent AOM and microorganisms detected in numerous habitats worldwide was established. A crucial gap in the understanding of anaerobic methane oxidation was closed.

Brunner et al. (2013), Deutzmann et al. (2014), Egger et al. (2015), Ettwig et al. (2016), Holler et al. (2011), Hu et al. (2014), Riedinger et al. (2014), Shen et al. (2016), Shen et al. (2014), Sivan et al. (2014), Wankel et al. (2012), Welte et al. (2016).

Test Your Knowledge

- What are energy-rich metabolites in glycolysis and TCC? What types of chemical bonding are present?
- Why do organisms produce fermentation products in the absence of external electron acceptors?
- What is a fermentation equilibrium?
- Which fermentation products do you know?
- What steps initiate the degradation of polymers?
- What is the function of the TCC, what is the function of the respiratory chain?
- What is substrate step phosphorylation?
- What is the ATP synthase needed for? What does it need to operate?
- What is the difference between starch and cellulose?
- What organisms are needed to produce methane from cellulose?
- What does syntrophy mean?
- Name substrates of methanogens.
- What are hydrogenotrophic, what are acetoclastic methanogens?
- Acidogenic and acetogenic bacteria are easily confused by their names: What is the metabolism of the two groups?
- Why does only little methane escape into the atmosphere at the continental margins of the oceans?
- Compare methane degradation in oxic and anoxic environments.
- Name CO_2—Fixation Mechanisms.
- What phases can the Calvin cycle be divided into?

- From which plant polymer is methanol released during degradation?
- Which are central metabolites in metabolism?

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Environmental Chemicals

Contents

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"**Environmental chemicals are substances** that are introduced into the environment by human activity and may **occur in quantities or concentrations that** are likely to **endanger humans and their living environment** (animals, plants and also micro-organisms). These include chemical elements or compounds of organic or inorganic nature, synthetic or natural origin. Human intervention may be direct or indirect, intended or unintended" (Umweltbundesamt, 1980).

Only as a result of accidents or environmental damage have harmful effects of chemicals been discovered and made visible. ◘ Table 5.1 summarizes the chronology of chemical-related environmental damage.

5.1 Chemicals in the Environment: Distribution and Concentration

The dispersion of all substances entering the environment takes place through natural **transport processes** (air and water currents) within the "mobile" environmental media, as well as through **transfer processes** between the environmental media or compartments. Chemical and biological **transformation processes** are also important. In transport and transfer processes, the substance remains unchanged, whereas in chemical and biological transformation processes, the structure

◘ **Table 5.1** Environmental chemicals and environmental degradation in public awareness

Period	Environmental chemicals and environmental degradation in public awareness
1950–1960	*DDT:* Damage to reproduction in birds of prey
	Organochlorine pesticides: bioaccumulation in food chains
	Methylmercury: poisoning of people in Minamata (Japan)
1960–1970	*Waste water, detergents, heavy metals:* pollution of waters
1970–1980	*Polychlorinated biphenyls:* bioaccumulation and reproductive damage (marine mammals)
	Polychlorodibenzo-p-dioxins: Seveso disaster with human and environmental exposure
	Acid rain: Acidification of waters and soils
	Detergent ingredients: Pollution of water bodies
	Chemical industry toxic waste landfill: Love Canal, Niagara Falls (USA)
1980–1990	*Air pollutants:* New types of forest damage (forest dieback)
	Organotin compounds: Harm to aquatic organisms
	CFCs: hole in the ozone layer
	Chemical release: death of thousands of inhabitants in Bhopal (India)
	Chemical fire in Basel: poisoning of the Rhine
Since 1970	*Tanker spills:* damage to marine coasts
	Chlorinated solvents: drinking water contamination
Since 1990	*Endocrine disrupting environmental chemicals:* adverse effects on reptiles, fish, marine snails

Modified and supplemented according to Fent (2003)

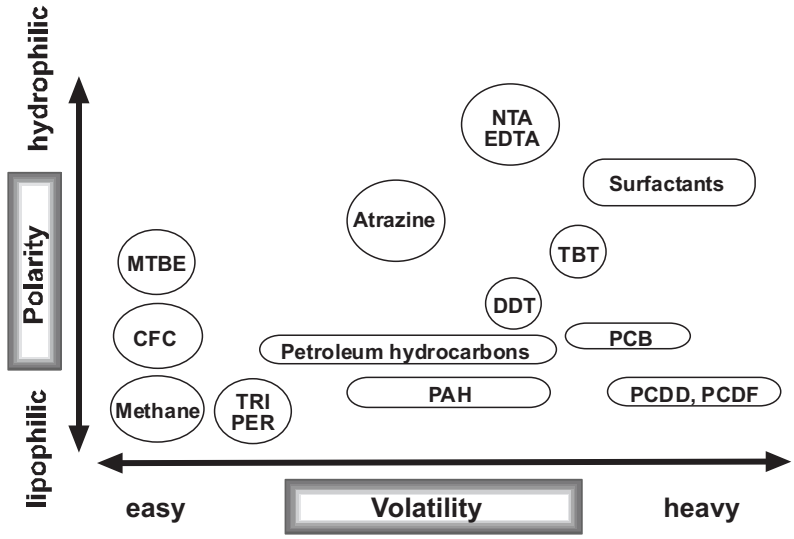
of the substance is changed. When assessing the fate of a substance, these processes must be considered in combination.

Substances spread in the environment when they escape human control during production, use or disposal, or through unintentional formation or release. In extreme cases of high mobility, ubiquitous, worldwide distribution can occur, i.e. the substances also appear in areas that are very far away from the emission areas.

The concentration and distribution of substances in the environment is related to the following variables:

1. Sources, input quantity and characteristics
2. Physico-chemical properties [molecular structure, specific gravity, vapour pressure, water and fat solubility, partition coefficients (octanol/water, air/water, sediment/water), adsorption capacity].
3. Physico-chemical and biological properties of the ecosystem (temperature, pH, salinity, suspended sediment content, sedimentation rate, nutrient cycles, redox ratios)
4. transformation processes (photolysis, hydrolysis, redox reactions and biotic degradation)

If environmental chemicals are classified according to volatility and polarity (◘ Fig. 5.1), the position in the resulting diagram can be used to roughly estimate the behaviour of a substance in the environment. Lipophilic and low-volatility chemicals are problematic substances in living organisms and sediments, while highly volatile ones such as methane, CFCs, trichloro- and tetrachloroethene are those in the air. However, trichloro- and tetrachloroethene are also substances known to be groundwater contaminants due to their higher specific gravity compared to water. They can lie as "Non-Aqueous Phase Liquids (NAPLs)" as a bubble at the bottom of a lake.



◘ **Fig. 5.1** Classification of environmental chemicals according to volatility and polarity. (Modified after Giger, 1995). *DDT* Dichlorodiphenyltrichloroethane, *EDTA* Ethylenediaminetetraacetate, *CFC* Chlorofluorocarbons, *PAH* Polycyclic aromatic hydrocarbons, *PCB* Polychlorinated biphenyls, *PCDD* Polychlorinated dibenzo-1,4-dioxins, *PCDF* Polychlorinated dibenzofurans, *MTBE* Methyl tert.butyl ether, *NTA* Nitrilotriacetate, *TBT* Tributyltin, *TRI* Trichloroethene, *PER* Tetrachloroethene

5.1.1 Transport Processes

5.1.1.1 Transport in the Water Body

In water bodies, chemicals generally spread to a greater or lesser extent by dissolution, adsorption to particles and uptake into organisms. They are distributed differently depending on water solubility, hydrological conditions and characteristics of the environmental system. The compounds are also distributed in different compartments: they can be released from water into the atmosphere and from there in turn reach the soil by deposition. Chemicals, for example, enter water bodies from fields via run-off, the atmosphere via drifting or evaporation, and return to the soil and water bodies with precipitation. If they are highly mobile, they reach groundwater by seepage and enter surface waters by surface runoff. Residual concentrations from sewage treatment plants and landfill leachates, together with surface runoff from agriculture and atmospheric deposition, are significant sources of chemical pollution of water bodies.

In the lake, chemicals are subject to various transport processes. With the inflows, they reach the upper water layer of the lake (see ◘ Fig. 10.18, ► Chap. 10, for stratification). There they are subject to the various water mixing processes. In the temperature-stratified lake (about April to November), they are distributed in the upper layer within a few days. As water drains from the lake, the chemicals are continuously removed. The much slower vertical water mixing transports a small fraction to deeper water layers. With winter circulation (approximately December to March), the remaining chemical is eventually distributed throughout the lake. In addition to these transport processes, gas exchange with the atmosphere, sorption to particles, sedimentation to deeper water layers and into the sediment, and **transformation processes** (chemical and biological degradation) are expected. The elimination processes can be estimated from physico-chemical properties (Henry's constant, sorption coefficient, reaction constants) and system variables (pH of the lake water, particle concentration, wind speed).

5.1.1.2 Atmospheric Transport

Atmospheric transport is a major pathway for the distribution of chemicals in the environment. Mixing and transport processes in the troposphere (see ◘ Fig. 1.2) are rapid, but exchange between the troposphere and stratosphere takes several years because only diffusion occurs. Thus it took years for the chlorofluorocarbons responsible for the destruction of the ozone layer to reach the stratosphere. Atmospheric pollution has assumed global dimensions. For example, **environmental chemicals** can be detected in **polar regions** completely untouched by human civilization. Traces of organic environmental chemicals can be found in the air, snow and ice of the Arctic and Antarctic. These chemicals reach these regions mainly from North America and Europe as gases and aerosols and condense out as a result of the cold temperatures. Highly volatile chemicals remain in the gas phase and are very mobile. Moderately volatile substances condense out and disperse in water, snow, ice and soil of polar regions. The combination of atmospheric transport, condensation and deposition, and re-volatilization from the ocean may explain the transport of organic compounds to polar regions. The atmosphere serves as a transport medium and the ocean as a storage medium. Persistent chemicals disperse from the tropics to the polar regions. The temperature gradient along the transport route to the polar regions causes an accumulation of moderately volatile compounds in the Arctic and Antarctic. We find this gradient clearly in the Pacific: in seawater the concentration of volatiles increases from the equator to 80° north latitude. Atmospheric transport processes also play a major role in the **acidification of** lakes in North America and Scandinavia.

5.1.2 Transfer Processes Between Environmental Media or Compartments

The exchange of chemicals between the individual environmental compartments is controlled by the tendency of the chemicals to enter into **phase transfer processes** (◘ Fig. 5.2). The rate of transfer depends on the properties of the substance and the environmental conditions. The tendency of chemicals to undergo transfer processes can be described by **equilibrium constants** (◘ Fig. 5.3), even though equilibrium will often not occur under real environmental conditions (dynamic, non-closed systems such as flowing waters).

5.1.2.1 Volatilisation: Transport from Water and Soil to Air

The exchange between water or soil and the atmosphere is the most important transport route for chemicals with low water solubility and low polarity. Chemicals with high vapour pressure and low water solubility are rapidly released into the atmosphere. The transfer of a substance by diffusion from water into the air is called **volatilization,** the reverse process is called **(**dry or wet) **deposition.**

The distribution between air and water at equilibrium is described by the Henry coefficient or **Henry constant**. It plays a decisive role for the volatility of chemicals from aqueous solution. The dimensionless constant K'_H represents the concentration ratio between the gas phase (c_g) and the solution (c_w). The constant K_H is often given, which is estimated approximately from the ratio between saturation vapour pressure and maximum water solubility.

$$K'_H = \frac{c_g}{c_w} \qquad K_H = \frac{p_i}{c_w}\left[\text{atm} \cdot \text{L} / \text{mol}\right]$$

P_i—Partial pressure of the compound in the gas phase (air) [atm]

c_g—Concentration of the compound in air [mol/L]

c_w—Concentration of the dissolved compound in water [mol/L]

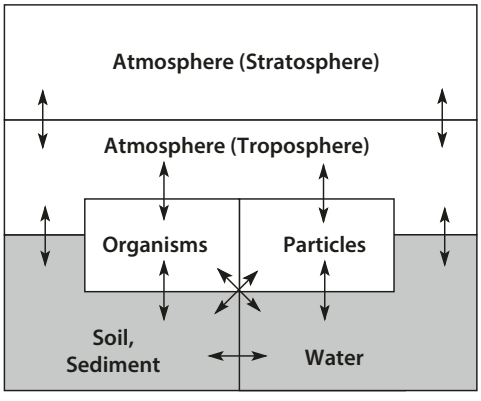
Data are often also given in Pa · m^3/mol, in which case:

$$1\text{atm} \cdot \text{L} / \text{mol} = 101.326\text{pa} \cdot \text{m}^3 / \text{mol}$$

A conversion of both constants is easily done by using the ideal gas law and the resulting relation

$$K'_H = \frac{K_H}{\text{RT}}$$

The greater the Henry coefficient, the faster the substance enters the atmosphere. However, even persistent substances with relatively small K_H are gradually released into the atmosphere due to long residence times in water and soil (for example DDT, PCB, PCDD, PCDF).



◘ **Fig. 5.2** Possible phase transfer processes of chemicals (grey-marked compartments are relevant for microorganisms)

5.1.2.2 Adsorption on Solids: Distribution Between Water and Particles

In water bodies, particles play an important role as adsorbents. The transfer of a compound from the aqueous phase to or into a solid phase (particles or sediment in a lake or river, soil material, aquifer material, activated sludge in a wastewater treatment plant) is called **sorption,** the reverse process

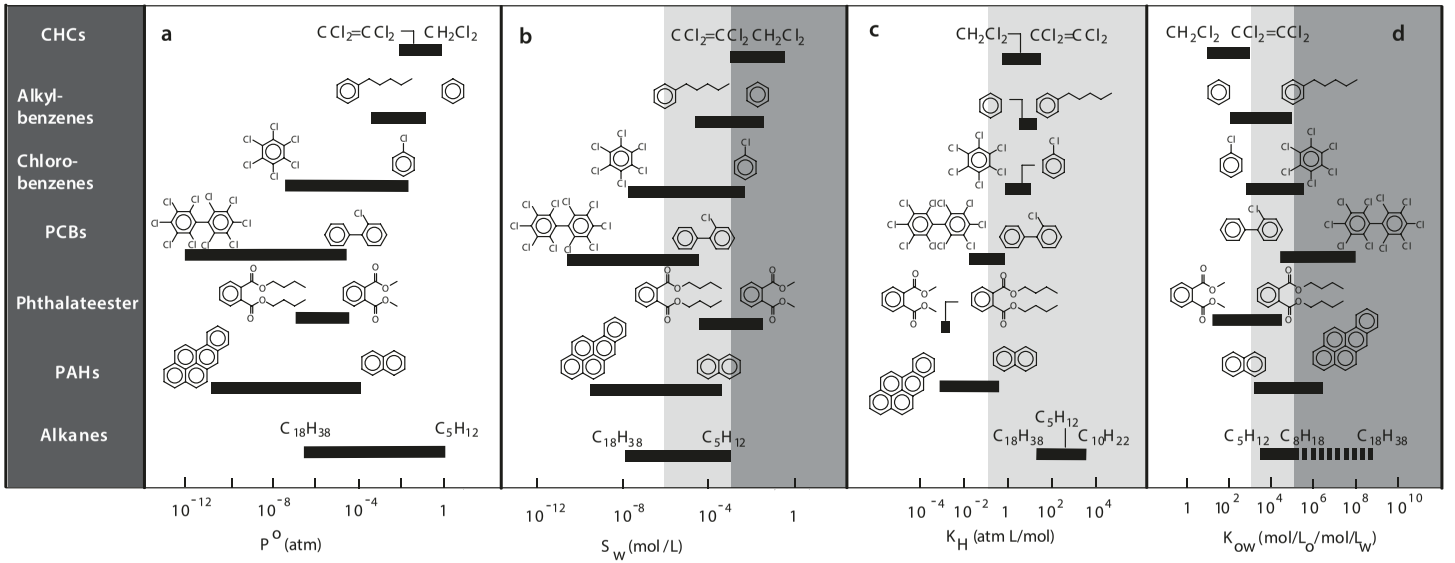


Fig. 5.3 Physicochemical properties of selected environmental chemicals sorted by substance class. (From Schwarzenbach et al., 2003; and Ballschmiter, 1992). **a** Vapour pressure (P^o), **b** Water solubility (S_W) [the plotted shades of grey mark high (dark grey), medium (light grey), poor solubility (white)], **c** Henry's constant (K_H) [preference of the chemical's residence in air (grey), in water (white)], **d** *n*-octanol-water partition coefficient (K_{ow}) [high (dark grey), medium (light grey) and low accumulation tendency of the chemical (white)]. Data can be determined with the program "EPI Suite" (see ► Chap. 18)

desorption. Sorption onto particles and their sedimentation can remove environmental chemicals from the water column of water bodies. In soil, the distribution between water and soil particles is also important for the transport of environmental chemicals. The distribution determines the extent to which a compound is washed off the surface or enters the groundwater.

The mobilization and migration of chemicals in and out of soil or sediment depend largely on the adsorption properties of the chemicals to soil or sediment. Many environmental chemicals are bound to a good extent to the particulate fractions in the water body. Examples are mainly hydrophobic compounds such as polychlorinated hydrocarbons, but also polar substances and heavy metals. Depending on the structure of the environmental chemical and the type and amount of solid phases, different sorption mechanisms may be important. On the one hand, hydrophobic partitioning may occur between the aqueous phase and organic material, and on the other hand, various specific interactions may occur at oxide and clay mineral surfaces.

In general, substances with low water solubility and low vapour pressure preferentially adsorb to particles, sediment and soil components.

The distribution between solid phase and solution is described in equilibrium by the **solid-water distribution constant** K_p

$$K_P = \frac{c_s}{c_w}$$

c_s—Concentration of the sorbed substance

c_w—Concentration of the solved substance

The sorption of hydrophobic compounds to particles, sediment and soil can be related to the lipid solubility (which is inversely proportional to the water solubility of the substances) and the organic carbon content of the sorbing solids: The greater the organic carbon content, the higher the adsorption. For adsorption in soil and sediment, therefore, the adsorption coefficient is often related to the organic carbon content. For all transitions of chemicals between soil and sediment and the aqueous phase, adsorption plays a decisive role. The more a substance is bound to the soil or sediment particles, the less it is mobilized. Many lipophilic substances (such as polycyclic aromatic hydrocarbons) are hardly bioavailable due to their firm binding. As a result, they are poorly degradable and there is a very long residence time in the soil and sediment.

5.1.2.3 Distribution Between Water and Biota: *n*-Octanol/Water Partition Coefficient

The adsorption of hydrophobic chemicals on particles can often be related to lipid solubility (lipophilicity), especially the distribution into organisms. As a measure of lipophilicity, the distribution coefficient K_{ow} of a substance in a mixture of *n*-octanol and water is mostly used today.

$$K_{ow} = \frac{c_o}{c_w}$$

c_o—Concentration in the *n*-octanol phase

c_w—Concentration in the water phase

The distribution coefficient K_{ow} is often given as a logarithm. *n*-octanol is used as a reference because it has similar properties to typical organic substances found in nature (for example humic substances) and to the organisms themselves, above all the cell membranes. The K_{ow} is of great importance as it gives information about the bioaccumulation tendency of substances. Lipophilicity is inversely proportional to the water solubility of a substance.

5.1.3 Transformation Processes

Structural changes of chemicals (**conversion** or **transformation processes**) in the environment are of great importance. The term "sink" is often used for this mechanism of reducing the concentration of an environmental chemical (◘ Table 5.2).

Table 5.2 List of the most important sinks for chemicals

A	**Mainly in the atmosphere:**
1	Indirect photochemical-oxidative degradation in the gas phase, mainly by OH radicals, ozone and nitrogen dioxide
2	Direct photochemical transformation in the atmosphere (also called "photolysis")
B	**Mainly in the water:**
3	Biological—predominantly microbial—oxidative degradation in surface waters
4	Chemical hydrolysis
5	Photochemical degradation in aqueous solution (direct and indirect)
6	Biological degradation/modification by aquatic plants (for example algae)
C	**Predominantly in soil/sediment:**
7	Biological—predominantly microbial—oxidative degradation in the soil
8	Photochemical-oxidative degradation at the surface
9	Degradation/modification by green land plants
10	Anaerobic-biological and reductive-abiotic degradation (for example in anoxic sediments)

5.1.3.1 Abiotic Transformations

Photolysis. Photolytic processes are particularly important in the atmosphere, but are also relevant at the surface of water and soil. By absorbing energy, certain chemicals are converted into an electronically excited state. They thus exhibit a higher reactivity and undergo transformations as a result. We speak of **direct photolysis** when the substance itself absorbs light and is subsequently transformed. **Indirect photolysis**, on the other hand, involves reactions of compounds with reactive species (mainly photooxidants such as hydroxy radicals ($OH^{\bullet}$), peroxy radicals ($R–O–O^{\bullet}$), singlet oxygen $^1O_2$) previously formed by exposure to light. Photolytic reactions depend on the energy of sunlight, the absorption spectrum of the molecule, and substances in the environment that favor these reactions. Many chemicals are degraded by photolytic processes on the surface of plants or soil and on the surface of water bodies.

Chemical hydrolysis (reactions of chemicals with water or hydroxyl ions) can, for example, produce C–OH bonds from C–Cl or C–C single bonds. Hydrolysis is of particular importance in waters, as it strongly determines the residence time of many chemicals. Hydrolysis reactions of organic compounds generally lead to less toxic products. Hydrolysis is strongly temperature- and—depending on the substance—pH-dependent.

Oxidative processes can occur through reactions with molecular oxygen or with reactive oxygen species, which are mostly produced by photochemical processes. In the troposphere, hydroxyl radicals and ozone are of particular importance for the transformation of chemicals. In waters, reactions with peroxy radicals and singlet oxygen are the most important.

Reductive processes occur particularly in the anaerobic environment of sediments and soil. Iron redox systems or porphyrins bound to proteins, which have been released by the decay of biological material, transfer electrons from the reduced organic substrate to the environmental chemicals.

5.1.3.2 Biotic Transformations

Biotic microbial transformation processes in waters, sediments and soil occur with the help of **enzymes.** Less toxic metabolites are usually formed, often followed by complete **mineralization** to carbon dioxide and water. Substances that are difficult or impossible to degrade by biotic processes are usually persistent, since biotic processes in water, sediment and soil probably play a much greater role overall than abiotic ones.

Persistence is the longevity of a chemical compound in the environment. Evidence for

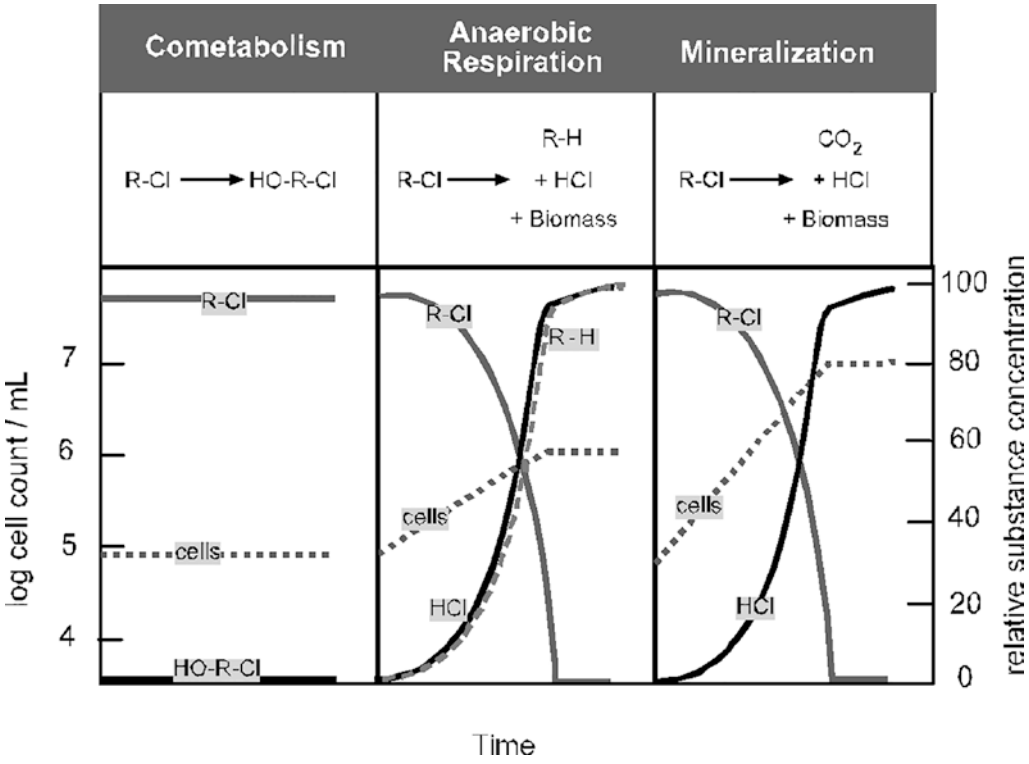
persistence can be obtained from the molecular structure and physico-chemical properties (see ► Sect. 18.2).

The difference between degradable and persistent compounds results from the kinetics of the most important biotic and abiotic degradation or transformation processes (biodegradation in water, soil and sediment, direct and indirect photochemical degradation in the atmosphere and hydrolysis). The kinetics of the transfer processes also play an important role. A substance is persistent if either all of the above degradation processes occur very slowly or not at all, or if a compartment containing an effective sink is generally not reached by the substance or the substance is rapidly removed from it by transfer. This explains the great stability of many substances in the mostly anaerobic sediments, although effective sinks are present in the air or even in the aerobic water body.

From a physiological point of view, the microbial degradation of environmental chemicals can be differentiated as follows (see ◘ Fig. 5.4):

The type of **complete metabolisation** involves the most extensive degradation and has been described for many substance groups only in bacteria. In this process, the substance in question is usually used by the microorganisms as a source of carbon and energy, i.e. biomass and CO_2 and H_2O are produced from the substance. During complete metabolization, small amounts of metabolites may temporarily accumulate. These metabolites are also found outside the cells under certain conditions.

Another productive degradation associated with biomass formation is the use of



◘ **Fig. 5.4** Comparison of degradation by cometabolism, use as an electron acceptor with dehalogenation and as a carbon and energy source using the example of a model compound (R = benzoate: 3-chlorobenzoate)

some environmental chemicals as **electron acceptors.** This metabolism has been shown for chlorinated chemicals, with dechlorination and usually accumulation of a dechlorinated product.

Cometabolic transformation has been described for a whole range of environmental chemicals. The essential criterion for this type of degradation is that the corresponding microorganisms cannot use the compounds as their sole source of carbon and energy or grow with them. Apart from a possible detoxification function, this type of degradation has no apparent growth advantage for the organisms. The organisms are therefore dependent on additional substrates to maintain their metabolism and to grow. For this type of "degradation", practically all known degradation pathways that do not lead to growth of the organisms (unproductive degradation) come into consideration. In part, primary oxidation products are formed, which can possibly be further metabolized by other organisms.

Another way of metabolizing environmental chemicals is by **non-specific radical oxidation.** This type is made possible by the properties of the ligninolytic enzyme systems of white rot fungi (see ► Sect. 4.4.4.5). In the degradation of wood, which is relatively persistent in nature, these organisms are specialised in the degradation of lignin. Due to the macromolecular structure and the lack of water solubility of lignin, degradation can only take place via extracellular enzymes. With these enzyme systems (lignin, manganese peroxidases and laccases), fungi are able to depolymerize lignin by radical-catalyzed cleavage reactions. These non-specific oxidation reactions predestine this group of fungi for the biodegradation of particularly persistent environmental pollutants.

REACH, the *New* EU Chemicals Regulation

The system used until 2007 for general industrial chemicals distinguishes between "existing chemicals", i.e. all chemicals declared to be already on the market in September 1981, and "new chemicals", i.e. those placed on the market after that date.

There are approximately 2700 new substances. These must be tested and assessed for their risk to human health and the environment in accordance with EU Directive 67/548 before they are placed on the market in quantities above 10 kg. For larger quantities, more thorough testing is required, focusing specifically on long-term and chronic effects.

In contrast, existing substances, which represented more than 99% of the total quantity of all substances on the market, were not subject to the same testing requirements. In 1981, a total of 100,106 substances were notified and it is estimated that 30,000 of these substances are still placed on the market in quantities of more than one tonne. About 140 of these substances are classified as priority substances and are subject to extensive risk assessments by Member State authorities.

Regulation (EC) No 1907/2006 of **the European Parliament and of the Council of 18 December 2006 states** that existing and new substances should be subject to the same procedure under a **single system**. Under the EU chemicals regulation REACH (an acronym for **Registration, Evaluation and Authorisation of Chemicals**), which came into force in 2007, manufacturers and importers of chemicals must prepare dossiers for their substances to demonstrate safe use. The

authority is the European Chemicals Agency (ECHA) in Helsinki.

The requirements, including testing requirements, of the REACH system depend on the proven or suspected harmful properties, uses, exposure and quantities of chemicals produced or imported. All chemicals placed on the market in quantities above one tonne are to be registered in a central database. For larger quantities, particular attention should be paid to long-term and chronic effects.

REACH fundamentally harmonises and simplifies the existing chemicals legislation. REACH relies on the industry's own responsibility. ECHA checks the dossiers for plausibility. National authorities in all EU states are to examine the dossiers of these substances in detail. In Germany, these are the Federal Institute for Occupational Safety and Health (BAuA), the Federal Institute for Risk Assessment (BfR) and the Federal Environment Agency (UBA).

Dossier evaluation: For every 20th registration dossier, the EU chemicals agency ECHA checks whether the dossiers submitted are complete and whether the animal tests proposed by manufacturers are appropriate and necessary. If dossiers are incomplete or incorrect, chemical manufacturers and importers must make improvements. However, ECHA cannot put companies under pressure—for example, by threatening to withdraw registration and thus the marketing opportunity.

Substance evaluation: National authorities review dossiers of individual substances. They decide whether the use of these substances should be regulated or whether further information should be requested in order to be able to evaluate them. Substance evaluation also plays a role in detecting and improving deficient dossiers.

Substances of Very High Concern (SVHCs)

SVHCs are chemical compounds (or part of a group of chemical compounds) identified under the **REACH Regulation** as having particularly hazardous properties. Substances are or will be included in the **candidate list** where the authorisation procedure has the long-term objective of progressively replacing them with less dangerous substances or technologies, provided that technically or economically viable alternatives are available (◘ Table 5.3).

Article 57 of the REACH Regulation

The following substances may be included in Annex XIV in accordance with the procedure referred to in Article 58:

(a) Substances meeting the criteria for classification as **carcinogenic** category 1A or 1B in accordance with Regulation (EC) No 1272/2008;
(b) Substances meeting the criteria for classification as **mutagenic** category 1A or 1B in accordance with Regulation (EC) No 1272/2008;
(c) Substances meeting the criteria for classification as **toxic for reproduction** category 1A or 1B in accordance with Regulation (EC) No 1272/2008;
(d) Substances that are **persistent, bioaccumulative** and **toxic (PBT substances)** in accordance with the criteria set out in Annex XIII to this Regulation;
(e) Substances that are **very persistent** and **very bioaccumulative (vPvB substances)** in accordance with the criteria set out in Annex XIII to this Regulation;
(f) substances, such as those with endocrine disrupting properties or those with persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative proper-

Table 5.3 Selected substances from the candidate list with particularly hazardous properties addressed in this book from various points of view

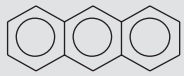
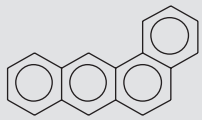
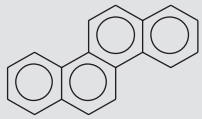
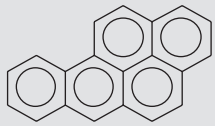
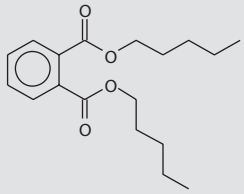
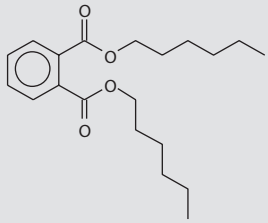
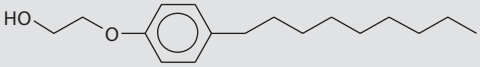
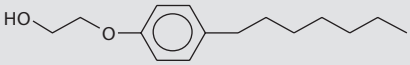
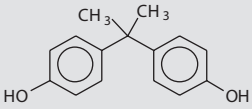
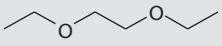
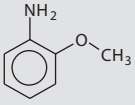
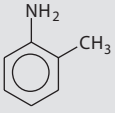
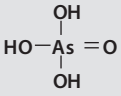
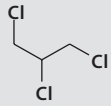
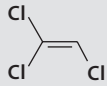
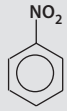
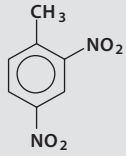
Substance	Property or properties referred to in Article 57
Anthracene 	PBT (Article 57d)
Anthracene oil	Carcinogenic (Article 57a) PBT (Article 57d) vPvB (Article 57e)
Benzo[a]anthracene 	Carcinogenic (Article 57a) PBT (Article 57d) vPvB (Article 57e)
Chrysen 	Carcinogenic (Article 57a) PBT (Article 57d) vPvB (Article 57e)
Benzo[a]pyrene 	Carcinogenic (Article 57a) Mutagenic (Article 57b) Toxic for reproduction (Article 57c) PBT (Article 57d) vPvB (Article 57e)
Furan 	Carcinogenic (Article 57a)
Dipentyl phthalate (DPP) 	Toxic to reproduction (Article 57c)
Dihexyl phthalate 	Toxic to reproduction (Article 57c)

Table 5.3 (continued)

Substance	Property or properties referred to in Article 57
4-Nonylphenol  Substances having a linear and/or branched alkyl chain with a carbon number of 9 covalently bonded at the 4-position to phenol, ethoxylated UVCB[a] and well-defined substances, polymers and homologues including each of the individual isomers and/or combinations thereof	Endocrine disrupting properties (Article 57(f) – Environment)
4-Heptylphenol  Substances having a linear and/or branched alkyl chain with a carbon number of 7 covalently bonded at the 4-position to phenol, ethoxylated UVCB and well-defined substances, polymers and homologues including each of the individual isomers and/or combinations thereof	Endocrine disrupting properties (Article 57(f) – Environment)
4,4′-Isopropylidenediphenol, bisphenol A; BPA 	Toxic to reproduction (Article 57c) Endocrine disrupting properties (Article 57(f) – Environment) Endocrine disrupting properties (Article 57(f) – human health)
1,2-Diethoxyethane 	Toxic for reproduction (Article 57c)
Methyloxirane (propylene oxide) 	Carcinogenic (Article 57a) Mutagenic (Article 57b)
2-Methoxyaniline, o-anisidine 	Carcinogenic (Article 57a)
o-Toluidine 	Carcinogenic (Article 57a)
Arsenic acid 	Carcinogenic (Article 57a)

(continued)

Table 5.3 (continued)

Substance	Property or properties referred to in Article 57
1,2-Dichloroethane 	Carcinogenic (Article 57a)
1,2,3-Trichloropropane 	Carcinogenic (Article 57a) Toxic to reproduction (Article 57c)
Trichloroethylene 	Carcinogenic (Article 57a)
Nitrobenzene 	Toxic to reproduction (Article 57c)
2,4-Dinitrotoluene 	Carcinogenic (Article 57a)

► https://echa.europa.eu/de/substances-of-very-high-concern-identification-explained

[a]**UVCB:** a UVCB substance is a chemical substance with an unknown or variable composition or a complex reaction product or biological material

ties, which do not meet the criteria in points (d) or (e), which, according to scientific evidence, are likely to have serious effects on human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure laid down in Article 59.

5.2 Assessment of Chemicals: General Principles and Concepts

The assessment of the environmental risk of chemicals and their evaluation is becoming increasingly important in practical environmental protection and environmental liability, especially since the Environmental Liability Act of 1991 came **into** force. According to the Chemicals Act (ChemG), there is an **obligation to test substances and**

products that are to be placed on the market for the first time, which is intended to allow the toxicological effect profile of a substance and its possible danger to the environment to be assessed before marketing. A distinction is thus made between substances newly placed on the market and existing substances: The notification requirement only applies to **new** substances, but it can be extended to particularly dangerous existing substances. Medicinal products and plant protection products were already subject to corresponding authorisation procedures in the past. New substances are all substances that have been placed on the market since 18.09.1981; approx. 100,000 existing substances were already in use before this date (listed in the European Inventory of Existing Commercial Chemicals, EINECS).

The **legal regulations** serve to protect humans, animals and plants, their biocoenoses and habitats (water, soil, air) from harmful effects caused by the handling of environmentally hazardous substances.

In addition to the degradation, dispersal and accumulation behaviour, the effects on selected organisms are to be examined. Test procedures are approved within the framework of the OECD (Organization for Economic Co-operation and Development, i.e. an international organization of industrialized countries) and by corresponding environmental protection agencies (USEPA, Environmental Protection Agency of the USA; UBA, Federal Environmental Agency Berlin; MITI, Ministry of International Trade and Industry, Japan). Standardized toxicity tests are also used for the classification and categorization of chemicals in water hazard classes.

Since 1981, the OECD has drawn up guidelines for testing chemicals (Chemical Testing Programme, 1979/1980, Premarket Testing System: Guidelines for Testing of Chemicals), which are continuously supplemented. These **standards,** which describe the organisms as well as the performance, conditions and evaluation of the tests, have contributed significantly to the emergence and harmonisation of the notification procedures for new substances. They are divided into four sub-areas:

1. Physico-chemical properties
2. Degradation and accumulation
3. Effects on biological systems
4. effects on human health (not discussed here)

Points 2 concerning environmental microbiology and parts of 3 are discussed here.

Standardized test methods are used to collect data on toxicity and ecotoxicity. The principle of the **German Chemicals Act** (ChemG) is based on a gradual increase in the test requirements with the production quantity (basic level, level I and level II). The amount of testing required thus increases with the quantity of a substance produced and the expected hazard (Box ► Step-By-Step Concept of the German Chemicals Act). REACH is structured in a similar way.

In addition to the ChemG, the German **Water Resources Act** (WHG) imposes extensive requirements on ecotoxicology data. The WHG mostly uses sum parameters (AOX, BOD, DOC and others) to regulate emissions of organic substances, which are predominantly present in a mixture in wastewater, whereas the ChemG refers to individual substances.

For practical purposes, the classification of chemicals according to their hazardousness plays an important role. One measure for assessing the toxic potential of a compound is the **water hazard class (WGK)** introduced in Germany**,** determined from the acute toxicity (rat, bacterial and fish toxicity), the biodegradability and the distribution behaviour (as *n*-octanol/water distribution coefficient K_{ow}). The effect on aquatic organisms is expressed as the water hazard number WGZ (negative logarithm of the toxicity threshold). Four water hazard classes are distinguished (■ Table 5.4).

Many of the pollutants to be detected in water have an ecotoxic potential in concentrations significantly <1 mg/L, which is not covered by a specific WGK.

Table 5.4 Water hazard classes and water hazard number with allocation of various petroleum fractions and generally known environmental chemicals

Water hazard classes (WGK)	Water hazard index (WGZ)	Examples of chemicals
0: generally not hazardous to water	0–1.9	
1: slightly hazardous to water	2–3.9	Crude oils (viscous and solid), fuel oil (heavy)
2: hazardous to water	4–5.9	Petrol, diesel fuel, heating oil (light), crude oils (light) Phenol, toluene, aniline
3: highly hazardous to water	≥6	Waste oils Benzene, 1,2,4-trichlorobenzene, Benzo[a]pyrene, tetrachloroethene, trichloroethene

Step-By-Step Concept of the German Chemicals Act

Production and marketing quantities determine which tests must be carried out in accordance with the Chemicals Act. If less than 1 tonne per year is placed on the market, the chemical does not have to be notified. For higher quantities, a three-tier system applies:

- **Basic level:** from 1 t/year or a total of 50 t
- **Stage I:** from 100 t/year or a total of 500 t
- **Stage II:** from 1000 t/year or a total of 5000 t
- The following data is required, with additional data to be provided at the next higher level:

Basic

1. Physico-chemical properties
2. Acute mammalian toxicity (rat), LD_{50} value
3. Subacute toxicity
4. Skin and eye irritation and sensitisation (hypersensitivity reaction) in mammals
5. Mutagenicity
6. Abiotic and biotic degradability
7. Acute toxicity (LC_{50}) for *Daphnia magna* (24–48 h)
8. Acute toxicity (LC_{50}) to fish (48–96 h)

Stage I

1. Subchronic mammalian toxicity
2. Influence on mammalian fertility (tests over 1–2 generations)
3. Cancerogenicity and teratogenicity (mammals)
4. Long-term biotic degradability (for poorly degradable basic stage substances)
5. Growth test (72 h) for unicellular green algae *(Scenedesmus subspicatus)*
6. Chronic toxicity (21 days) to *Daphnia magna* (mortality, reproduction), NOEL
7. Chronic toxicity (14–21 days) to fish (*Brachydanio rerio*) (lethal and sublethal effects)
8. Plant toxicity: growth inhibition of seeds for 14 days (oats, beet)
9. Earthworm toxicity (*Eisenja foetida*): Lethal effect during 14 days

Stage II

1. Toxicokinetics and biotransformation (mammals)
2. Chronic toxicity (mammalian)
3. Cancerogenicity (long-term study) (mammalian)
4. Behavioural toxicity (mammalian)
5. Fertility alteration and damage (3 generation test) (mammals)
6. Environmental fate: Mobility and additional degradability test

7. Bioaccumulation (fish)
8. Long-term toxicity to fish (zebrafish, *Brachydanio rerio*) including effect on reproduction
9. At bioconcentration factor >100, acute and subacute toxicity to birds.
10. Additional toxicity test on other organisms (clawed frog, springtails)

5.2.1 Degradability Tests

Knowledge of biodegradability is a key criterion for assessing the environmental relevance of substances and substance mixtures. Degradation tests can be used to determine whether substances are likely to persist in the environment and thus also represent a risk potential in the long term.

Due to the large number of chemicals used in society, a cost-effective approach is needed that provides adequate knowledge for decision-making on environmental protection. Ideally, a system of simple tests for "ultimate biodegradability" (degradation of a substance to CO_2) is used to identify, through preliminary screening, those chemicals for which further data and thus more costly tests are required. Degradability is currently determined mainly in the presence of oxygen, while anaerobic tests are still being developed.

A distinction is made between test methods for determining **ultimate degradability** (mineralisation under oxygen consumption to CO_2) and **eliminability** (decrease in sum parameters such as COD or DOC). By contrast, substance-specific analysis is used to determine **primary degradation.**

5.2.1.1 Methods for Tracking Substance Turnover

In principle, the following process takes place during the aerobic degradation of a substance:

$$\text{Substrate} + \text{Oxygen} \rightarrow \text{Biomass} + \text{Products of degradation}\left(CO_2, \text{chloride and others}\right)$$

This results in the following possibilities for tracking the degradation of a chemical, which are applied separately or in combination, depending on the experimental setup:

- Measurement of the concentration of the test substance.
- Measurement of oxygen consumption.
- Measurement of carbon dioxide evolution.
- Measurement of other degradation products (intermediates) (for example chloride in the case of chlorinated hydrocarbons).

For the **tracking of the concentration decrease of a test substance** all specific methods of chemistry (HPLC, HPLC-MS, GC, GC-MS and others) are to be mentioned. However, the determined decrease of a substance only tells something about the **primary degradation** and nothing about the final degradation (complete metabolization). Today, however, it is generally required that complete degradation takes place, since intermediates that are difficult to degrade or environmentally problematic may still be formed after primary degradation.

A variety of methods are available for **tracking the decrease in concentration by means of sum parameters.** The **determination of chemical oxygen demand** (COD) is a commonly used method. This generally uses the consumption of potassium dichromate in hot sulphuric acid solution or another strong oxidant such as potassium permanganate as a measure of oxidisable carbon. Problems with this are that other oxidizable substances (such as NH_4^+) give incorrect values. Also, different values are obtained depending on the degree of oxidation of the compound. The oxidation is not complete for some substances (for example glycerol, pyridine, nicotinic acid). Otherwise, the determination of COD (expressed in g of oxygen per g of

substance) is a method to determine the degree of mineralisation based on the concentration of organic substances.

Furthermore, the **theoretical oxygen demand** (ThOD) is determined. It is the total amount of oxygen required for the complete oxidation of a substance. It is calculated from the empirical formula and expressed as g oxygen demand per g test substance.

Other determination methods analyze the carbon present. **Total organic carbon** (TOC) is the sum of the organic carbon in solution and in suspension in a sample. Here, the carbon in the sample is completely converted into CO_2 or CH_4. These are then determined (measuring range up to approx. 1 mg/L). In the case of TOC, as with COD, the concentration or the degree of degradation is determined, since metabolites with the starting compound give identical values, provided that no carbon has been split off. In contrast to COD, the degree of oxidation of the compound has no influence on TOC.

In the determination of **dissolved organic carbon** (**DOC**), the sample is previously filtered (membrane filter 0.45 µm pores) or centrifuged for 15 min, so that only the elimination of the substance from the aqueous phase is determined. The substance may also have been adsorbed, precipitated or incorporated into biomass. However, experience with the OECD screening test shows that adsorption or flocculation are only of secondary importance and that, as a rule, a DOC decrease of soluble compounds is due to conversion into CO_2 and biomass.

Biological oxygen demand (BOD) can be used as a measure of the oxidative degradation of a substance. The consumption of oxygen is measured. The degree of degradation is then calculated from BOD/COD or BOD/TOD. Nitrification processes, which also consume oxygen, are disturbing.

The biological oxygen demand during aerobic degradation for five days at 20 °C without exposure to light is referred to as BOD_5. **BOD** is generally expressed in g of oxygen per g of substance. For readily biodegradable substances it is approximately as high as the COD, for poorly biodegradable substances it is significantly lower.

There are several methods for measuring oxygen:

1. Start-end measurement in completely filled, closed vessel by titration.
2. Use of an oxygen electrode in a closed vessel.
3. Use of a Sapromat, which indicates the consumption on the basis of the oxygen to be regenerated.

Another determination using sum parameters involves the development of CO_2. It is a measure of the mineralisation of a substance and is therefore very suitable as a measure of ultimate degradability in die-away tests where the test substance is the only carbon source. CO_2 can be absorbed by KOH or NaOH solution, for example. By back titration after the end of the test, remaining caustic and thus formed CO_2 can be determined. Alternatively, precipitated $BaCO_3$ can be determined. The measurement of CO_2 formation is not suitable for tests with complex substrates.

Theoretical carbon dioxide (**$ThCO_2$**) is the amount of carbon dioxide calculated from the known or measured carbon content of the test substance at complete mineralisation. It is also expressed as g of carbon dioxide per g of test substance.

For some compounds, degradation can be determined by tracking **specific inorganic ions,** for example chloride from chlorinated hydrocarbons, nitrate from nitro compounds, sulphite from sulphonates or phosphate from some herbicides. However, this method only tells us something about the removal of the specific group in question, but nothing about complete degradation.

The use of **radiolabelled compounds** is often the only way to fully account for the fate of a compound in complex systems. This involves tracking the radioactivity in the various compartments (CO_2, biomass and others).

5.2.1.2 OECD Testing Strategy

In the evaluation of substances according to the Chemicals Act and the Plant Protection Act as well as in the classification of chemicals into the "hazard characteristic environmentally hazardous" or into water hazard classes (WGK), the OECD test strategy is applied. The tests for degradability of chemicals are organised in such a way that the three-stage testing strategy includes different complexity, reality of the environment and also costs:

- Aerobic degradability is first investigated by means of screening tests for **"ready biodegradability".**
- In the case of a negative result in the "ready degradability" test, the degradability of a chemical is tested in a **simulation test** to obtain data describing degradation rates in the environment. Alternatively, or as a supplement, screening tests for **"inherent biodegradability"** are performed to obtain results indicating "ultimate degradability" under optimal aerobic conditions, such as in a biological sewage treatment plant (STP).

Finally, the "possible degradability under anaerobic conditions" is investigated by means of screening tests for anaerobic degradability.

Terms Used in the Assessment of Chemicals for Microbial Degradability

"Primary biodegradability" (or "partial biodegradability") is the change in structure of a chemical by microorganisms associated with its disappearance.

"Ultimate biodegradability" (or "total biodegradability") means complete mineralization and assimilation. The chemical is completely degraded by the microorganisms, producing CO_2 (under aerobic conditions) or methanes (under anaerobic conditions), as well as mineral salts and biomass. This means that there is no residue of the degradation or accumulation of transformation products.

"Ready biodegradable" is determined in narrowly defined tests, which offer limited opportunity for biodegradation and adaptation. **Ready biodegradability** means ready degradability under aerobic conditions, specifying that the test substance must be practically completely degraded in 28 days.

"Inherently biodegradable" is determined in those microbial tests that expose a test compound to microorganisms for an extended period of time or provide other conditions favorable for degradation.

The **"potential degradability"** is determined under optimal aerobic conditions, without time limit. The degradation shown under the optimised conditions does not necessarily occur under normal test conditions.

■ Tests for "Ready Degradability"

These tests are performed under aerobic conditions and are very stringent. In them, **high concentrations of the test substance** (in the range of 2–100 mg/L) are used. The degradation rate is determined by means of non-specific parameters such as Dissolved Organic Carbon (DOC), Biochemical Oxygen Demand (BOD) and CO_2.

Tests for "ready degradability" must be designed so that a positive result is unequivocal. If there is a positive result in a "ready degradability" test, it can be assumed that a chemical is subject to rapid and "complete mineralisation" in nature. In such a case, no further investigation of a chemical's degradability or potential environmental problems from resulting transformation products is normally necessary. However, the fact that a chemical has been found to be "readily degradable" does not preclude concerns about degradation rates and transfor-

mation products in the event of a high influx into an ecosystem.

A negative result in a 'ready degradability' test does not necessarily mean that the chemical will not degrade under relevant environmental conditions, but it does mean that it should be taken to the next stage of testing, either a simulation test or a 'possible degradability' test.

5

Six tests that can be used to determine the "ready degradability" of organic chemicals are described in the OECD Test Guidelines: DOC Die-Away Test, CO_2 Evolution Test, Modified MITI Test (I), Closed Bottle Test, Modified OECD Screening Test and Manometric Respirometry Test (◘ Table 5.5).

A typical approach with the necessary parallel vessels is shown in ◘ Fig. 5.5 for the modified OECD screening test.

A chemical is rated as "readily degradable" if the **arbitrarily defined threshold value** for degradation is reached (see ◘ Table 5.4). The threshold must be reached in the 10-day window within the 28-day duration of the test. The 10-day window begins when the degradation level has reached 10% DOC, ThOD or $ThCO_2$. It must be completed before the end of the 28-day test. As an example, ◘ Fig. 5.6 shows a degradation curve with the relevant parameters.

If, in a toxicity control with test and reference substances, less than 35% degradation (DOC) or less than 25% (ThOD or $ThCO_2$) is achieved within 14 days, an inhibitory effect of the test substance can be assumed and the test series must be repeated with a lower concentration of the test substance and/or a higher concentration of the inoculum.

The standardised test duration is 28 days, although tests may be extended beyond this 28-day period if degradation has started and the plateau has not yet been reached. However, only the degree of degradation within the 28 days should be used for the estimation of "ready degradability".

Thresholds of either 60% (ThOD or $ThCO_2$) or 70% DOC mean that the test substance is practically completely degraded, as the remaining amount of test substance of 30–40% goes into biomass.

The test guidelines addressed are similar in many respects: In all tests, the test substance—offered as the sole source of carbon and energy (with the exception of biomass carbon)—is diluted in the medium in such a way that a **relatively low concentration of biomass is** produced. In all tests, a **non-specific analysis method** is used for the course of degradation. This has the advantage that the methods can be used for a wide range of different organic compounds and there is no need to develop specific analytical methods. Since these methods also respond to degradation residues or transformation products, an indication of the extent of "complete mineralization" is provided.

It has been found that standardisation of the inoculum increases the comparability of the methods. However, it was noted that this is not possible without significantly reducing the number of species in the test system. A mixed inoculum is therefore proposed to have a variance of degrading organisms in the test. To ensure stringent conditions in the tests, it was decided **not to** allow preadaptation of the inoculum to the test substance.

All the methods mentioned above are fresh water tests. For the marine environment, the OECD test TG 306 "Biodegradability in Seawater" is proposed, which includes the seawater variant of the Closed Bottle Test (TG 301 D) and the Modified OECD Screening Test (TG 301 E). It has been found that degradation of organic chemicals in seawater is slower than that in fresh water, sewage sludge and sewage treatment plant effluent. A positive result in 28 days in a "Biodegradability in Seawater test" (>60% ThOD; >70% DOC) is therefore a very clear indication of "ready degradability".

Table 5.5 Overview of degradation methods for substance testing (the OECD designations as well as those commonly used in the EU are listed)

Name of the test	Guidelines		Model for	Test substance concentration (TS) (mg per L test)	Limit value for evaluation of degradability or measurement parameter	Cells per L; test inoculum	Test duration (in days)	Applicability for	
	OECD	EU						Poorly volatile substance	Soluble
Test for ready biodegradability									
DOC DIE-AWAY Test	301 A	C.4-A		10–40 DOC	70% DOC	10^4–10^5, sewage sludge, sewage treatment plant effluent, surface water, soil or mixture	28	–	–
CO_2 Evolution Test (Modified Storm Test)	301 B	C.4-C	Surface water	10–20 DOC or TOC	60% $ThCO_2$	10^4–10^5, sewage sludge, sewage treatment plant effluent, surface water, soil or mixture	28	+	–
M.I.T.I. Test (I) (Icon)	301 C	C.4-F		100 TS	60% ThOD	10^4–10^5, mixture from 10 different locations, incl. industrial wastewater treatment plant outlet, adjustment in laboratory 1–3 months	28	–	±
Closed Bottle Test (Icon)	301 D	C.4-E	Surface water	2–10 TS = 5–10 ThOD	60% ThOD	10–10^3 , secondary wastewater treatment plant effluent (municipal) and/or surface water	28	±	+
Modified OECD screening test	301 E	C.4-B	Surface water	5–40 DOC	70% DOC	10^2, secondary wastewater treatment plant outfall (municipal)	28	–	–

(continued)

Table 5.5 (continued)

Name of the test	Guidelines		Model for	Test substance concentration (TS) (mg per L test)	Limit value for evaluation of degradability or measurement parameter	Cells per L; test inoculum	Test duration (in days)	Applicability for	
	OECD	EU						Poorly volatile substance	Soluble
Manometric respiration test	301 F	C.4-D		100 TS = 50–100 ThOD	60% ThOD	10^4–10^5, sewage sludge, sewage treatment plant effluent, surface water, soil or mixture	28	+	±
Test for potential inherent biodegradability									
Semi Continuous Activated Sludge Test (SCAS)	302 A	C.12		20 TS	DOC	>10^5, sewage sludge from a suitable plant, adaptation not allowed	approx. 180	–	–
Mod. Stand test according to Zahn-Wellens/EMPA	302 B	C.9	Industrial wastewater treatment plant	50–400 DOC = 10–100 TS	DOC	Sewage sludge, adjustment not allowed	28	–	–
Mod. M.I.T.I. Test (II)	302 C			30 TS	BOD	10^4–10^5, mixture from 10 different locations, incl. industrial wastewater treatment plant outlet, adjustment in laboratory 1–3 months		+	–

Simulation test									
Coupled Units Test (Waste Water Treatment Simulation Test) OECD Confirmation Test: Activated sludge	303 A	C.10	Municipal wastewater treatment plant	10–20 DOC	DOC/special analytics		approx. 180		
Coupled Units Test (Waste Water Treatment Simulation Test) OECD Confirmation Test: Biofilm	303 B								
Aerobic and anaerobic transformation in soil	307			1–100 µg					
Aerobic and anaerobic transformation in aquatic sediment systems	308			1–100 µg					

Functional check with diethyl glycol or aniline. If no entry, then the tests are not yet official or in sample, or information not necessary (Icon)are referred to as the two most stringent tests

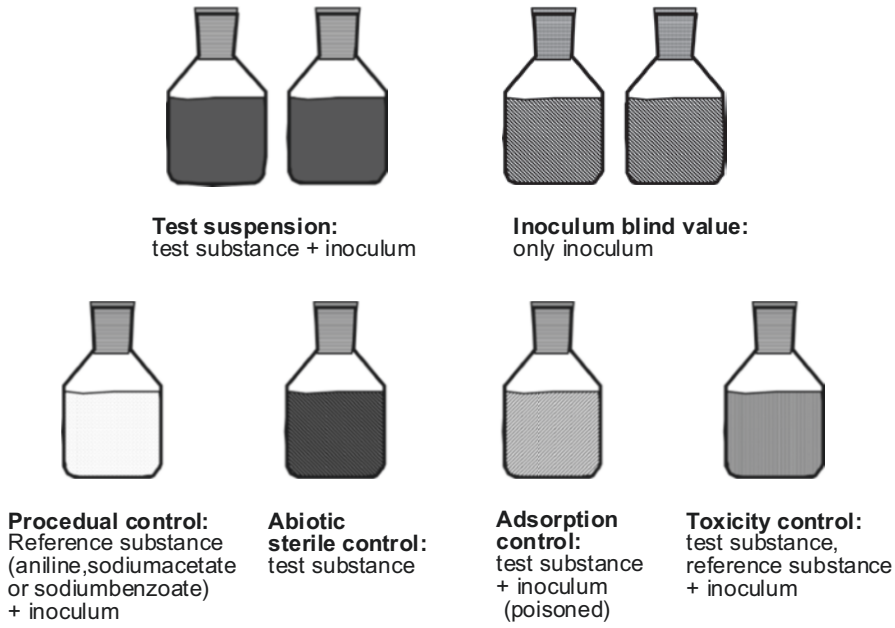


Fig. 5.5 Necessary test vessels in the modified OECD screening test (OECD301E). The large number of different control approaches required to obtain a result is striking

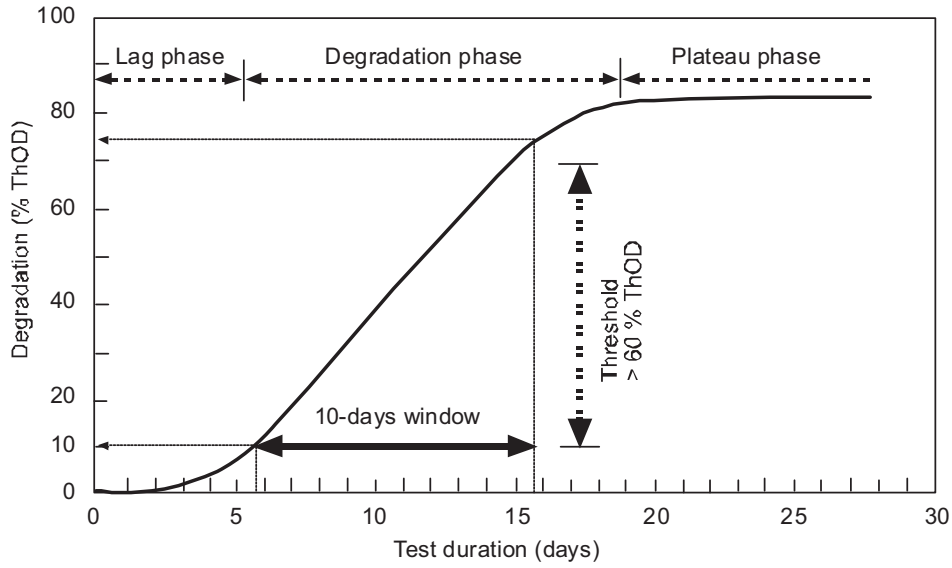


Fig. 5.6 Degradation curve and parameters for "easy degradability"

5.2.1.3 Simulation Tests

Simulation tests are generally aimed at testing the rate and extent of degradation in laboratory systems to simulate either wastewater treatment plants or specific environmental areas such as soil, aquatic sediments and surface water. Therefore, the biomass present in each case, relevant solids (soil, sediment, or other surfaces) that allow sorption of the chemical, and a typical temperature are used. A low concentration of the test chemical is used to determine degradation rates. High concentrations are typically used to identify and quantify the major transformation products. A low concentration in these tests means a concentration (less than 1–100 µg/L) low enough that the degradation kinetics resulting in the tests approximate those expected in the environment. Degradation rates are determined either by ^{14}C-labeling method or by specific chemical analyses. When testing for total degradation, if ^{14}C-labeled chemicals are used, the ^{14}C-label should be in the most poorly degradable part of the molecule. If the most stable part does not contain the functional and environmentally relevant parts of the molecule, then a chemical with different ^{14}C-labeling may be considered.

- **Wastewater Treatment Plant Simulation**

The fate of a chemical in wastewater treatment plants can be investigated in the laboratory using the Aerobic "Sewage Treatment: Activated Sludge Units (TG 303 A)" and "Biofilms (TG 303 B)" simulation tests. The degradation of the test substance is monitored by means of DOC and/or COD. In the basic test method (TG 303 A and TG 303 B), addition of the test substance at a concentration between 10 and 20 mg/L DOC is suggested. However, many chemicals are also present in wastewater at much lower concentrations, so a reasonable low concentration of (<100 µg/L) is listed in the appendix to TG 303.

Threshold values for the elimination of chemicals in the **WWTP simulations** are not defined. It is noted that such values are only useful in relation to the specific operating conditions and design of the plant. However, the test results are useful for estimating elimination in a WWTP and thus predicting the resulting concentration in the effluent to the receiving water given an existing influent concentration.

- **Simulation of Degradation in Soil, Sediment or Surface Water**

The following tests can be used to mimic the degradation of organic chemicals under realistic environmental conditions in soil, sediment, or surface water: Aerobic and Anaerobic Transformation in Soil (TG 307); Aerobic and Anaerobic Transformation in Aquatic Sediment Systems (TG 308); and Aerobic Mineralisation in Surface Water—Simulation Biodegradation Test (TG 309).

Generally, low concentrations of the substances are used for the determination of degradability. A low concentration (not more than 1–100 µg/L) allows degradation kinetics that correspond to those expected in the environment. The tests should be carried out at a temperature characteristic of the environment to be simulated.

5.2.1.3.1 Tests for "Possible Degradability"

The tests for "possible degradability" are designed to allow degradation to take place due to the use of favourable aerobic conditions and thus to estimate "possible degradability". The powerful methods allow a sustained exposure of the test substance to the micro-organisms and a low test substance to biomass ratio. Some of the tests can be performed with microorganisms that have been previously exposed to the substance. This often leads to an adaptation and thus to significantly higher degradation of the substance.

The following four methods listed in the OECD Test Guidelines allow the measurement of "ultimate degradability" of organic chemicals (see ◘ Table 5.4): Modified SCAS Test (TG 302 A), Zahn-Wellens/EMPA Test (TG 302 B) and Modified MITI Test (II) (TG 302 C).

Since **"potential degradability" is** considered to be **a specific property of a chemical**, it is not necessary to define limits on test duration or degradation rates. Degradation rates above 20% (measured as BOD, DOC or COD) can be taken as an indication of "possible, primary degradation", while rates above 70% are considered as evidence of "possible, complete mineralisation".

A substance that gives a positive result in such a test is classified as "possibly degradable", which should desirably be labelled "with preadaptation" or "without preadaptation". Since these tests provide very favourable conditions, "inherently biodegradable" chemicals cannot generally be expected to degrade rapidly in the environment. Thus, it does not mean that persistence in the environment cannot exist.

If the results indicate that "possible, complete mineralisation" is not present, it may lead to the preliminary assessment for **persistence in the environment** and the judgement of possible adverse effects from the transformation products.

5.2.1.3.2 Screening Tests for Anaerobic Degradability

The possible anaerobic degradability of an organic chemical can be determined by the following test: "Anaerobic Biodegradability of Organic Compounds in Digested Sludge/Method by Measurement of Gas Production (TG 311)". The test substance, which is the only organic substance added to the test mixture, is exposed at a relatively low concentration to a dilute anaerobic sewage sludge. The degradation rates are determined in a closed vessel with non-specific parameters such as formation of total inorganic carbon, CO_2 and CH_4.

A test duration of 60 days is suggested, but the test may be continued beyond this period or terminated earlier if degradation has reached a plateau indicating a sufficient level of degradation (>60%).

No formal decision criteria for "anaerobic degradability" have yet been made, but provisionally the lowest value for "ready anaerobic biodegradability" is given as 60% ThOD or $ThCO_2$.

The test (TG 311) has been developed for the estimation of "ultimate anaerobic biodegradability of organic chemicals in heated digesters for anaerobic sludge treatment" in a certain concentration range. It is therefore not applicable to anoxic environmental compartments such as anoxic sediments and soils.

5.2.2 Toxicity and Mutagenicity Testing with Microbial Systems

For the rapid detection of toxic environmental chemicals in water, soil and air, the use of microorganisms in toxicity tests has found wide application. Microbial methods are important screening methods because of their speed, economy, ease of use and sensitivity. For example, newly synthesized compounds are routinely screened for mutagenicity (mutagenic effects) using short-term mutagenicity tests, especially in the chemical and pharmaceutical industries.

5.2.2.1 Toxicity Tests for Aquatic Ecosystems

The **parameter** determined in acute toxicity tests is survival rate or mortality, chronic effects are measured with more sensitive and versatile parameters. The **aim** and **benefit** of toxicity tests are the detection, characterisation and evaluation of the ecotoxicity of individual chemicals, chemical mixtures and environmental samples and, as a result, a definition of hazard classes for the environment.

In principle, it is only possible to assess the effects of chemicals on ecosystems using **several toxicity tests and organisms of different trophic levels.** Details of the individual toxicity tests are set out in corresponding OECD guidelines, DIN standards and "EU Testing Methods". Recommended standard tests are bacterial, algal, daphnia and fish toxicity. Within the scope of this book, the presentation of microbial tests must be limited.

5.2.2.1.1 Algae Test

The algal toxicity test aims to determine **chronic toxic effects of** test substances or environmental samples on the growth of planktonic freshwater algae. For this purpose, the algae (*Selenastrum carpricornutum* or *Scenedesmus subspicatus*) are cultivated with the test substance in a defined medium over several generations.

After 24, 48 and 72 h incubation under specific light and temperature conditions, the cell number is determined microscopically as a measure of biomass. From the dose-response relationship, the 50% effect concentration on growth (EbC_{50}), related to biomass, is determined. In addition, the inhibition of the growth rate (ErC_{50}), related to cell number at the beginning and at the end of the test, as well as the highest concentration at which no growth inhibition was observed compared to the control approach (No Effect Concentration, NOEC) is given.

5.2.2.1.2 Pseudomonas putida Growth Inhibition Test

The Pseudomonas cell proliferation inhibition test is a test for **determining the chronic toxicity of** water-soluble substances to bacteria. *Pseudomonas putida* is used as a representative for heterotrophic microorganisms in freshwater. The test bacteria are cultured in a defined nutrient solution with a concentration series of the test substance over several generations. After 16 h, the cell concentration is determined by turbidimetry. The 10 and 50% effect concentration on growth (EC_{50}) is given as the result. The reference substance is 3,5-dichlorophenol (EC_{50} 10–30 mg/L).

5.2.2.1.3 Luminescent Bacteria Test

The luminescent bacteria test **(Microtox test)** is a **static short-term test** for the examination of waste water and aqueous solutions of test substances. Today, it is one of the most commonly used toxicity tests and has been included in the legal regulations of some countries (e.g. Germany).

Marine bacteria of the genus *Vibrio fischeri* (also *Photobacterium phosphoreum*) are used. They emit a cold glow **(bioluminescence)** as a product of their metabolism (see ◘ Fig. 11.7). Uniform luminescence indicates undisturbed metabolism, whereas when disturbed (for example, by exposure to chemicals) the bacterial luminescence intensity is reduced. After a contact time of 30 min, the decrease in luminescence intensity is determined compared to control mounts without chemicals. The EC_{50} value obtained is the concentration at which light emission is reduced by 50%.

In addition to chemicals, **environmental samples** (wastewater, leachate, sediment pore water, sediment extracts) are also tested. The test is also used to assess the **bioavailability** of pollutants in soils and to monitor the success of soil and contaminated site remediation.

The Microtox test is well reproducible and a broad data base with several hundred chemicals exists. For soluble organic chemicals, the Microtox test sometimes shows clear correlations with acute daphnia or fish tests. For poorly water-soluble, lipophilic organic chemicals and for substances that poorly penetrate the cell wall, the test appears to show lower sensitivity.

5.2.2.1.4 Nitrification Inhibition Test

The nitrification inhibition test with activated sludge investigates the potential effect of test substances or wastewater samples on the bacterial group of nitrifiers, which oxidize ammonium to nitrate. These are particularly

susceptible due to their relatively long generation time, which can lead to disturbances in nitrifying wastewater treatment plants.

In the test, a dilution series in the relevant range including the control without test substance is examined. In parallel, the reference substance allylthiourea is also tested as a nitrification inhibitor (inhibits ammonia monooxygenase). After 4 h the concentration of ammonium, nitrite and nitrate in the filtered sample is determined. The percentage inhibition is calculated for each test concentration by comparing the oxidized nitrogen compounds in the test samples with those in the blank. The result is the 50% effect concentration on nitrification (EC_{50}).

5.2.3 Mutagenicity Testing with Bacterial Systems

5.2.3.1 Ames Test (OECD 471, DIN 38415-4)

The short-term mutagenicity test developed by Ames and co-workers in the 1970s is routinely carried out primarily in the chemical and pharmaceutical industries to test newly synthesized compounds for their mutagenicity (mutagenic effect).

The strains of *Salmonella enterica* serovar Typhimurium used in the Ames test can no longer grow on agar plates without the amino acid histidine due to mutations (*his*$^-$). The mutants TA100 (base substitution) as well as TA98 (grid thrust) are used. Under the influence of a mutagenic substance, the respective mutation can be partially reversed (re-mutations to the *his*$^+$ genotype), so that a corresponding number of colonies grow on the nutrient medium and can be counted (in the case of revertants, a lost gene activity is thus restored).

An increase in the revertant numbers compared to the strain-specific spontaneous rates (controls) is a direct measure of the mutagenic effect of the test substance, i.e. the reversion rate determined must be above the spontaneous mutation rate also determined.

To simulate mammalian metabolism, an organ extract (so-called S9 fraction) from rat liver is added. This extract contains a large proportion of the mammalian cytochrome P-450 enzymes responsible for processing foreign substances. These may be able to convert mutagenic substances into non-mutagenic compounds, but they can also convert a non-mutagenic substance so that a "premutagen" becomes a "mutagen".

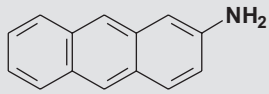
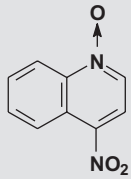
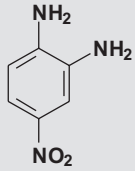
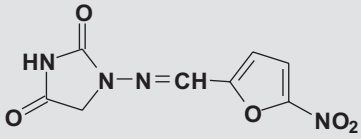
◘ Table 5.6 shows the substances used as positive controls.

A positive Ames test is taken as an indication of the carcinogenicity of a substance, since most mutagenic substances are also carcinogenic in mammals. The test has generally good significance, although further investigation is required, particularly in the absence of increased reverse mutation by the chemical or its activation products in other tests (◘ Fig. 5.7).

SOS Repair

The SOS functions are complex biochemical processes that counteract the damage of the bacterial genetic material. These functions include, for example, an error-prone DNA repair system (error-prone-repair), the synthesis of an excision repair complex (*uvrABC*), an inhibitor of cell division (*sfiA*) and the gene products RecBCD, which are necessary for the execution of homologous recombination, the only repair system that works completely error-free. The SOS functions are

Table 5.6 Control substances in mutagenicity tests

Substance		Mutagenic effect	Test organisms
	2AA, 2-aminoanthracene	With S9 mix	TA98, TA100, TA1535/ pSK1002
	NQO, 4-nitroquinoline N-oxide	Without S9 mix	TA1535/ pSK1002
	4-NPDA, 4-Nitro-1,2-phenylenediamine	Without S9 mix	TA98
	NF, Nitrofurantoin	Without S9 mix	TA100

controlled by the *recA* and *lexA* genes. The cell's SOS response to DNA damage is activated by interaction of the RecA protein with the LexA repressor protein, which closes many operons in the undamaged cell. Interaction with activated RecA triggers proteolytic cleavage of the repressor. This leads to the coordinated induction of all operons to which LexA was bound, resulting in the activation of SOS functions. Single-stranded regions in the DNA are thought to be the triggering signal for the activation of the RecA protein. Among the target genes of LexA repression is the *umuC* gene, which is involved in mutagenic DNA repair (*umu*, UV mutagenesis) (◘ Fig. 5.8).

UmuC is an UmuD′-, RecA*-, and SSB-activated DNA polymerase specialized for bridging DNA damage (SSB, single strand **binding** protein) (◘ Fig. 5.9).

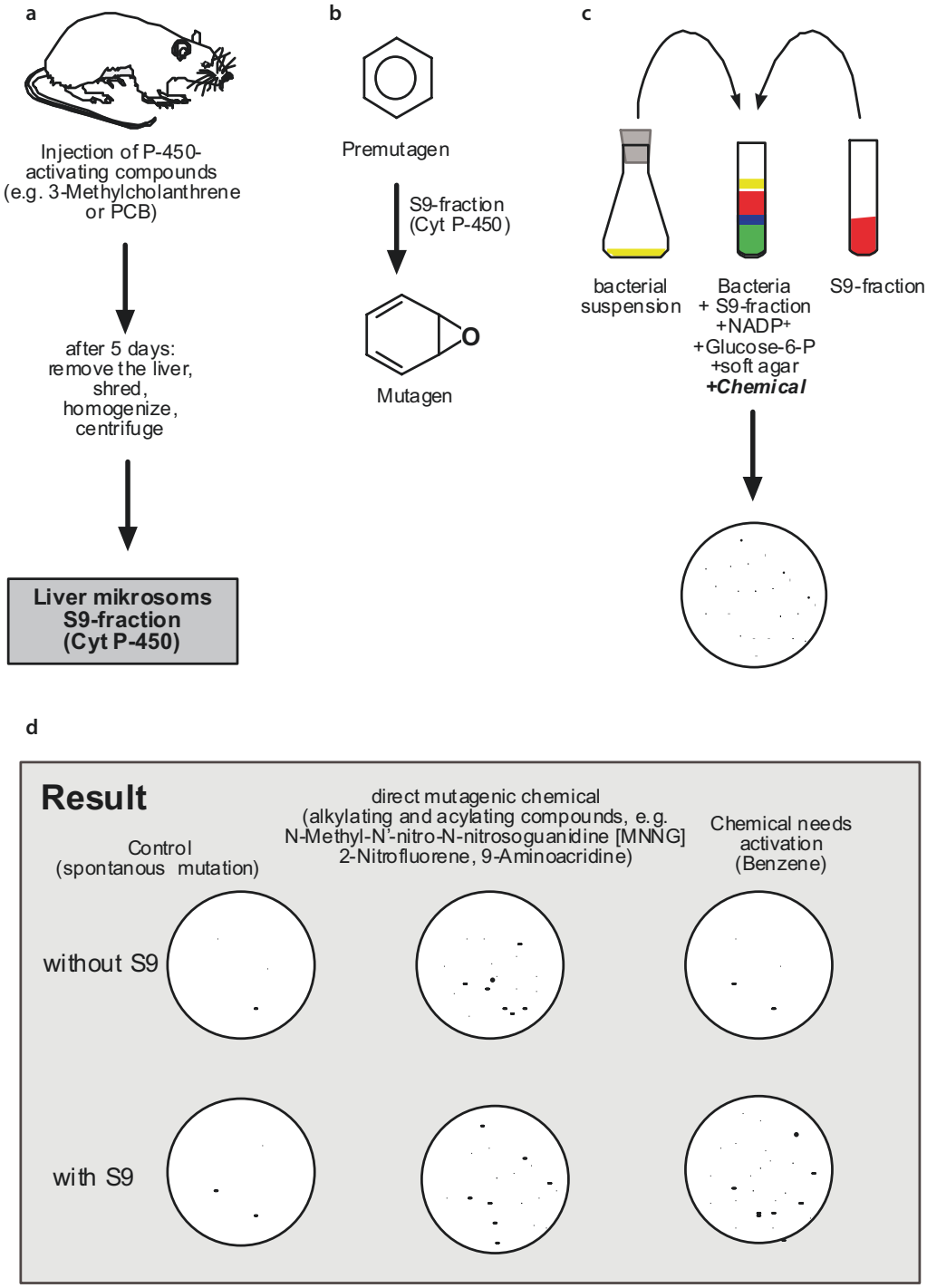


Fig. 5.7 Mutagenicity testing with the Ames test. **a** Isolation of the S9 fraction, **b** Example of the conversion of a premutagen into a mutagen by cytochrome P-450, **c** Experimental procedure, **d** Result with different chemicals

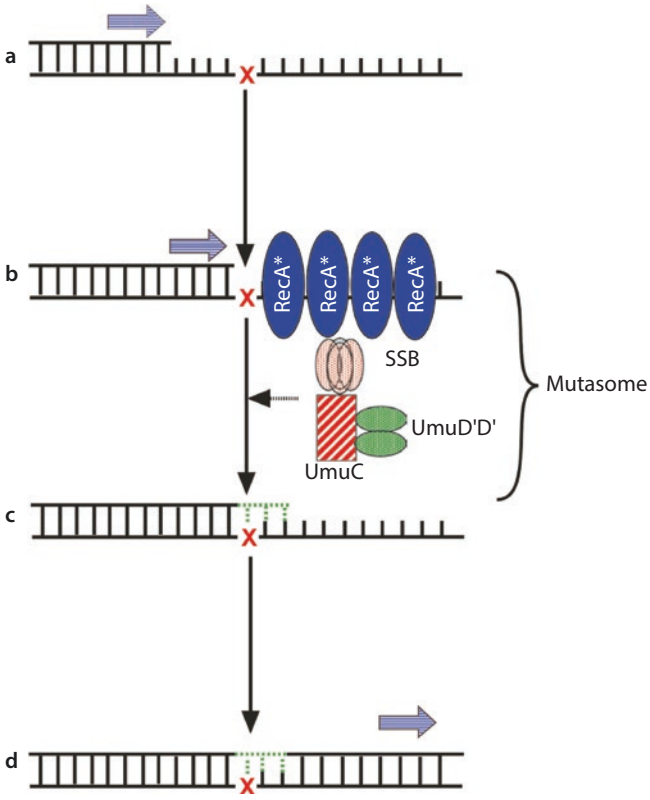


Fig. 5.8 The model of SOS mutagenesis by "translesion" synthesis, an important cellular mechanism that overcomes the blockage of replication due to DNA damage, albeit often defective. **a** The DNA polymerase replicates the DNA template normally (active replication is indicated by the arrow). **b** The polymerase encounters a damaged nucleotide (X). It cannot replicate over this "lesion" (wound). **c** RecA*, UmuD′D′, UmuC and SSB are necessary to perform replication over the damage (translesion synthesis). **d** If the nucleotide inserted opposite the lesion is incorrect, "translesion" synthesis fixes a mutation in the organism's genome

5.2.3.2 Umu Test (DIN 38415-3)

The *umu* test indicates damage to DNA caused by genotoxins by detecting the induction of cellular DNA repair mechanisms. The test organism *S. enterica* serovar Typhimurium TA1535/pSK1002 reacts to damage to its genetic material by inducing the SOS repair system (see Box ► SOS Repair).

The bacteria are exposed to different concentrations of the test substance. In this process, genotoxins induce the so-called *umuC* gene, which belongs to the cell's SOS repair system, which counteracts damage to the bacterial genetic material.

Since the *umuC* gene in the strain used is cloned directly upstream of the *lacZ* gene on plasmid pSK1002, which codes for

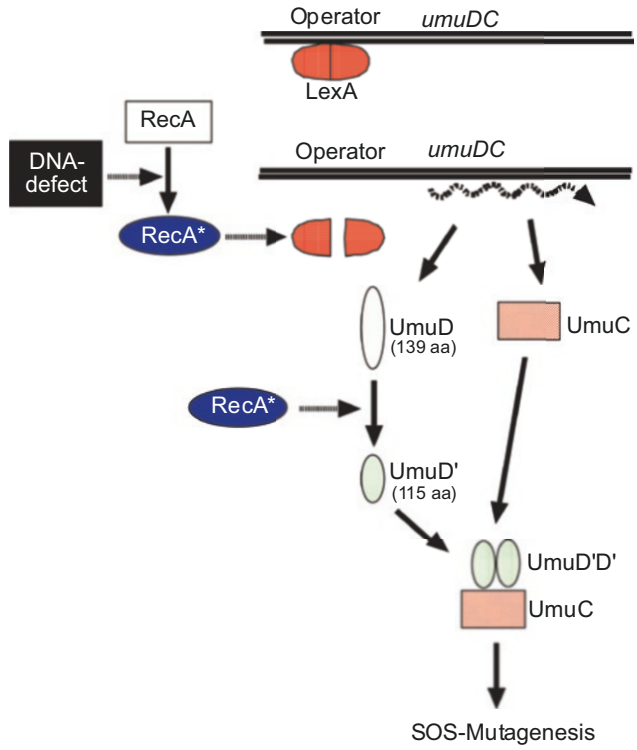


Fig. 5.9 Regulation of the *umuDC* operon by RecA and LexA. DNA damage generates a signal that converts RecA to RecA*. RecA* mediates cleavage of the LexA repressor, which leads to induction of the *umuDC* operon as well as the rest of the SOS response genes. RecA* may also mediate the processing of UmuD to the shorter UmuD′ molecule. UmuD and UmuD′ can interact with UmuC in various combinations. The UmuD′D′C complex is active in SOS mutagenesis (translesion synthesis)

β-galactosidase, the measurement of the induction rate of the *umuC* gene can be made by determining the *β*-galactosidase. Thus, the formation of the yellow dye *o*-nitrophenol from *o*-nitrophenyl-*β*-D-galactoside is the signal indicating the genotoxic effect of the test substance. The induction rate relative to the negative control is reported as a measure of genotoxicity. The test is performed in microtiter plates both with S9 extract for metabolic activation of genotoxins (from promutagen to mutagen) and without.

The positive control substances are listed in ◘ Table 5.5 (◘ Fig. 5.10).

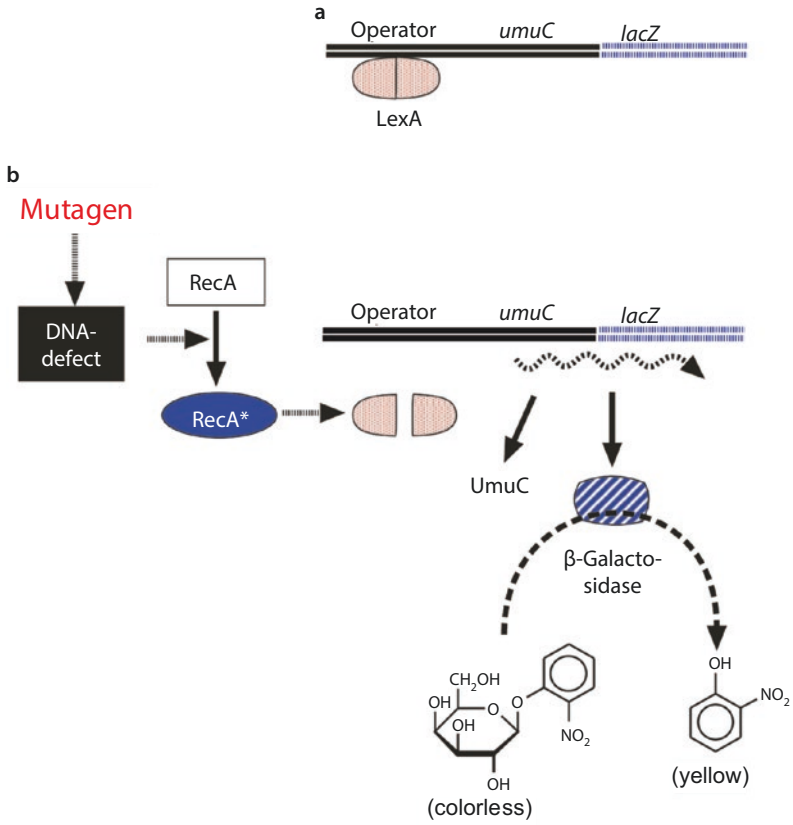


Fig. 5.10 Mutagenicity testing with the Umu test. **a** LexA blocked β-galactosidase synthesis, **b** reaction cascade of mutagen induced synthesis of β-galactosidase

Test Your Knowledge

- What do you associate with the names "Seveso" and "Love Canal"?
- What poisoned the inhabitants of Minamata? What influence do microorganisms have on the formation of the poison (see also ► Chap. 9)?
- What three processes are responsible for the spread of chemicals in the environment?
- Which physico-chemical constant describes the distribution of a chemical between air and water?
- What does the octanol/water coefficient of a chemical tell you?
- What does the environmental chemist mean by a sink?
- Outline using an organochlorine compound as an example: cometabolism, dehalorespiration, and use as a carbon and energy source.
- Chemicals assessment through REACH: What is intended?
- Which measurement parameters are suitable for detecting the biodegradability of a chemical?

- What is meant by "ready", "inherent", "ultimate" and "primary" biodegradability?
- Which test approaches are needed to obtain a reliable statement on degradability in the OECD screening test? Think about the controls! Is "degradability" an absolute value?
- Say something about the claims made in the test regarding degradability and the behavior of a chemical in the environment.
- What is the so-called "10 day window", what is the "threshold value" to be reached in the degradability tests?
- Describe simple tests to test for mutagenicity of a chemical.

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Microbial Degradation of Pollutants

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_6

A distinction must be made between the potential and actual degradability of environmental chemicals. In the following sections, **potential degradability** is discussed. The strategy by which degradation takes place, the enzymatic steps through which a chemical must pass in order to be degraded, and the principles that can be identified are discussed. Information is given on the extent to which prokaryotes as well as eukaryotes carry out degradation. Furthermore, the differences in degradation in the presence of "air" oxygen and in the absence of this "co-substrate" are discussed. Oxygen has not only the function to act as an electron end acceptor in respiration, but is also an indispensable substrate of important degradation steps.

6.1 Degradation of Hydrocarbons

With the industrial development of the past hundred years, components and products from petroleum and coal have increasingly entered the environment. Petroleum contamination occurs during extraction, transportation, processing into fuels and petrochemicals, and improper use of fuels and lubricants.

While the public assumes that tanker accidents are the main source of marine pollution, they account on average for only about 12% of the total input (see ◘ Table 6.1).

The development of carbochemistry (coal chemistry) began with the extraction of coal gas and coke in gas works, which produced tars as by-products. Tar waste, rich in phenol and polycyclic aromatic hydrocarbons (PAHs), was often deposited around gas plant sites. Some fractions were used as wood preservatives (creosote), which is the reason for the contamination of railway sleepers with PAHs. With the development of coal hydrogenation in the first decades of the twentieth century, the extensive extraction of hydrocarbons as fuels and carbochemicals began, largely superseded by petrochemicals in the 1960s. Microorganisms also come into contact with petroleum hydrocarbons under natural conditions, as in some areas of the world, for example in the Near East, petroleum seeps out at the surface without extraction measures. Petroleum, like coal, is of biogenic origin, so many components are similar to natural products. Nevertheless, many petroleum hydrocarbons are difficult to degrade. This is due to the fact that in many cases the conditions for the realization of the degradation services are not given.

◘ **Table 6.1** Input of hydrocarbons to the sea

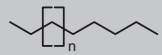
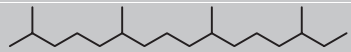
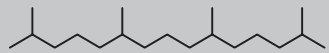
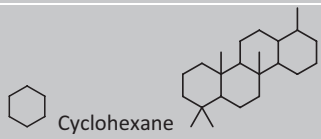
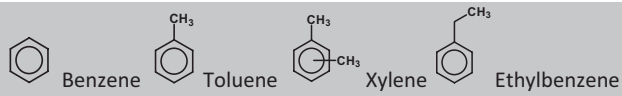
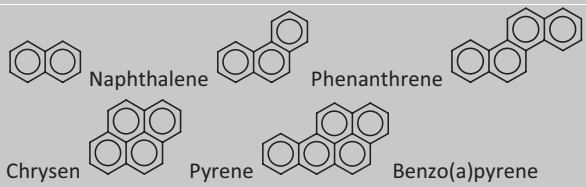
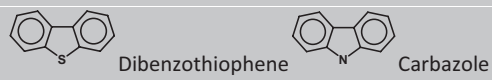
Entry form	Tons per year
Transport by sea	1,070,000
Tanker accidents	400,000
Accidents during offshore oil production	50,000
Refineries and industry on the coast	300,000
Urban drainage and waste disposal	820,000
river input	40,000
Dumping[a]	20,000
Natural oil wells and sediment erosion	250,000
Rainfall	300,000
Total	3,250,000

National Research Council (1985)
[a]According to Fent (2003): 20–30 cases of illegal oil discharges at the Cape of Good Hope annually

6.1.1 Petroleum: Composition and Properties

Crude oil is currently the most important source of energy and raw materials. The **composition of** petroleum varies greatly depending on its origin. This is due to the nature of the biogenic starting compounds and the biogeochemical formation processes. ◘ Figure 6.1 summarizes the main components of crude oil. It becomes clear that it is a rather heterogeneous mixture of substances.

Significant changes in composition occur during processing. Thus, a decrease in cycloalkanes and an increase in aromatics is

Class	Example
Alkanes (up to C40) 10 - 70 % Portion	
Isoalkanes, branched, Isoprenoidalkanes	 Phytan (2,6,10,14-Tetramethylhexadecane)  Pristan (2,6,10,14-Tetramethylpentadecane)
Cycloalkanes 30 - 80 % Portion	 Cyclohexane Hopan
Aromatics, single ring, BTXE 20 - 30 % Portion	 Benzene Toluene Xylene Ethylbenzene
Aromatics, multi ring, PAK	 Naphthalene Phenanthrene Chrysen Pyrene Benzo(a)pyrene
Heterocycles 2 - 15 % Portion	 Dibenzothiophene Carbazole
Asphaltene	Fraction not soluble in pentane

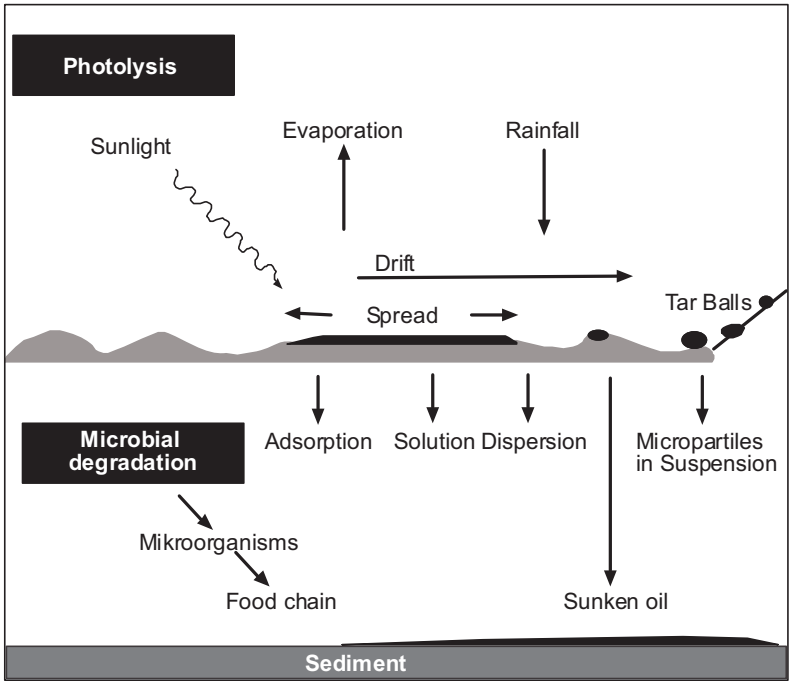
◘ **Fig. 6.1** Class of substances occurring in crude oil with information on proportions

due to catalytic hydrogenation processes. The volatility and solubility of the individual fractions are significant for the degradation problem. The lower alkanes (C_1–C_4) are gaseous and soluble in water (20–390 mg/L). As the chain length of the alkanes increases further, the volatility and solubility in water decreases sharply. Pentane has a solubility of about 40 mg/L and hexane of 10 mg/L, whereas it is around 0.1 µg/L for the components of kerosene (C_{10}–C_{17}). The low water solubility leads to slow mass transfers and thus to low bioavailability.

6.1.2 The Process of Oiling in the Sea

Although each oil spill in the sea has its own particularities, there are a number of processes that all oil spills have in common. In a highly simplified manner, this is illustrated in ◘ Fig. 6.2. The oil is immediately subject to **spreading** on the water surface and, within a very short time, will thereby usually be present only in a layer thickness of less than 1 mm. The speed of spreading depends on the type of oil, the water temperature as well as wind and current. Also of importance is whether the oil reaches the water surface in a thin trickle or whether larger quantities are released, for example by the break-up of a tanker.

The spilled oil is subject to **drift** simultaneously with the dispersion. If the current as well as the wind speed and direction are known, fairly accurate information about the drift path can be obtained. From the moment the oil is released, the low-boiling components begin to **evaporate.** Within a few hours, most of the components boiling below 160 °C go into the atmosphere. Evaporation also depends on the composition of the oil, the water temperature and the wind speed. Evaporation increases the viscosity of the oil. Some of the oil passes



◘ **Fig. 6.2** Schematic representation of a marine oil spill

into the body of water. Primarily the aromatics with low boiling point go into real **solution. Adsorption** to the organic and inorganic particles of the seawater and **dispersion** also occur. These processes lead to a segregation of the oil.

The formation of water-in-oil emulsions affects the part of the oil floating on the water. The volume increases due to the water absorption. The water content of these emulsions can be up to 80%. Favoured by the wave motion, simultaneously with the water absorption, the water-in-oil emulsion changes into a particulate form. The particles can be of different sizes, depending on the physical conditions. They have a water content of 50%, their consistency is relatively solid. The properties of at least the surface of these particles are modified so that they do not cause oiling of birds even in close contact.

Crude oil, which is driven onto the seawater on the beach a short time after the spill, has also absorbed considerable amounts of water. Because of its coloration and consistency, it is called "chocolate mousse".

The more or less large oil clumps floating on the water are subject to an aging process and eventually form the so-called **"Tar Balls".** They are dark and hard. Their consistency changes from "tar-like" to "asphalt-like", they become brittle and can absorb inorganic turbidity, which can increase their specific weight to such an extent that they are distributed in the form of microparticles in the water column or sink. Stranding of the oil can occur at any time. The residence time of the "tar balls" floating in the sea can be several years.

Two processes are responsible for the removal of some of the oil in the marine environment, **photolysis** and **biodegradation.** Most of the evaporated hydrocarbons are thought to undergo photolysis. However, in the case of stranded oil or oil floating at sea, photolysis plays only a minor role.

Figure 6.3 schematically shows a mass balance of an oiling in the marine environment. The time axis is logarithmic. It is assumed that after 100 days most of the conversion has taken place and then only relatively minor changes occur. After 1000 days, all hydrocarbons that have entered the atmosphere have been photolytically degraded. In contrast, the part floating on the water is subject to photolysis only to a very small extent. About 45% of the oil is biodegraded, with 44% of the degradation occurring in the water column and only 1% in the sediment. Depending on the composition of the oil, the water temperature, the nutrient content and the distance from the coast where the oiling takes place, other distributions will occur, but this compilation allows an idea of the course of an oiling.

6.1.3 Degradation of Alkanes, Alkenes and Cyclic Alkanes

6.1.3.1 Alkanes/Alkenes

In the **presence of oxygen, unbranched alkanes** up to a chain length of C_{30} are microbially degradable. Due to the good fat solubility and thus high affinity to cell membranes, the short-chain compounds, for example hexane, are toxic in higher concentrations. *n*-Alkanes of medium chain length (C_{12}–C_{20}) are very well degradable, with further increasing chain length the degradability decreases due to the decreasing bioavailability. Waxes are relatively persistent, so a paraffin candle will remain in the compost for years. Lumpy and resinous lubricating oil residues are very persistent. Naturally occurring asphalts still hold together the ruins of Babylonian structures.

The main pathway of alkane degradation is **terminal oxidation** (Fig. 6.4). One of the terminal methyl groups is oxidized to the alcohol by an alkane monooxygenase, so atmospheric oxygen is essential for degradation. The respective alcohol is then

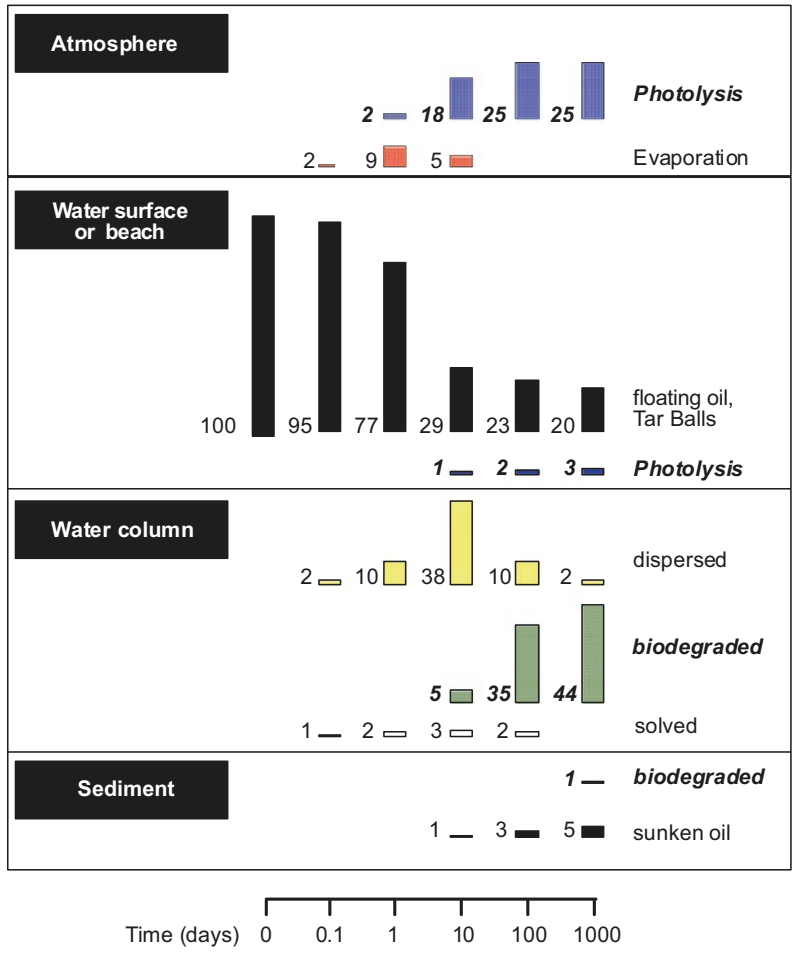


Fig. 6.3 Mass balance of a marine oil spill. (Adapted from Gunkel, 1988; data in italics refer to the proportion of oil that is removed, while data in normal type only indicate a distribution)

oxidized by dehydrogenases via the corresponding aldehyde to the fatty acid, which is gradually degraded by β-oxidation to C_2 units and enters intermediary metabolism as acetyl-CoA (Fig. 6.5). Further utilization as a carbon and energy source can be seen in ► Fig. 4.12. The capacity for terminal oxidation is widespread among bacteria and fungi. Particularly active representatives of bacteria are species of *Acinetobacter, Bacillus, Mycobacterium, Nocardia, Pseudomonas* and *Rhodococcus.* Among the yeasts and molds are representatives of the genera *Candida* and *Rhodotorula,* as well as *Aspergillus, Cladiosporum, Penicillium* and *Trichoderma.*

Some species of *Nocardia* and *Pseudomonas,* as well as yeasts and fungi, have other degradation pathways, **subterminal oxidation** and **diterminal** or ω-oxidation (Fig. 6.4).

Microorganisms degrade both even and odd *n*-alkanes. The end products of the degradation of odd-numbered alkanes are acetyl- and propionyl-CoA. The higher fatty acids and alcohols formed intermediately

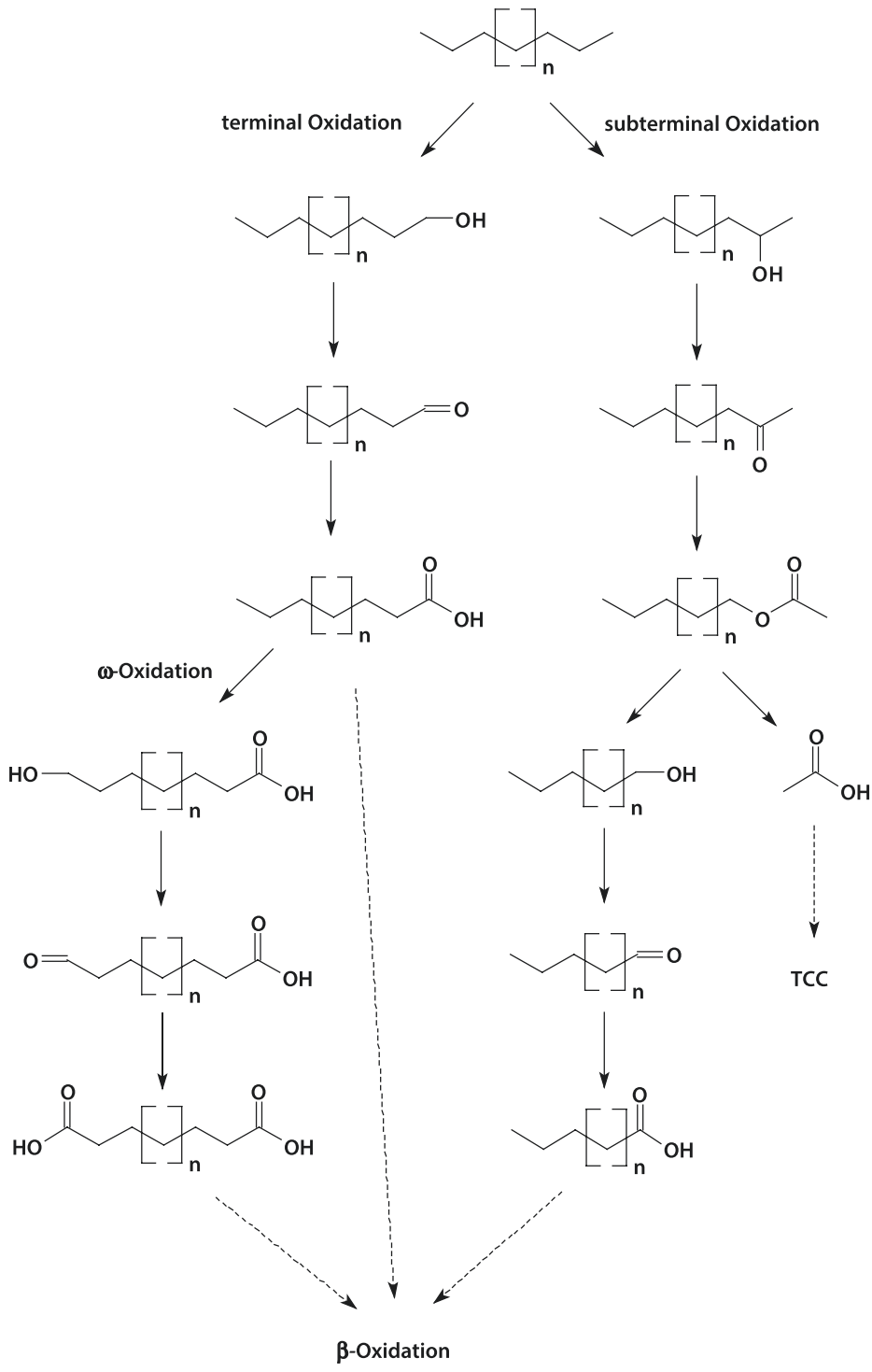


Fig. 6.4 Steps in the formation of fatty acids from alkanes with three types of oxidations

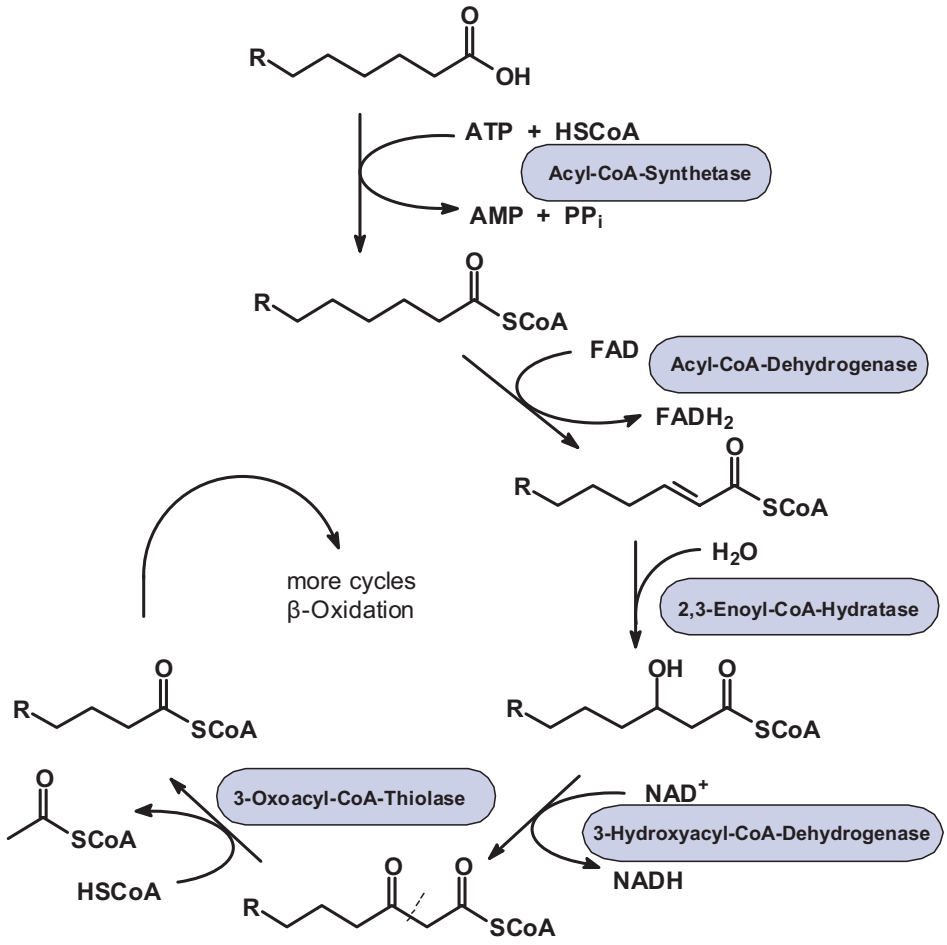


Fig. 6.5 ***β*-Oxidation** of fatty acids

during degradation can react to form long-chain esters, as they also occur in the waxes of plants.

If a fatty acid carries a double bond, the *β*-oxidation cycle must be slightly modified, as Fig. 6.6 shows using oleic acid as an example.

Anaerobic n-alkane degradation (for example of hexane) has been described by denitrifying pseudomonads as well as by sulfate-reducing bacterial consortia. Elucidation of the mechanisms and estimation of the distribution of these processes are in their infancy. An initial proposal for the anaerobic degradation sequence for hexane was presented using the denitrifying strain HxN1 (Fig. 6.7). Similar to the anaerobic degradation of toluene (see later Fig. 6.22), the first step postulates a hydrocarbon-fumarate adduct that undergoes *β*-oxidation after activation to the CoA ester and rearrangement of the carbon chain.

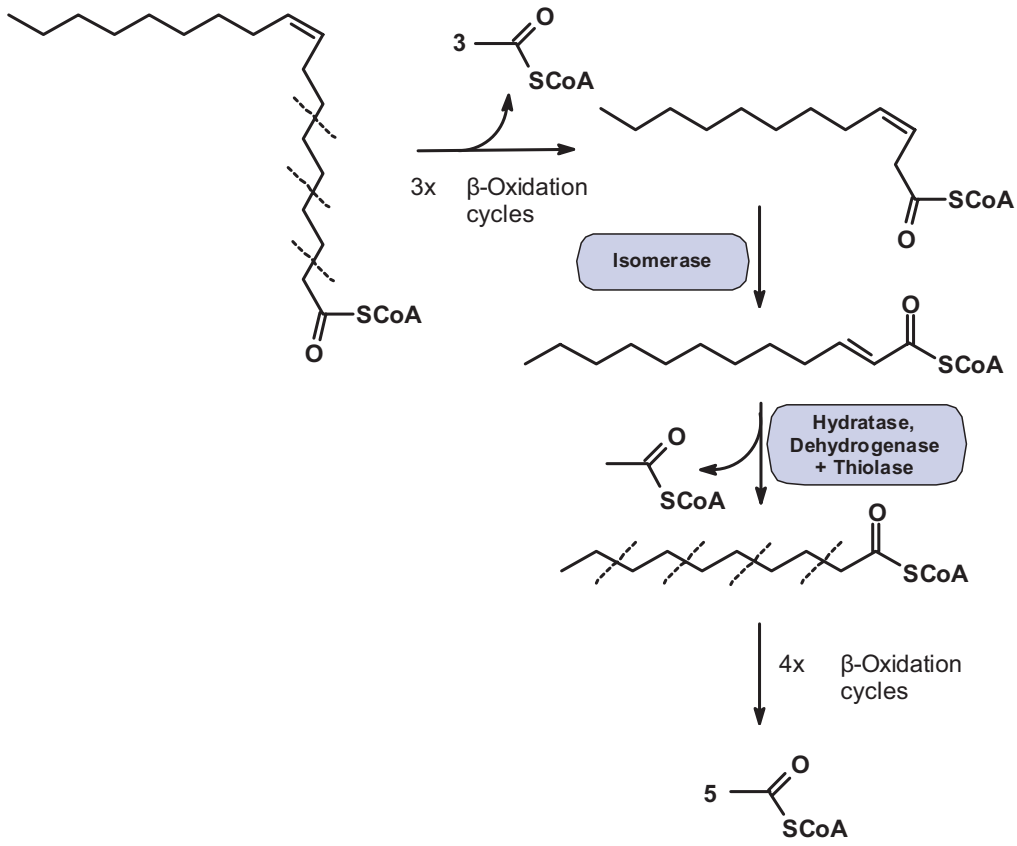


Fig. 6.6 ***β*-Oxidation** of fatty acids with double bond: *cis*-Δ3-*trans*-Δ-2-enoyl-CoA isomerase

Anaerobic Alkane Degradation with Methane Formation on the Seabed

In many places, crude oil and natural gas emerge from the seabed at so-called "seeps". There, the hydrocarbons migrate from the source rock through cracks and sediments towards the sediment surface. In the upper sediment layers, many hydrocarbons, primarily alkanes, are degraded, enabling a variety of densely populated habitats to develop on the dark seabed. Deep down in the sediment, where no oxygen is present, they also form an important energy source for subsurface microorganisms, including some *Archaea.*

Sediment samples from oil and asphalt seeps from the Gulf of Mexico were analyzed by combining geochemical methods, metagenomics, and fluorescence in situ hybridization. Based on genome composition and environmental observations, a preliminary model of the metabolism of the target organism "*Ca* Methanoliparia" was developed. Phylogenetic analysis of the detected divergent methyl-coenzyme reductase (div. MCR) type suggests that Methanoliparia can activate alkanes and form a CoM-linked alkyl moiety as a primary intermediate. This CoM-linked alkyl moiety is further degraded to CO_2 and methyl-H_4 MPT, with this disproportionation occurring through an acetyl-CoA decarbonylase/synthetase complex (ACDS) (Fig. 6.8). A methyl

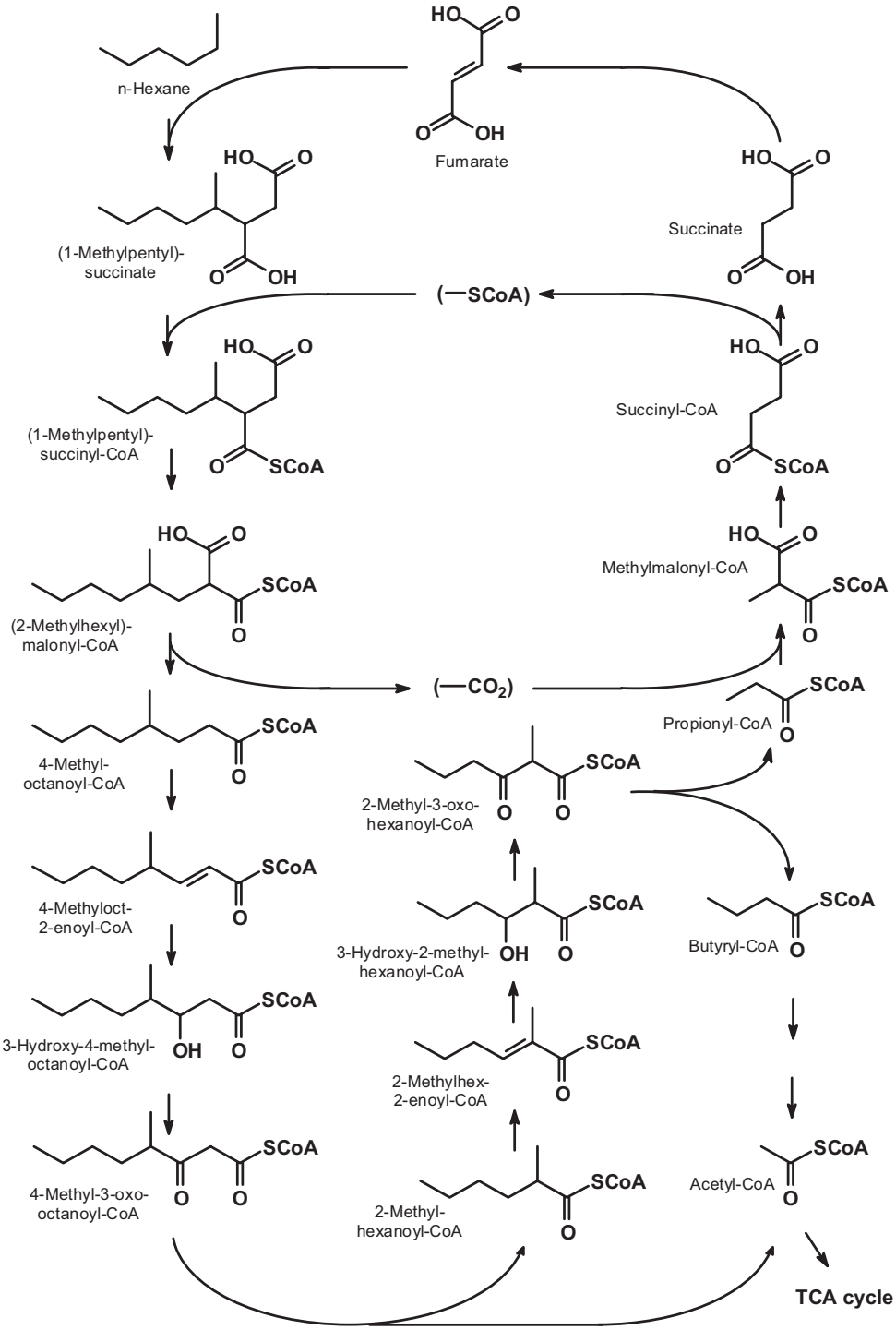


Fig. 6.7 Degradation of hexane by a denitrifier

transferase (MTR), generating a sodium gradient, transfers the methyl group from H_4 MPT to CoM, forming methyl-CoM, which can eventually be reduced to methane by canonical MCR (can. MCR). The reduction equivalents released during the cleavage are used to reduce CO_2 release, thus resulting in additional methane.

Based on metagenomic data, the range of alkanes that Methanoliparia can utilize cannot be defined. Under standard conditions, methanogenic alkane degradation is exergonic for all multicarbon alkanes and energy yields increase with alkane chain length.

In methanoliparia, divergent MCRs can couple the activation of medium- or long-chain alkanes to methanogenesis. Remarkably, in the oily sediment, alkanes with less than 20 carbon atoms are almost completely depleted. Therefore, Methanoliparia can be expected to degrade alkanes to methane. The process can be discussed using hexadecane as an example, one of the most abundant compounds in the saturated portion of the non-biodegraded asphalt stream. Without biosynthesis, the overall catabolic reaction for four hexadecane molecules would be as follows:

$$4\,C_{16}H_{34} + 30\,H_2O \rightarrow 49\,CH_4 + 15\ CO_2 \quad \Delta G^{0'} = -339.2\,\text{kJ / mol Alkane}$$

The reaction is exergonic under standard conditions, but also under a variety of environmental conditions, including those in the collected oily deep-sea environment.

The ability of microbial consortia to methanogenically degrade hydrocarbons has long been described, but was previously thought to rely on the syntrophic interaction of hydrocarbon-degrading bacteria with methanogenic *Archaea*. Methanoliparia does not appear to form associations with a bacterial partner organism, it does not encode a dissimilatory sulfate reduction pathway, and it lacks essential genes for cytochromes involved in direct electron transfer. Therefore, the complete reaction must occur in a single cell. In a first step, alkane oxidation generates methyl groups bound in equal amounts to H_4MPT and CO_2, releasing electrons. Some of these are used for the reduction of methyl-H_4MPT to methane in an energy-conserving step by the translocation of sodium ions to the outside of the membrane. The remaining electrons require an electron sink, such as the methanogenic CO_2 reduction.

The results suggest that "Ca. Methanoliparia" can be a key player in methanogenic oil degradation without the need for a syntrophic partner. This implies a previously overlooked role for *Archaea* in hydrocarbon degradation (Laso-Pérez et al., 2019; Borrel et al., 2019).

Branched alkanes are degraded much more slowly than the unbranched *n*-alkanes, even under aerobic conditions. Branched alkanes with an isoprenoid structure such as pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane), which carry a methyl group on every fourth C atom, are degradable. These isoprenoid alkanes undergo mono- or diterminal oxidation and alternate cleavage of C_2 and C_3 units (◘ Fig. 6.9). This ability has been demonstrated for *Brevibacterium, Corynebacterium* and *Rhodococcus* species. More branched isoalkanes with more methyl substituents and longer side chains are much more persistent, as the branches sterically prevent the attack of oxidative enzymes.

As ◘ Fig. 6.10 makes clear, a side group in the "wrong" position that interferes with *β*-oxidation can also be eliminated. Carboxylation is an important step so that

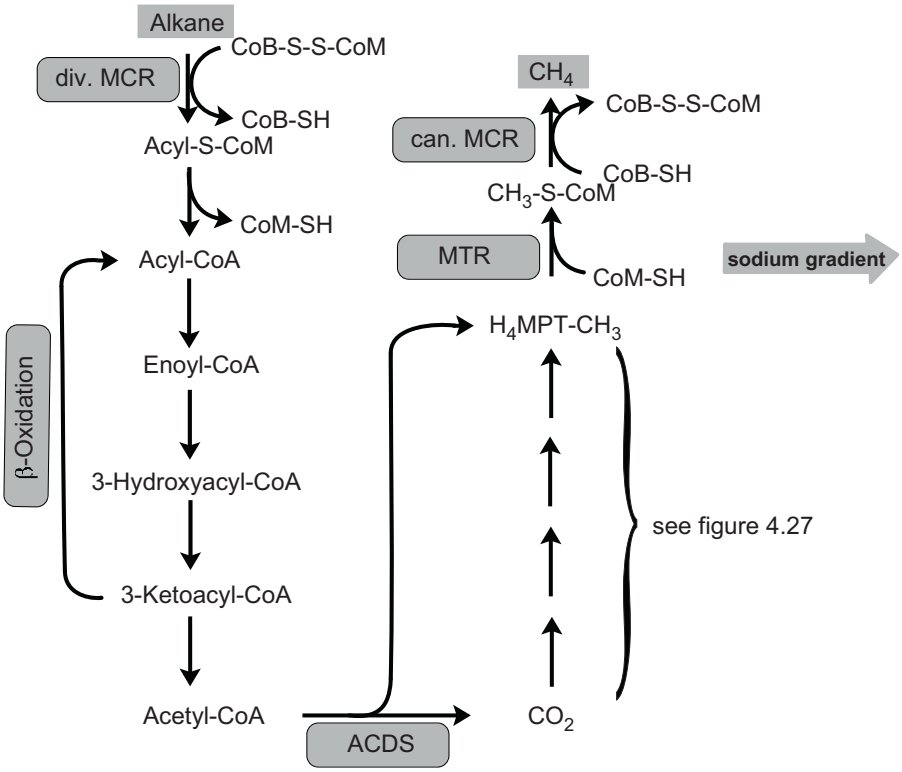


Fig. 6.8 Anaerobic degradation of alkanes in sediment samples from oil and asphalt seeps by Methanoliparia. Detected genes of possible key enzymes: methyl transferase *(mtr)*, acetyl-CoA decarbonylase/synthetase complex *(acds)*, divergent methyl-CoM reductase (div. *mcr*), canonical methyl-CoM reductase (can. *mcr*)

the interfering side group can be cleaved off as acetic acid.

Alkenes or olefins, which only occur in small quantities in crude oil, can be degraded both from the saturated end and via intermediate epoxide formation at the double bond (Fig. 6.11).

Another strategy aimed at producing acetyl-CoA is shown in Fig. 6.12. Thus, the C_3 compounds propenes and acetone are converted to even-numbered metabolites by carboxylation, producing two acetyl-CoAs.

6.1.3.2 Cycloalkanes

Cycloalkanes, also known as naphthenes, cycloparaffins or alicycles, occur in petroleum primarily as cyclohexane, methyl- and ethyl-substituted cycloalkanes, and cyclopentane and their substituted derivatives. Since it is difficult to obtain pure cultures on these substrates, the metabolism of cycloalkanes, in contrast to alkane degradation, has been relatively little studied.

From studies with mixed cultures of bacteria of the genera *Flavobacterium, Nocardia, Pseudomonas* and *Rhodococcus,* the reaction steps shown in Fig. 6.13 for cyclohexane were derived. Two oxygenase reactions occupy a key position, hydroxylation of the cycloalkane and oxidation to a lactone (analogous to a Baeyer-Villiger oxidation). The lactone is opened by hydrolysis and the metabolite is converted via several dehydrogenation reactions in such a way that further degradation via *β*-oxidation can lead to acetyl-CoA.

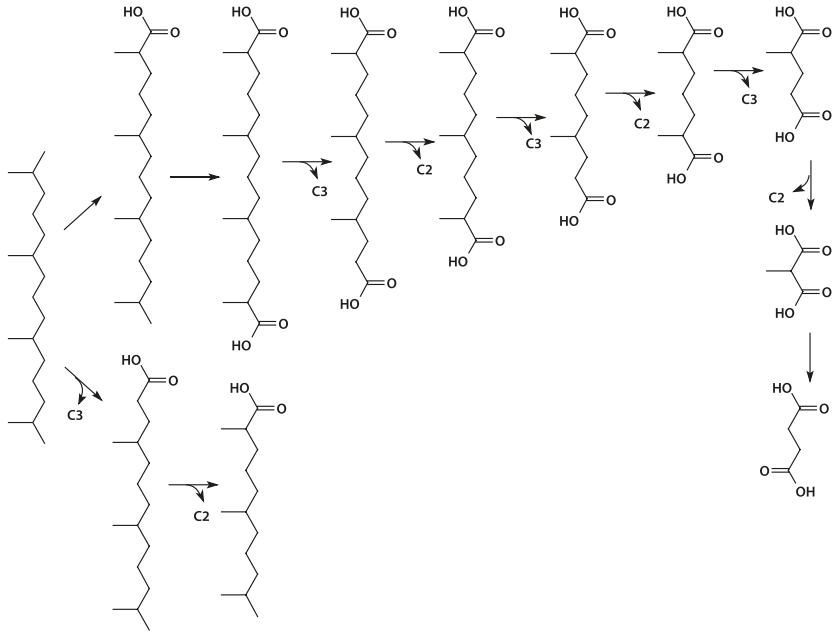


Fig. 6.9 Degradation sequence of a branched alkane using pristane as an example. (The degradation will probably take place as CoA ester. It is only shown schematically here)

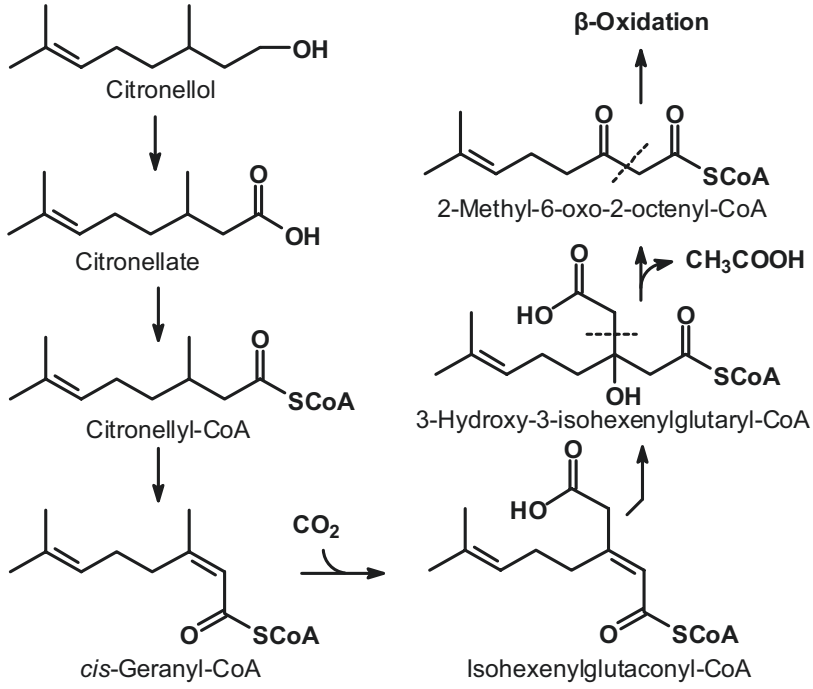


Fig. 6.10 Degradation sequence of a branched alkane using the example of citronellol

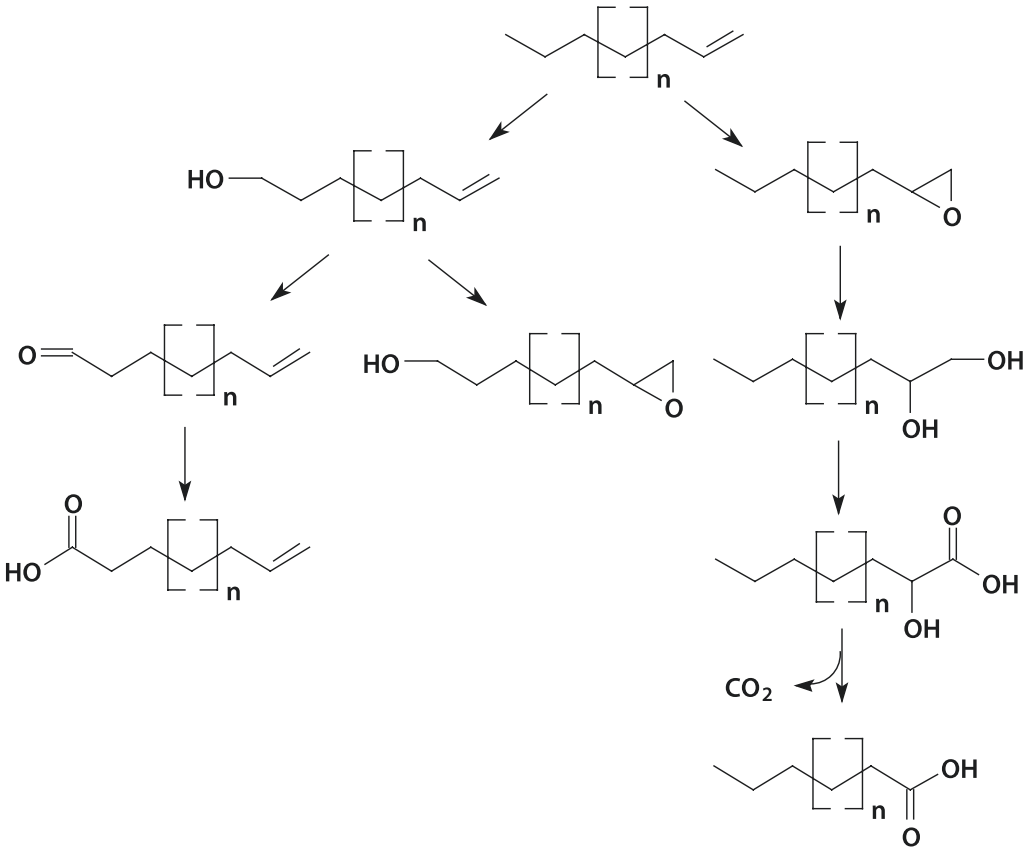


Fig. 6.11 Principles of the degradation of alkenes

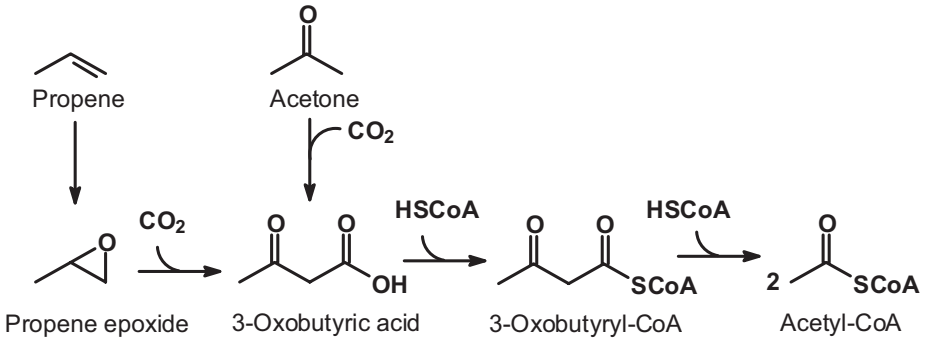


Fig. 6.12 Degradation of propene and acetone

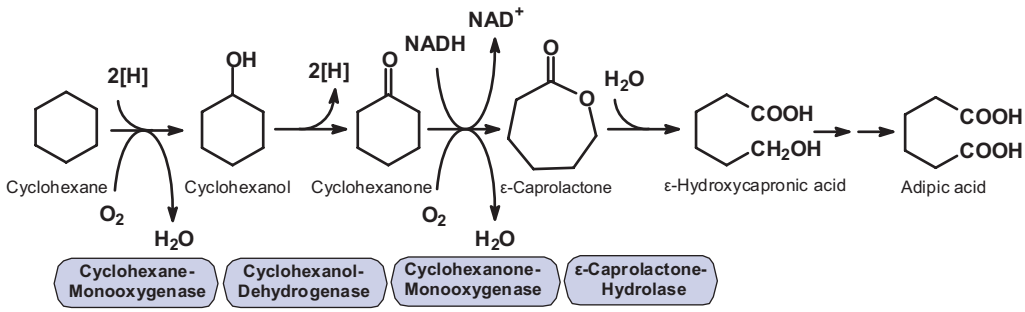


Fig. 6.13 Degradation of cyclohexane

6

6.1.4 Degradation of Monoaromatic Hydrocarbons

Substituted aromatic compounds are often found as plant constituents. These widespread natural substances include phenols, benzoic acids, phenylacetic acid, phenylpropane bodies, coumarins, tannins, flavonoids and the aromatic amino acids. Monoaromatic hydrocarbons, especially the BTEX compounds benzene, toluene, ethylbenzene, and xylenes, also occur as contaminants in the environment due to their extensive use as petrochemicals and as components of gasoline. From carbochemistry, phenols are added as common environmental chemicals.

The large number of aromatic natural products has led to the fact that bacteria have developed manifold enzymatic processes in order to introduce them into the intermediary metabolism. Aromatic structures are generally quite stable due to the delocalization of electrons in the aromatic ring. A key problem of aromatic degradation is therefore the question of how it is possible biochemically to activate and ultimately cleave the ring despite this stability. Aerobic and anaerobic microorganisms use very different mechanisms for this.

6.1.4.1 Aerobic Aromatics Degradation

Under aerobic conditions, aromatic compounds are generally degraded by bacteria much faster than under anaerobic conditions. This is of course due to the fact that oxygen is the best **electron acceptor** and allows particularly effective energy production. However, the faster degradation under aerobic conditions is due in particular to the fact that oxygen is used **as a reagent** in the degradation processes: First, it is largely oxygen-incorporating enzymes, called **oxygenases, that activate the various aromatic compounds,** converting them to a small number of ring cleavage substrates. Secondly, it is **oxygenases that cleave the aromatic ring.** The metabolism of aromatic compounds leading to the intermediate products of the tricarboxylic acid cycle can thus be divided into the following stages:

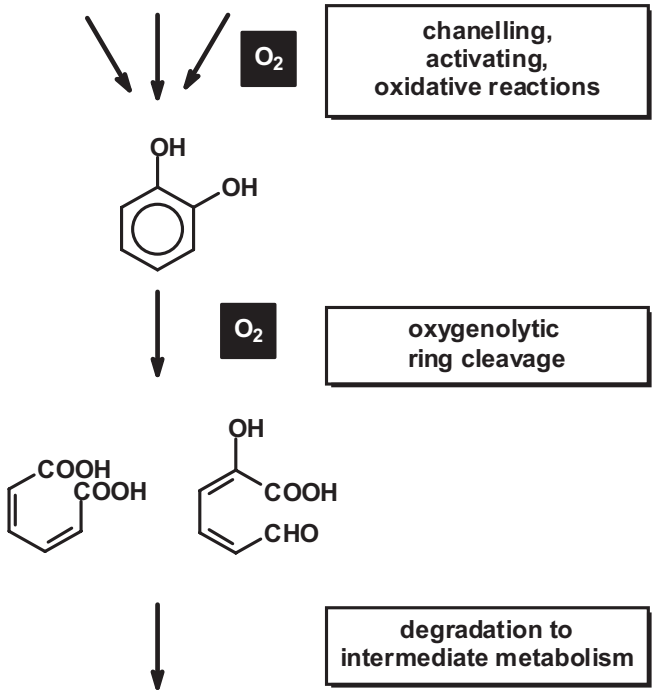
1. Peripheral degradation pathways: ring activation and preparation for ring cleavage by conversion of the substrate into a derivative with two *ortho* or *para* hydroxyl groups.
2. Oxidative cleavage of the aromatic ring by dioxygenases.
3. Metabolism of ring gap products into intermediates of central metabolism.

The **reactions preparing** the **ring cleavage** are mostly oxidations leading to three key compounds, **catechol, protocatechuate** and **gentisate.**

Activation of the aromatic ring occurs through **dioxygenase** and **dehydrogenase reactions**. **Dioxygenases** incorporate both atoms of atmospheric oxygen into the substrate and a non-aromatic *cis*-dihydrodiol is formed. Subsequent dehydrogenation produces the key compound catechol (◘ Fig. 6.14). **Phenol,** which already contains a hydroxyl group, is converted to catechol by a monooxygenase reaction. **Monooxygenases** catalyse the incorporation of one of the atoms of atmospheric oxygen into the substrate, the second oxygen atom is found in the water.

◘ Figure 6.15 shows that peripheral degradation pathways convert different aromatic compounds to the same dihydroxyaromatic compound. This phenomenon, termed **convergence of degradation pathways,** is particularly pronounced in the reactions leading to catechol and protocatechuate. For example, many of the mononuclear aromatics released during lignin degradation, such as cinnamate, coniferyl alcohol, ferulate, vanillinate and coumarate, are converted to catechol and protocatechuate (for enzymatic attack on lignin, see Sect. ► 4.4.4.5). In addition to the two compounds mentioned, there are others that serve as **substrates of the ring cleavage enzymes** (for example, gentisate, homogentisate). Practically all of these compounds carry two hydroxyl groups in *ortho*- or *para*-position to each other. Since many degradation pathways converge in ring cleavage **substrates**, they can be called catabolic hubs. To a lesser extent, however, pathways also converge in other compounds, including benzoate. This convergence in the chemistry of the degradation pathways is of central importance for their genetic organisation and regulation.

Catechol and the acid derivative protocatechuate can be cleaved by oxidation in



◘ **Fig. 6.14** Degradation principle for aromatics: aerobic by bacteria

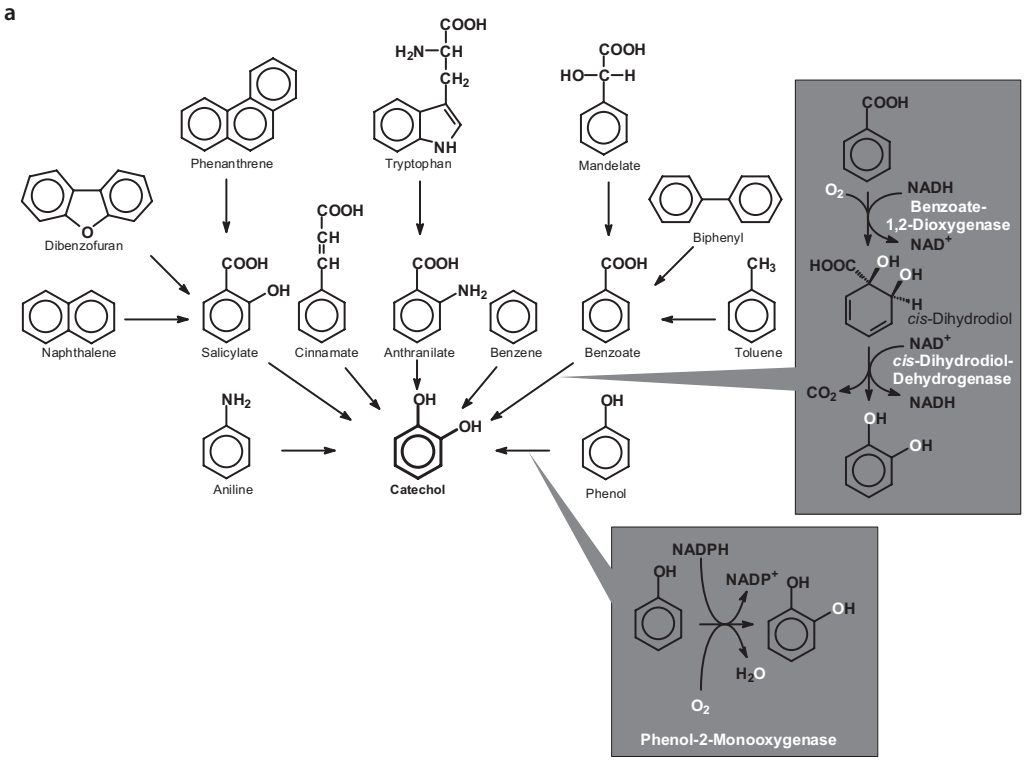
the *ortho*- or *meta*-position (◘ Fig. 6.16a, b), resulting in two different degradation pathways.

A third degradation pathway exists for gentisate, and another for the homogentisate produced during phenylalanine and tyrosine degradation.

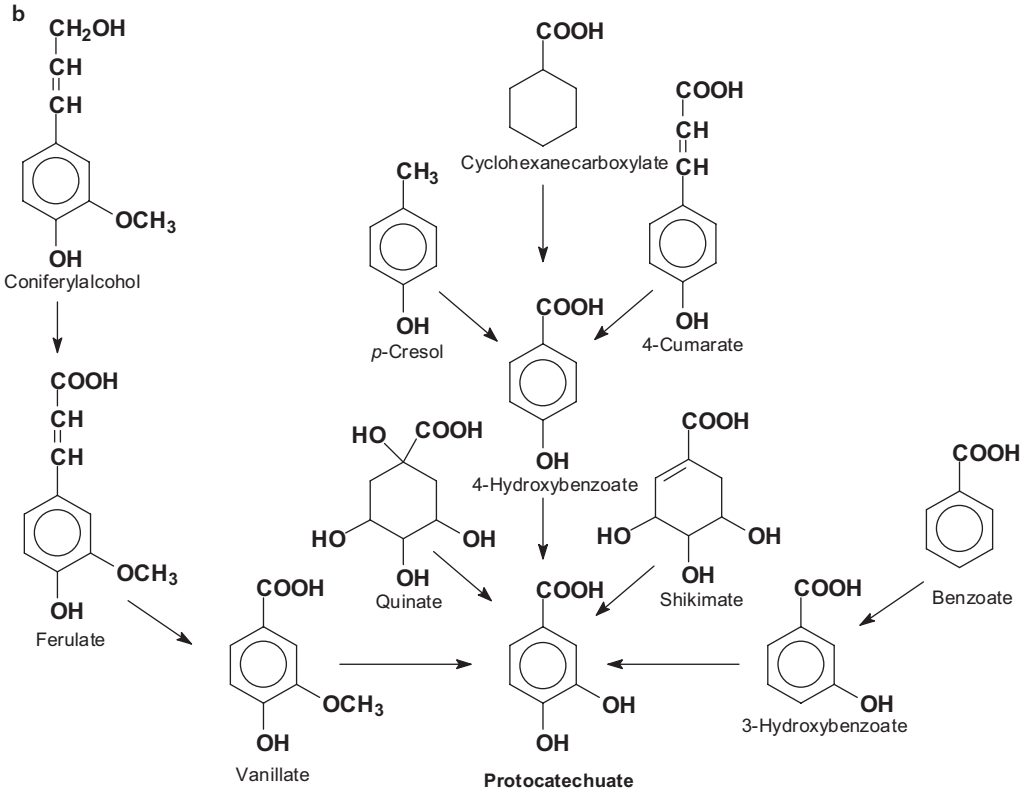
The **ring cleavage of** the 1,2-diphenol (catechol or protocatechuate) can occur in two positions, between the two adjacent hydroxyl groups, in the *ortho*-position (**intradiol)** (◘ Fig. 6.16a) or next to the two hydroxyl groups, in the *meta*-position (**extradiol)** (◘ Fig. 6.16a).

Both reactions are catalyzed by dioxygenases. The ring cleavage reactions have led to the naming of the degradation pathways, *ortho*- and *meta*- pathway. The ***ortho*-pathway** is also often named after the characteristic intermediate product *β*-ketoadipate pathway (oxoadipate pathway). The main reactions of the *ortho*-pathway are lactonization of the dicarboxylic acid, isomerization of the muconolactone to the *β*-ketoadipate enolactone, and thiolytic cleavage of the lactone opening product *β*-ketoadipate to succinyl-CoA and acetyl-CoA (◘ Fig. 6.16a).

The ring cleavage product of the ***meta*-pathway** is 2-hydroxymuconate semialdehyde, from which formate is hydrolytically cleaved to give 2-oxopentenoate. Alternatively, a reaction sequence via 2-hydroxymuconate and oxalocrotonate also leads to the C_5 compound. This becomes amenable to aldol cleavage after the introduction of a hydroxyl group by water addition, leading to pyruvate and acetaldehyde. Acetaldehyde is converted into acetyl-CoA by a CoA-dependent dehydrogenase and, like pyruvate, enters the intermediary metabolism.



◘ **Fig. 6.15** Convergence of peripheral degradation pathways of aromatic compounds



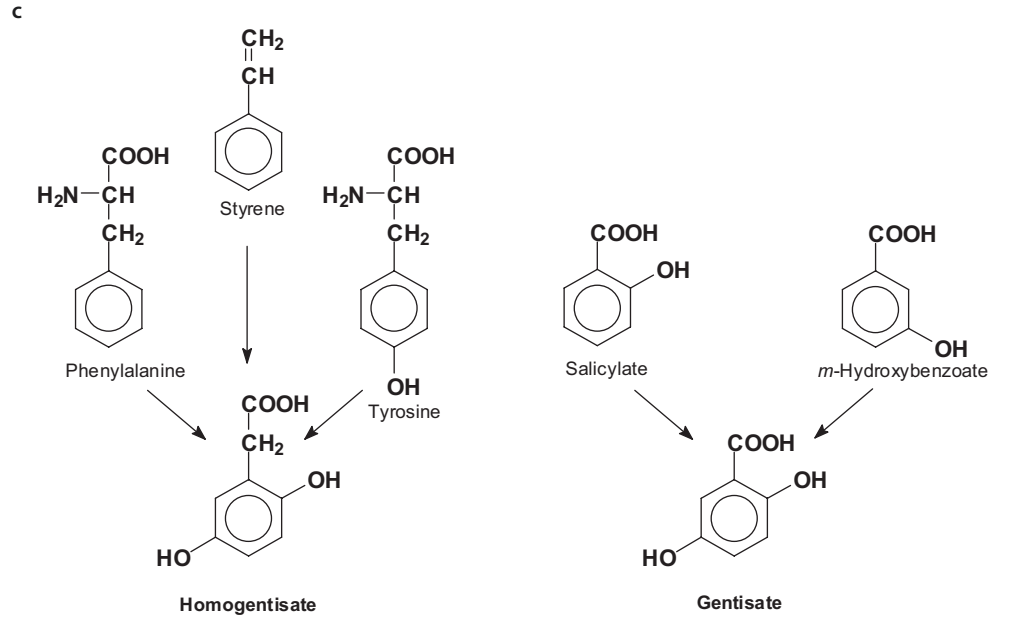


Fig. 6.15 (continued)

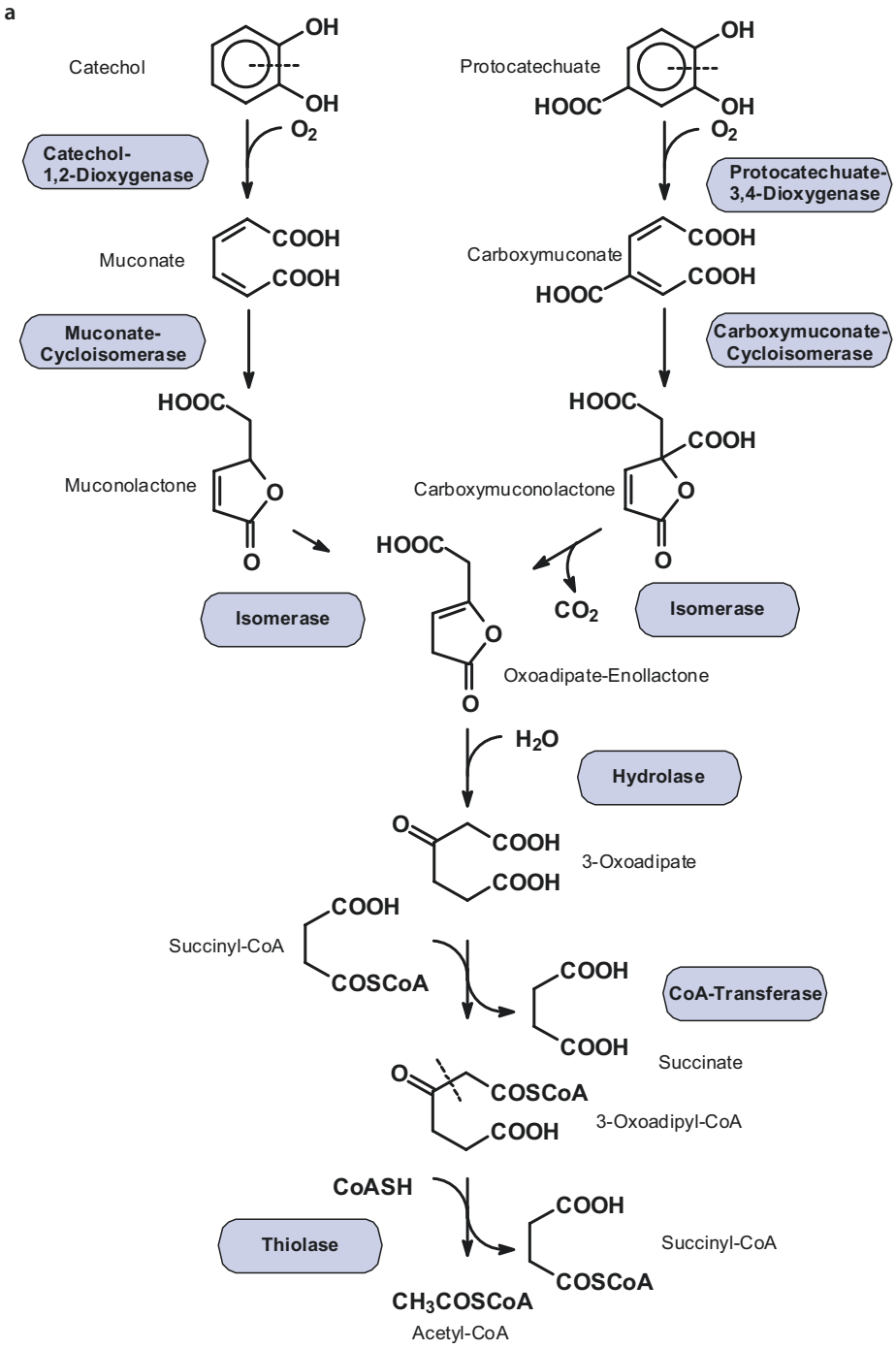


Fig. 6.16 **a** Degradation pathways for catechol and protocatechuate: *ortho*-pathway. **b** Degradation pathways for catechol and protocatechuate: *meta*-pathway

b

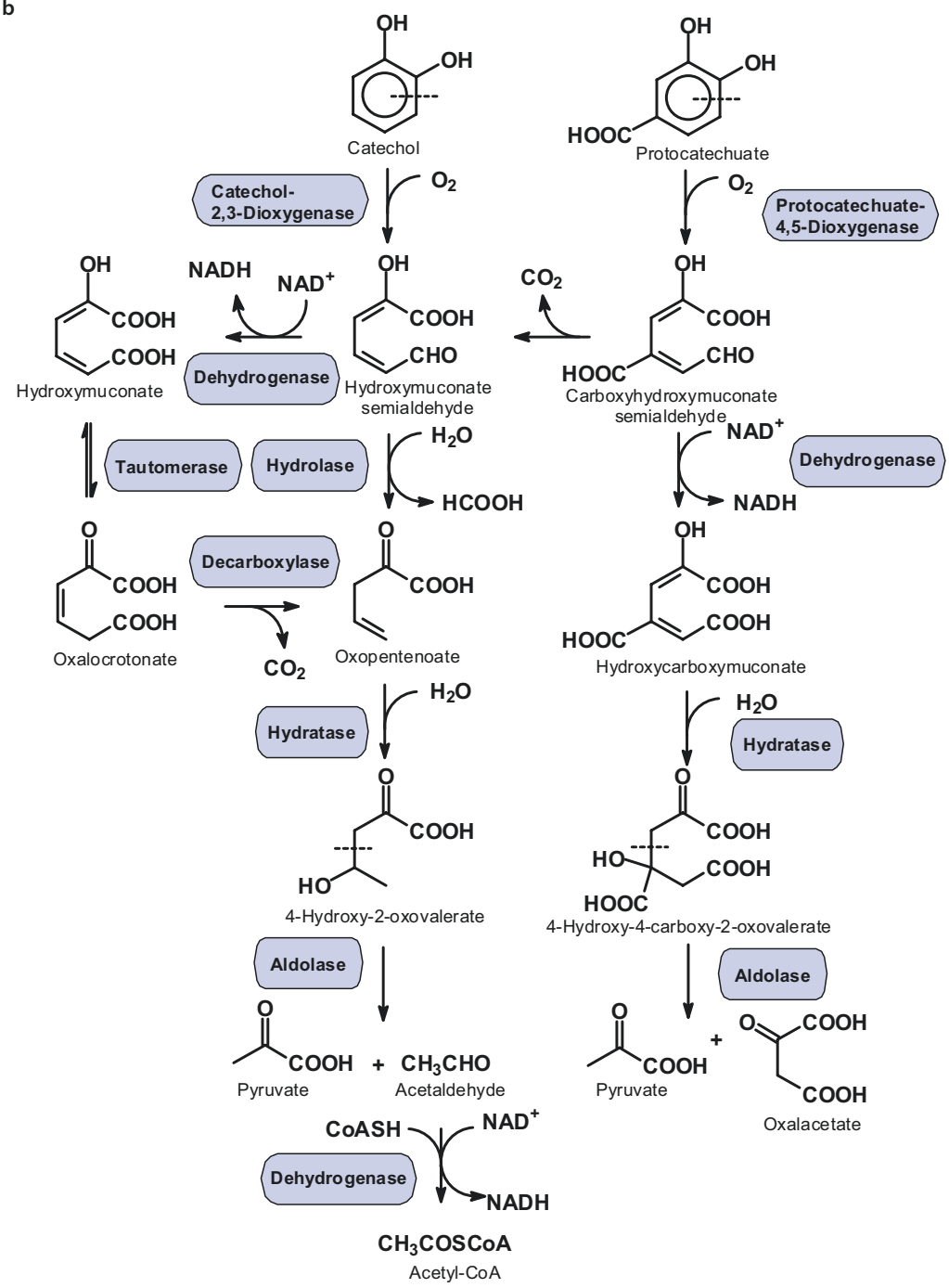


Fig. 6.16 (continued)

The enzymes of the *meta*-pathway also catalyze the degradation of **alkyl aromatics**. During xylene degradation, a methyl group is oxidized stepwise to the carboxyl group. Methyl pyrocatechol is formed after dioxygenation and dehydrogenation. Toluene can be converted to 3-methylcatechol by toluene dioxygenase as well as subsequent dehydrogenase. Analogous reactions to those shown in ◘ Fig. 6.16a, b are observed in the degradation of methylcatechols (◘ Fig. 6.17). Instead of formate, acetate is formed in the hydrolase reaction and instead of acetaldehyde, propionaldehyde results.

Salicylate degradation can proceed via catechol but also via gentisate (◘ Fig. 6.18). The **gentisate pathway,** in which the substrate of ring cleavage, gentisate, is hydroxylated in the *para*- position, cleavage occurs by a dioxygenase between a hydroxylated and a ring-substituted aliphatic C atom. The gentisate 1,2-dioxygenase produces a 1,3-dioxometabolite, which undergoes hydrolysis directly or after isomerization. The hydrolysis yields fumarate and maleate, respectively, both of which lead to the intermediate of the tricarboxylic acid cycle, malate, by water addition.

In the **homogentisate pathway,** in which the substrate of the ring cleavage, the homogentisate, is hydroxylated in the *para*-position, the cleavage is carried out by a dioxygenase between a hydroxylated and an aliphatic C atom substituted on the ring. The degradation of **styrene,** the basic building block of polystyrene, occurs in this way. The end products of this aromatic degradation are fumarate and acetoacetate.

The ability to degrade monoaromatics is not as common among microorganisms as that to degrade aliphatics. However, it is again representatives of the same genera that are characterized by a high degradation potential: *Acinetobacter, Bacillus, Burkholderia, Corynebacterium, Cupriavidus, Flavobacterium, Pseudomonas, Rhodococcus* and *Sphingomonas*.

6.1.4.2 Anaerobic Aromatics Degradation

For many years, there has been evidence that mono-aromatic natural products are degraded under anaerobic conditions. Benzene is very stable due to the homogeneous electron distribution in the molecule, so that anaerobic degradation is difficult. There are also few positive findings on the anaerobic degradation of polycyclic aromatic hydrocarbons.

The mechanism of degradation has only been elucidated in outline in recent years. Since there is no oxygen that can be used for the activation and cleavage of the aromatic ring, other mechanisms must be present for the cleavage of the aromatic. Anaerobic aromatic degradation can be divided into four phases:

1. Via modification reactions, a wide variety of aromatic compounds are converted into only a few key intermediates: Benzoyl-CoA, resorcinol, phloroglucin, hydroxyhydroquinone. The aromatic character of the respective compound is not abolished in the process.
2. This cancellation occurs in the de-aromatization reactions of the key intermediates and prepares for ring cleavage to form a non-aromatic 1,3-dioxo and 1,4-dioxo compound, respectively.
3. Hydrolytic ring cleavage follows.
4. The cleavage products are converted into intermediates of the central metabolism. This occurs primarily via reactions similar to those of the β-oxidation of fatty acids (◘ Fig. 6.19).

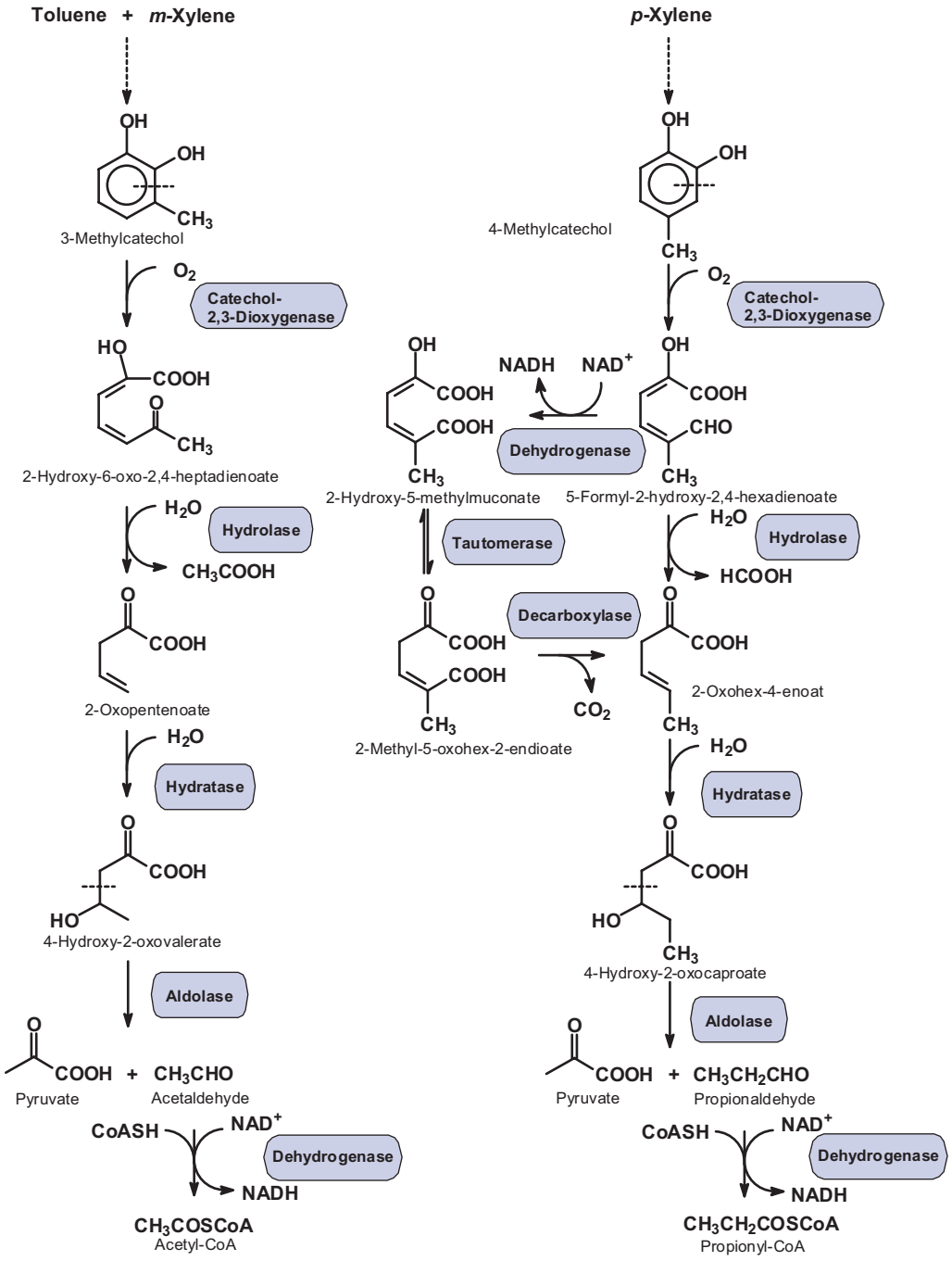


Fig. 6.17 Degradation of alkyl aromatics via the *meta*-pathway

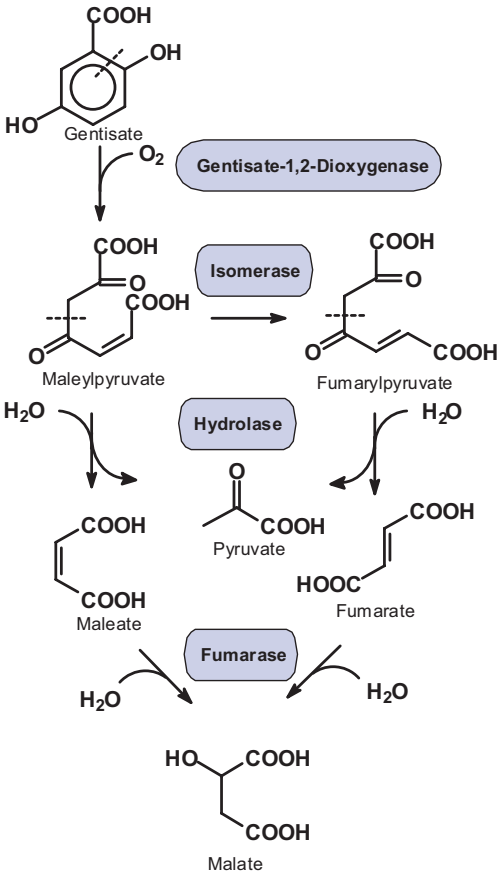


Fig. 6.18 Degradation of aromatics via the gentisate pathway

6.1.4.2.1 Formation of the Central Key Intermediates

The following reactions are involved in the formation of key intermediates:

1. Formation of a carboxylic acid by carboxylation or oxidation of a methyl group.
2. Decarboxylations.
3. Reductive Eliminations.
4. Hydroxylations.

The individual degradation pathways with leading modification reactions are explained in detail in a series of review articles by the Schink, Fuchs and Harwood working groups.

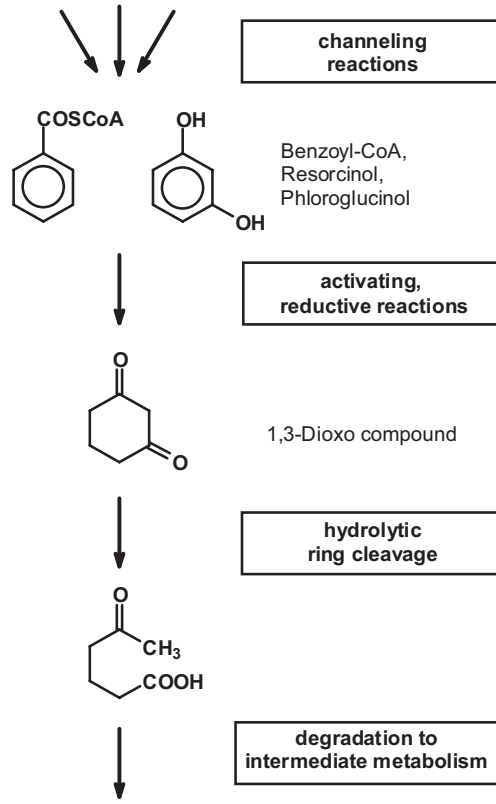


Fig. 6.19 Degradation principle for aromatics under anaerobic conditions by bacteria

6.1.4.2.1.1 Formation of Benzoyl-CoA

Many aromatics are degraded via benzoyl-CoA (Fig. 6.20). An important characteristic of such reactions of anaerobic aromatic degradation is a sequence at the coenzyme A thioester stage. Reasons for the role of the coenzyme appear to lie primarily in the mechanism of the subsequent reactions.

In the case of aromatic carboxylic acids, the necessary carboxyl group is already present. In the other case, this carboxyl group must be introduced or generated, i.e. carboxylation of the aromatic as for example with phenol, carboxylation of the alkyl side group with ethylbenzene, coupling with fumarate with toluene or another way of oxidation of a methyl group as with *p*-cresol takes place.

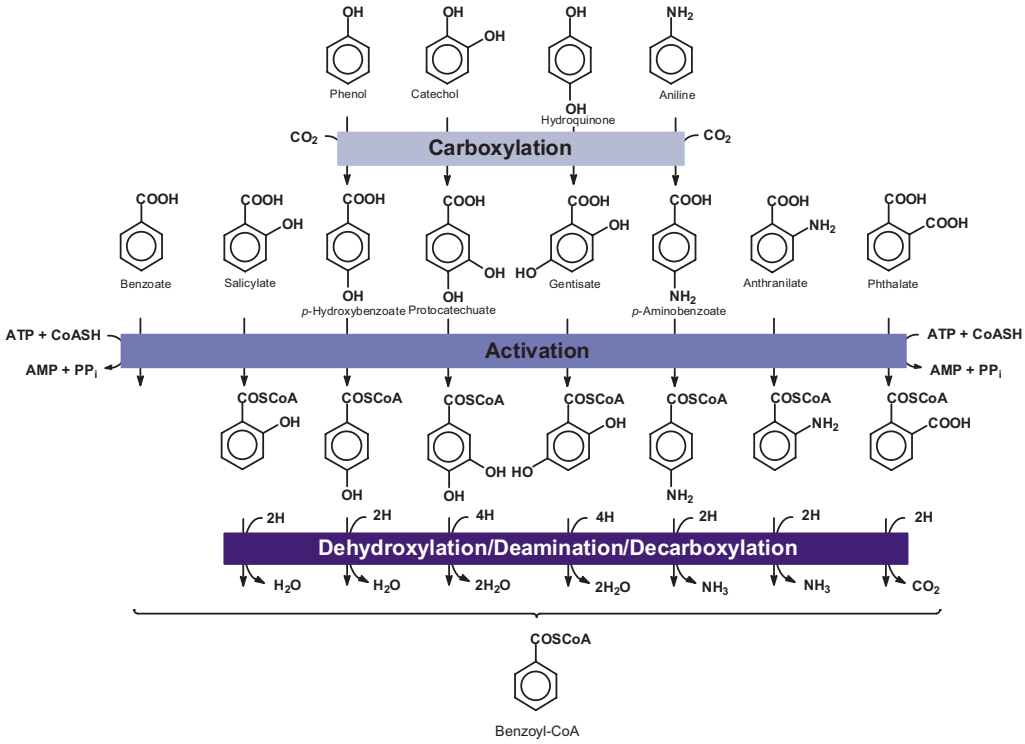


Fig. 6.20 Reaction sequences for the formation of benzoyl-CoA

Thus, the reaction sequence to form the benzoyl-CoA involves the following steps:

1. Formation of the aromatic carboxylic acid by carboxylation.
2. Activation of the carboxylic acid to form the coenzyme A thioester. The esterification of the respective substrate with CoASH is catalyzed by ligases, which hydrolyze ATP to PP_i and AMP.
3. Elimination of hydroxyl, amino and carboxyl groups (reductive dehydroxylation) to form benzoyl-CoA.

A number of phenolic compounds are first carboxylated to allow esterification with coenzyme A. This reaction has been shown for the anaerobic degradation of phenol, hydroquinone, catechol and also aniline.

In the degradation of ethylbenzene, the carboxylation of the side group occurs later (Fig. 6.21). Here, two dehydrogenases are upstream of the carboxylase. This is followed by activation to form the coenzyme A thioester and cleavage of acetyl-CoA to form benzoyl-CoA.

The example of toluene illustrates the extraordinary biochemical reactions involved in the oxidation of a methyl group (Fig. 6.22). The following steps take place:

1. Coupling of toluene in a radical reaction with fumarate.
2. Activation of the resulting carboxylic acid to the coenzyme A thioester.
3. Steps of β-oxidation and formation of β-oxo coenzyme A thioester.
4. Thiolytic cleavage of succinyl-CoA and formation of benzoyl-CoA.

There is evidence that an analogous reaction involving the addition of fumarate is also present as an initiating step in the degradation of *o*-, *m*- and *p*-xylene and *m*- and *p*-cresol.

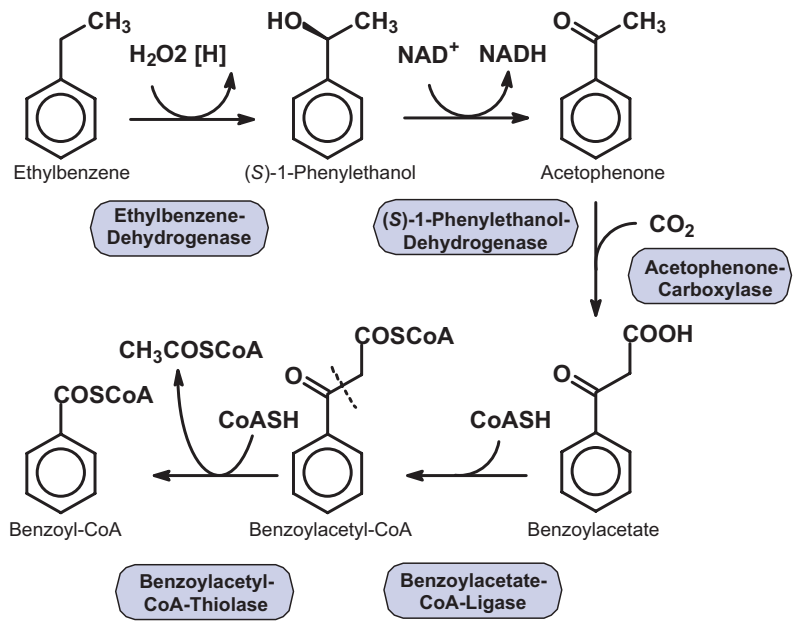


Fig. 6.21 Ethylbenzene activation

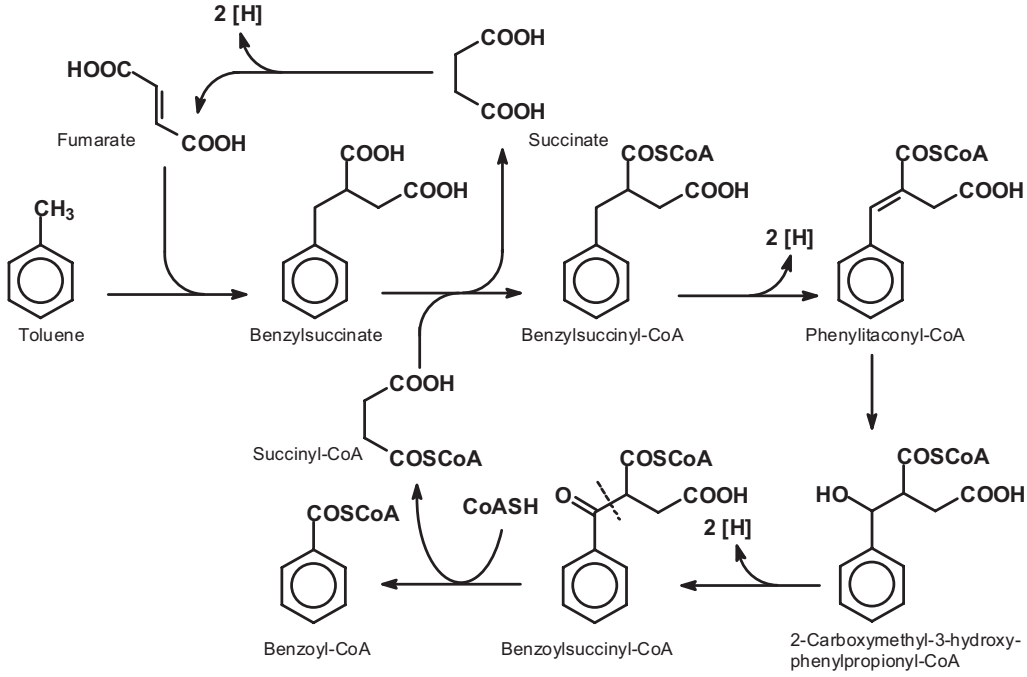


Fig. 6.22 Toluene activation

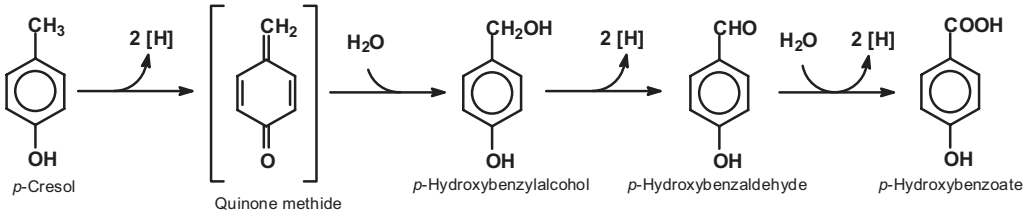


Fig. 6.23 *p*-Cresol activation

p-Cresol can be oxidized relatively easily to a quinone methide, to which water can be readily attached. Further oxidation of the alcohol then takes place by means of dehydrogenases to 4-hydroxybenzoate (Fig. 6.23).

Reductive eliminations of carboxyl, hydroxyl and amino groups play a key role in the conversion of a number of benzoate derivatives to benzoyl-CoA. Decarboxylation to benzoate and benzoyl-CoA, respectively, has been postulated for phthalate. In particular, phenolic compounds that are not degraded via one of the hydroxybenzenes (resorcinol, phloroglucin, hydroxyhydroquinone) undergo elimination reactions. All reductive dehydroxylations described so far are catalyzed at the CoA ester step. The reductive dehydroxylation of 4-hydroxybenzoyl-CoA in *Rhodopseudomonas palustris* and also *Thauera aromatica* has been studied in detail. Amino substituents can also be reductively cleaved. For example, 4-aminobenzoyl-CoA, an intermediate of the degradation of aniline, is deaminated to benzoyl-CoA. For anthranilate, reductive cleavage of the amino group has also been postulated.

6.1.4.2.1.2 Formation of 1,3-Diphenols

Multiple hydroxylated benzoate derivatives are degraded via resorcinol, phloroglucin or hydroxyhydroquinone.

Decarboxylation leads to products with a 1,3-diol system such as resorcinol and pyrogallol from di- and trihydroxybenzoate (for example 2,4-dihydroxy- and 2,6-dihydroxybenzoate or gallate).

In pyrogallol, a reaction cascade of transhydroxylations then occurs to form phloroglucin. Unusual reactions involving 1,2,3,5-tetrahydroxybenzene as a cosubstrate are involved. A hydroxyl group of tetrahydroxybenzene is transferred to position 5 of pyrogallol to form phloroglucin and a new molecule of tetrahydroxybenzene. Phloroglucin is also formed directly from phloroglucinate by decarboxylation.

Oxidation reactions on the aromatic ring follow in the degradation of α-resorcylate and resorcinol in the denitrifying bacteria *Azoarcus anaerobius* and *Thauera aromatica* strain AR-1. In both cases, the aromatic ring is further destabilized by the introduction of another hydroxyl group. Products of these reactions are hydroxyhydroquinone from resorcinol and 2,3,5-trihydroxybenzoate from α-resorcylate. 2,3,5-Trihydroxybenzoate can be readily decarboxylated, also forming hydroxyhydroquinone as a product (Fig. 6.24).

6.1.4.2.2 Dearomatisation Reactions

6.1.4.2.2.1 Degradation of Benzoyl-CoA

Detailed work with *Rhodopseudomonas palustris* and *Thauera aromatica* shows that benzoyl-CoA is converted to a diene derivative. Analogous to the Birch reduction of benzene, the sequential sequence of one-electron reduction—proton addition is assumed for this reaction. With the physiological electron donor ferredoxin, this is an endergonic process driven in *Thauera aromatica* by the hydrolysis of 2 moles of ATP per mole of substrate, that is, one ATP for each electron added.

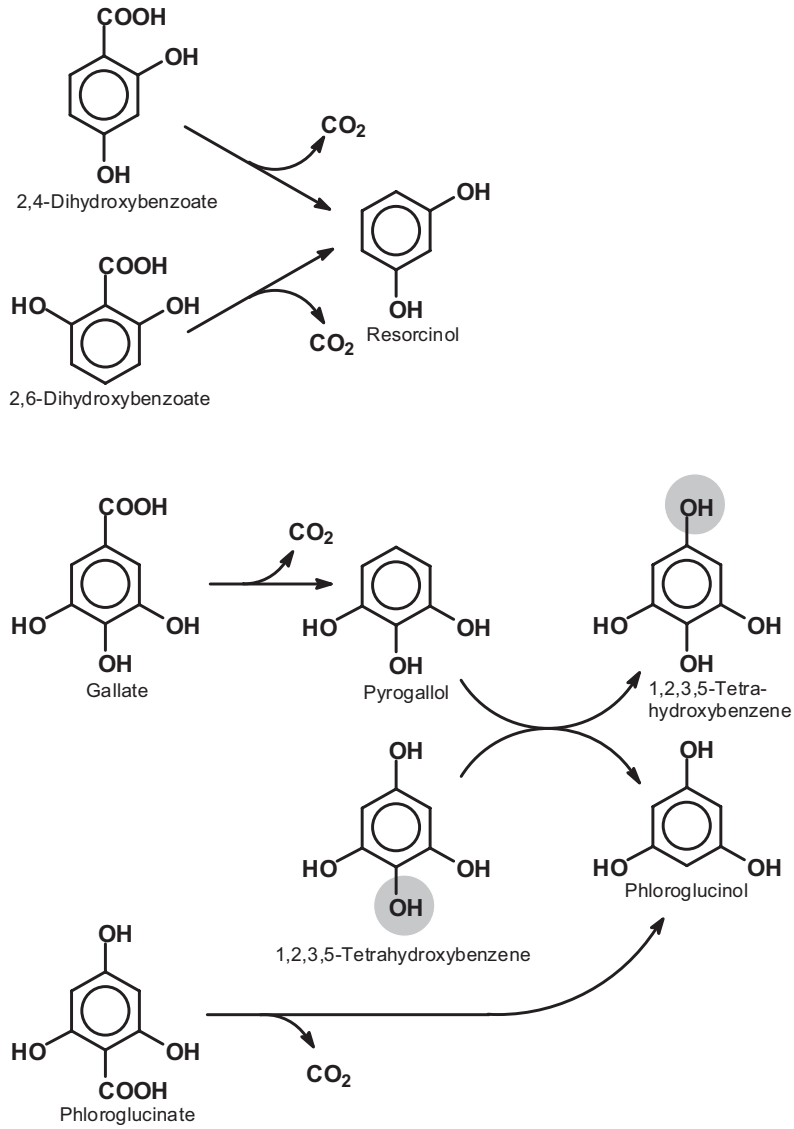


Fig. 6.24 Resorcinol and phloroglucin formation

Different routes were found in the denitrifying *Thauera aromatica,* the phototrophic *Rhodopseudomonas palustris* and a fermenter (Fig. 6.25).

In *Thauera aromatica,* the next intermediate that follows from the diene is 6-hydroxycyclohex-1-ene-1-carboxyl-CoA due to the addition of water. Then there is a gap in knowledge between 6-hydroxycyclohex-1-ene-1-carboxyl-CoA and 3-hydroxypimelyl-CoA, the first non-cyclic intermediate. The simplest way to explain the formation of the intermediate is the addition of water to the double bond in 6-hydroxycyclohex-1-ene-1-carboxyl-CoA, the oxidation of the resulting alcohol to the carbonyl compound, and the hydrolytic ring cleavage.

In *Rhodopseudomonas palustris,* the cyclic diene is further reduced to cyclohex-1-ene-1-carboxyl-CoA. Subsequent β-oxidation

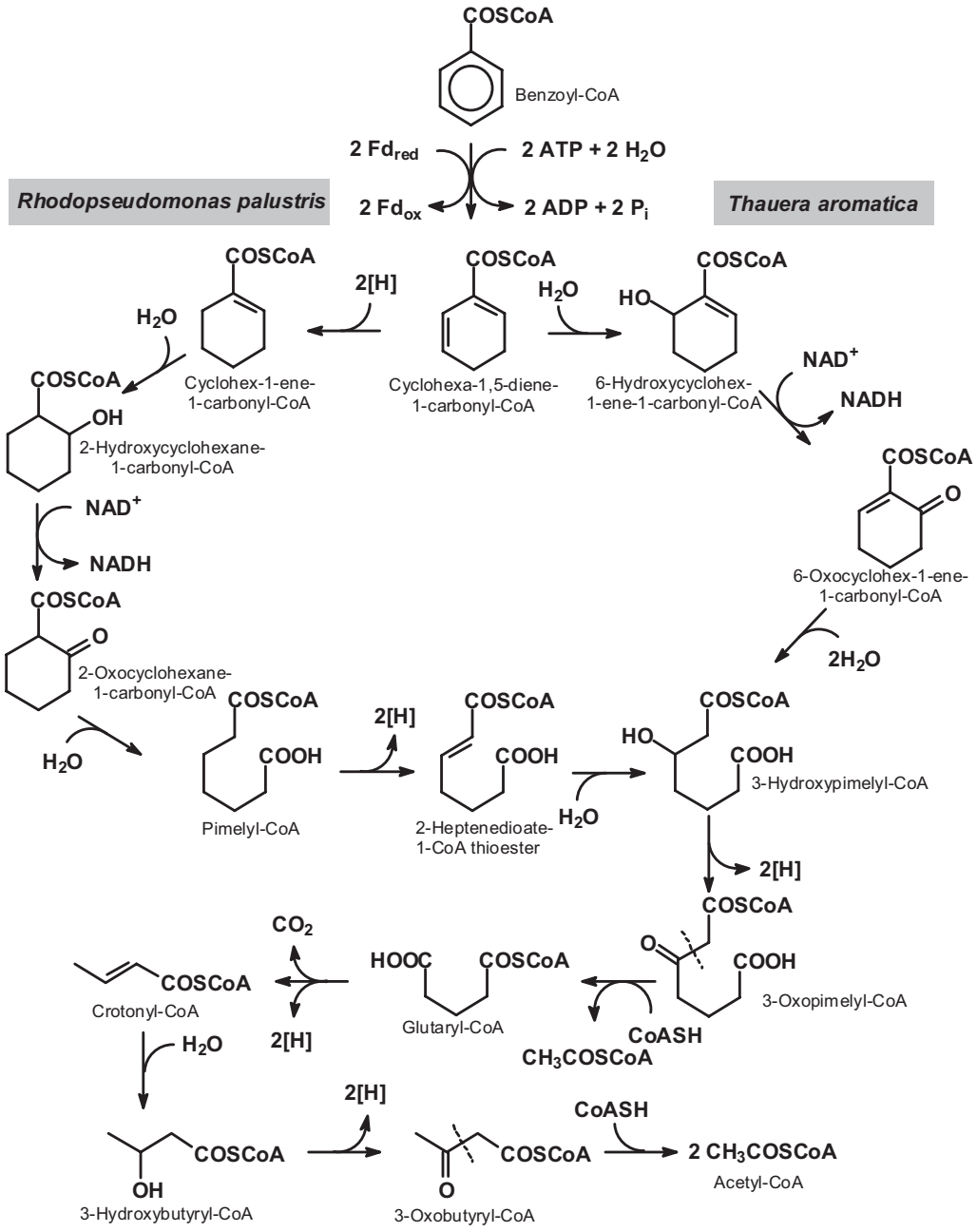


Fig. 6.25 Degradation of benzoyl-CoA to acetyl-CoA by a phototrophic and a denitrifying bacterium

leads to the formation of a cyclic *β*-oxo compound. This is followed by hydrolytic ring opening to pimelyl-CoA, which is further oxidized via 3-hydroxypimelyl-CoA as in *Thauera aromatica.* The same route via cyclohex-1-ene-1-carboxyl-CoA is also used by *Syntrophus gentianae,* which undergoes fermentation with benzoate.

Further *β*-oxidation of 3-hydroxypimelyl-CoA leads to glutaryl-CoA as well as acetyl-CoA. The oxidation of glutaryl-CoA to 2 acetyl-CoA as well as CO_2 proceeds via glutaconyl-CoA and crotonyl-CoA and is catalyzed by a glutaryl-CoA dehydrogenase.

6.1.4.2.2.2 Degradation of Resorcinol, Phloroglucin and Hydroxyhydroquinone

No CoA thioester activation occurs in the degradation pathways for resorcinol, phloroglucin, and hydroxyhydroquinone.

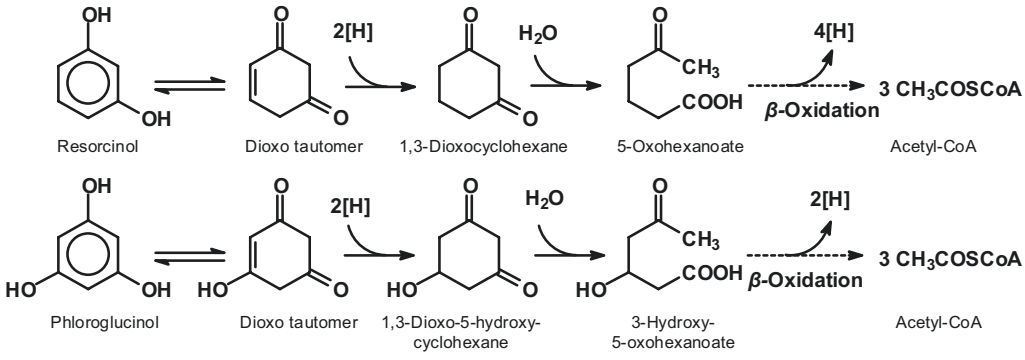
Reductive dearomatization initiates ring cleavage in the resorcinol and phloroglucin pathways.

In resorcinol, the two *meta*-positional hydroxyl groups polarize the π-electron cloud in such a way that, with the formation of the oxotautomer, an "isolated" double bond can exist in the ring, which can be relatively easily reduced selectively with two electrons. Dihydroresorcinol is formed and thus the aromatic character of the molecule is eliminated. The three hydroxyl groups in phloroglucin polarize the π-electron cloud even more strongly than the two of resorcinol. Therefore, in aqueous solution, the trioxotautomer is encountered. Consequently, phloroglucin has no aromatic character and can be easily reduced with mild reducing agents.

Resorcinol-degrading fermenting bacteria (*Clostridium* sp.) follow this degradation strategy and 1,3-dioxocyclohexane is produced as the final alicyclic compound. Apparently, the C-3 atom of 1,3-dioxocyclohexane carries sufficient positive charge to allow hydrolytic cleavage to 5-oxocaproate.

Anaerobic phloroglucin-degrading bacteria, such as *Coprococcus* sp., *Eubacterium oxidoreducens, Pelobacter acidigallici* or *Rhodopseudomonas gelatinosa,* first reduce phloroglucin to dihydrophloroglucin (1,3-dioxo-5-hydroxycyclohexane) in an NADPH-dependent reaction. Nucleophilic attack on one of the carbonyl groups of dihydrophloroglucin opens the ring and generates 3-hydroxy-5-oxocaproate. Further degradation of the partially oxidized caproate no longer presents any fundamental biochemical problems (◘ Fig. 6.26).

A fundamentally different pathway of de-aromatization is found in the degradation of resorcinol by *Azoarcus anaerobius* and of *α*-resorcylate by *Thauera aromatica* AR-1. Here, hydroxyhydroquinone, intermediate of both degradation pathways, is oxidized by hydroxyhydroquinone dehydro-



◘ **Fig. 6.26** Proposed degradation sequences for resorcinol (top) and phloroglucin (bottom)

genase to 2-hydroxybenzoquinone. This means that the aromatic character is eliminated by oxidation and not by reduction. The further degradation pathway has not yet been clarified.

The ability to anaerobically degrade aromatic compounds occurs in facultative and obligate anaerobic bacteria. It has been demonstrated in nitrate-reducing *Moraxella* and *Pseudomonas* strains, sulfate-reducing *Desulfobacterium* and *Desulfomaculum* species, phototrophic *Rhodopseudomonas* and *Rhodospirillum* species, and methanogenic consortia. Many of the above-mentioned representatives can use aromatic compounds as their sole carbon and energy source; the growth rate is low and the yield is low.

6.1.4.2.2.3 Hypothesis: Anaerobic Degradation of Naked Aromatic Compounds

Of great interest and therefore worked on for a long time is the anaerobic degradation of benzene, which has been detected in many anaerobic sediments. Here, other mechanisms are to be expected, since there is no attachment point for the generation of a carboxyl group as, for example, in toluene. A sequence via phenol and then benzoate is proposed for benzene, whereby the carboxyl group should also originate from benzene and not CO_2.

Formation of Oxygen Under Anaerobic Conditions from Perchlorate and Degradation of Aromatics

The oxyanions chlorate (ClO_3^-) and perchlorate (ClO_4^-) are widely used for various purposes. Chlorate is used as a herbicide or defoliant. It is released when chlorine dioxide (ClO_2) is used as a bleaching agent in the paper and fiber industry. Perchlorate is produced in large quantities as a high-energy compound in solid rocket propellant. Incorrect handling of these compounds and the fact that they are chemically stable in water have led to concentrations in surface and ground waters that are hazardous to health.

Various microorganisms are known to have the ability to reduce (per)chlorate, either to chlorite (ClO_2^-) or completely to chloride. The former reaction has long been known and is carried out by denitrifying bacteria through the enzymes nitrate or chlorate reductase. These organisms are probably not able to respire with (per)chlorate as electron acceptor.

However, the complete reduction of **(per)chlorate** to chloride is coupled to growth in newer isolates, **anaerobic respiration** takes place. Chlorate has a redox potential that is even more positive than that of the O_2/H_2O-pair.

$$ClO_3^- \rightarrow Cl^- + 3H_2O$$
$$\left(6e^- + 6H^+; E^{0'} = +1030\,mV\right)$$

The various chlorate-reducing bacteria are mostly facultative anaerobes and therefore capable of aerobic growth.

Since all perchlorate reducers also reduce chlorate, an identical degradation pathway can be assumed. The pathway for (per)chlorate reduction was proposed as follows:

$$ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$$

Organisms have been shown to dismutate harmful chlorite into chloride and oxygen using chlorite dismutase. The complete degradation pathway also includes a (per) chlorate reductase.

Organisms have been isolated that carry out the degradation of benzene and other monoaromatics with perchlorate under anaerobic conditions. Whether the mechanisms of oxic degradation with ring-activating and -cleaving dioxygenases, i.e. the utilization of molecular oxygen, play a role in this will have to be

clarified in the future. Or does the oxygen produced during dismutation also function as an electron acceptor of the respiratory chain?

6.1.4.3 Strategies of an Unorthodox Aerobic Degradation of Aromatics

An elegant strategy to cope with the high resonance energy of aromatic substrates is shown in so-called hybrid degradation pathways, which have mostly been overlooked so far (see ◘ Fig. 6.27 for benzoate and phenylacetate). There, a CoA thioester is used as in anaerobic metabolism. Oxygen is also used, but unlike in the classical aerobic degradation pathway, an epoxide is formed by monooxygenase, preparing the ring cleavage, which then requires no further oxygen.

6.1.4.3.1 Hybrid Pathway for Benzoate

The complicated degradation pathway for benzoate, studied in the denitrifying facultative aerobes *Azoarcus evansii* and *Bacillus stearothermophilus,* requires the formation of benzoyl-CoA followed by an oxygenase reaction and non-oxygenolytic ring cleavage. The benzoyl-CoA transformation is carried out by the iron-containing benzoyl-CoA oxygenase (BoxB). It obtains electrons from NADPH through reductase (BoxA), which uses iron-sulfur centers and FAD for transfer. Benzoyl-CoA oxygenase forms a non-aromatic 2,3-epoxide. Enoyl-CoA hydratase (BoxC) then needs two molecules of water to first hydrolytically open the ring of 2,3-epoxybenzoyl-CoA, which runs off via its tautomeric seven-membered oxepin ring form. Then the C2 of the ring is dehydrolyzed as formate to give 3,4-dehydroadipyl-CoA-semialdehyde. The formation of formate also explains the old finding that genes for enzymes of benzoate and formate metabolism are co-induced. The semialdehyde is oxidized to 3,4-dehydroadipyl-CoA by an $NADP^+$-dependent aldehyde dehydrogenase (BoxD). The subsequent degradation sequence yields acetyl-CoA and succinyl-CoA in analogy to fatty acid degradation.

The overall stoichiometry of aerobic benzoate degradation via CoA ligation yields the following equation:

$$\text{Benzoate} + \text{ATP} + 2\text{CoASH} + \text{O}_2 + 3\text{H}_2\text{O} + \text{NAD}^+ \rightarrow \text{Acetyl-CoA} + \text{Succinyl-CoA} + \text{Formiate} + \text{AMP} + \text{PP}_\text{i} + \text{NADH} + \text{H}^+$$

In contrast, aerobic benzoate degradation via the 3-oxoadipate pathway shows the following equation:

$$\text{Benzoate} + \text{CoASH} + 2\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Acetyl-CoA} + \text{Succinate} + \text{CO}_2$$

This means that the "new" degradation pathway needs less oxygen and forms the reduced products NADH and formate.

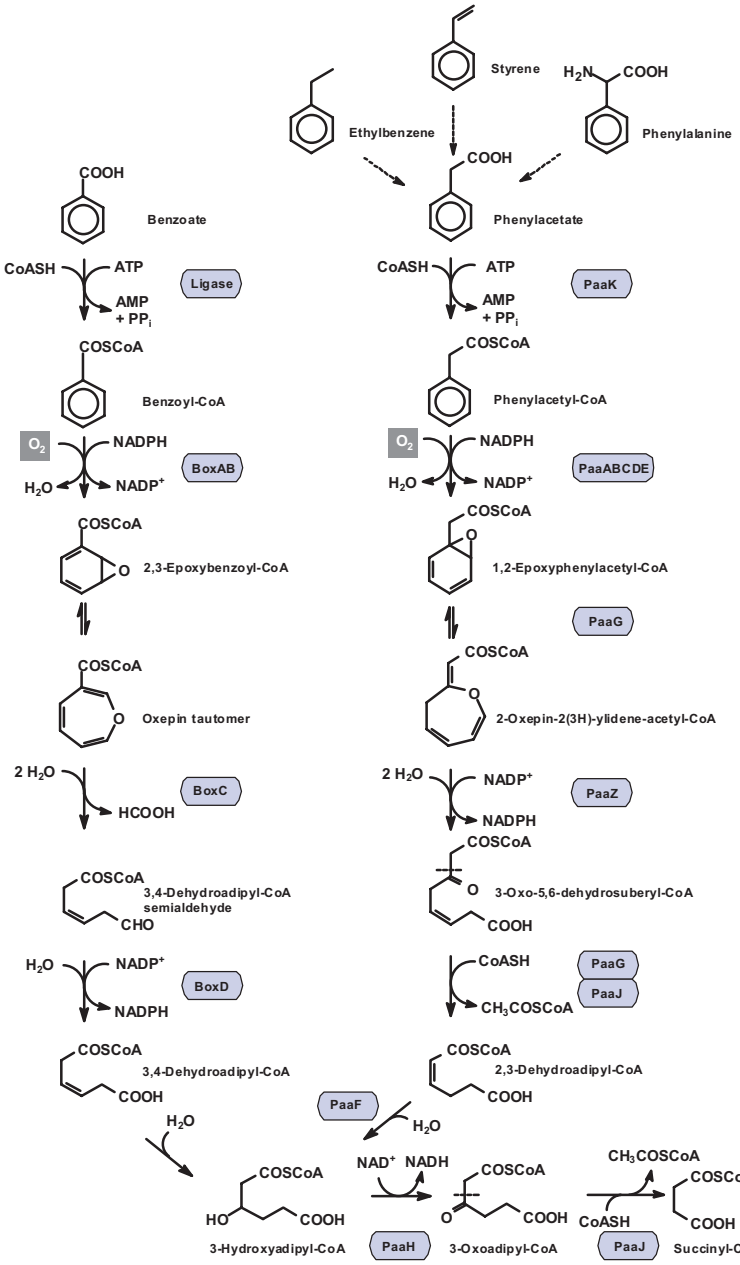


Fig. 6.27 CoA-dependent aerobic degradation pathways for aromatics. Reactions and intermediates of aerobic benzoate degradation have been studied in *Azoarcus evansii* (*left*). Reactions and intermediates of aerobic phenylacetate degradation have been studied in *E. coli* K12 and *Pseudomonas* sp. strain Y2 (*right*). *BoxAB* Benzoyl-CoA, *NADPH* Oxygen oxidoreductase (2,3-epoxide-forming); trivial name CoA 2,3-epoxidase, *BoxC* 2,3-Epoxybenzoyl-CoA dihydrolase, *BoxD* 3,4-Dehydroadipyl-CoA-semialdehyde dehydrogenase, *PaaK* Phenylacetate-CoA ligase (AMP-forming), *PaaABCDE* 1,2-Phenylacetyl-CoA epoxidase (NADPH), *PaaG* 1,2-Epoxyphenylacetyl-CoA isomerase (oxepin-CoA-forming), postulated 3,4-dehydroadipyl-CoA isomerase, *PaaZ* Oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA-semialdehyde dehydrogenase ($NADP^+$), *PaaJ* 3-Oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase, *PaaF* 2,3-Dehydroadipyl-CoA hydratase, *PaaH* 3-Hydroxyadipyl-CoA dehydrogenase (NAD^+)

6.1.4.3.2 Hybrid Pathway for Phenylacetate

A substantial part of aromatic compounds (such as phenylalanine or styrene) is metabolized by bacteria via phenylacetate. The unusual strategy listed above for benzoate, a characteristic typical of anaerobic rather than aerobic aromatic degradation, is also found for phenylacetate: The first enzyme in the degradation pathway, phenylacetate CoA ligase (PaaK), converts phenylacetate into phenylacetyl-CoA. The other intermediates are then processed as CoA thioesters. The aromatic ring of phenylacetyl-CoA is activated to a 1,2-epoxide by a special multicomponent oxygenase (PaaABCDE). The reactive non-aromatic epoxide isomerizes by 1,2-epoxyphenylacetyl-CoA isomerase (PaaG) to a seven-membered O-heterocyclic enol ether, an oxepin. This isomerization is followed by hydrolytic ring cleavage catalyzed in an unprecedented reaction by oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA-semialdehyde dehydrogenase ($NADP^+$) (PaaZ). Oxepins are amenable to hydrolysis. Water is added to the double bond of the side chain, the ring is opened and the 3-oxo-5,6-dehydrosuberyl-CoA-semialdehyde is oxidized to 3-oxo-5,6-dehydrosuberyl-CoA.

It must be emphasized that the product of thiolytic cleavage of 3-oxo-5,6-dehydrosuberyl-CoA by 3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase (PaaJ) is not the 2,3-dehydro compound, as in benzoate degradation, but 3,4-dehydroadipyl-CoA. The 3,4-2,3 isomerization reaction is likely catalyzed by enoyl-CoA isomerase (PaaG), although PaaJ itself may also carry out the reaction.

The subsequent β-oxidation steps leading to acetyl-CoA and succinyl-CoA are catalyzed by enoyl-CoA hydratase (PaaF), alcohol dehydrogenase (PaaH), and β-ketothiolase (PaaJ).

In summary, the equation below shows the formation of two molecules of acetyl-CoA and one molecule of succinyl-CoA from phenylacetate:

$$\text{Phenylacetate} + O_2 + 2H_2O + 3\text{CoASH} + \text{ATP} + NAD^+ \rightarrow 2\text{Acetyl-CoA} + \text{Sccinyl-CoA} + \text{AMP} + PP_i + \text{NADH} + H^+$$

6.1.4.3.3 Are the Hybrid Degradation Pathways Significant?

The previously overlooked benzoate degradation pathway occurs in 4–5% of all bacteria whose genomes have been sequenced, demonstrating the wide use of the listed strategy. For comparison, 7% of species possess the genes for a benzoate-1,*2*-dioxygenase (BenABC) and a *cis*-diol dehydrogenase (BenD), which are characteristic of the classical benzoate degradation pathway with ring-cleaving dioxygenase. Some organisms possess both types of benzoate degradation pathways.

In the case of phenylacetate degradation, it is even clearer as the pathway is the only one for aerobic degradation by bacteria. The high significance is illustrated by the fact that phenylacetate is a central key intermediate in aromatic degradation and the amino acid phenylalanine is metabolized mainly via it. Conserved genes of the phenylacetate degradation pathway have been found in 16% of sequenced bacterial species to date, clearly indicating relevance. These species include many members of the *Proteobacteria,* such as the model organisms *Escherichia coli* or *Pseudomonas putida,* pathogens such as *Bordetella pertussis* and *Shigella dysenteriae,* or opportunistic pathogens such as *Burkholderia xenovorans* and *Burkholderia cenocepacia.* Gram-positive organisms

such as *Rhodococcus* sp. or members of the *Deinococcus*/*Thermus* group also use this degradation pathway. The degradation pathway appears to have evolved in the bacterial domain, as the only archaea carrying the key genes of the degradation pathway are some members of the Halobacteria.

Attempts to Explain/Interpret Hybrid Degradation Pathways with CoA Intermediates (After Georg Fuchs, Freiburg)

The use of CoA esters in an aerobic aromatic degradation pathway does not appear to make sense at first glance. However, the energy invested in CoA thioester formation is not lost, but is later recovered in the form of acetyl-CoA.

A CoA thioester-dependent degradation pathway has an advantage over the classical oxic and anoxic pathways, respectively, under fluctuating oxic/anoxic conditions. Such a hybrid degradation pathway allows flexibility and rapid adaptation to changing oxygen levels, as CoA-thioester substrates are required in both the oxic and anoxic situations. If the classical anaerobic pathway were run for benzoate, the switch from anoxic to oxic conditions would lead to the accumulation of benzoyl-CoA because the ring-reducing enzyme, benzoyl-CoA reductase of the anoxic pathway, is oxygen-sensitive and consequently would be inactivated. All cellular CoA would be trapped in this dead-end product, since the benzoate-CoA ligase is oxygen-insensitive and continues to function under these conditions. CoA deficiency would eventually be lethal. In addition, benzoyl-CoA oxygenase, the enzyme that dearomatizes benzoyl-CoA, has a high affinity for oxygen and would therefore function under microaerobic conditions.

Isomerase PaaG-mediated oxepin formation is facilitated by the CoA thioester, so that subsequent ring cleavage can occur hydrolytically and no further oxygen is required.

CoA thioester intermediates appear to be less toxic than the analogous metabolites of classical aerobic degradation pathways, especially those of meta-degradation *sequences.*

Furthermore, CoA thioester formation appears to indirectly facilitate the transport of aromatic acids. In addition, CoA-bound intermediates are retained in the cell. They can be rapidly recognized and bound via the CoA-binding motifs of processing enzymes. This rapid recognition and binding may be important for the highly reactive intermediate 1,2-epoxyphenylacetyl-CoA, since free epoxides would damage the cell.

6.1.5 Degradation and Humification of Polynuclear Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are compounds whose backbone consists of two or more fused (anellated) benzene rings. The simplest PAH with two condensed benzene rings is **naphthalene.** Further condensation can be linear or angular, resulting in two different trinuclear systems, **anthracene** and **phenanthrene.** As the number of rings increases, so does the number of possible arrangements and hence PAH structures. Common tetranuclear compounds are **benzo[a]anthracene** and **pyrene.** The pentonuclear **benzo[a]pyrene** is one of the best known environmental toxins due to its carcinogenic properties. The list is to be concluded with the hexonuclear **perylene.**

In addition to the unsubstituted ring systems, there are a large number of substituted PAHs, especially methyl-substituted compounds and fused systems with a cyclopentadiene ring, for example **fluorene** and **fluoranthene.**

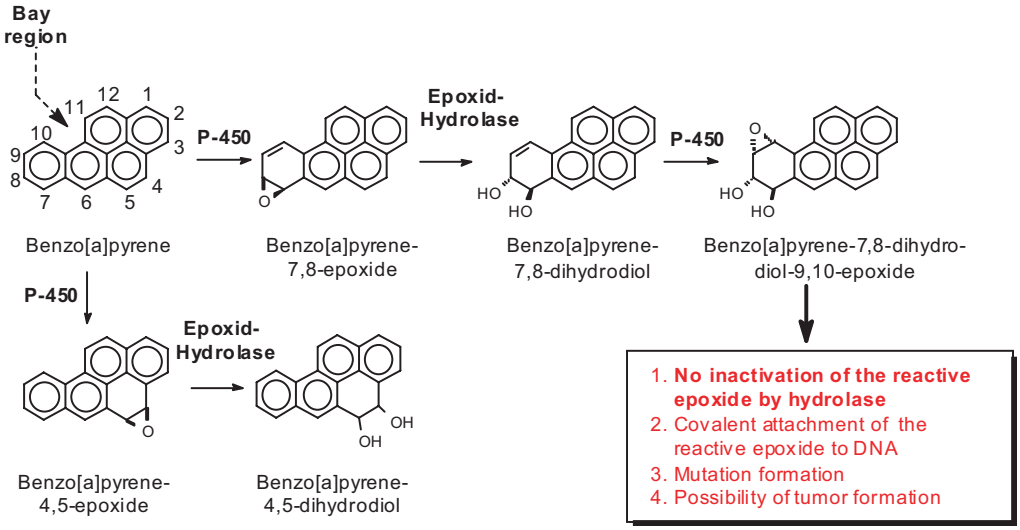
PAHs are ubiquitously distributed in low concentrations, as they are formed during combustion processes of organic material (forest fires, cigarette smoke). They are present in low concentrations in petroleum, but are formed during incomplete combustion, especially in engines (for example diesel soot).

High concentrations are present at coking plant and gas works sites. In the past, PAH-rich tar residues were deposited uncontrolled at these sites. Contamination from carbochemicals and coal combustion is due to the coal structure. Coal is an amorphous polymer composed of mono- and polynuclear aromatic compounds and heterocycles cross-linked by carbon and oxygen bridges. When heated, the macromolecule breaks down into fragments. In addition to phenols and xylenes, coal tar mainly contains polynuclear PAHs.

The high environmental relevance of PAHs is due to the carcinogenic properties of some compounds. The mutagenic and carcinogenic effect in the liver results from metabolic activation of compounds such as benzo[a]pyrene, which possess the so-called **Bay region.** The epoxide formed at the Bay region is not inactivated by epoxide hydrolase, but can react with DNA as a reactive metabolite (◘ Fig. 6.28).

Part of ◘ Fig. 6.28 Activation and inactivation of benzo[a]pyrene and its metabolites in the liver.

As the number of condensed rings and the size of the molecule increase, the solubility in water decreases sharply. For naphthalene it is 31.7 mg/L, for phenanthrene 1.6 mg/L, for benzo[a]pyrene only 0.003 mg/L. Microbial **degradability** also decreases with increasing ring number. While naphthalene, anthracene and phenanthrene are readily bacterially degradable, no microorganism has yet been isolated that can use benzo[a]pyrene as its sole carbon and energy source. However, the high persistence does not mean that the higher-nuclear PAHs are not metabolized.



◘ **Fig. 6.28** Reaction sequence for benzo[a]pyrene to explain the carcinogenic property

6.1.5.1 Bacterial Aerobic Degradation of PAHs

The complete bacterial degradation, by which a PAH can be used as the sole source of carbon and energy, will be explained in detail using the example of naphthalene. Phenanthrene, anthracene and, by a few bacteria, pyrene are degraded in a similar manner. Bacterial naphthalene degradation occurs ring by ring (◘ Fig. 6.29).

Characteristic reactions of the degradation of the first ring are:

1. Formation of a cis-dihydrodiol by a dioxygenase.
2. Dehydrogenation to a dihydroxy derivative.
3. Extradiole ring fission by a second dioxygenase reaction.
4. Cleavage of pyruvate, which leads to the single-ring intermediate.

There are differences between the various bacterial species. Further degradation of salicylate occurs either via catechol and the *meta*-pathway (see ◘ Fig. 6.16) or via gentisate (see ◘ Fig. 6.18).

The degradation sequence found for phenanthrene makes it clear that reaction sequences repeat after the dihydroxy derivative stage is reached (◘ Fig. 6.30). The sequence shown is characteristic of *Pseudomonas*. Other bacterial species that mineralize PAHs are representatives of the genera *Beijerinckia, Flavobacterium, Mycobacterium* and *Rhodococcus.*

6.1.5.2 Degradation of PAHs by Fungi

Fungi can convert PAHs in the presence of a growth substrate. Representatives of the molds, which include phycomycetes such as *Cunninghamella* and *Rhizoctonia as* well as deuteromycetes such as *Aspergillus,* oxidize low and higher molecular weight PAHs by cytochrome P450 monooxygenases to epoxides, which are further converted to *trans*-dihydrodiols as the main metabolites. The attack can occur in different positions of the ring system, thus a broad spectrum of diols is formed. In addition, phenolic and methoxylated derivatives are also formed. Conjugates with glucuronate and sulfate have also been detected. ◘ Fig. 6.31 shows the reaction sequence presented using the example of naphthalene.

The wood-degrading white-rot-fungi, which predominantly belong to the Basidiomycetes, possess an additional enzyme-system, that among other things also to the PAH-degradation is capable. The enzymes of the ligninolytic system above all the lignin-peroxidase, manganese-dependent peroxidase, Laccase and probably also the lipid-peroxidases (Lipoxygenase) catalyze the oxidation of PAH.

Lignin peroxidase from *Phanerochaete chrysosporium*, the best-studied white rot fungus, directly catalyzes the one-electron oxidation of aromatic compounds. The resulting aryl cation radical then undergoes spontaneous rearrangement and degradation. It was observed that transformation by lignin peroxidase proceeds only with those PAHs whose ionization potential (IP) is <7.6 eV. The isolated lignin peroxidase is not able to react with substances with IP above this limit. Consequently, the turnover shown with whole cells of substances such as triphenylene, phenanthrene, fluoranthene, chrysene, benzo[b]fluoranthene and benzo[e] pyrene cannot be explained by the direct action of lignin peroxidase. Manganese-dependent peroxidase-mediated lipid peroxidation has been reported to lead to the oxidation of phenanthrene by *Phanerochaete chrysosporium.* The degradation of three- to

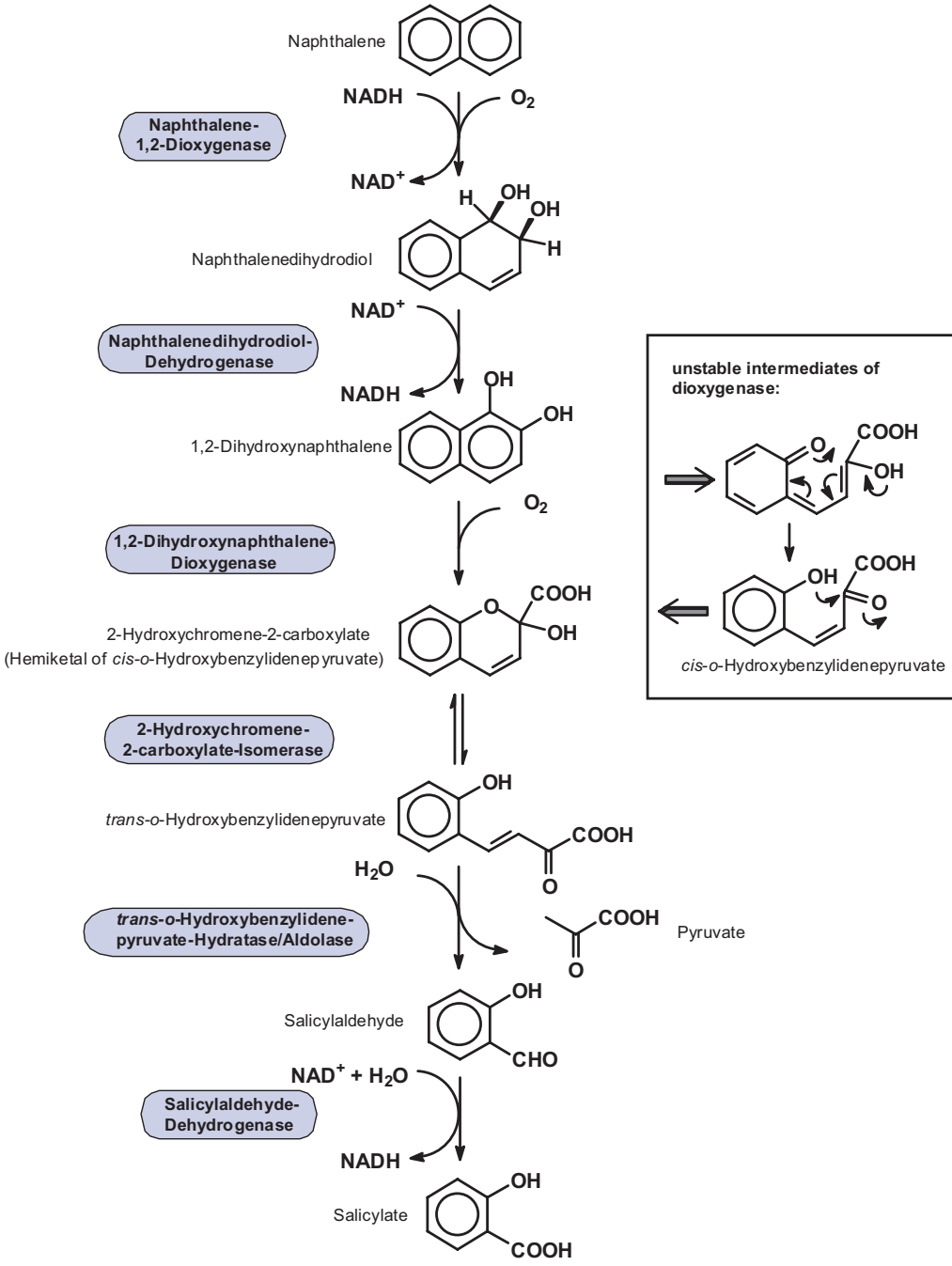


Fig. 6.29 Bacterial degradation sequence for naphthalene

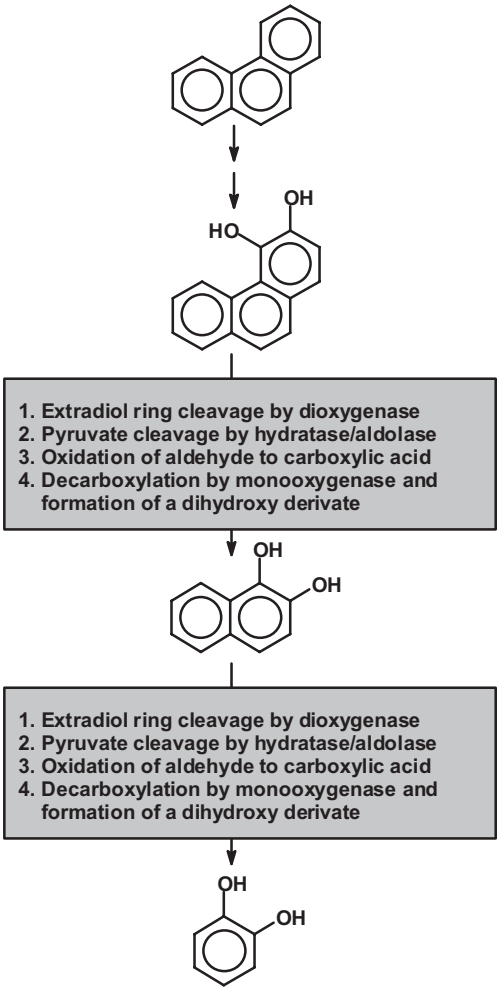


Fig. 6.30 Reaction sequences of the degradation of phenanthrene by *Pseudomonads*

six-ring PAHs with IPs between 7.2 and 8.1 eV is IP-dependent during *in vivo* and *in vitro* lipid peroxidation. This suggests that a single-electron oxidant is more involved than lignin peroxidase or Mn^{3+}.

Figure 6.32 shows the reaction carried out by lipoxygenase. It catalyzes the formation of an allylic hydroperoxide of a polyunsaturated acid or ester if they have a *cis,cis-1*,4-pentadiene system. The lipid radical formed first is further converted to lipid hydroperoxide via the peroxyl radical. Homolytic cleavage of the lipid hydroperoxide by lignin peroxidase or manganese-dependent peroxidase then leads to the alkoxyl radical ($RO^{\bullet}$), which is known to be a very strong oxidant.

The primary peroxidase reaction is thus a one-electron deprivation. Oxygen, which originates from the water, is added to the resulting cation radical by nucleophilic attack. Further electron withdrawal leads to hydroxylated PAHs, which are converted to quinones by further water addition and electron withdrawal. In the case of pyrene, the formation of pyrene-1,6- and 3,6-quinone has been demonstrated. This is shown schematically in Fig. 6.33. The degradation of PAHs can sometimes lead to CO_2. While the formation of the quinones has been observed to be a rapid process, the transformation to CO_2 is very slow. In nature, the reactive intermediates of PAH degradation will react with components of the soil matrix (Table 6.2).

Since white rot fungi possess intracellular monooxygenases, cytochrome P450 enzymes, in addition to extracellular ligninolytic enzymes, they have a high and complex degradation potential. In addition to the well-studied white rot fungus *Phanerochaete chrysosporium*, other white rot fungi and degraders of litter (plant debris on the forest floor) have ligninolytic enzymes, for example *Bjerkandera* sp., *Crinipellis stipitaria, Pleurotus ostreatus, Stropharia rugosa-annulata* and *Trametes versicolo.*

6.1.5.3 Bacterial Anaerobic Degradation of PAHs

PAHs have been detected in a variety of anaerobic sediments. The anaerobic degradation of polycyclic aromatic hydrocarbons is consequently of great interest, although there have been few positive findings to date. For a long time, the anaerobic degradation of naphthalene has been studied as a model PAH by sulfate-reducing bacteria. Overall, however, few data describing the degradation sequence have been identified. In the case

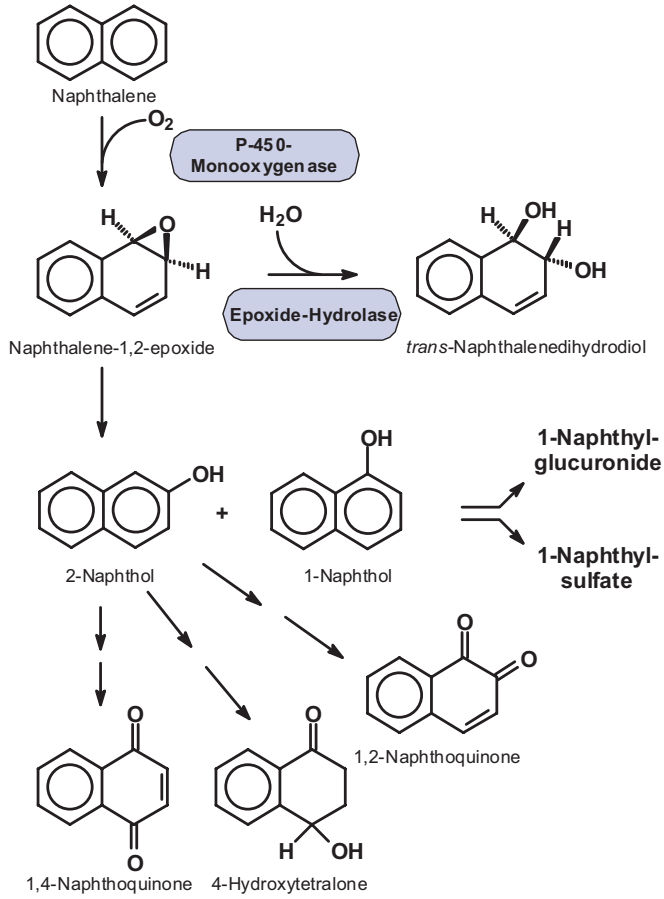


Fig. 6.31 Transformation reactions by moulds using the example of naphthalene

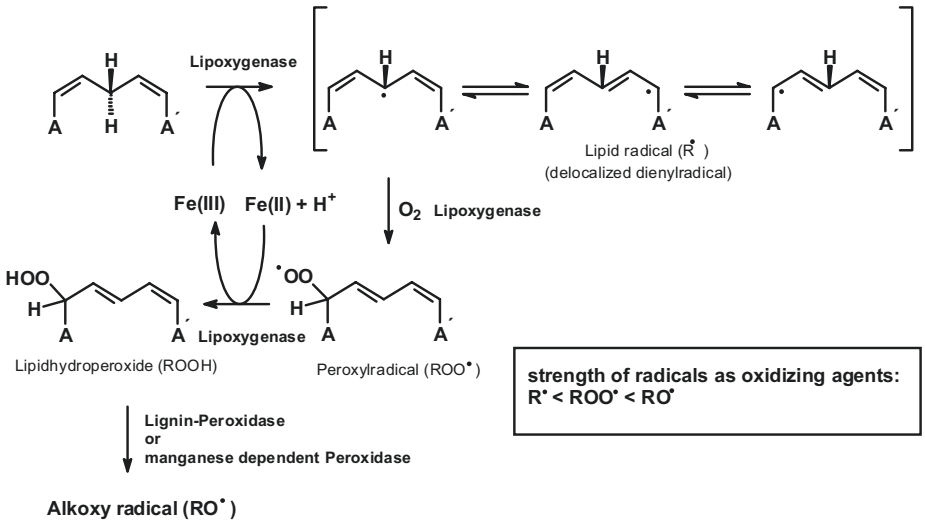


Fig. 6.32 Lipoxygenase catalyzed formation of the very strong oxidant, the alkoxyl radical

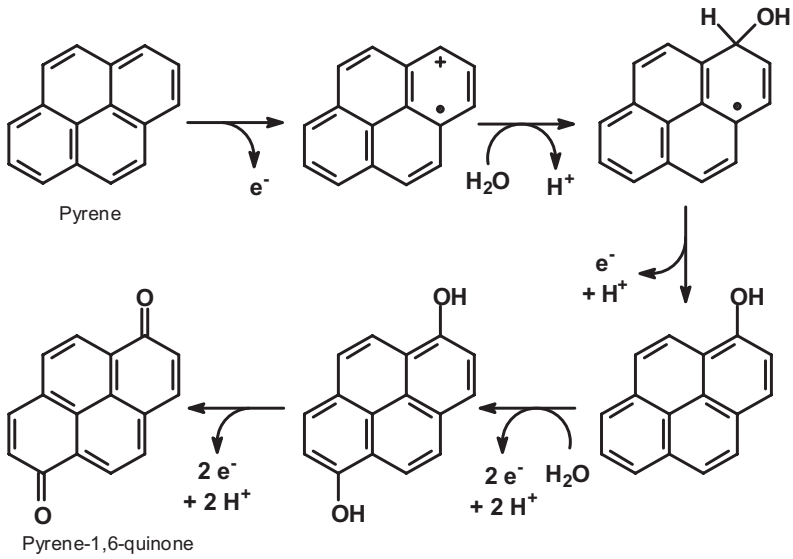
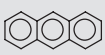
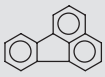
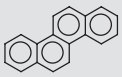
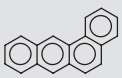


Fig. 6.33 Schematic representation of the reaction sequence on pyrene to pyrene-1,6-quinone by ligninase

Table 6.2 Polycyclic aromatic hydrocarbons and physico-chemical properties important for their evaluation

Compound	Structure	Water solubility (mg/L) at 25 °C	Octanol/ Water coefficient (log K_{ow})	Ionization potenial (eV)
Naphthalene		31.7	3.37	8.12
Acenaphthene		3.42	4.33	7.61
Anthracene		0.075	4.45	7.43
Phenanthrene		1.6	4.46	8.03
Fluoranthene		0.265	5.33	7.85
Pyrene		0.148	5.32	7.53
Chrysene		0.002	5.61	7.81
Benzo[a] anthracene		0.014	5.61	756

of naphthalene, carboxylation using CO_2 appears to initiate the degradation. Further degradation of the 2-naphthalenecarboxylic acid formed is then thought to proceed reductively analogous to the anaerobic benzoyl-CoA pathway.

6.1.6 Degradation of Heterocycles

Groundwaters of former coking plants, soot factories, gas works and wood impregnation plants are contaminated with tar oil constituents (over 10,000 different compounds according to estimates). In addition to PAHs and BTEX, homocyclic and heterocyclic compounds are also found, which can be present in up to 15% of the tar oil. Heterocycles, compounds with ring-shaped molecules that, in addition to carbon atoms, also contain one or more atoms of other elements in the ring, such as nitrogen, oxygen or sulfur atoms, include substances such as mononuclear volatile compounds like furan, pyridine, pyrrole and thiophene, and polynuclear ones like acridine, benzo[b]thiophene, carbazole, quinoline and dibenzofuran. S-heterocycles are responsible for the sulfur content in petroleum.

Due to the heteroatom in the molecule, there is an increase in water solubility compared to analogous PAHs, which is an important criterion for mobility in groundwater. Another parameter for assessing mobility is the octanol-water coefficient. Substances with $\log P_{ow}$ values above 4 adsorb quite strongly to soil matrix and thus are not transported far in the aquifer. Almost all mononuclear and dinuclear heterocycles have values below 4. Especially the mononuclear heterocycles show a strong volatility from aqueous environments, which is characterized by the Henry coefficient K'_H. Substances with $K'_H > 0.04$ (dimensionless, corresponding to $K_H \sim 1$ atm L/mol) are called highly volatile, and up to a value of 0.0004 they still exhibit significant volatility.

The reports on the biodegradation of heterocycles do not show a clear picture regarding the degradation under aerobic and anaerobic conditions. Complete aerobic degradation was reported, but in some cases long lag phases were present. For anaerobic conditions, extensive persistence as well as degradation of individual substances under nitrate- and sulfate-reducing and methanogenic conditions was reported in laboratory experiments. The nitrogen-containing heterocycles such as pyridine, indole and quinoline appear to be more easily converted than the corresponding sulfur- and oxygen-containing compounds.

6.1.6.1 Sulfur-Containing Heterocycles

Two types of metabolism with sulfur-containing heterocycles were described using the example of aerobic degradation of dibenzothiophene: (a) the substance is a carbon and energy source, (b) the substance serves as a sulfur source, so that biodesulfurization of the petroleum component occurs.

The so-called "Kodama pathway" was reported for *Burkholderia* and *Pseudomonas.* It corresponds in its reaction sequence to the pathway found for aromatic compounds such as naphthalene (see ◘ Fig. 6.34) with activating dioxygenase, dehydrogenase, ring-cleaving 2,3-dioxygenase, an isomerase and the water-attaching hydratase followed by the aldolase, so that an aldehyde (3-hydroxy-2-formylbenzothiophene) and pyruvate are formed. Mineralization of the total molecule of dibenzothiophene has not been described so far, so it can be concluded that only the pyruvate is used for growth. Biodesulfurization does not appear to occur.

With dibenzothiophene as a model compound for organic sulfur in crude oil, however, in contrast to the described aim of degradation, it is not the achievement of intermediate metabolism that is intended, but desulfurization.

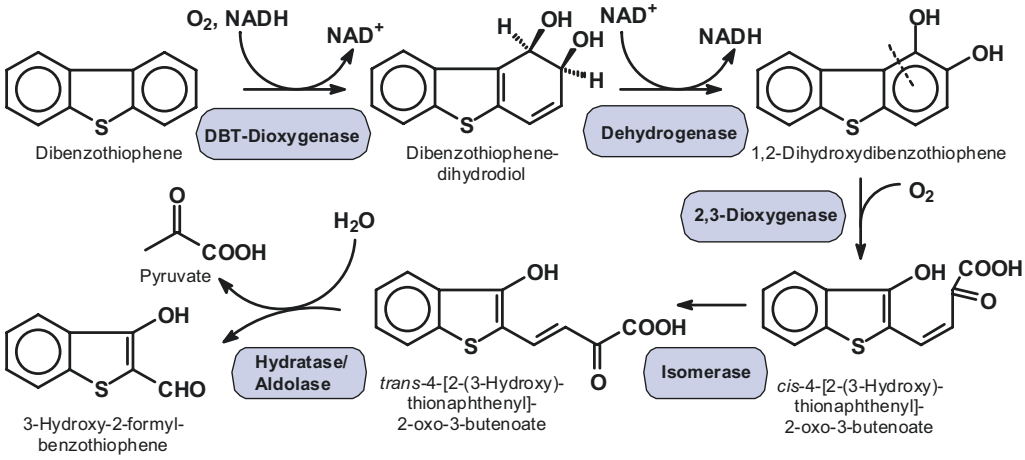


Fig. 6.34 Kodama degradation pathway for dibenzothiophene

Rhodococcus causes a conversion to 2-hydroxybiphenyl, which is excreted into the medium. This is also intended, since, as mentioned, only the desulfurization of the fossil energy carrier and not the degradation should take place. The transformation into a sulfur-free product, which is to function further as an energy carrier, is thus the goal in the use of the organism. The *Rhodococcus* and many other organisms such as *Arthrobacter, Bacillus subtilis, Brevibacterium, Corynebacterium, Gordonia desulfuricans, Gordonia amicalis* and the thermophilic *Paenibacillus* use the released sulfur as a source of sulfur.

The degradation sequence shown in Fig. 6.35 has been described for *Rhodococcus erythropoli, Corynebacterium* sp. and *Paenibacillus polymyxa.* It involves three monooxygenase steps in sequence, followed by a hydrolase that forms sulfite as well as 2-hydroxybiphenyl. The genes encoding it are located on an operon: *Bacillus subtilis (bdsABC),* thermophilic *Paenibacillus (tdsABC), Rhodococcus erythropolis (dszABC).*

In *Rhodococcus* sp. the operons *dszABC* (**desulfurization**) or *soxABC* (sulfur oxidizing) are localized on plasmids.

The expression of the enzymes DszABC is repressed by sulfide, sulfate, methionine, cysteine, indicating that in natural environment desulfurization is thus rather unlikely.

The aforementioned genes have been detected in environmental samples: *dszABC* is always present in oil-contaminated soils, while detection in clean soils was partially negative.

There are few reports of the degradation of sulfur-containing heterocycles under anaerobic conditions. For thiophene and benzothiophene, only cometabolic degradation with benzene or naphthalene as cosubstrate has been reported so far. Carboxythiophenes have been identified as degradation products under anaerobic conditions and have now also been detected in groundwater at one site.

6.1.6.2 Nitrogen-Containing Heterocycles

The degradation of N-heteroaromatic compounds can be initiated by two types of reactions: (1) at the heterocycle by nucleophilic attack with water (molybdenum-dependent hydroxylase) or (2) electrophilic at the aromatic ring with oxygen as electrophile (mono- or dioxygenase).

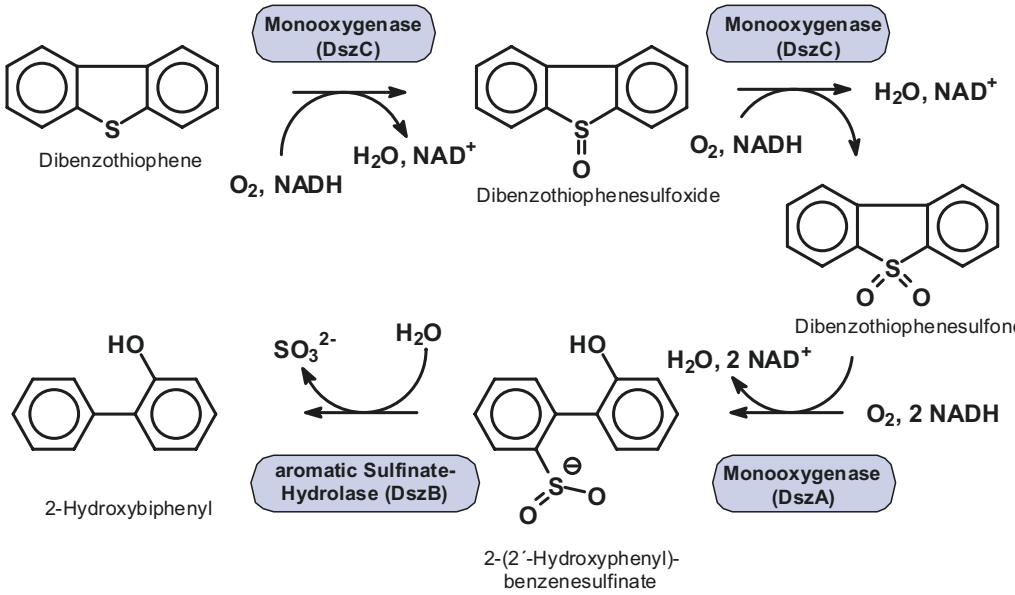


Fig. 6.35 Desulfurization of dibenzothiophene when used as a sulfur source for *Corynebacterium* sp., *Paenibacillus polymyxa,* and *Rhodococcus erythropolis*

The degradation of N-heteroaromatic compounds with initiating hydroxylation adjacent or in the *para*-position to the N-heteroatom has been shown for pyridine, picolinate, nicotinate, isoquinoline, quinoline and methylquinoline. The reaction is catalyzed by a molybdenum-dependent hydroxylase by a nucleophilic attack of water. Thus, the oxygen of the product is not derived from atmospheric oxygen. Tautomerization of the product of hydroxylase leads to the oxo compound, which predominates in the aqueous environment. Further degradation is often an unusual dioxygenolytic cleavage of the heteroaromatic ring with formation of carbon monoxide. The overall reaction sequence is well studied for methylquinoline (Fig. 6.36) and links the hydroxylase, which incorporates oxygen from water into the substrate and uses molecular oxygen as an electron acceptor, and the unusual 2,4-dioxygenase, which cleaves two C–C bonds to release CO, to the common 3-oxoadipate pathway. The suspected amide hydrolase that converts N-acetyl-anthranilate to anthranilate has not been studied. The formation of catechol from anthranilate may be catalyzed by an anthranilate 1,2-dioxygenase, although this step has also only been postulated. The degradation of catechol is accomplished by *ortho*-ring cleavage by catechol 1,2-dioxygenase and then follows the known 3-oxoadipate pathway, yielding acetyl-CoA and succinyl-CoA.

Pyridine degradation is widespread among microorganisms. Since a large number of mostly aliphatic intermediates have been detected, a clear degradation sequence is difficult to identify. The mechanism of cleavage of the pyridine ring is not yet known.

Some bacteria have been isolated that can metabolize isoquinoline. All isolates metabolize isoquinoline via isoquinolinone (1-oxoisoquinoline). For *Brevundimonas diminuta,* a possible pathway via phthalate, 4,5-dihydroxyphthalate and protocatechuate has been suggested (Fig. 6.37).

The 8-hydroxycoumarin pathway has been described for the degradation of quinoline, which also means that the pyridine ring is attacked first before cleavage of the

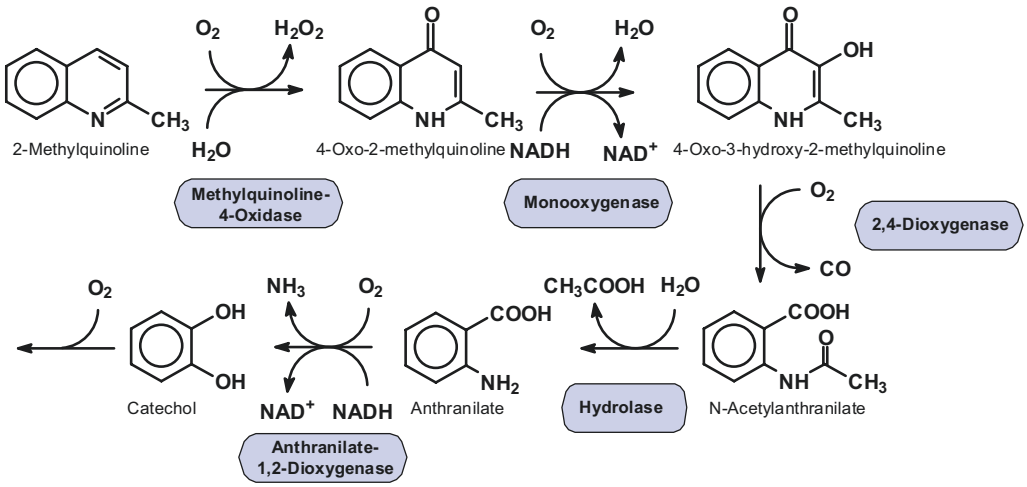


Fig. 6.36 Anthranilate pathway for methylquinoline

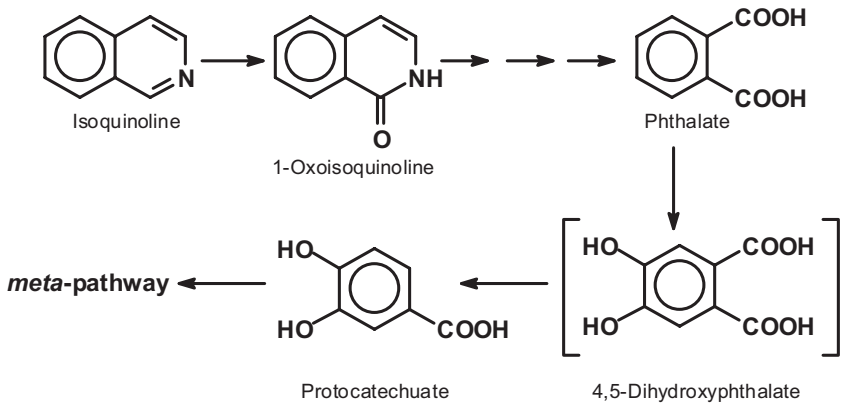


Fig. 6.37 Degradation of isoquinoline by *Brevundimonas diminuta*

benzene ring occurs. However, the reaction sequence of quinoline degradation is still unclear: only a few metabolites, 2-oxoquinoline, 8-hydroxycoumarin and 3-(2,3-dihydroxyphenyl)propionic acid, have been identified as true intermediates. The mechanism for cleavage of the N-heterocyclic ring is unknown (Fig. 6.38).

The degradation of carbazole follows a reaction sequence as described for other aromatics such as biphenyl (Fig 6.39). Here, as with dibenzofuran and dibenzodioxin, angular dioxygenation leads to cleavage of the carbon-heteroatom bond. While the cleavage of the ether bond in the O-heterocycles occurs due to the formation of the unstable hemiacetal, here a **hemiaminal** is the product of dioxygenation followed by the spontaneous formation of the aminodihydroxybiphenyl. The hydroxylated ring is opened by a *meta*-cleaving dioxygenase. This is followed by hydrolytic cleavage of the vinylogous 1,3-dioxo compound to anthranilate and the C_5-body known from the *meta*-pathway, 2-hydroxypenta-2,4-dienoate. The further degradation has already been presented in Fig. 6.16b in ► Sect. 6.1.4.1.

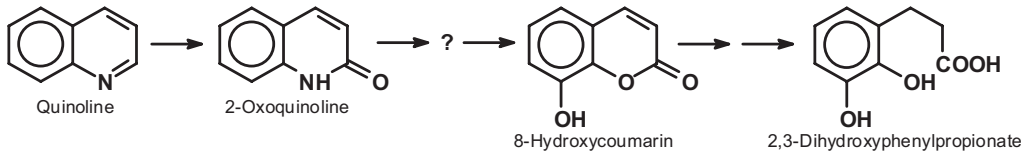


Fig. 6.38 Degradation of quinoline

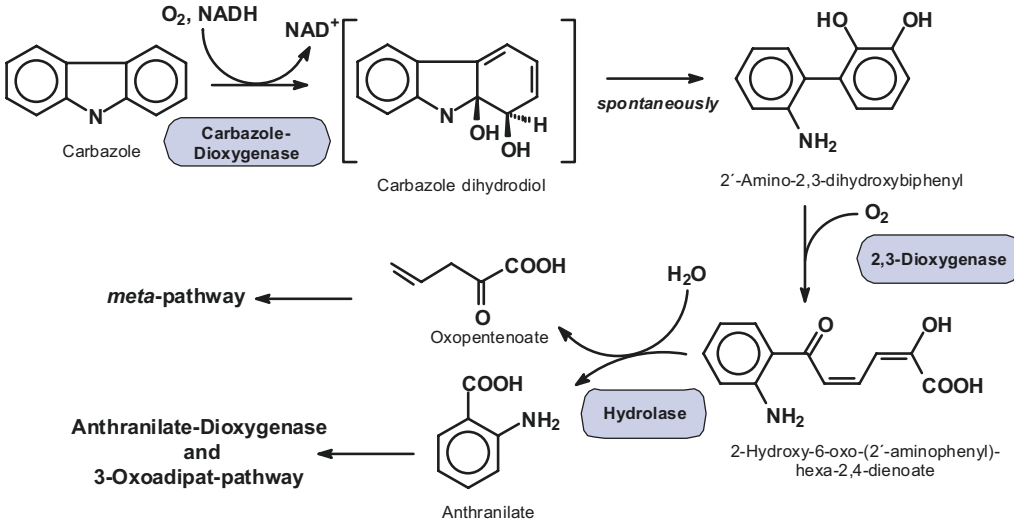


Fig. 6.39 Degradation pathway for carbazole (identification of metabolites and by analogy with the biphenyl degradation pathway)

In the absence of oxygen as a reaction partner in central degradation reactions, a degradation of N-heterocycles is described as shown in Fig. 6.40. With the exception of one study with *Desulfobacterium indolicum*, most analyses on anaerobic degradation were carried out with environmental samples as catalyst. Details on the enzymology of the degradation sequences are missing.

6.1.6.3 Oxygenated Heterocycles

The aerobic degradation of the O-heterocycles dibenzofuran and dibenzo-p-dioxin is analogous to the degradation of carbazole and thus to the general reaction sequence for aromatics. An important step is again the angular dioxygenation, which incorporates both atoms of atmospheric oxygen into the respective substrate in such a way that an unstable **hemiacetal** is formed, which spontaneously leads to the cleavage of the ether bond. Thus, unlike the "normal" aromatic degradation sequence, no dehydrogenase reaction follows. The trihydroxybiphenyl formed in the degradation of dibenzofuran subsequently undergoes *meta*-cleavage of the dihydroxylated ring, resulting in a vinylated 1,3-dioxo compound, which is hydrolyzed to 2-oxopentenoate and salicylate. In the degradation of dibenzo-p-dioxin, *meta*-cleavage of the trihydroxyphenyldihydroxyphenyl ether leads to an ester. Catechol and 2-hydroxymuconate and the tautomeric 2-oxocrotonate, respectively, are the hydrolysis products. The carboxylic acids formed in both pathways are metabolites of the *meta*-pathway and are degraded accordingly. The degradation of salicylate and catechol occurs as described above (see ► Sect. 6.1.4.1) (Fig. 6.41).

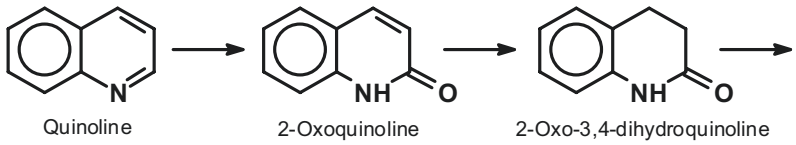


Fig. 6.40 Initiating steps in the anaerobic degradation of quinoline by *Desulfobacterium indolicum*

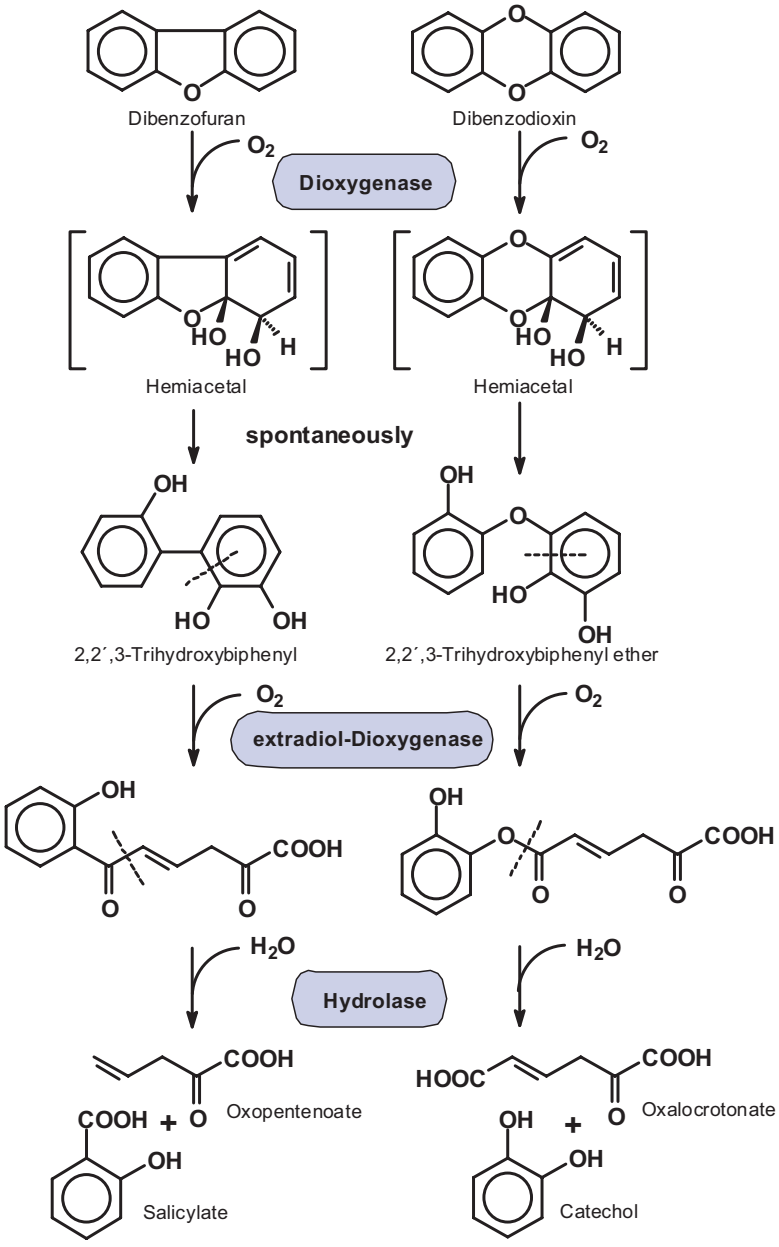


Fig. 6.41 Degradation pathways for O-heterocycles

6.1.7 Formation of Biosurfactants/ Absorption of Mineral Oil Hydrocarbons

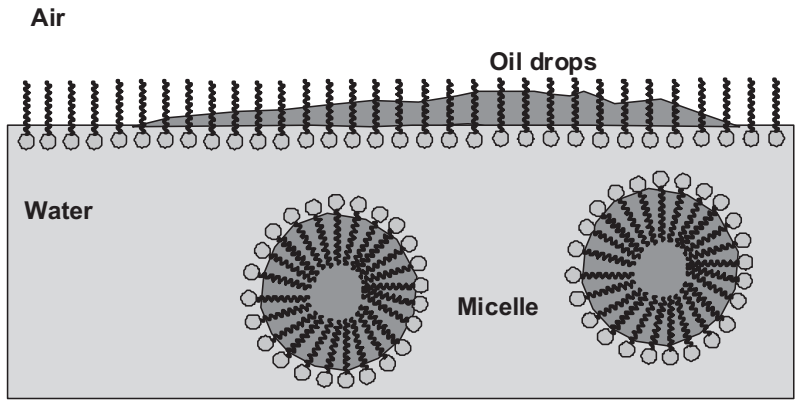
Hydrocarbons (long-chain alkanes and PAHs) are lipophilic compounds that are only sparingly water-soluble and thus poorly bioavailable. For uptake by the predominantly hydrophilic cells, **phase boundaries** must be overcome. In the course of evolution, bacteria have developed strategies to increase the bioavailability of such poorly accessible substances. First, they can change the fluidity of their cell envelope, resulting in improved contact with solid substrates. A second strategy is the production and release of surface-active compounds, the biosurfactants.

6.1.7.1 Surface-Active Substances (Biosurfactants)

Like synthetic surfactants, biosurfactants have an amphiphilic structure, i.e. they consist of a hydrophilic (water-soluble, fat-repellent) and a hydrophobic (water-insoluble, fat-loving) component. These structures align themselves with interfaces of different polarity (liquid/liquid, liquid/gaseous, liquid/solid) and cause a reduction in the interfacial tension prevailing there (◘ Fig. 6.42).

This manifests itself in macroscopically visible phenomena such as wetting, emulsification and foam formation. If the concentration of the biosurfactant reaches a specific threshold value (the critical micelle concentration), spherical surfactant structures (micelles) are formed in which individual molecules or molecular aggregates of the hydrophobic compound are enclosed. The compound is virtually dissolved, "pseudosolubilized". If such substrate-loaded micelles reach the cell envelope, they "fuse" with the cell membrane and the hydrophobic component diffuses into the cell interior. A second possible mechanism for enhancing substrate uptake is based on the increased hydrophobization of the cell surface by attachment of the biosurfactant. This allows the cell to have increased contact with substrate particles.

Microbial surfactants, formed by bacteria, yeasts, or fungi, have recently generated a great deal of interest, not only because they are less toxic and more biodegradable than synthetic surfactants, but also because they work effectively at extreme temperatures, pH levels, and salinity.



◘ **Fig. 6.42** Behaviour of amphiphilic molecules at interfaces and at concentrations above the critical micelle concentration

6.1.7.1.1 Structure of Biosurfactants

Biosurfactants characterized so far are exclusively neutral and anionic in nature. The hydrophilic structural components are mono-, di- or polysaccharides, amino acids or peptides. The hydrophobic structural component usually consists of a saturated or unsaturated, branched or unbranched, hydroxylated or non-hydroxylated fatty acid.

Biosurfactants are classified according to their biochemical character and the microorganisms by which they are formed. Typically, microbial surfactants are divided into the following classes:

- Fatty acids
- Glycolipids
- Lipopeptides and lipoamino acids
- Polymeric biosurfactants

▪ Fatty Acids

The simplest form of biosurfactants are the fatty acids, which can occur either as simple, saturated fatty acids with 12–14 carbon atoms or as complex fatty acids containing hydroxyl groups and alkyl branches.

▪ Glycolipids

Glycolipids are the most commonly isolated and studied biosurfactants. They contain various sugar moieties (for example, rhamnose, trehalose, and sophorose) that are linked to long-chain fatty acids or hydroxy fatty acids. The following figure shows structural formulas of the three glycolipids that have been best studied to date.

In **rhamnolipids,** one or two rhamnose molecules are linked to one or two molecules of *β*-hydroxydecanoic acid. Two types of rhamnolipids have been most commonly isolated to date, namely rhamnolipid R-1 (L-rhamnosyl-L-rhamnosyl-*β*-hydroxydecanoyl-*β*-decanoate, ◘ Fig. 6.43) and rhamnolipid R-2 (L-rhamnosyl-*β*-hydroxydecanoyl-*β*-decanoate). The first type consists of two rhamnoses, the second of one, to each of which is attached a chain of two *β*-hydroxydecanoic acids. Both types are produced by *Pseudomonas aeruginosa.*

Trehalolipids consist of two glucose molecules (=trehalose), each of which is linked to mycolic acids at the sixth carbon atom. Mycolic acids are long-chain *α*-branched *β*-hydroxy fatty acids whose number of C atoms and double bonds may vary. The trehalolipids are associated with the cell wall structures of most species of the genera *Corynebacterium, Mycobacterium*, *Nocardia* and *Rhodococcus.*

Sophorolipids are predominantly produced by yeasts. They consist of the dimeric carbohydrate sophorose and the associated long-chain hydroxycarboxylic acids.

▪ Lipoproteins and Lipopeptides

Lipopeptides consist of short polypeptides (3–12 amino acids) which are connected with a hydrophobic chain. One of the most effective biosurfactants is the lipopeptide surfactin, which is formed by *Bacillus subtilis.* It consists of seven amino acids and contains a bridging hydroxy fatty acid (◘ Fig. 6.44). A salient feature of the lipopeptides mentioned is their pronounced antibiotic broad-spectrum effect.

▪ Polymeric Biosurfactants

Among the best studied polymeric biosurfactants are emulsan (◘ Fig. 6.45) and liposan. Emulsan is produced by *Acinetobacter lwoffii* RAG-1 (◘ Fig. 6.46) and has less the property of lowering interfacial tensions than of stabilizing emulsions. It is a polyanionic, amphiphilic, heteropolysaccharide compound, the heteropolysaccharide character being derived from an n-times repeating trisaccharide consisting of *N*-acetyl-D-galactosamine, *N*-acetylgalactosaminuronate and 2,4-diamino-6-deoxyglucosamine. The fatty acids (C_{10} to C_{18}, saturated and mono-unsaturated) account for 5–23% of the total weight of an emulsan molecule. They are linked to the

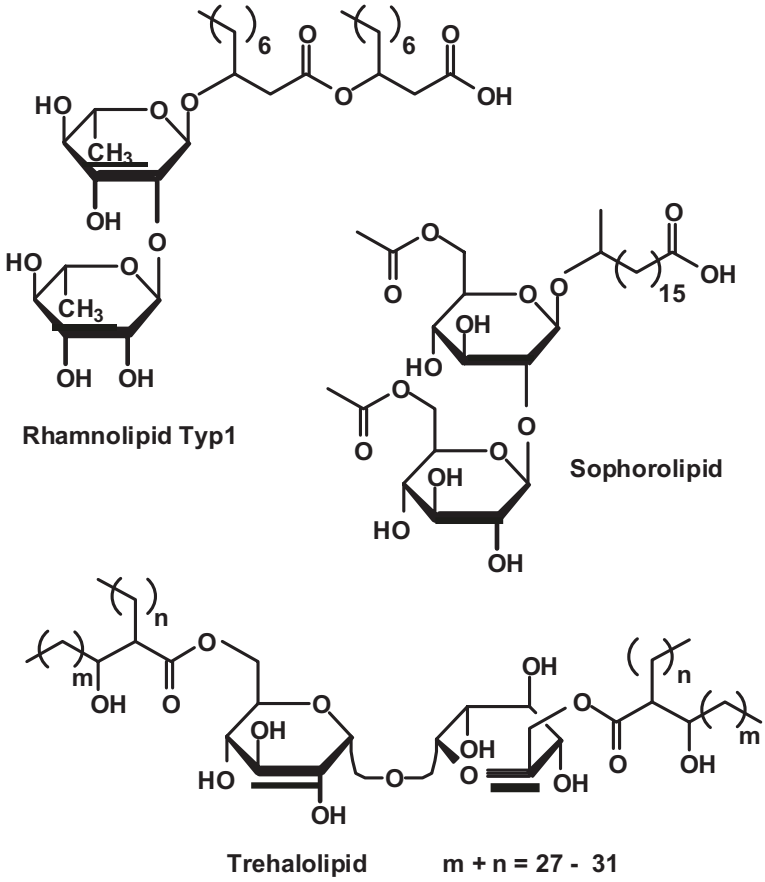


Fig. 6.43 Structure of the most common glycolipid biosurfactants

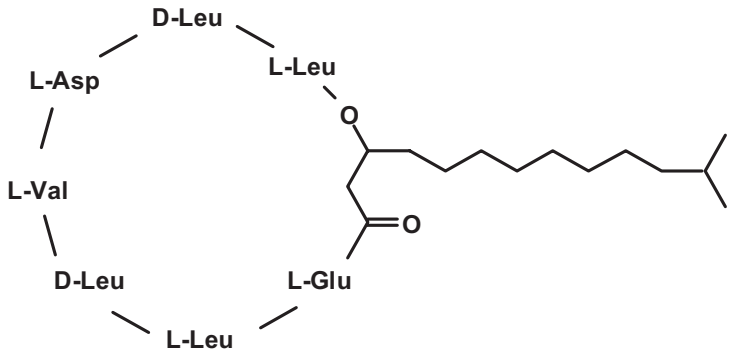


Fig. 6.44 Structure of surfactin produced by *Bacillus subtilis*

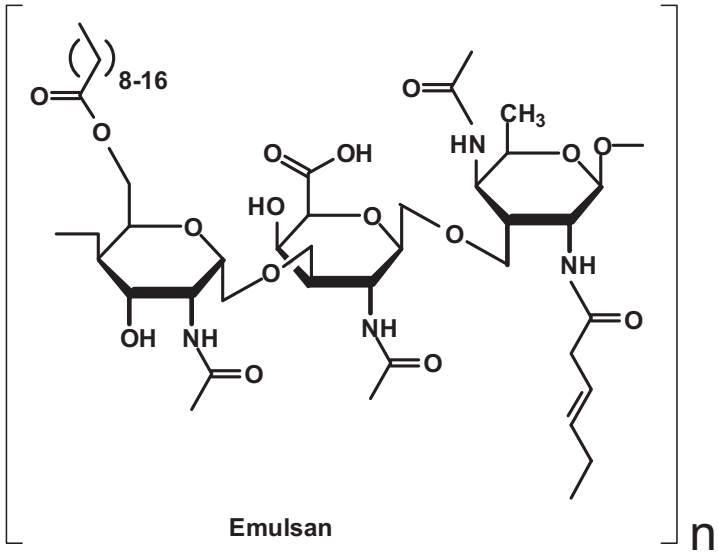


Fig. 6.45 Proposed structure of a polymeric biosurfactant

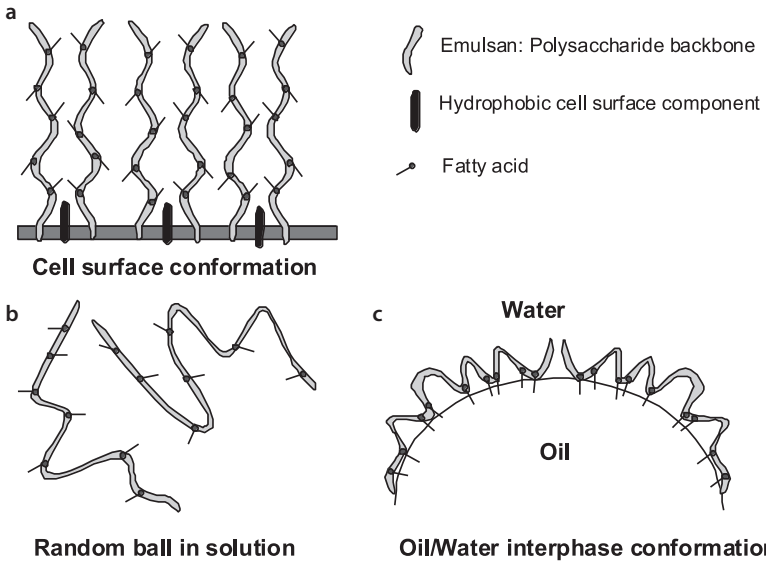


Fig. 6.46 Model showing the similarity between emulsan on the cell surface of strain RAG-1 and emulsan on the surface of an oil droplet. Sequence: **a** → **c** (According to Gutnick lecture)

polysaccharide via ester and amide bonds. Already in concentrations of 0.001–0.01%, **emulsan** is a very efficient emulsifying substance for hydrocarbons in water and is at the same time one of the best known emulsion stabilizers. Liposan, which consists of 83% carbohydrates and 17% proteins and is formed by *Candida lipolytica,* has similar emulsifying properties. The carbohydrate part is a heteropolysaccharide and consists of glucose, galactose, galactosamine and galacturonic acid.

6.1.7.2 Sequence of Colonisation of an Oil Droplet

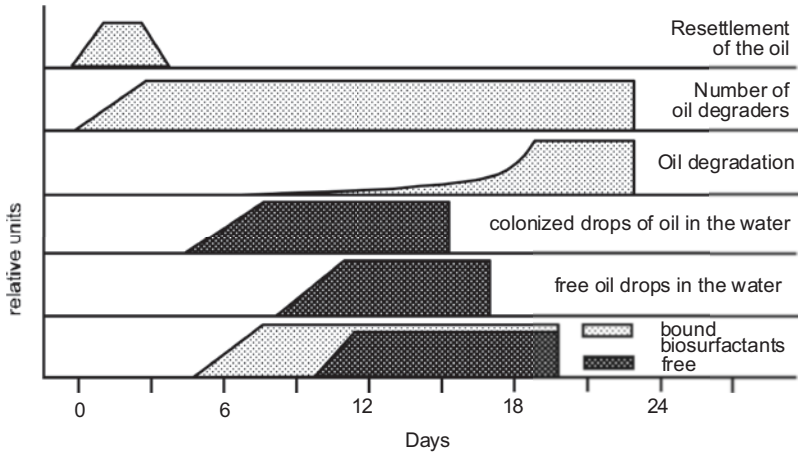
The process of colonization of an oil drop can be described as follows: First, rapid, non-specific colonization of the new habitat by hydrophobic bacteria takes place (see ◘ Fig. 6.47). The oil droplets are occupied by microorganisms in several layers. After a short time, this process is completed. Somewhat delayed, but rapidly increasing, the oil degraders multiply and reach maximum cell numbers after only a few days. Then oil droplets colonized and stabilized by bacteria appear in the aqueous phase and later, increasingly, free oil droplets. Thus, cell-bound biosurfactants are synthesized first, allowing contact and intimate colonization with the oil. After a time delay, free biosurfactants are formed, which increase the attackable oil surface without direct participation of bacterial cells.

6.1.7.3 Use of Biosurfactants

Under culture conditions, biosurfactant quantities of up to 1 g/L are formed by the microorganisms.

Biosurfactants are readily microbially degradable, which is why they are also important for detergents (rinsing agents) and for tertiary oil production. They are used in environmental protection, plant protection, crude oil production and in the cosmetics and pharmaceutical industries.

The formation of the biosurfactants is difficult to detect in the soil. Biosurfactants have been added in soil remediation studies. Promoting effects are controversial, however, as they were obtained under conditions that were difficult to reproduce. Biosurfactants have been successfully used in conjunction with suitable nitrogen and phosphorus sources in tanker spills and pollution of sandy coasts.



◘ **Fig. 6.47** Sequence of colonization of an oil droplet. (Adapted from Poremba et al., 1989)

6.2 Degradation of Chlorinated Pollutants

6.2.1 Degradation of Chlorinated Aromatics

6.2.1.1 Chlorinated Aromatics as an Environmental Problem

6.2.1.1.1 Production and Use

Chlorinated aromatics are important end products and synthesis intermediates of the chemical industry. For chlorinated aromatic compounds, 200,000 tonnes per year were reported for chlorophenols and about 1 million tonnes per year for chlorobenzenes. In contrast, the annual quantities of polychlorinated biphenyls (PCBs) were lower. Industrial production of PCBs began in 1929, and the total quantity produced worldwide up to 1983 was about 1.5 million t. In 1991, the figures for worldwide annual production quantities of pentachlorophenol (PCP) varied between 25,000 and 90,000 t. In Germany, over 1000 t were still produced in 1985. In Germany, more than 1000 t of PCP were still produced in 1985. ◘ Table 6.3 clearly shows how universally chlorinated benzenes, phenols and biphenyls were and are used.

For some chlorinated aromatics there are bans on production and use. For example,

◘ **Table 6.3** Examples for the use of chlorinated aromatics

Link	Use
Polychlorinated biphenyls (PCBs, mixtures of up to 70 congeners)	*In closed systems:* Transformers, condensers, heat transfer, hydraulic fluid (especially in mining)
	In open systems: Cutting oil, drilling oil, plasticizers, printing inks, putties, adhesives, nail varnish
Chlorobenzene	Solvent, intermediate in syntheses
1,2-Dichlorobenzene	Solvent, intermediate in syntheses, heat transfer agent
1,4-Dichlorobenzene	Odour eliminator, insect repellent, intermediate in syntheses
1,2,4-Trichlorobenzene	Intermediate, heat transfer medium, dielectric in transformers, capacitors, intermediate in syntheses
1,2,4,5-Tetrachlorobenzene	Intermediate, dielectric in transformers, capacitors
Hexachlorobenzene	Intermediate in syntheses, fungicide, wood preservative, additive in PVC and rubber production
2-Chlorophenol	Disinfectant, fungicide, bactericide, intermediate in syntheses
3-Chloro- and 4-chlorophenol	Antiseptic, bactericide, intermediate in syntheses
2,4-Dichlorophenol	Antiseptic, mothicide, intermediate in syntheses of herbicides
2,4,5-Trichlorophenol	Intermediates in syntheses of herbicides
2,4,6-Trichlorophenol	Fungicide, acaricide, intermediate in syntheses
Pentachlorophenol	Disinfectant and preservative for leather and textiles, wood preservative, insecticide, herbicide

the PCP Prohibition Ordinance has been in force in the Federal Republic of Germany (FRG) since 1989, and the Chemicals Prohibition Ordinance since 1993. The production, marketing and use of PCP and PCP-containing materials are completely prohibited.

Due to legal regulations, the production of PCBs no longer takes place today. In 1973, a restriction to closed systems was enacted. Production was discontinued in the USA in 1977 and in the FRG in 1983. The contamination of environmental media by PCBs is therefore a problem whose cause was generally prior to 1983.

On 17 May 2004, the Stockholm Convention on the Prohibition of the Twelve Most Hazardous Chemicals in the World, the so-called "POP Convention" (Persistent Organic Pollutants—POPs), entered into force. The Convention provides for a worldwide ban on the production and use of twelve of the most dangerous chemicals ("The ban on the so-called *Dirty Dozen*"). These include 8 pesticides, as well as DDT, dioxins, furans, polychlorinated biphenyls and hexachlorobenzene (■ Table 6.3).

6.2.1.1.2 Physico-Chemical Properties and Evidence

The physico-chemical properties of chloroaromatics are largely determined by the degree of chlorine substitution. With increasing substitution, water solubility and vapor pressure are reduced. The water solubility of monochlorophenols is in the range of 20–30 g/L and decreases to about 10 mg/L until pentachlorophenol. Monochlorobenzene shows a solubility of about 0.5 g/L, while hexachlorobenzene dissolves to only 5 µg/L. Analogous to the two groups of chloroaromatics mentioned above, the solubility of polychlorinated biphenyls also decreases with increasing number of chlorine substituents from monosubstituted biphenyls with solubility in the mg range to the more highly substituted ones with solubilities in the 1 µg/L range.

Due to the relatively high vapour pressure, air is a preferred environmental compartment for chlorobenzenes. The risk of "stripping" (pollutants are transferred from the liquid phase to the gas phase) from wastewater is given.

When looking at the distribution coefficient K_{ow} it is noticeable that the solubility in organic solvents increases with increasing chlorine content. This is an indication of the increasing tendency to accumulate in biological material or the organic matrix of a soil with increasing chlorine substitution. PCBs can therefore be expected to accumulate. The tendency to bioaccumulate is higher for PCBs than for chlorobenzenes.

Due to their physico-chemical properties, chlorinated aromatics are found in varying concentrations in almost every environmental medium and accumulated in biological material. In addition to being detected in a variety of sediments and soils, this class of compounds is found in landfill wastewater and as contaminants in groundwater. Chlorobenzenes and chlorophenols have been detected in contaminated soil and activated sludge up to concentrations of 1000 mg/L and mg/kg, respectively. While monochlorobenzene is found in waters at concentrations up to 5 mg/L, it was 2 orders of magnitude lower for hexachlorobenzene. Due to their better solubility, the chlorophenols show an average pollution of waters rather in the range of mg/L.

6.2.1.2 Possibilities of Microbial Degradation of Chlorinated Aromatics

Chloraromatics can be rendered harmless by the activity of microorganisms, in the better case the chemicals are a **benefit to** the **organisms,** or in the other they are transformed **randomly,** i.e. **without benefit,** by the enzymes present. The latter metabolism is **cometabolism**, whereas use as a **source of carbon and energy** or as an **electron acceptor** is associated with growth of the organisms (see ► Fig. 5.4).

6.2.1.2.1 Cometabolic Degradation

6.2.1.2.1.1 Cometabolic Conversions by Aerobic Bacteria After Growth on Aromatics

A few examples will be used to show which dead-end metabolites can be formed from chloroaromatics, some of which are toxic to the microorganisms that form them. In many cases it was observed that cells grown with biphenyl can cooxidize 4-chlorobiphenyl as substrate analogue up to 4-chlorobenzoate. Further degradation usually does not occur because benzoate-1,2-dioxygenases are often a bottleneck, especially for the benzoates substituted in the 4-position (■ Fig. 6.48).

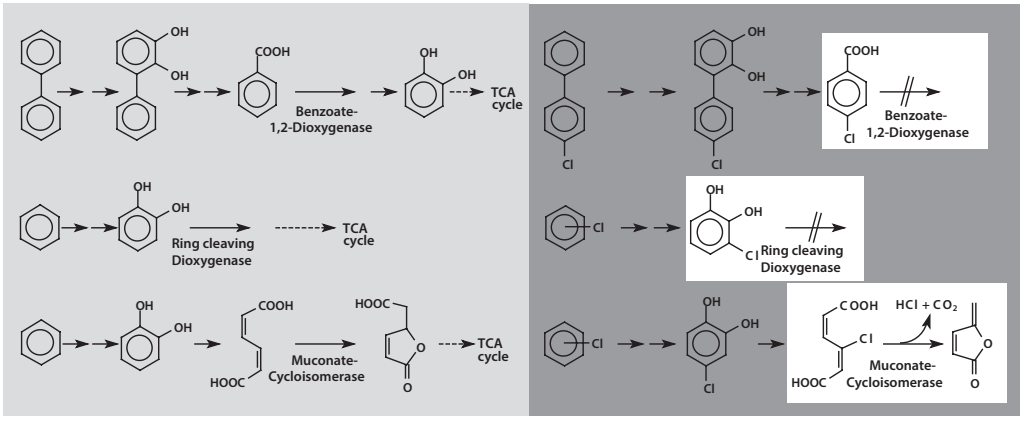
Cometabolic transformations can also form **toxic metabolites**. Thus, chloroaromatics are transformed by many aromatic degrading enzyme sequences up to the stage of **chlorocatecholss**. The lack of or insufficiently rapid conversion by ring-cleaving dioxygenases then causes an accumulation of, for example, **3-chlorocatechol**, which subsequently ends after autoxidation with generally toxic effects on the microorganisms.

Another example is the conversion of chloroaromatics via 4-chlorocatechol to **protoanemonin**, a metabolite toxic to a variety of microorganisms**,** as shown by soil populations.

In organisms with modified *ortho*-pathway (see later, also hybrid strains with such pathways) the last two poisonings do not occur.

6.2.1.2.1.2 Cometabolic Degradation by Ligninolytic Fungi

Unlike bacteria, fungi cannot use chloraromatics as a source of carbon and energy. Thus, the degradation of chloraromatics is carried out by enzymes synthesized for a different purpose. The ligninolytic fungi such as *Phanerochaete chrysosporium* are organisms that have degradation potential for a wide range of environmental chemicals such as chlorinated anilines, benzenes, phenols, phenoxyacetates, biphenyls, and dibenzo-p-dioxins due to their non-specific extracellular enzymes. However, the available data are insufficient to properly estimate the degradation potential of fungi for chloroaromatics. The degradation rates achieved vary greatly and are often very low.



■ **Fig. 6.48** Cometabolic conversion of chloroaromatics by biphenyl recyclers, aromatic recyclers and soil populations: Formation of neutral and toxic end products (*left*: "natural" reactions; *right*: cometabolic reactions with chloroanalogue). End products: 4-chlorobenzoate (top), 3-chlorocatechol (middle), protoanemonin (bottom)

6.2.1.2.1.3 Cometabolic Dechlorination by Anaerobic Bacterial Populations

It has been found that dechlorination of chloroaromatics can occur through environmental material (contaminated sediment, groundwater) when denitrifying, sulfate-reducing, or even methanogenic conditions are present. The slow, anaerobic dechlorination leading to the non-chlorinated or low-chlorinated aromatics has been demonstrated for chlorinated anilines, benzoates, benzenes, biphenyls, catechols, dibenzo-p-dioxins, phenols and phenoxyacetates. That these are microbial processes could be concluded from the following findings:

1. No dechlorination takes place with autoclaved environmental material.
2. Reductive dechlorination can be stimulated by the addition of organic electron donors such as lactate, acetate, pyruvate, ethanol or glucose. H_2 can act as an electron donor. In some cultures, the addition of electron donors is essential for dechlorination.
3. Dechlorination is very specific and varies through different environmental samples: only certain congeners are used as substrates such as *meta*-substituted benzoates or *meta*- and *para*-substituted PCBs.

According to current knowledge, the described processes could also be **dehalorespiration**, which will be discussed below.

6.2.1.2.2 Chlorinated Aromatic Compounds Beneficial to Microorganisms

Microorganisms can use chlorinated aromatics in two ways, and dechlorination occurs in both:

1. The chloroaromatic serves as an electron acceptor for anaerobic bacteria, and
2. the Chloroaromatic serves as a carbon and energy source for aerobic bacteria.

6.2.1.2.2.1 Dehalorespiration, an Anaerobic Respiration

In addition to inorganic nitrogen and sulfur compounds or CO_2, a variety of other substances, both organic and inorganic, can serve as electron acceptors for anaerobic respiration. These include, in particular, Fe^{3+}, Mn^{4+}, but also humic substances and various chloroaromatics. During dehalorespiration, the chloroaromatic acts as an electron acceptor for metabolic oxidation processes. The reduction of the chloroaromatic that occurs leads to the elimination of at least one chlorine substituent. The formation of a non-chlorinated or low-chlorinated aromatic is coupled to the generation of a proton gradient. A scalar mechanism is responsible for this, shown in ◘ Fig. 6.49: protons are consumed inside the cell and released outside. ATP generation then occurs subsequently by the reflux of protons into the cell by ATP synthase. Dehalorespiration of chloroaromatics has been demonstrated with *Desulfitobacterium chlororespirans, D. dehalogenans, D. frappieri, Desulfomonile tiedjei* and *Desulfovibrio* sp.

According to calculations by Jan Dolfing, the redox potentials for the Ar-X/Ar-H redox pairs lie in the range between 286 and 478 mV. This means that halogenated aromatics can be suitable electron acceptors for microorganisms under anaerobic conditions.

If **a** chlorine substituent is eliminated from a halogenated aromatic compound, the Gibbs free energies available for dehalogenation are in the range 130–171 kJ/mol (◘ Table 6.4). For chlorobenzoates, 130–171 kJ/mol were determined, for chlorophenols it is 131–168 kJ/mol and for chlorobenzenes 146–171 kJ/mol.

The reductive dechlorination of hexa-, penta-, and tetrachlorobenzene releases

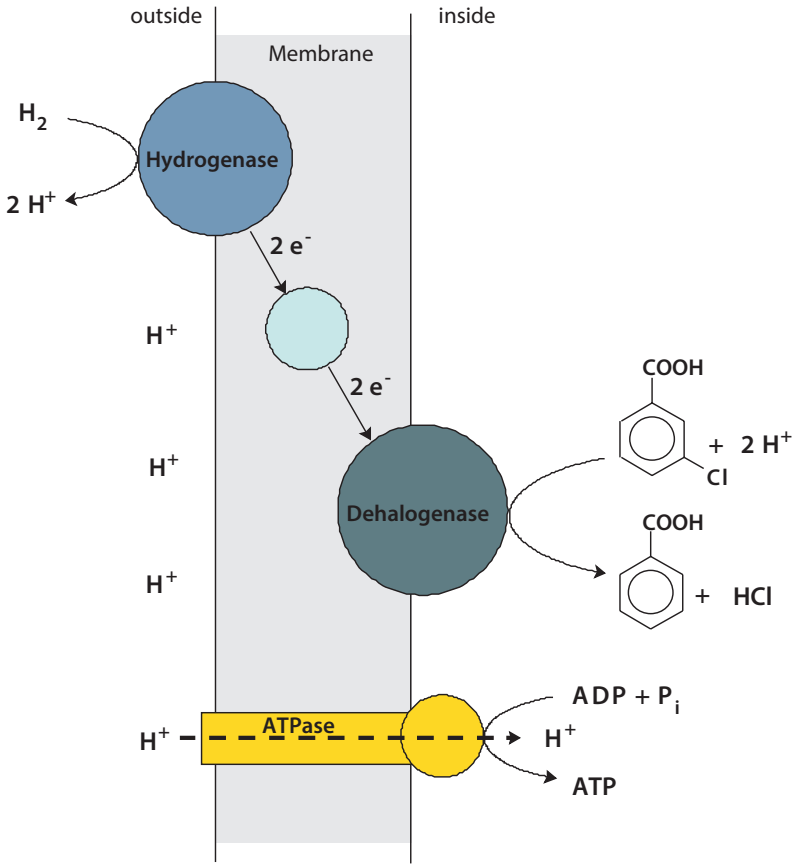


Fig. 6.49 Schematic representation of the respiratory chain using a chloroaromatic as an electron acceptor using the example of 3-chlorobenzoate

~20 kJ/mol more per dechlorination step than the dechlorination of tri-, di-, and monochlorobenzene.

An advantage for the use of 3-chloro-4-hydroxyphenylacetate as electron acceptor over *ortho*-substituted chlorophenols is seen in the comparable structure but lower toxicity towards the microorganisms.

Since aeration of soils and aquifers is rather difficult and expensive, dehalorespiration metabolism is a possible alternative to aerobic degradation of chlorinated aromatics during purification, as presented below.

6.2.1.2.2.2 Chloroaromatics as Carbon and Energy Source of Aerobic Bacteria

It has been known for some time that chloroaromatics can serve as a source of carbon and energy for aerobic bacteria, with mineralization leading to CO_2, chloride and biomass.

The chloroaromatics, like the non-halogenated analogues, must first be converted (activated) to dihydroxy- or trihydroxyaromatics, which are then cleaved by dioxygenases. The degradation pathways for single-ring chloroaromatics can be classified as follows:

Table 6.4 Usable electron donors, acceptors and carbon sources in bacteria with dehalorespiration (with Gibbs free energies of dechlorination according to Jan Dolfing)

Electronics donor	**H_2, formate, acetate, lactate, pyruvate, crotonate, butyrate, succinate, ethanol, benzoate, methoxybenzoate**
Electron acceptor (in parentheses ΔG^0 ' in kJ/mol of the reaction: Substrate + H_2 → Product + H^+ + Cl^-)	***meta*-substituted chlorobenzoates:** 3-Chloro- (−137.3) 3,5-Dichloro- (−143.7)
	***ortho*-substituted chlorophenols:** 2-Chloro- (−156.9) 2,3-Dichloro- (−144.3 or −147.9)[a] 2,6-Dichloro- (−135.3) 2,4,6-Trichloro- (−141.5 or −146.0) Pentachloro- (−156.9, −167.8 or −166.0)
	Chlorobenzenes: 1,2,3-Trichloro- (−158.6 or −161.2) 1,2,4-Trichloro- (−147.3, −149.9 or −153.4) 1,2,3,4-Tetrachloro- (−155.2 or −166.5) 1,2,3,5-Tetrachloro- (−148.6, −163.5 or −159.9) 1,2,4,5-Tetrachloro- (−164.3.) Pentachloro- (−161.1, −167.7 or −163.4) Hexachloro- (−171.4)
Carbon source	CO_2 and organic compounds

[a]The Gibbs free energy depends on the position of the dechlorination: for example, it leads to 2-chloro- or 3-chlorophenol as product

1. on the basis of the elimination of the chlorine substituent(s) **before ring cleavage, i**.e. from an aromatic step, or **after ring cleavage,** i.e. from a non-aromatic step, or both,
2. according to the **type of ring cleavage substrate** formed, catechol, hydroquinone, protocatechuate, and
3. according to the **type of ring cleavage reaction** that cleaves the chlorinated ring cleavage substrate as a **metabolite**, ***ortho*- or *meta*-cleavage.**

In bicyclic systems such as chlorobiphenyls, the non- or low-substituted ring is usually opened to give chlorobenzoates, thus reaching the single-ring stage.

▪ Elimination Before Ring Splitting

The mechanism of dechlorination prior to ring cleavage may involve hydrolytic, oxygenolytic, or reductive elimination of the chlorine substituent.

▪ Hydrolytic Elimination

Hydrolytic dechlorination has been observed in the degradation pathways of 4-chlorobenzoate and some chlorophenols. The reaction with 4-chlorobenzoate as utilized in *Alcaligenes, Arthrobacter, Micrococcus, Nocardia* and *Pseudomonas strains has* been particularly well studied. The 4-chlorobenzoate dehalogenase system consists of three components. The role of each component was elucidated by cloning

the corresponding genes and by studies with purified enzymes (◘ Fig. 6.50). Activation of the substrate to the CoA derivative requires ATP and is carried out by a ligase. Hydrolytic elimination of the chlorine substituent by dehalogenase follows. Based on sequence similarity, the ligase and dehalogenase are thought to originate from the *β*-oxidation of fatty acids. The final step in the formation of 4-hydroxybenzoate is carried out by 4-hydroxybenzoate:coenzyme A thioesterase, one enters the protocatechuate degradation pathway (◘ Fig. 6.50).

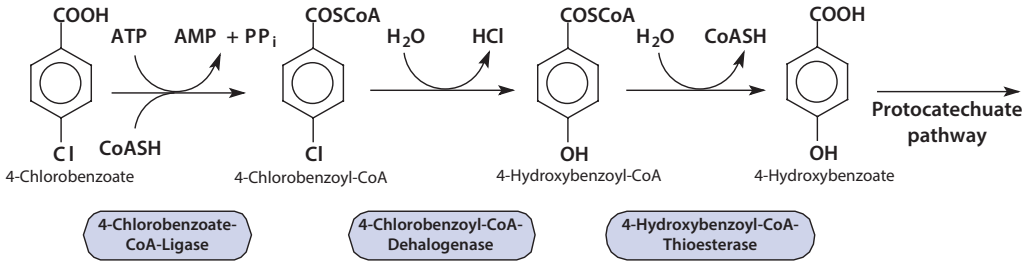
Dehalogenation appears to be limited to halogenated benzoates with substitution in the *para*position.

Hydrolytic eliminations have also been described for other chloroaromatics such as chlorophenols in the so-called chlorohydroquinone degradation pathway.

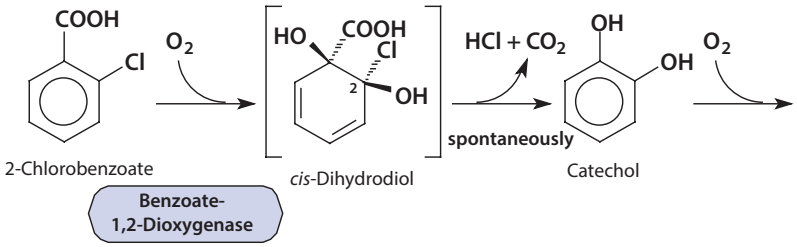
▪ Oxygenolytic Elimination by Dioxygen ases

Dechlorination by dioxygenases is another mechanism that causes removal of a chlorine substituent, forming 1,2-diphenols. The initiating dioxygenase responsible for ring activation forms a *cis*-dihydrodiol, which is normally converted to the 1,2-diphenol by a dehydrogenase. Here, however, the molecular oxygen is placed on the aromatic ring by the dioxygenase in such a way that one of the vicinal hydroxyl groups of the *cis*-dihydrodiol in question is on the same carbon atom as the chlorine substituent (◘ Fig. 6.51 for 2-chlorobenzoate). From this **unstable dihydrodiol,** the chlorine substituent eliminates spontaneously, i.e. without further enzyme action, and the *ortho*-diphenol is formed.

Because of the site-specificity of the insertion of oxygen, for example by benzoate 1,2-dioxygenase into positions 1 and 2, elimination of the chlorine substituent can only occur from position 2 of the benzoate. A similar narrow elimination potential was observed for other dioxygenases such as phenylacetate 3,4-dioxygenase. While 4-chlorophenylacetate is a substrate, the enzyme fails with all other chlorinated phenylacetates.



◘ **Fig. 6.50** Activation and hydrolytic dechlorination of 4-chlorobenzoate



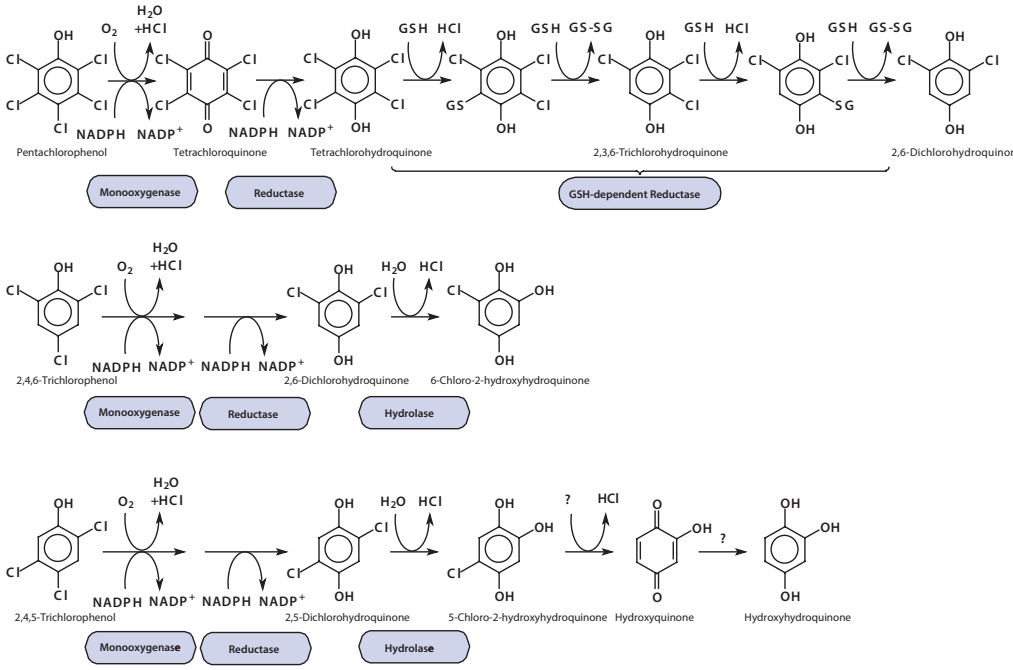
◘ **Fig. 6.51** Oxygenolytic dechlorination of 2-chlorobenzoate by benzoate-1,2-dioxygenase

The Hydroquinone Pathway

In the hydroquinone pathway, all three mechanisms of elimination of chlorine substituents **prior to ring cleavage** are realized, hydrolytically, oxygenolytically, or reductively from the aromatic. The degradation of pentachlorophenol is best realized in *Sphingobium chlorophenolicum* (older names: *Sphingomonas chlorophenolica, Flavobacterium chlorophenolicus*). The pathway is initiated with **oxidative dechlorination by a monooxygenase** to tetrachloro-p-quinone. This is followed by reduction to tetrachloro-*p*-hydroquinone. **Reductive dechlorination** of tetrachloro-*p*-hydroquinone is carried out by a glutathione-dependent reductase. The first chlorine substituent of tetrachloro-*p*-hydroquinone is substituted by glutathione. Glutathione is then removed from the aromatic by means of a second glutathione molecule, resulting in oxidized glutathione. This reaction sequence proceeds a second time, leading to the formation of 2,6-dichlorohydroquinone (◘ Fig. 6.52). The overall reaction from tetrachloro-*p*-hydroquinone to 2,6-dichlorohydroquinone is comparable to reductive dechlorination as it occurs in anaerobic organisms. With 2,6-dichlorohydroquinone, the ring cleavage substrate is reached.

2,6-Dichlorohydroquinone is also produced from 2,4,6-trichlorophenol by a monooxygenase reaction. In contrast to the degradation of pentachlorophenol, a hydrolytic elimination and formation of the ring cleavage substrate 6-chlorohydroxyhydroquinone was detected in *Cupriavidus necator* JMP134 (formerly: *Alcaligenes eutrophus, Ralstonia eutropha, Wautersia eutropha*).

The degradation of 2,4,5-trichlorophenol is initially analogous to the other two sub-



◘ **Fig. 6.52** Hydroquinone pathway for pentachloro-, 2,4,5-trichloro- and 2,4,6-trichlorophenol in *Sphingobium chlorophenolicum, Burkholderia phenoliruptrix* AC1100 (formerly: *Burkholderia cepacia*), *Cupriavidus necator* JMP134 up to the stage of ring cleavage: oxidative, reductive and hydrolytic dechlorination

strates, namely a monooxygenase-catalyzed elimination followed by reduction to 2,5-dichlorohydroquinone. A hydrolase causes the substitution of a chlorine substituent by a hydroxyl group, i.e. formation of 5-chlorohydroxyhydroquinone, which is further converted via hydroxyquinone to hydroxyhydroquinone, the ring cleavage substrate.

The following conclusion can be drawn for hydrolytic dechlorination from the aromatic ring: Removal of the substituent by nucleophilic substitution from a system rich in π-electrons is difficult. Therefore, the ring must be activated by coenzyme A or the presence of hydroxyl or further halogen substituents.

■ Elimination After Ring Splitting

The following chlorinated hydroxylated aromatics occur as central metabolites in the degradation of chloroaromatics and also lindane as ring cleavage substrates: chlorinated catechols, hydroquinones, hydroxyhydroquinones.

The reactions that carry out the formation of the chlorinated catechols are similar to the peripheral reactions used for the degradation of the non-chlorinated aromatics. In part, they are catalyzed by the same enzymes, and in part by specialized enzymes. In the course of the peripheral reactions, partial dechlorination (for example, oxygenolytic by dioxygenases) may occur, depending on the strain and compound. However, it can also be completely absent. This means that several reactions with chlorinated metabolites may have to take place.

Dechlorination of chlorocatechols and chlorohydroxyhydroquinones occurs after *ortho*-cleavage, but also in the course of *meta*-ring cleavage (◘ Fig. 6.53).

■ The Ortho Pathway for Chlorocatechols

Chlorocatechols formed by peripheral enzymes are degraded as follows: Ring cleavage, using molecular oxygen, by chlorocatechol1,2-dioxygenase (usually referred to as chlorocatechol1,2-dioxygenase, abbreviated CC12O, in the literature) leads to the formation of the corresponding chloro-*cis,cis*-muconates. These are subsequently converted by a chloromuconate cycloisomerase to a lactone, i.e. an intramolecular ester. In most cases, the chloromuconate cycloisomerases are not only able to catalyze the actual cycloisomerization, but also to perform an elimination of the chloride. Thus, an additional exocyclic double bond is formed in the product, the so-called *cis*- or *trans*-diene lactone. In the case of the degradation of 3-chloroprene catechol via 2-chloro-*cis,cis*-muconate, (+)-5-chloromuconolactone can also be released first and then dechlorinated by a 5-chloromuconolactone dehalogenase to *cis*-diene lactone. Due to the exocyclic double bond, the dienelactones can be directly hydrolyzed to form maleylacetate and 2-chloromaleylacetate, respectively. Maleylacetate reductase leads to the formation of 3-oxoadipate by reducing the double bond, thereby introducing chloroaromatic degradation into the pathway for conventional aromatic degradation (see ◘ Fig. 6.16a). In addition, maleylacetate reductases are capable of reductively eliminating the chlorine substituent from position 2 of chloromaleylacetates, thus contributing to the dehalogenation of higher chlorinated catechols.

The degradation pathway described works for chlorocatechols with up to 4 chlorine substituents, as described in the degradation of 1,2,3,4-tetrachlorobenzene. To date, however, only the two dechlorination steps shown in ◘ Fig. 6.54 are well understood. Where the third and fourth substituents are eliminated is not yet known (◘ Fig. 6.55).

■ The *Ortho* Cleavage Of Chlorohydroxyhydroquinone

In the degradation of 2,4,6-trichlorophenol, chlorohydroxyhydroquinone is formed as a ring cleavage substrate. This is converted

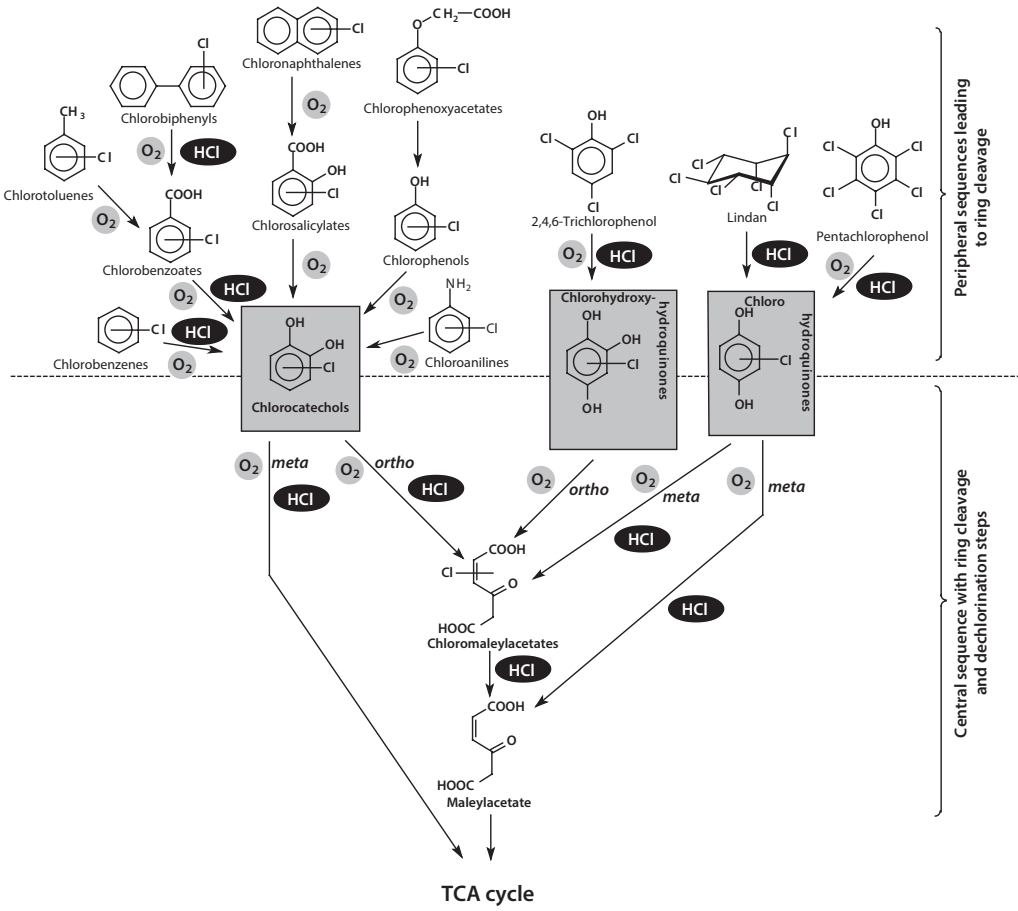


Fig. 6.53 Schematic representation of the mineralization of chloroaromatics and lindane with chlorocatechols, chlorohydroquinones, and chlorohydroxyhydroquinones as key metabolites. A large number of the compounds shown avoid fixing the chlorine substituent to a specific position in the ring

to 2-chloromaleylacetate by chlorohydroxyhydroquinone-1,2-dioxygenase, an enzyme with sequence similarity to typical *ortho*-cleaving enzymes such as catechol-1,2-dioxygenase. Dechlorination occurs as shown above for dichlorocatechols by maleylacetate reductase.

■ Dechlorination as Part Of The *Meta* Pathway

For a long time, the degradation of chloroaromatics via the *meta*-pathway was considered impossible. One reason for this was the observation that a so-called suicide product, a reactive acyl chloride, is formed from 3-chlorocatechol by catechol 2,3-dioxygenase, which leads to the inactivation of the ring cleavage enzyme (Fig. 6.56). In addition, it had been shown with another *Pseudomonas putida* strain that 3-chlorocatechol itself can also reversibly inactivate a catechol 2,3-dioxygenase because of its ability to complex the Fe^{2+}. Oxidation of Fe^{2+}, which is essential for the reaction, to Fe^{3+} in the presence of catechols is another mechanism leading to inactivation.

Recent publications describe growth using the *meta*-pathway of such chloroaromatics degraded via catechols with a chlo-

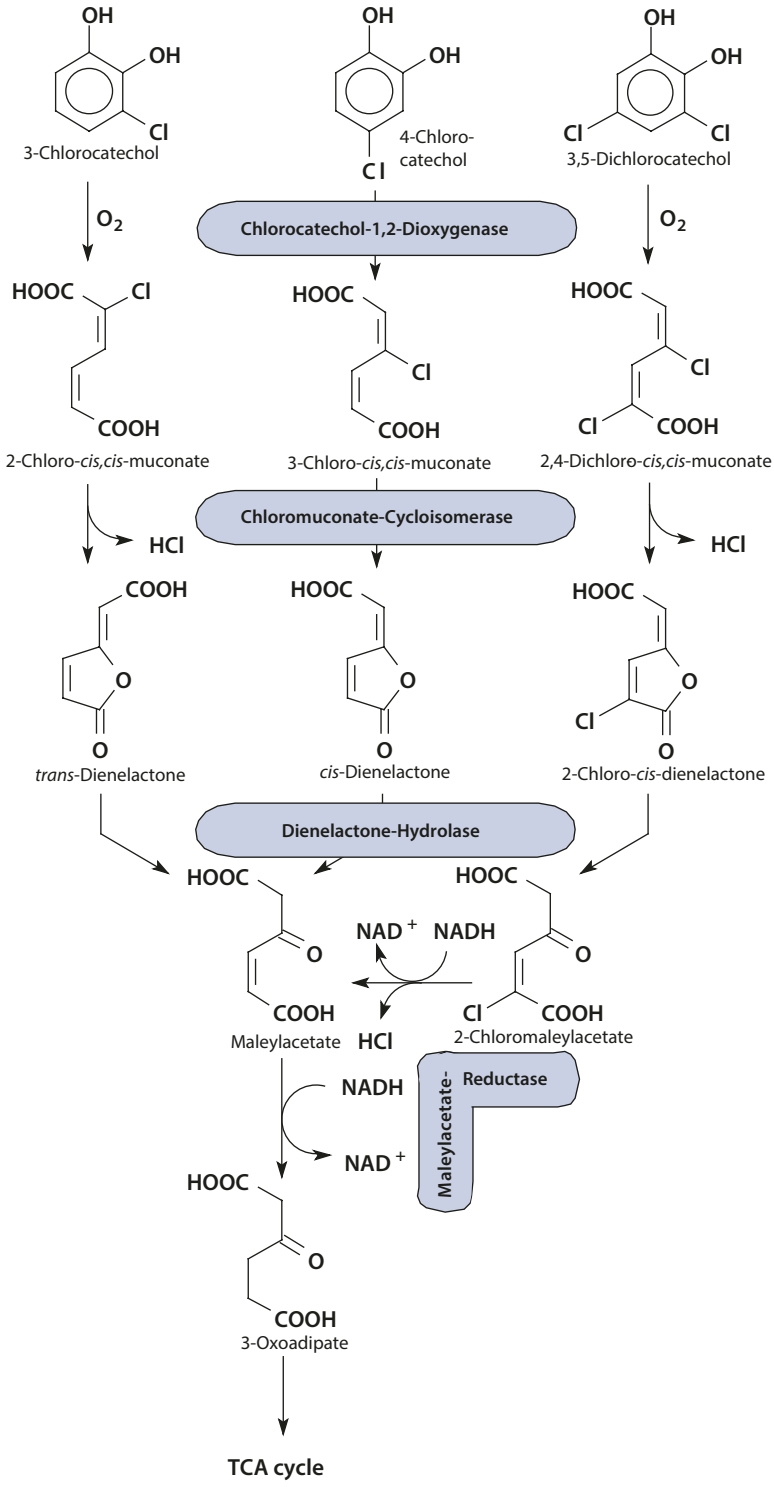


Fig. 6.54 *Ortho*-pathway for the degradation of 3-chloro-, 4-chloro- and 3,5-dichlorocatechol

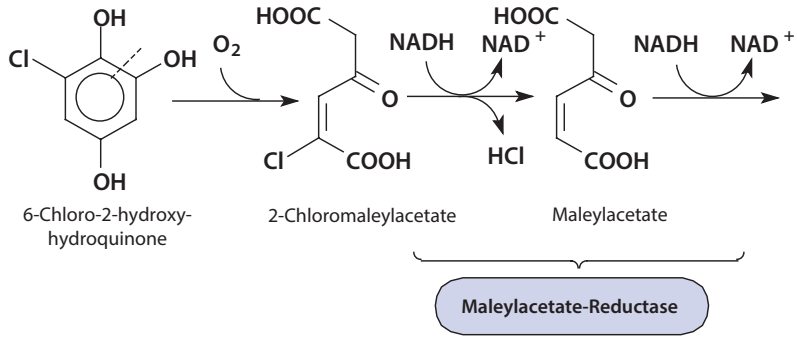


Fig. 6.55 Chlorohydroxyhydroquinone degradation: ring opening

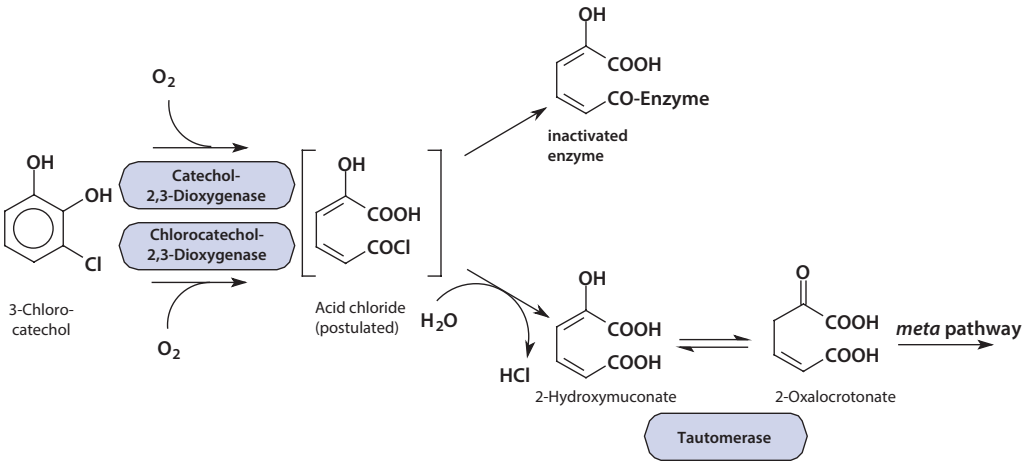


Fig. 6.56 3-Chlorocatechol and the *meta*-pathway. top: unproductive reaction with inactivation; bottom: productive degradation

rine substituent in the 4-position. However, more detailed information on the elimination of the chlorine substituent is lacking so far. Further, it was found that some *Pseudomonas putida* strains can grow rapidly on chlorobenzene using the *meta*-pathway. Surprisingly, 3-chlorocatechol was found to be a metabolite (Fig. 6.56). Unlike other catechol 2,3-dioxygenases, which are subject to inactivation, the so-called chlorocatechol 2,3-dioxygenase productively converts 3-chlorocatechol. Stoichiometric release of chloride leads to the formation of 2-hydroxymuconate, which is then further degraded via the *meta*-pathway.

Dechlorination as a Result of the *Meta*-Cleavage of Chlorohydroquinone

The 2,6-dichlorohydroquinone formed from pentachlorophenol in the chlorohydroquinone pathway is opened by an oxygen-dependent reaction (Fig. 6.57). Sequence comparisons show high similarity of the enzyme to *meta*-cleaving enzymes such as catechol 2,3-dioxygenase. Hydrolysis of the resulting acid chloride leads to the formation of 2-chloromaleylacetate, which is dechlorinated by maleylacetate reductase as described in the degradation pathway for chlorinated catechols, converted to 3-oxoadipate and thus fed to the citrate cycle.

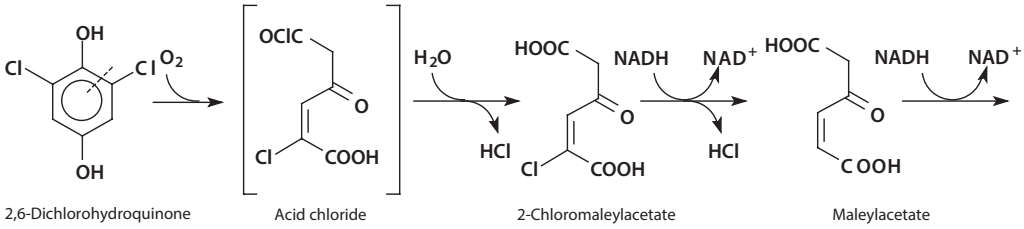
6.2.2 Degradation of Hexachlorocyclohexane

Hexachlorocyclohexanes (HCHs, $C_6H_6Cl_6$) are chlorinated cyclohexanes with eight stereoisomers, designated α- to ϑ-HCH (alpha- to theta-HCH) depending on the position of the chlorine substituents. The proportionally most important isomers formed in the production of the insecticide lindane (γ-HCH) are α-, β- and γ-HCH (◘ Fig. 6.58).

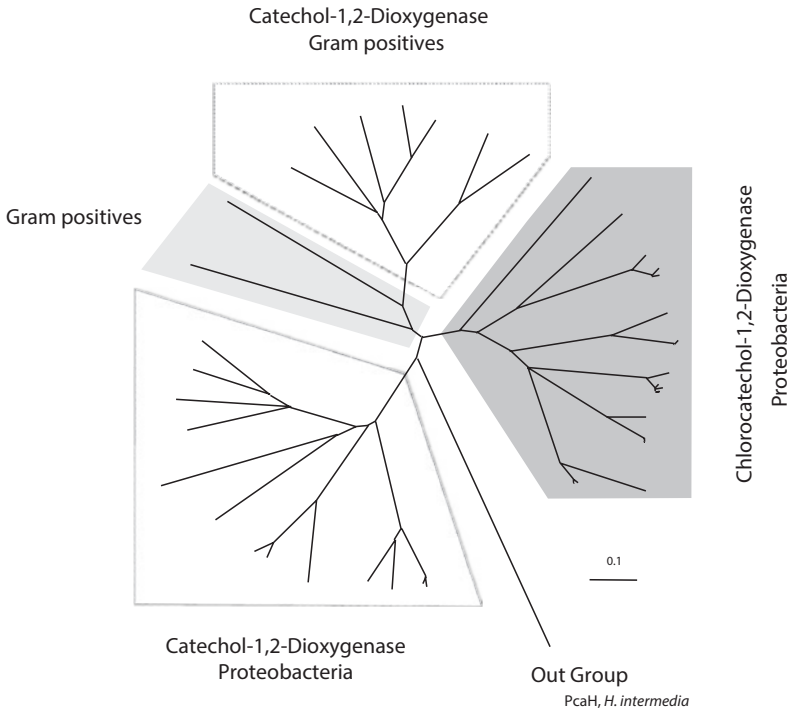
Lindane is produced by chlorination of benzene in UV light and isolated from the mixture of isomers by extraction with methanol.

Lindane, which acts as a feeding and contact poison, was one of the most widely used insecticides worldwide until the early 1980s. It was used against soil pests, to treat seeds, to control parasites in livestock, for wood preservation and in insect sprays.

HCHs are poorly soluble in water, hardly volatile and persistent. Because of this, they



◘ **Fig. 6.57** 2,6-dichlorohydroquinone degradation



◘ **Fig. 6.58** Dendrogram illustrating the relationship of catechol 1,2-dioxygenases (CatA, Phe) and chlorocatechol 1,2-dioxygenases (Clc, TfdC, CplC, CbnA, TcbC, TetC). (Scale: changes per digit; Out-Group: Protocatechuate-3,4-dioxygenase, PcaH, from *Hydrogenophaga intermedia*)

can be enriched in the food chain. They are sorbed in the soil, so that transport through leachate into groundwater occurs only to a small extent. Lindane and its by-products represent a potential hazard due to their persistence and ecotoxicity. This is particularly true for former production sites.

Lindane was not included in the POP list. The use of the substance is banned today.

The rates of biological conversion are limited by water solubilities. As in the case of PAHs, the rate of desorption or redissolution limits the possibilities of improving degradation rates. The biodegradability of HCHs is influenced by the spatial position of the chlorine atoms on the ring. In addition, significant differences in the rate of degradation as well as in the degree of degradation depending on the environmental conditions (oxic or anoxic) can be observed. The focus of studies on the biodegradation of HCHs was predominantly on agriculturally oriented studies on the fate of lindane, with the result that it is dechlorinated more slowly under aerobic conditions than under anaerobic conditions.

There is no evidence of biodegradation of β-HCH (here all chlorine substituents are equatorial).

Anaerobically, γ-HCH is reductively dechlorinated relatively rapidly. The product formed is 1,3,4,6-tetrachlorocyclohexene by cleavage of 2 HCl and formation of double bonds, which is further oxidized to chloroaromatics (◘ Fig. 6.59). α-HCH is less degradable than γ-HCH under anaerobic conditions and therefore accumulates relatively in soils. It can be reductively dechlorinated like γ-HCH but apparently only slowly to form chloroaromatics.

Aerobically, HCl is also split off from α-HCH in the first step by dehydrodehalogenation. The α-pentachlorocyclohexene formed in this process appears to be more labile than the γ-isomer, because it can be mineralized. However, mineralization may not occur if nutrients that can be readily utilized are added.

The important question for bioremediation, whether the degradable HCH isomers can be better converted anaerobically or aerobically and to which end products, cannot be answered on the basis of the available results from practice.

There are bacterial isolates that can use lindane as a carbon and energy source. The degradation pathway is shown for *Sphingomonas paucimobilis* in ◘ Fig. 6.60, excluding the formation of the dead-end products 1,2,4-trichlorobenzene and 2,5-dichlorophenol. Initially, two dehydrohalogenations are followed by two hydrolytic dechlorination steps. Rearomatization to 2,5-dichlorohydroquinone and glutathione-dependent reductive dechlorination follow.

6.2.3 Degradation of Triazines

An important group of active ingredients in herbicides are the triazines, with the representatives atrazine, simazine and terbuthylazine. These herbicides are used in corn cultivation. Their action as herbicides is based on the inhibition of electron transfer during photosynthesis. However, in the corn plant, the herbicide is rapidly deactivated by conversion to the hydroxy compound, which does not affect photosynthesis; therefore, the triazines are used as selective total herbicides in corn crops. Atrazine has also been used for weed control in asparagus, potato and tomato crops (◘ Fig. 6.61).

Atrazine has a relatively high water solubility (33 mg/L) and a low partition coefficient (K_{ow} 25–155). Both result in the herbicide exhibiting relatively high mobility from soil to surface water and groundwater. Atrazine does not sorbed much to soil and sediment particles, it is considered to be poorly degradable and therefore may leach into groundwater. Atrazine has been shown to migrate more rapidly than terbuthylazine in saturated soil using soil colonisers.

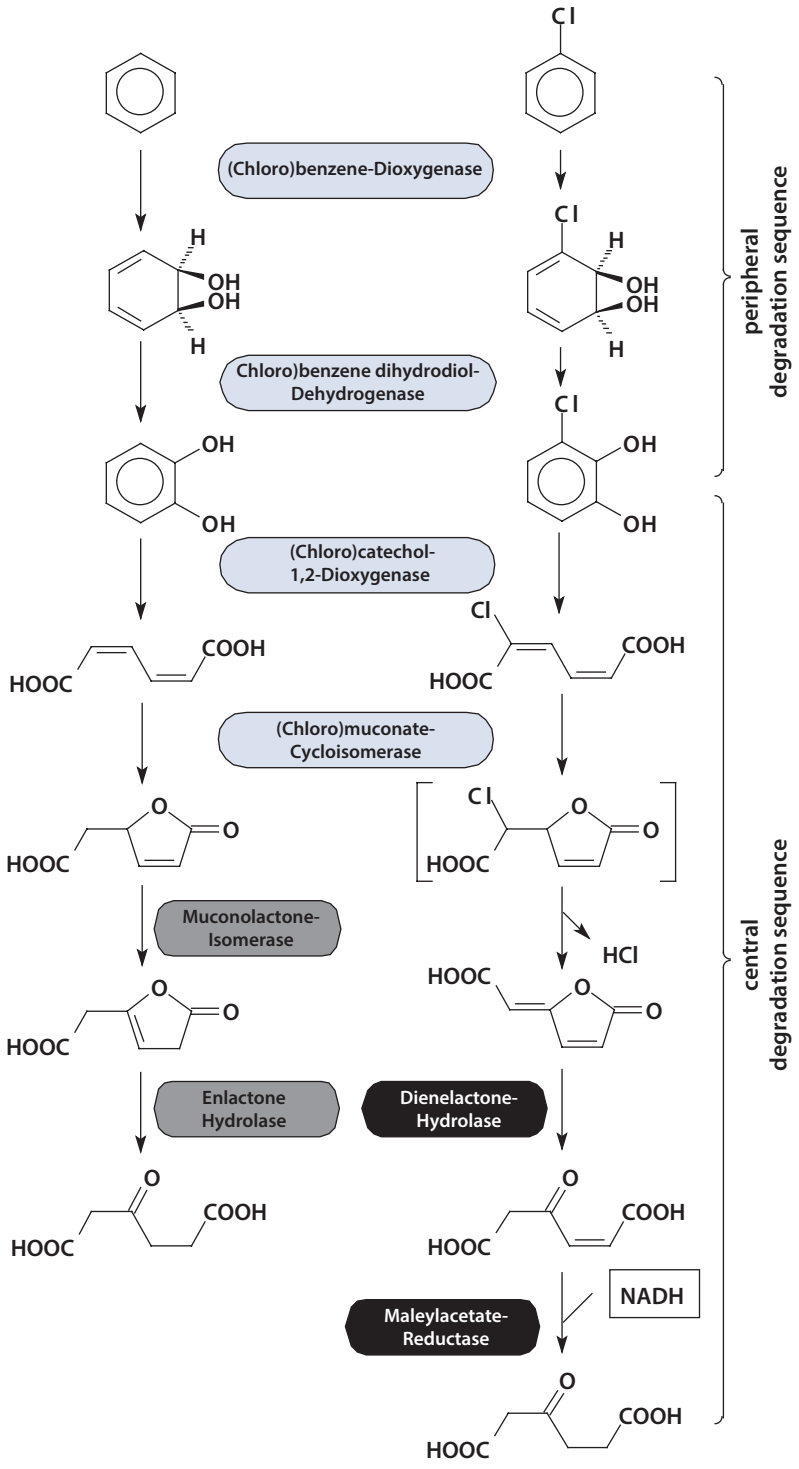


Fig. 6.59 Comparison of the degradation pathways: aromatic versus chloroaromatic using the example of benzene/chlorobenzene

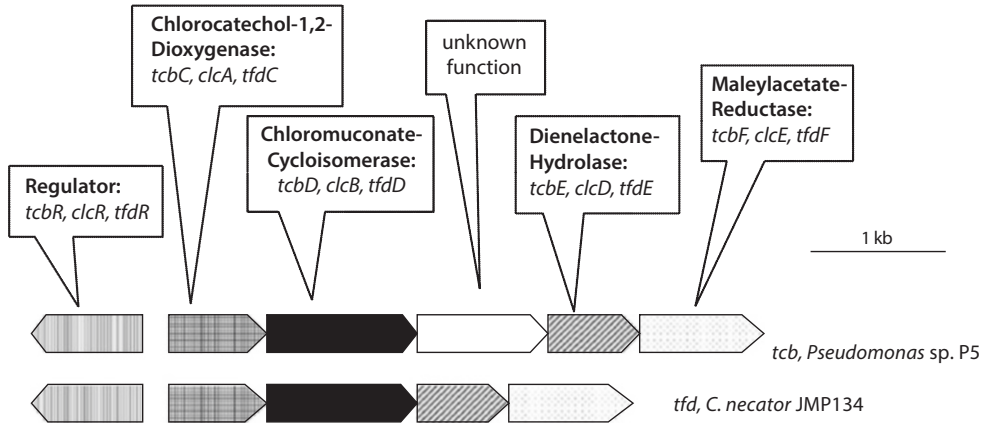


Fig. 6.60 Operon structure of the *ortho*-degradation pathway for chlorocatechols

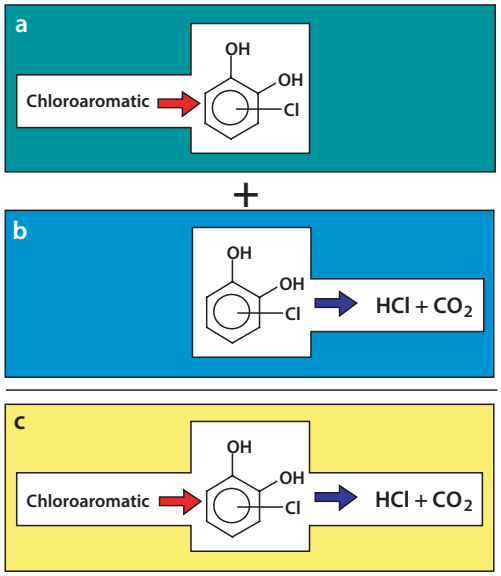


Fig. 6.61 General principle of the construction of the degradative pathways

Since 1991, there has been a complete ban on the use of atrazine and a restricted ban on simazine in Germany and France. Terbuthylazine is approved and has partially replaced atrazine. At the European level, the inclusion of the active substance on a positive list of active substances that can be approved is being examined.

In Germany, despite the ban on use, the substances atrazine, simazine, as well as the metabolite deethylatrazine, have been detected in groundwater in concentrations exceeding the respective limit value of 0.1 µg/L of the Drinking Water Ordinance. Two thirds of the pesticides found in German groundwater belong to the herbicide group of 1,3,5-triazines (Umweltbundesamt, 2000).

Isolation of microorganisms for atrazine degradation is possible because some can use the N-heterocyclic ring structure as a source of carbon and energy as well as nitrogen. Organisms that grow with atrazine are *Agrobacterium radiobacter, Pseudoaminobacter* sp., *Pseudomonas* ssp. and *Ralstonia* sp. In *Pseudomonas* sp. strain ADP runs the sequence shown on the left in ◘ Fig. 6.62. Initiating hydrolytic reactions that eliminate the chlorine substituent as well as the two alkyl side groups form cyanuric acid, the central metabolite. The genes *atzA-C* encoding the hydrolytic enzymes are known. A further three hydrolytic steps continue the mineralization of cyanuric acid to CO_2 and NH_3.

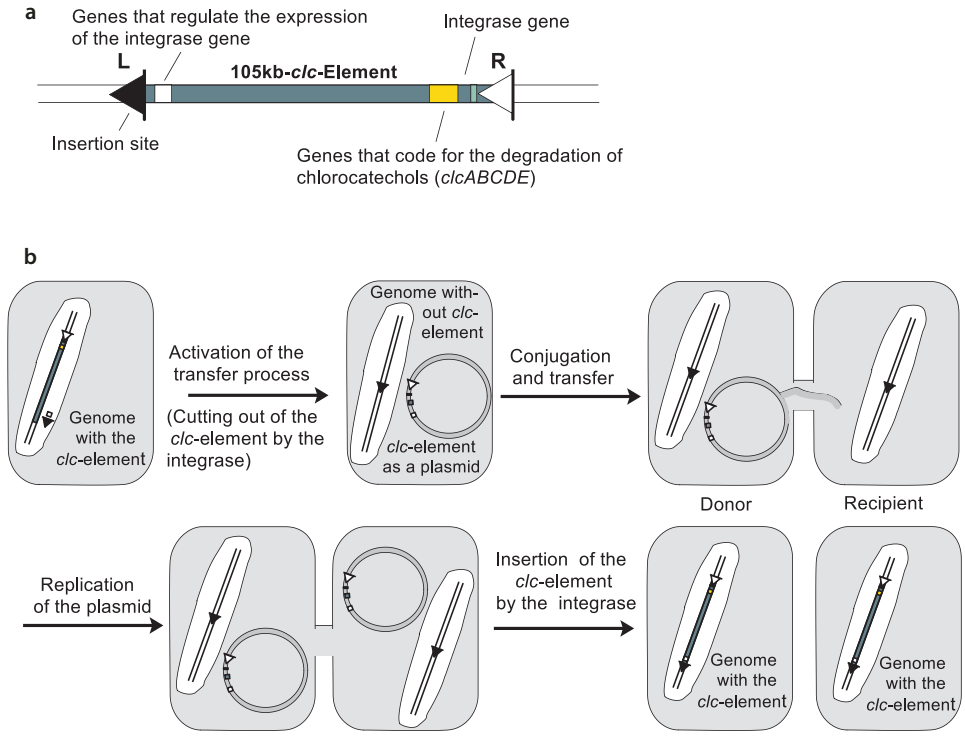


Fig. 6.62 The genes encoding the chlorocatechol degradation pathway in *Pseudomonas* sp. strain B13 are located on the *clc element* integrated in the chromosome ("genomic island")

The dealkylated metabolites (deethyl- and deisopropylatrazines) shown on the right were found in studies with soil.

Thoughts on the Evolution of Degradation Pathways

1. Comparison between enzymes and genes of catechol and chlorinated catechol degradation

A comparison of the enzymes and genes of the catechol and chlorocatechol pathways provides an example of how new degradation pathways for foreign substances arise or have arisen.

The chlorocatechol-1,2-dioxygenases and the chloromuconate cycloisomerases catalyze reactions analogous to those of catechol-1,2-dioxygenase and muconate cycloisomerase. Sequence analyses have shown that the catechol and chlorocatechol 1,2-dioxygenases arose from a **common enzyme of origin** (Fig. 6.63).

The same applies to the muconate and chloromuconate cycloisomerases. The chlorocatechol-1,2-dioxygenases differ from the corresponding catechol-1,2-dioxygenases in **substrate specificity,** i.e. in that they also convert 3-chloro- and 4-chloro- as well as 3,5-dichlorocatechol with high affinities and high relative activities in addition to catechol. Similarly, the chloromuconate cycloisomerases accept in addition to *cis,*cis-muconate 2-chloro-, 3-chloro-, or 2,4-dichloromuconate as substrates, whereas the muconate cycloisomerases exhibit very unfavorable affinities and low relative activities toward the latter substrates. The chloromuconate

cycloisomerases from some 2,4-D metabolizers also show activity but very low affinity toward *cis,cis*-muconate and, in some cases, 2-chloro-*cis,cis*-muconate. Thus, they possess relatively pronounced substrate specificity for the 2,4-dichloromuconate formed in 2,4-D degradation (◘ Fig. 6.64).

Recent findings highlight that in addition to the adaptation of chloromuconate cycloisomerases to better utilize chlorinated substrates, the ability to **eliminate chloride** has also emerged. Indeed, the conversion of 2-chloromuconate by muconate cycloisomerases does not result in chloride release, but rather in the formation of the stable intermediate 5-chloromuconolactone and often 2-chloromuconolactone. Chloromuconate cycloisomerases also promote chloride elimination during 3-chloromuconate and 2,4-dichloromuconate turnover. From these substrates, normal muconate cycloisomerases apparently first form 4-chloro- and 2,4-dichloromuconolactone, respectively, from which toxic protoanemonin and chloroprotoanemonin, respectively, are presumably formed by non-enzymatic reaction.

Sequence comparison reveals that the dienelactone hydrolases of the chlorocatechol pathway appear to be only very distantly related to the analogous enzymes of catechol and protocatechuate degradation, the 3-oxoadipatenollactone hydrolases. The dienelactone hydrolases do not convert the corresponding metabolite of the 3-oxoadipate pathway, 3-oxoadipateenollactone, any more than the 3-oxoadipateenollactone hydrolases hydrolyze *cis*- and *trans*-dienelactone. Thus, the dienelactone hydrolases are not simply 3-oxoadipateenollactone hydrolases with low substrate specificity. Thus, the specialization of some enzymes of chloroaromatic degradation to the metabolites of this pathway is such that they are not applicable to normal aromatic degradation, and they cannot readily be considered enzymes of lower specificity. The maleylacetate reductases have no equivalent in the 3-oxoadipate pathway. Dienelactone hydrolases and maleylacetate reductases are necessary in the degradation of those compounds in which an additional double bond is formed by elimination of a chlorine substituent during or after cycloisomerization. Both types of enzymes must have been recruited to chlorocatechol degradation from degradation or synthesis pathways other than catechol degradation.

While the genes of catechol degradation, the *cat* genes, are usually located on the bacterial chromosome, those of chlorocatechol degradation are usually encoded by plasmids. Very well studied are plasmid pAC27 from the 3-chlorobenzoate degrader *Pseudomonas putida* AC858 with the *clc* genes (stands for chlorocatechol), plasmid pJP4 from the 2,4-D degrader *Cupriavidus necator* JMP134 with the *tfd genes* (stands for two-four-D), and plasmid pP51 from the 1,2,4-trichlorobenzene degrader *Pseudomonas* sp. P51 with the *tcb* genes (stands for trichlorobenzene).

Despite the different origins of the bacteria, the structure of the corresponding operons is almost the same, as documented in ◘ Fig. 6.65, where the genes and the respective enzymes are assigned. Thus, the *clc* and *tcb* genes are organized identically, and the *tfdCDEF* operon differs from them only in the absence of an open reading frame (ORF) between *tfdD* and *tfdE*. The putative regulatory genes *tfdT, clcR* and *tcbR* are oriented opposite to the structural genes. In contrast, the *cat* genes of *Acinetobacter calcoaceticus* and *Pseudomonas putida* are organized completely differently.

2. Evolution and time of origin of the chlorocatechol operons

The question arises as to how and when the operons of chlorocatechol degradation arose. In addition to the high

similarity of the sequences, the similarity of the operon structure on the investigated plasmids also speaks for a single origin of the chlorocatechol degradation pathways. In the case of multiple origins of these pathways, it would not be expected that, for example, the dienelactone hydrolase gene (*clcD, tcbE* and *tfdE*) would be located at the same position in the operon in each case. The origin of the plasmids shows that the original operon was spread worldwide after its creation. In the process, it was combined with various peripheral degradation pathways, and the enzymes evolved certain differences in substrate specificity, some of which were striking. All three chlorocatechol operons discussed contain a gene for a dienelactone hydrolase and one for a maleylacetate reductase. Both enzymes have a function only if an exocyclic double bond is formed on the lactone after cycloisomerization by an elimination reaction. This suggests that the operons probably did indeed arise in the context of chlorocatechol degradation.

Was the evolution of these degradation pathways a response to the release of large amounts of chloroaromatics by humans in the last hundred years, or did this process occur before then? The time elapsed since, for example, the cycloisomerases encoded by pJP4 and pAC27 diverged from a common precursor should provide a clue to the age of the chlorocatechol degradation pathways. This time period can be estimated from the frequency of so-called synonymous differences. These are mutations that do not result in a change in the protein sequence because of the degeneracy of the genetic code and are therefore not subject to selection at the protein level. Assuming an absolute evolutionary rate of 7×10^{-9} mutations per base pair per year, it can be estimated that the chlorocatechol degradation enzymes encoded by plasmids pJP4 and pAC27 diverged about 140 million years ago. A problematic aspect of such calculations is the question of the correctness of the assumed mutation rate, in particular whether mutations due to specific mechanisms or due to the presence of mutagenic agents may not also occur more frequently than assumed. However, since there are six orders of magnitude between the calculated value and the development of the chemical industry, it can be considered certain that the degradation pathways for chlorocatechols developed long before humans produced large quantities of chloroaromatics and polluted the environment with these substances. Thus, while the worldwide distribution of chlorocatechol degradation pathways is not recent, it can be assumed that the frequency of occurrence is closely related to chlorine chemistry.

3. Gene transfer and degradation pathways

It is well known that gene transfer between bacteria can occur by uptake of DNA **(transformation),** by bacteriophages acting as carriers **(transduction),** or by a type of sexual process called **conjugation.** Transformation has been observed under natural environmental conditions, but it is severely limited by the barrier of restriction (cutting of foreign DNA by restriction endonucleases) and is generally restricted to bacteria related to the donor bacteria. Similar performance and limitations were also found for transduction, as the spectrum of infection of most bacteriophages is not particularly broad.

Conjugation is a gene transfer mediated by plasmids. Plasmids, like the chromosome of their host cells, have a circular shape and can be replicated autonomously. However, they are usually much smaller than the chromosome and are dispensable, at least for the important func-

tions associated with the vegetative growth of their hosts. In addition, they often possess the ability to self-transfer into other bacteria (*tra* functions).

In addition to the genetic basis for their self-replication and self-transfer, plasmids can carry genes for a variety of functions that often give their hosts a certain survival advantage (resistance functions) and allow the colonization of specific biotopes (for example, catabolic plasmids for the degradation of organic compounds such as aromatics or chloroaromatics). As ◘ Table 6.5 shows, a variety of degradation sequences are encoded on plasmids, both genes for peripheral degradation pathways and those for central ones.

With the above knowledge, chloroaromatic recyclers can be generated in the laboratory when suitable partner strains with complementary degradation sequences are brought together on solid culture media. Horizontal, conjugative gene transfer results in strains that can use new substances as growth substrates. This in *vivo* construction of degradation strains by transfer of gene sequences is relatively easy to achieve for chloroaromatic degradation (see ◘ Fig. 6.66). In strains from nature that use aniline, benzene, benzoate, biphenyl, phenol, salicylate, and toluene as carbon and energy sources, aromatic degradation converges on the catechol step. Such strains can be transformed into specialists that utilize chloroanilines, chlorobenzenes, chlorobenzoates, chlorobiphenyls, chlorophenols, chlorosalicylates, and chlorotoluenes. While the wild-type strains convert the chlorinated aromatics to only the chlorocatechols using the degradation pathways for the aromatics, the hybrid strains can perform total degradation of the chloroaromatics by acquiring a chlorocatechol-degrading enzyme sequence. *Pseudomonas* sp. B13 has been shown to be a donor strain for the genes encoding the degradation of chlorocatechols, but here the degradation genes are located on a **genome island** called the 105 kb *clc* element, rather than on a conjugative plasmid. Genome islands are unstable regions on the bacterial chromosome that sometimes insert themselves from one bacterium directly into the genome of another. Genome islands are characterized by having a gene for an integrase, which is responsible for cutting out and reinserting the element. Furthermore, insertion into a specific location in the chromosome is one of the characteristics of a genome island. Since the element is only present for a short moment as a plasmid in the sequence (see ◘ Fig. 6.67), the difficulty in detecting a suspected plasmid is also explained.

Thus, one way of generating hybrid strains is to transfer the chlorocatechol degradation genes into strains that cometabolize chloroaromatics to the chlorocatechol stage. Another involves transferring the genes encoding the peripheral degradation sequences into the chlorocatechol-utilizing strains such as strain B13.

There are examples where a peripheral enzyme sequence is not able to convert a chloroaromatic to the chlorocatechol stage. Then not only is the crossing of two strains necessary, but the "new" degradation pathway must be accumulated from partial segments from three strains in one strain. This is shown schematically in ◘ Fig. 6.68 for various chlorobiphenyls. It is important to note that not only must the degradation sequences be unified, but the regulatory genes must also be transferred alongside the structural genes, and the regulatory elements must harmonize with the structurally analogous chlorinated aromatic compounds.

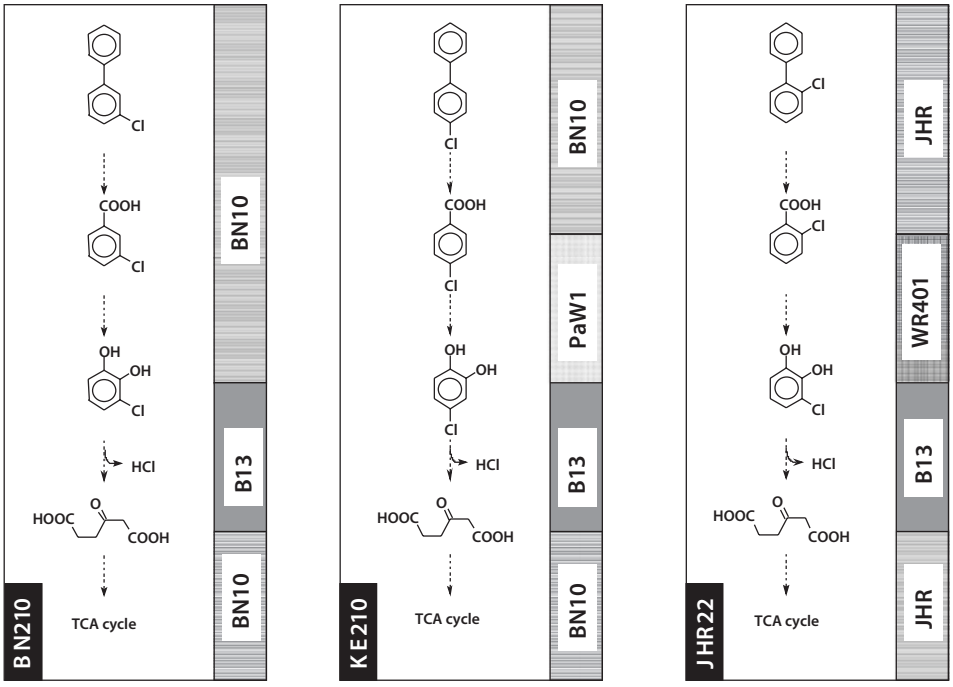
It has also been found in isolates from nature that gene transfer is responsible for

the evolution of degradation pathways. The example of the degradation of chlorobenzenes by *Pseudomonas* sp. P51 illustrates this particularly well. The organism possesses the identical chlorobenzene catechol degradation pathway on plasmid pP51 with the same gene cluster as strain B13. However, the degradation pathway is initiated by an operon that normally encodes toluene degradation (see ◘ Fig. 6.69).

However, in strain P51, the genes *tcbAaAbAcAdB,* which encode toluene dioxygenase and dihydrodiol dehydrogenase, are now also located on plasmid pP51. In contrast, in toluene utilizers such as *P. putida* F1, this sequence is located in the chromosome. However, unlike in the original, the sequence of the part of the toluene degradation pathway leading into the *meta*-pathway is no longer present in strain P51. In strain P51, the operon *tcbAaAbAcAdB* encoding the peripheral degradation sequence is located at a different position on plasmid pP51 than the *tcbCDEF* encoding the central degradation (◘ Fig. 6.70).

Using the example of *Pseudomonas* sp. strain PS12, the above observation becomes even clearer. The upper operon still shows the "remains" of the *meta*-pathway gene cluster with a mutated, inactive *tecE* gene.

Degradation pathways are a **mosaic of different sources** in hybrid strains from the laboratory as well as in isolates from nature. Indications of different sources for degradation subsequences are easily obtained from the different GC contents of the fragments.



◘ **Fig. 6.63** Mosaic structure of pathways for the degradation of chlorobiphenyls by laboratory-generated "new" strains

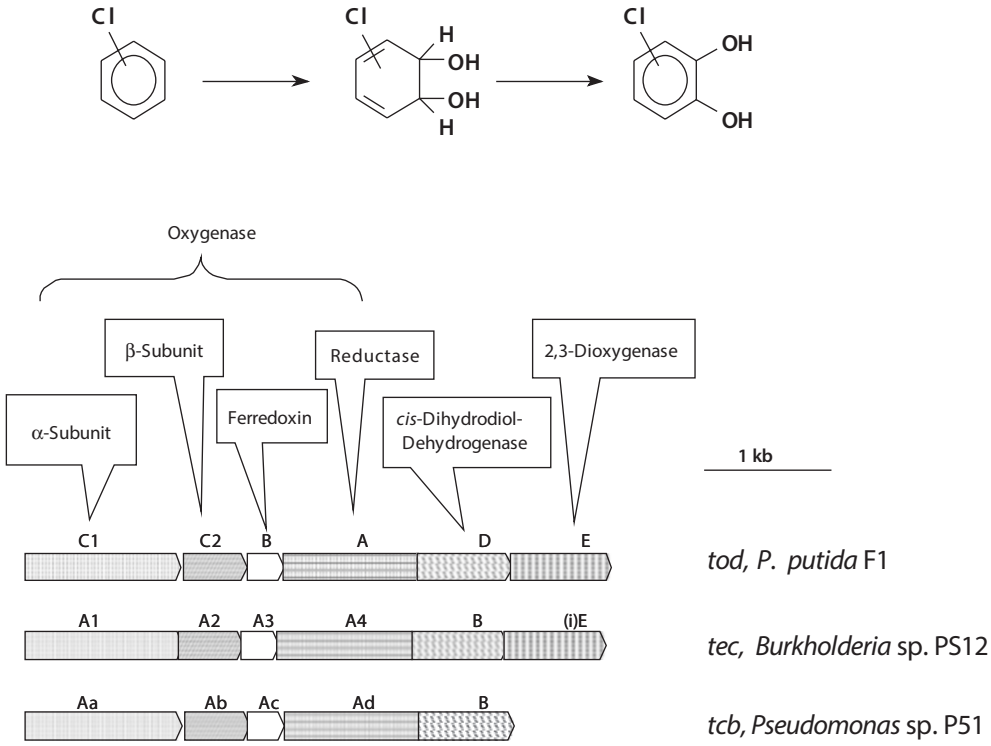


Fig. 6.64 Upper pathway gene cluster encoding (chloro)benzene dioxygenase, (chloro)benzenedihydrodiol dehydrogenase and catechol-2,3-dioxygenase with function in toluene and chlorobenzene degradation, respectively

6.2.4 Degradation of Chloroaliphatic Compounds

6.2.4.1 Environmental Problem Using the Example of Volatile Halogenated Organic Compounds

The volatile halogenated organic compounds (abbreviated VOX**) are** among the problematic chemicals with risks for the environment. They include chloroethenes, chloroethanes and chloromethanes. Due to their physicochemical properties (VOX have excellent oil- and grease-dissolving properties and, with the exception of the incombustible tetrachloroethene and 1,1,2-trichloroethane, are only hardly combustible), the areas of application of VOX are extremely diverse (Table 6.6).

The following areas can be regarded as the main areas of application for the most common **VOX** outside the chemical industry: Metal degreasing, dry cleaning or—in small quantities—as solvents in adhesives and paints. They are also important synthetic chemicals.

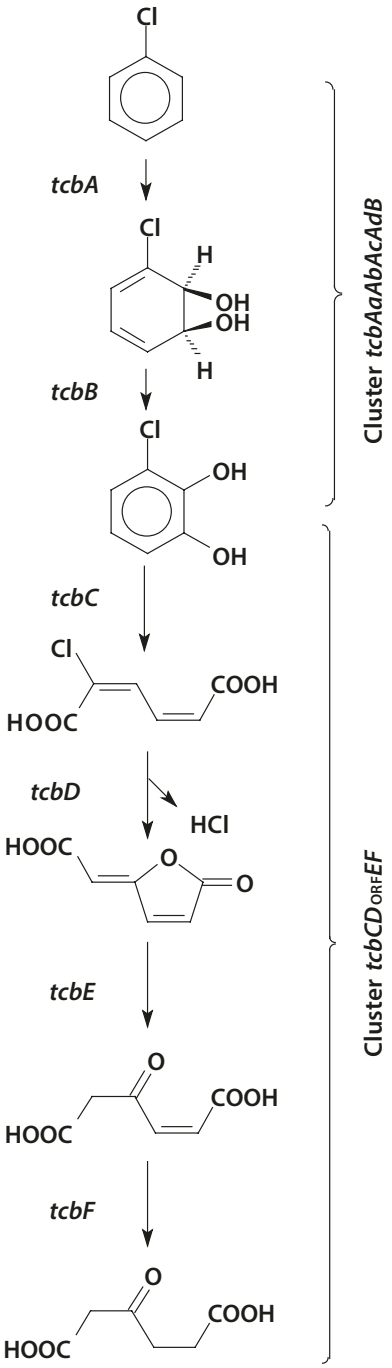


Fig. 6.65 Upper pathway gene cluster encoding (chloro)benzene dioxygenase, (chloro)benzenedihydrodiol dehydrogenase and catechol-2,3-dioxygenase with function in toluene and chlorobenzene degradation, respectively

Due to the high application quantities and the fields of application, PER and TRI are widely used in the environment. The behaviour in and between environmental compartments is determined by the rather good water solubility and high volatility. The compounds are characterized by relatively high mobility in water and atmosphere. Although simple distribution models predict that they should reside predominantly in the atmosphere, they are shown to pose a hazard to groundwater.

They are very mobile in the subsoil. Their high density means that they are rapidly transported into deeper soil layers. Sorption to soils occurs depending on the organic carbon content, but overall substance retention in the soil is low.

The kinematic viscosity of **VOX**, which is significantly lower than that of water, together with their relatively high density, means that they penetrate and permeate other materials—including fine-pored ones such as water-impermeable concrete. As a result, they can easily and rapidly enter the grown soil.

The ecological risks of this group of substances thus include:

- Groundwater contamination from industrial contaminated sites (chemical and metal processing industries, dry cleaners, rendering plants) and resulting drinking water contamination,
- the high aquatic toxicity and the slow degradation rates.

6.2.4.2 Possibilities of Microbial Degradation of Chloroaliphatic Compounds

Five different types of interaction between microorganisms and chloraliphatic compounds have been observed:

1. The substance serves as the sole **carbon and energy source** for the growth of aerobic bacterial pure cultures (Table 6.7).

Table 6.5 Degradative plasmids relevant for the construction of chloroaromatic degradative hybrid strains. Peripheral sequences encode degradation enzymes for the conversion of chloroaromatics up to the stage of chlorocatechols, central sequences those for the turnover up to the citrate cycle

Plasmid	Size (kb)	Conjugative	Incompatibility group	Substrates	Landlord
Peripheral sequence					
TOL, pWWO	117	+	P-9	Xylenes, toluene, toluate	*Pseudomonas putida*
NAH7	83	+	P-9	Naphthalene via salicylate	*Pseudomonas putida*
SAL1	85	+	P-9	Salicylate	*Pseudomonas putida*
pKF1	82	–	NU	Biphenyl over benzoate	*Rhodococcus globerulus*
pCITI	100	NU	NU	Aniline	*Pseudomonas* sp.
pEB	253	NU	NU	Ethylbenzene	*Pseudomonas fluorescens*
pRE4	105	NU	NU	Isopropylbenzene	*Pseudomonas putida*
pWW174	200	+	NU	Benzene	*Acinetobacter calcoaceticus*
pVI150	mega	+	P-2	Phenol, cresols, 3,4-dimethylphenol	*Pseudomonas* sp.
Central sequence					
pAC25	117	+	P-9	3-Chlorobenzoate	*Pseudomonas putida*
pJP4	77	+	P-1	3-Chlorobenzoate, 2,4-D	*Cupric Necator*
pBR60	85	+	NU	3-Chlorobenzoate	*Alcaligenes* sp.
pP51	100	–	NU	1,2,4-Trichlorobenzene	*Pseudomonas* sp.

NU Not studied
2,4-D 2,4-Dichlorophenoxyacetate
Incompatibility: two plasmids of the same group are not stable in one cell, one plasmid is lost or a cointegrate plasmid is formed

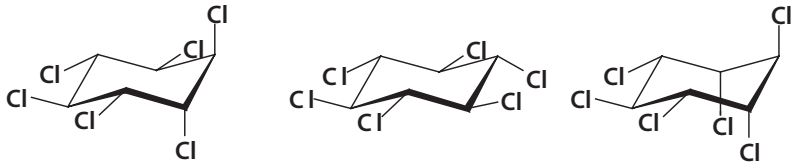


Fig. 6.66 Main stereoisomers of HCHs in lindane. (Conformation: a = axial, e = equatorial)

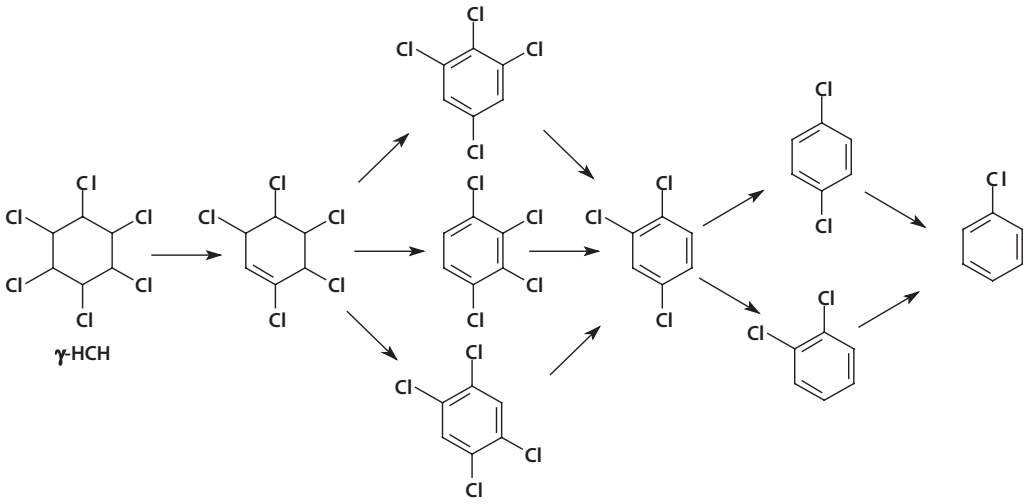


Fig. 6.67 Anaerobic degradation of γ-HCH

2. The substance serves as a **growth substrate** for organisms that use an electron acceptor other than oxygen **(nitrate respiration).**
3. The substance serves as a growth substrate in an **acetogenesis.**
4. The substance serves only as a substrate for enzymes in aerobic and anaerobic bacteria, whereby the microorganisms grow on another compound, **cometabolism** (Tables 6.8 and 6.9).
5. The substance is used as an **electron acceptor** under anaerobic conditions (Table 6.10).

Thus, the chloroaliphatics may be used by microorganisms or only transformation may occur randomly without benefit, and dechlorination may occur in either case.

Most VOX can be degraded both oxically and, in the absence of oxygen, anoxically. The site conditions then determine

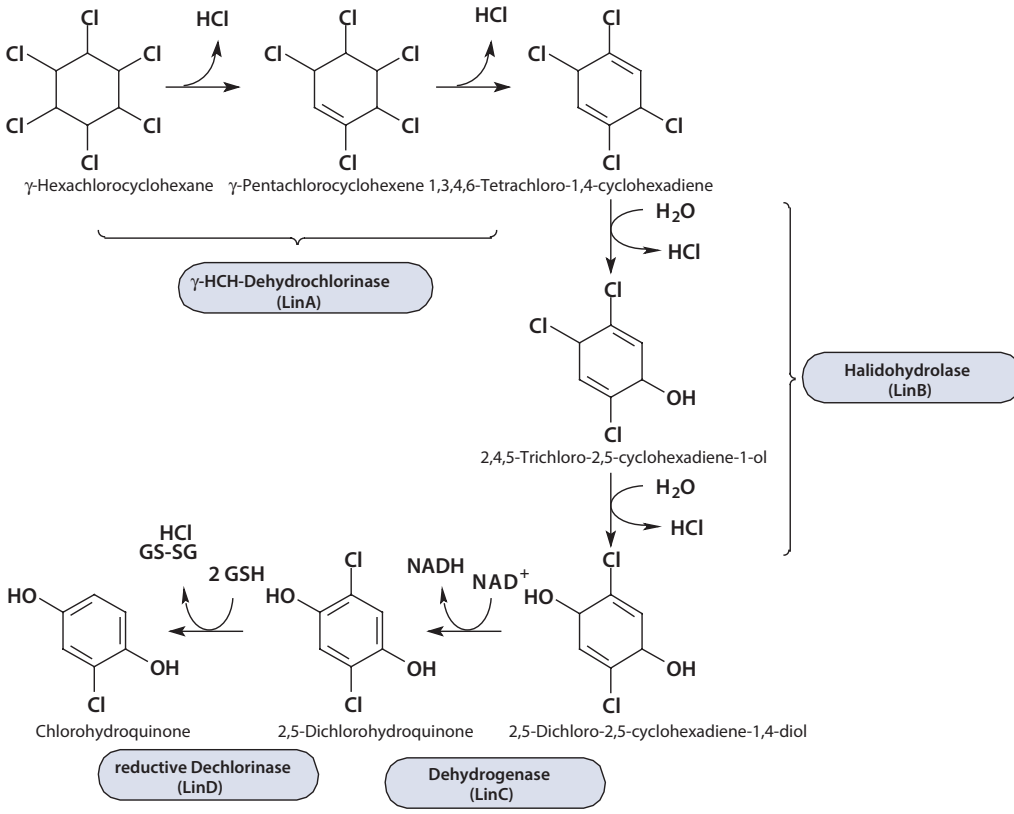


Fig. 6.68 Degradation pathway for γ-HCH by *Sphingomonas paucimobilis*

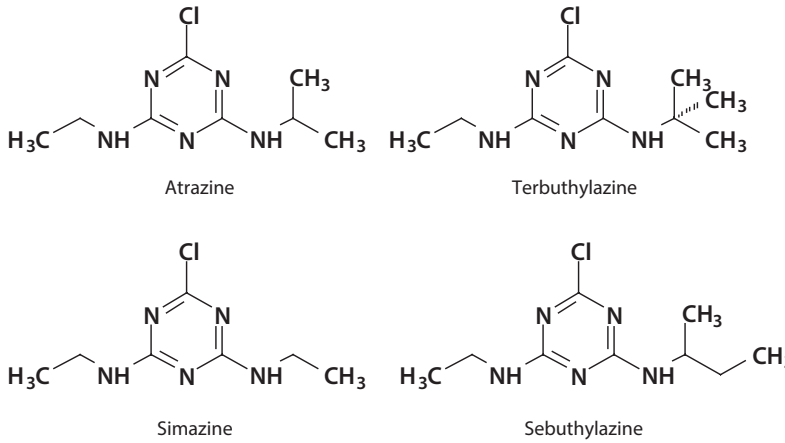


Fig. 6.69 Important triazines

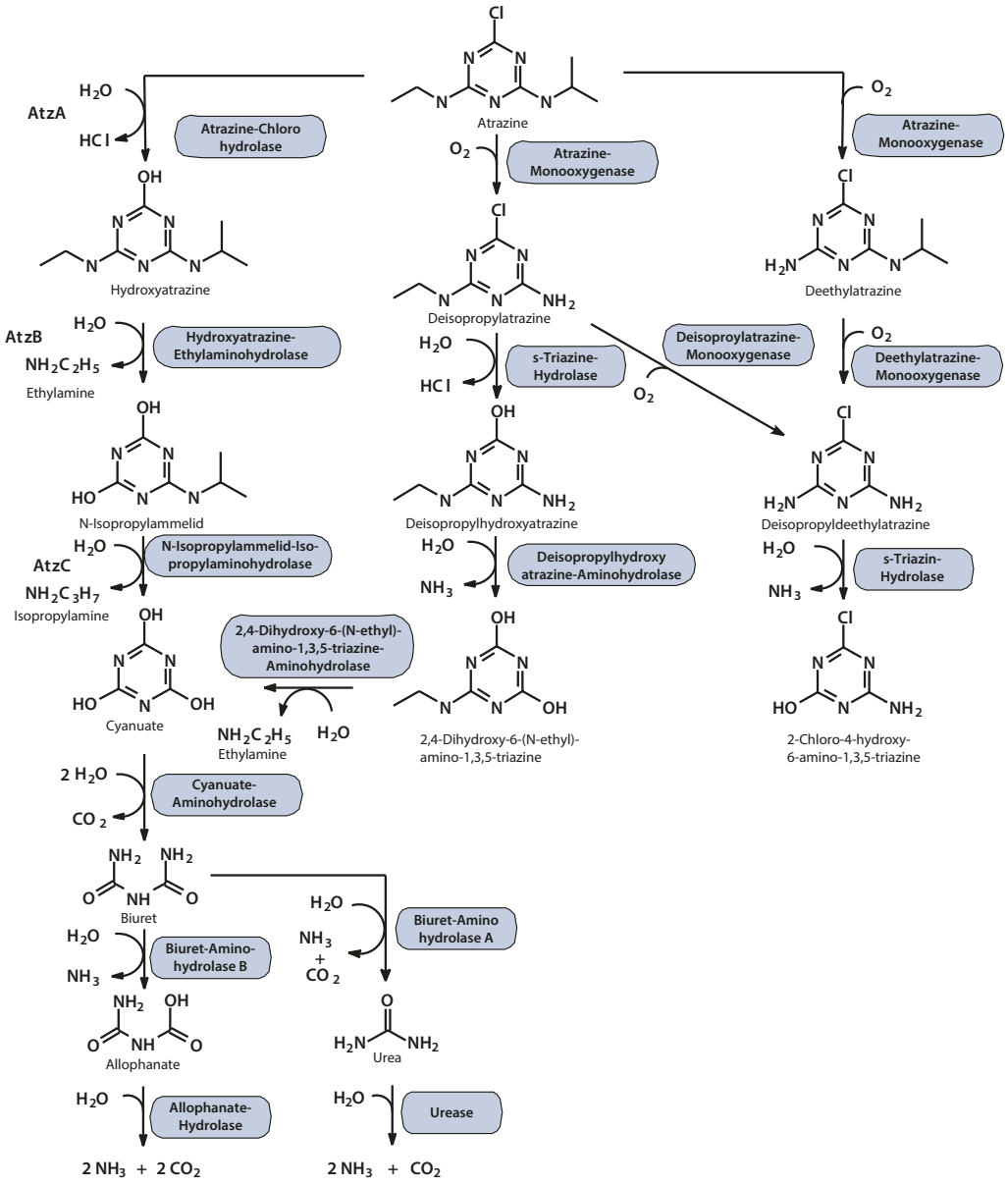


Fig. 6.70 Degradation of atrazine

Table 6.6 VOXin use with their substance properties and production quantities

Material property	Trichloroethene (Trichloroethylene, Tri, TRI)	Tetrachloroethene (perchloroethylene, TCE, Per, PER, PCE, tetrachloroethylene)	Dichloromethane (methylene chloride)	Trichloromethane (chloroform)	1,1,1-Trichloroethane (methyl chloroform)
Vapour pressure at 20 °C (mmHg)	60	14	349	155	100
Water solubilityat 20 °C (g/L)	1.1	0.149	13.2	8.22	4.4
K_H (atm m^3/mol)	0.0103	0.0096	0.003	0.0042	0.0049
Density at 20 °C	1.46	1.626	1.3255	1.489	1.35
logP_{ow}	2.29	2.53	1.25	1.97	2.17
Production (1000 t/a)	74 EU (2000) 93 US (2001)	70 EU (2000) 152 US (2001)	147 EU (2000) 90.7 US (2003)	297 US (2003)	**Forbidden**[a]

Common abbreviations and other designations are given in brackets
US data: ► http://www.the-innovation-group.com/chemprofile.htm and
EU data: ► http://ecb.jrc.it/existing-chemicals
[a]According to EU regulation for ozone depleting substances

Table 6.7 Halogenated aliphatics as growth substrates for aerobic bacterial pure cultures

Substrates		Other common names	Formulas
Haloalkane	Chloromethane	Methyl chloride	CH_3Cl
	Dichloromethane	Methylene dichloride	CH_2Cl_2
	Chlorethane	Ethyl chloride	CH_3CH_2Cl
	1,2-Dichloroethane	Ethylene dichloride	CH_2ClCH_2Cl
	1-Chloropropane		$CH_3CH_2CH_2Cl$
	1-Bromoctane		$CH_3(CH_2)_6CH_2Br$
	1-Chloroctane		$CH_3(CH_2)_6CH_2Cl$
	1,3-Dichloropropane		$CH_2ClCH_2CH_2Cl$
	1,9-Dichloronane		$CH_2Cl(CH_2)_7CH_2Cl$
Haloalkenes	Chlorethene	Vinyl chloride	$CH_2{=}CHCl$
	1,3-Dichloropropene	Allyl dichloride	$CH_2ClCH{=}CHCl$
Haloalkanols	2-Chloroethanol		CH_2CH_2OH
	2,3-Dichloro-1-propanol		$CH_2ClCHClCH_2OH$
	1,3-Dichloro-2-propanol		$CH_2ClCHOHCH_2Cl$
Haloalkenols	2-Chlorallyl alcohol		$CH_2{=}CClCH_2OH$
	3-Chlorallyl alcohol		$CHCl{=}CHCH_2OH$
Haloalkanoates	Chloroacetate		$CH_2ClCOOH$
	Dichloroacetate		$CHCl_2COOH$
	Trichloroacetate		CCl_3COOH
	2,2-Dichloropropionate		CH_3CCl_2COOH
Haloalkenoate	3-Chloroacrylate		$CHCl{=}CHCOOH$
	3-Chlorocrotonate		$CH_3CCl{=}CHCOOH$

which of the possible degradation pathways is followed. PER is an exception: to date, it can only be degraded anaerobically.

6.2.4.2.1 Aerobic Growth with Chloroaliphates

Table 6.7 summarises the chloraliphatics that can be used by aerobic bacteria as a carbon and energy source. TRI has **not** been found to be **readily degradable in** the closed-bottle test (OECD 301D). No pure cultures are found that grow with this substance either.

Several types of enzymes are able to function as dehalogenases: Hydrolases, lyases, hydratases, glutathione transferases, monooxygenases and methyltransferases. This will be shown by the example of dichloroethane, 1,3-dichloropropene, dichloromethane, chloromethane and chlorocrotonate.

Table 6.8 Examples of halogenated aliphatic compounds as substrates for cometabolic processes: *aerobic transformation*

Transformation Substrate	Growth substrate	Organisms: Enzyme
Trichloroethene	Methane	*Methylosinus trichosporium, Methylocystis* ssp., *Methylomonas methanica, Methylococcus capsulatus*
	Propane	*Mycobacterium vaccae*, *Rhodococcus* ssp.
	Phenol	*Burkholderia cepacia*, *Ralstonia eutropha*
	Toluene	*Pseudomonas putida*: toluene-2,3-dioxygenase *Burkholderia cepacia*: toluene-2-monooxygenase *Burkholderia pickettii:* toluene-3-monooxygenase *Pseudomonas mendocina:* toluene-4-monooxygenase
	Cumol	*Rhodococcus erythropolis*
	Propene	*Xanthobacter* sp.
	Isoprene	*Alcaligenes denitrificans* spp. *xylosoxidans*
	Ammonia	*Nitrosomonas europaea*
Chloroform	Toluene	*Pseudomonas* sp.: toluene-2-monooxygenase *Pseudomonas mendocina:* toluene-4-monooxygenase
Vinyl chloride	Propane	Actinomycetales, *Rhodococcus* ssp.

Table 6.9 Examples of halogenated aliphatic compounds as substrates for cometabolic processes: *anaerobic transformation by pure cultures*

Transformation substrate	Proven products	Organisms
Carbon tetrachloride	Trichloromethane, Dichloromethane, Chloromethane, CO_2	*Methanobacterium thermoautotrophicum, Methanosarcina barkeri, Desulfobacterium autotrophicum, Acetobacterium woodii, Clostridium thermoaceticum, Shewanella putrefaciens*
Trichloromethane	Dichloromethane, Chloromethane	*Methanosarcina* spp.
1,2-Dichloroethane	Chloroethane, Ethene	Methanogenic
1,1,1-Trichloroethane	1,1-Dichloroethane	*Methanobacterium thermoautotrophicum, Desulfobacterium autotrophicum, Acetobacterium woodii, Clostridium* sp.
Bromethane	Ethan	Methanogenic
1,2-Dibromoethane	Ethene	Methanogenic
Tetrachloroethene	Trichloroethene	Methanogens, *Desulfomonile tiedjei, Methanosarcina* sp., *Acetobacterium woodii*
1,2-Dibromoethylene	Acetylene	Methanogenic

Table 6.10 Bacteria that use tetrachloroethene as an electron acceptor and the products formed in the process

Tribe	Electronics Donor	Electron acceptor	Dechlorination product from PER
Sulfospirillum (Dehalospirillum) multivorans	H_2, formate, lactate, pyruvate, ethanol, glycerol	PER, TRI	*c*DCE[a]
Dehalobacter restrictus PER-K23[b]	H_2	PER, TRI	*c*DCE
Desulfitobacterium sp. PCE-1	Formate, Lactate, Pyruvate, Ethanol, Butyrate, Succinate	PER	TRI
Desulfitobacterium frappieri PCE-S	H_2, acetate, pyruvate	PER, TRI	*c*DCE

(continued)

Table 6.10 (continued)

Tribe	Electronics Donor	Electron acceptor	Dechlorination product from PER
Dehalococcoides ethenogenes 195	H_2	PER, TRI	Ethene
Desulfuromonas chloroethenica TT4B	Acetate, pyruvate	PER, TRI	*c*DCE
Desulfitobacterium frappieri TCE1	H_2, formate, lactate, ethanol, butyrate, crotonate	PER, TRI	*c*DCE

[a]*cis*-Dichloroethene
[b]A peculiarity of *Dehalobacter restrictus* is that so far only PER can be used as electron acceptor. Humic substances could act as a "natural" acceptor

Dichloroethane

Two hydrolytic dehalogenases are involved in the degradation of 1,2-dichloroethane in *Ancylobacter aquaticus* and *Xanthobacter autotrophicus.* The compound is degraded to glycolate via 2-chloroethanol, chloroacetaldehyde and chloroacetate in four sequential steps before reaching the central pathways (Fig. 6.71). The dehalogenase (DhlA) that converts 1,2-dichloroethane belongs to the so-called *α*/*β*-hydrolase group. A specific aldehyde dehydrogenase is then necessary for the conversion of the toxic aldehyde. The further conversion is carried out by chloroacetate dehalogenase (DhlB), an L-specific halocarboxylic acid dehalogenase.

In *Pseudomonas* sp. DCA1, a monooxygenase reaction initiates the degradation of 1,2-dichloroethane. The organism shows very high affinity for the substrate. Oxidation leads to the formation of the unstable intermediate, 1,2-dichloroethanol, which spontaneously releases chloride, thus forming chloroacetaldehyde Further degradation proceeds via the pathway described for *Ancylobacter aquaticus* and *Xanthobacter autotrophicus*.

1,3-Dichloropropene

A haloalkane dehalogenase that hydrolyzes 1,3-dichloropropene to 3-chloroallyl alcohol has been characterized in *Pseudomonas cichorii.* The overall degradation pathway with the second dechlorination step is shown in Fig. 6.72.

Dichloromethane

In the degradation of halogenated compounds, glutathione transferases carry out the nucleophilic removal of a halogen substituent and further the spontaneous decay of the resulting glutathione adduct (Fig. 6.73). Initial evidence for the involvement of glutathione transferases in bacterial dehalogenation was obtained with the facultative and obligate methylotrophs of the genera *Hyphomicrobium* and *Methylobacterium,* which use dichloromethane as a carbon source for growth.

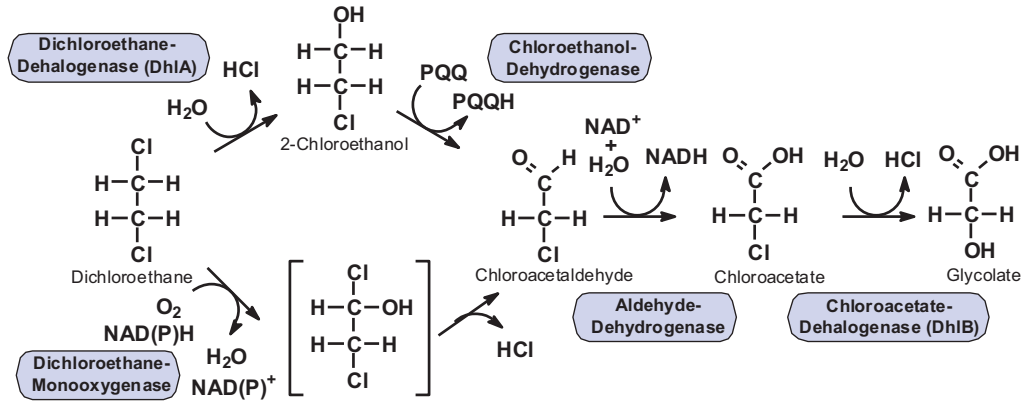


Fig. 6.71 Degradation pathway for 1,2-dichloroethane in *Ancylobacter aquaticus* and *Xanthobacter autotrophicus* (top) and *Pseudomonas* sp. strain DCA1 (bottom)

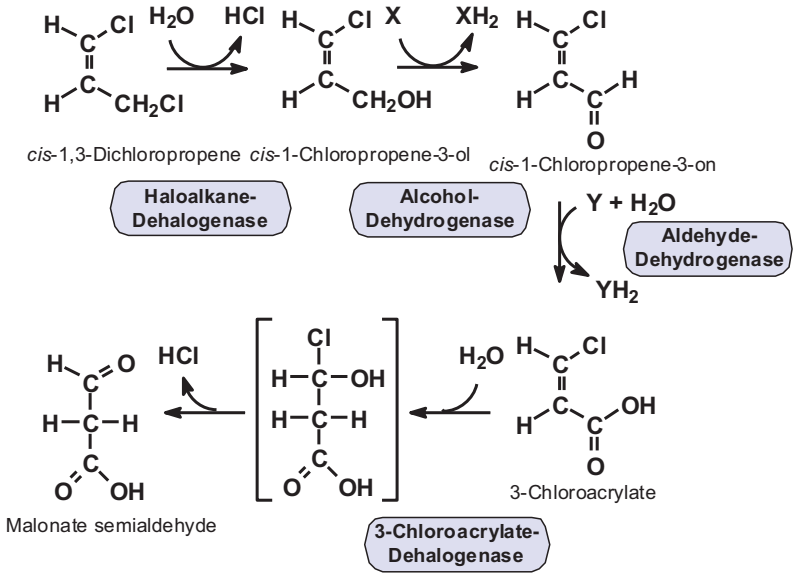


Fig. 6.72 *cis*-1,3-Dichloropropene degradation. The pathway also works for the *trans* isomer (X and Y = unknown)

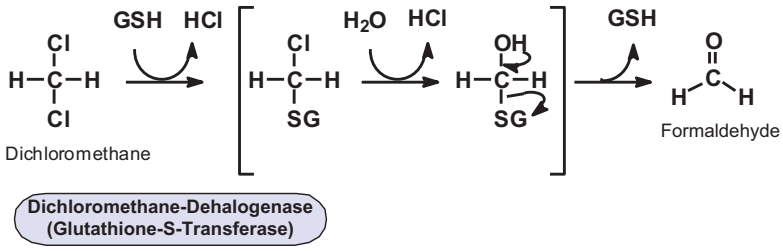


Fig. 6.73 Dichloromethane degradation

Dichloromethane dehalogenases are atypical enzymes among the glutathione S-transferases. In contrast to most enzymes of the glutathione S-transferase superfamily, their substrate spectrum is very narrow and limited to dihalomethanes. Halomethanes and dihaloethanes are not converted.

▪ Chloromethane

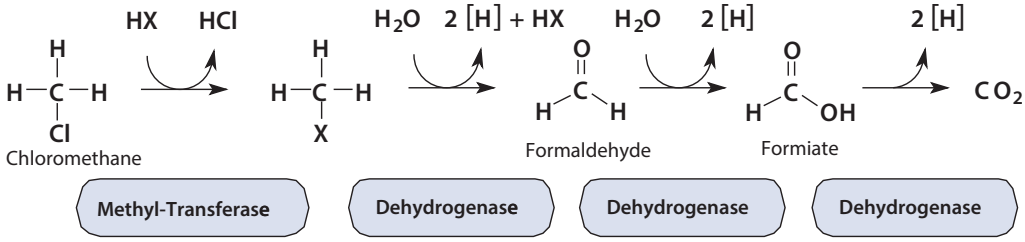
Aerobic methylotrophic bacteria of the genera *Hyphomicrobium* and *Methylobacterium* have been characterized that can utilize chloromethane as a growth substrate. Physiological and genetic studies showed that *Methylobacterium* sp. metabolizes chloromethane by initiating dehalogenation via a methyl transfer reaction. A series of dehydrogenase-dependent steps follows, which are different from the subsequent steps of methanol metabolism in the same organism. Dichloromethane and trichloromethane are not growth substrates nor are they dechlorinated by cells of *Methylobacterium* sp. illustrating the high specificity of the dechlorination step (◘ Fig. 6.74).

▪ Chlorocrotonate and Chlorobutyrate

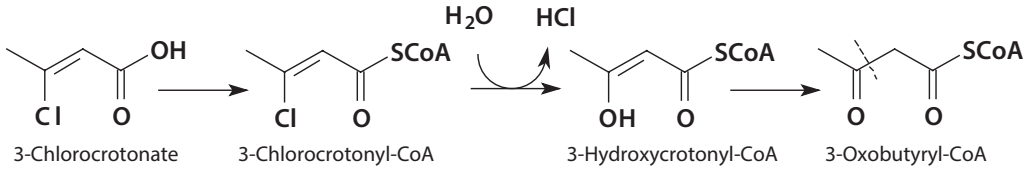
There is evidence that dehalogenation of chlorinated carboxylic acids can occur after the formation of the CoA derivatives (◘ Fig. 6.75). It has been shown that dechlorination of *trans*-3-chlorocrotonate and 3-chlorobutyrate, which are used as carbon and energy sources by *Alcaligenes* sp., occurs after activation of the acid to the respective coenzyme A derivative. The dechlorination reaction is not understood. The same mechanism could be used in organisms growing with chloroallyl alcohols.

6.2.4.2.2 Cometabolic Degradation

No organisms have yet been isolated as pure cultures that can use TRI or chloroform as carbon and energy sources. Consequently, alternative conversions are being sought, for example co-oxidation reactions.



◘ **Fig. 6.74** Chloromethane degradation (X = unknown)



◘ **Fig. 6.75** Proposed degradation sequence for *trans*-3-chlorocrotonate

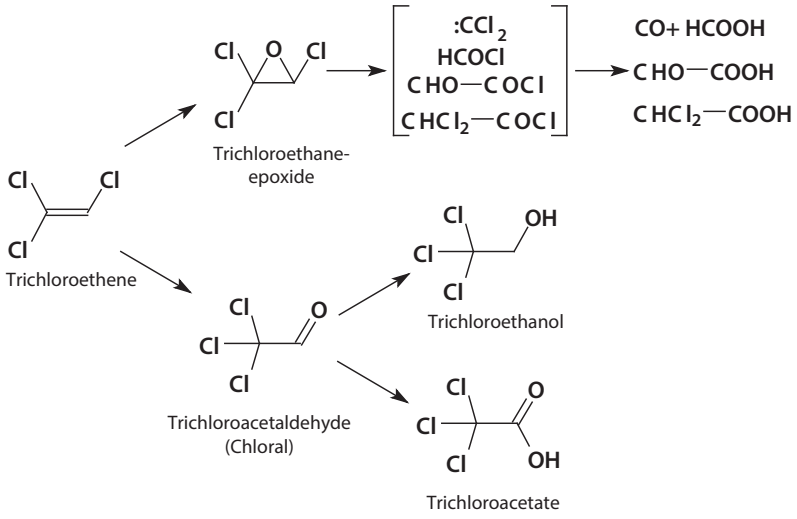
Aerobic Cometabolic Processes

Monooxygenases whose function is the oxidation of natural products such as methane, propane, alkylarenes (toluene, cumene) or alkenes (propene, isoprene) but also phenol or ammonia were found to be effective for the dechlorination of chloroethenes other than PER. Furthermore, dioxygenase acting in the degradation of toluene or cumene have been investigated for the ability of dechlorination of TRI. Overall, the majority of studies are concerned with the possibility of eliminating TRI.

The turnover of TRI with the methane monooxygenases, which were originally the most commonly studied, resulted in a variety of products and also partial dechlorination (◘ Fig. 6.76). However, rapid inactivation of the enzymes of the methylotrophs during the transformation of TRI was observed.

Isoprene-degrading bacterial strains have proven to be particularly effective. The 3,4-epoxy-3-methyl-1-butene (isoprene oxide) formed by monooxygenase in isoprene degradation is hydrolysed to the diol (◘ Fig. 6.77). This enzyme activity appears to allow the isoprene-degrading cells to tolerate high concentrations of TRI, unlike methylotrophic bacteria. It has been suggested that the methane monooxygenase of methylotrophic bacteria is rapidly inactivated by the epoxide formed during the cooxidation of TRI. However, other intermediates formed from the epoxide by nonenzymatic hydrolysis or isomerization such as glyoxyl-, formic- and dichloroacetic acid chloride or dichlorocarbene appear to be the actual agents of enzyme damage in the methylotrophic bacteria. In isoprene-degrading cells, after diol formation, subsequent reactions yield three equivalents of chloride without loss of activity. The advantage of isoprene over methane as a cosubstrate is seen in the fact that three equivalents of chloride are cleaved by epoxide hydrolase and inactivation is largely absent.

A stoichiometric release of chloride from TRI is also observed when the substance is reacted by bacteria isolated with cumene (isopropylbenzene) as energy substrate. Apparently, the direct formation of the



◘ **Fig. 6.76** Oxidation of trichloroethene by the monooxygenase of *Methylosinus trichosporium.* top: Main reaction, bottom: Secondary pathway; in parentheses: reactive products of isomerization and hydrolysis of the epoxide, respectively

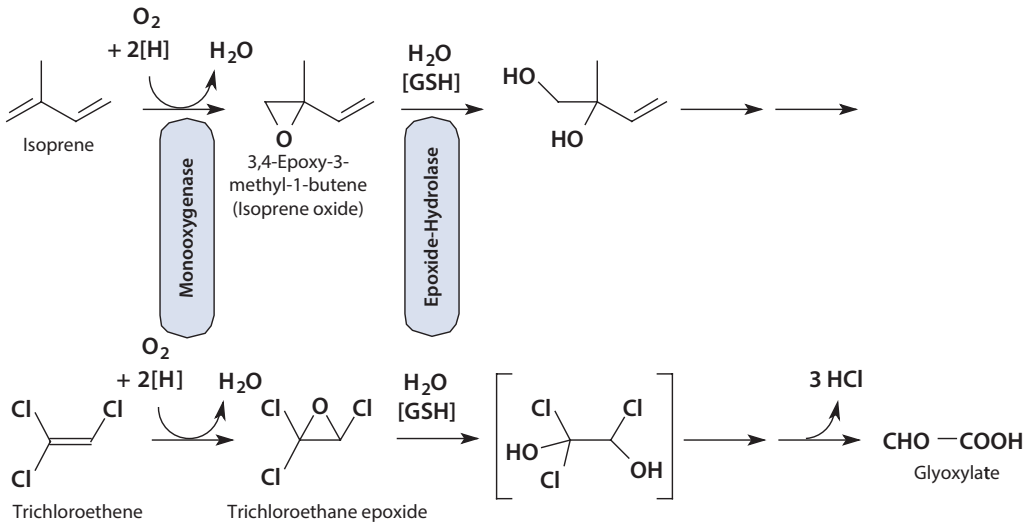


Fig. 6.77 Initial reactions of the degradation of isoprene (top) and the cometabolism of trichloroethene by *Rhodococcus erythropolis* (bottom)

unstable diol (1,2-dihydroxy-1,1,2-trichloroethane) is due to a non-specific isopropylbenzene 2,3-dioxygenase.

Less inactivation was also observed with toluene-2-monooxygenase in the turnover of TRI, products are CO, formate and glyoxylate.

It should be noted that the natural substrate must always be fed. Only if new enzyme is always formed, the transformation can take place. TRI is normally not an inducer for the cooxidizing enzymes, efforts are being made to eliminate this problem.

▪ Anaerobic Cometabolic Processes

Cometabolic conversions under anaerobic conditions were investigated for a variety of chlorine/bromine C_1 and C_2 compounds. Table 6.9 summarizes data with pure cultures.

Furthermore, dechlorinations of PER and TRI in particular were investigated in the environment. Successively, the chlorine substituents can be eliminated from the environmental samples by means of microbial populations. TRI is formed from PER. In the second degradation step, TRI is then converted to *cis*-dichloroethene .The other stereoisomer, *trans*-dichloroethene, is only formed to any significant extent in abiotic processes, i.e. processes that are not linked to the presence of organisms. Biodegradation of VOX, on the other hand, leads mainly to *cis*-dichloroethene , so its formation is an indication of the presence of microbial degradation activity for TRI and PER in an environmental medium.

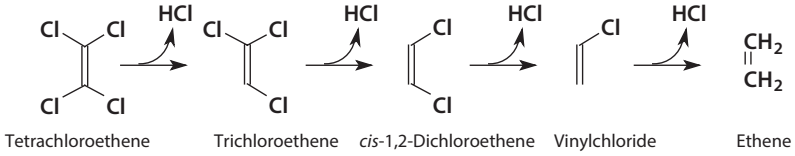
The third step of the dechlorination sequence converts *cis*-dichloroethene into vinyl chloride. This is a problematic metabolite, as it is carcinogenic. Biodegradation must therefore not be allowed to come to a standstill at this point. This is prevented by the final step of dechlorination, in which vinyl chloride is converted to a chlorine-free compound, namely ethene. In many cases, however, the degradation of TRI and PER stops at the *cis*-dichloroethene stage.

6.2.4.2.3 Chloroaliphates Beneficial to Anaerobic Microorganisms

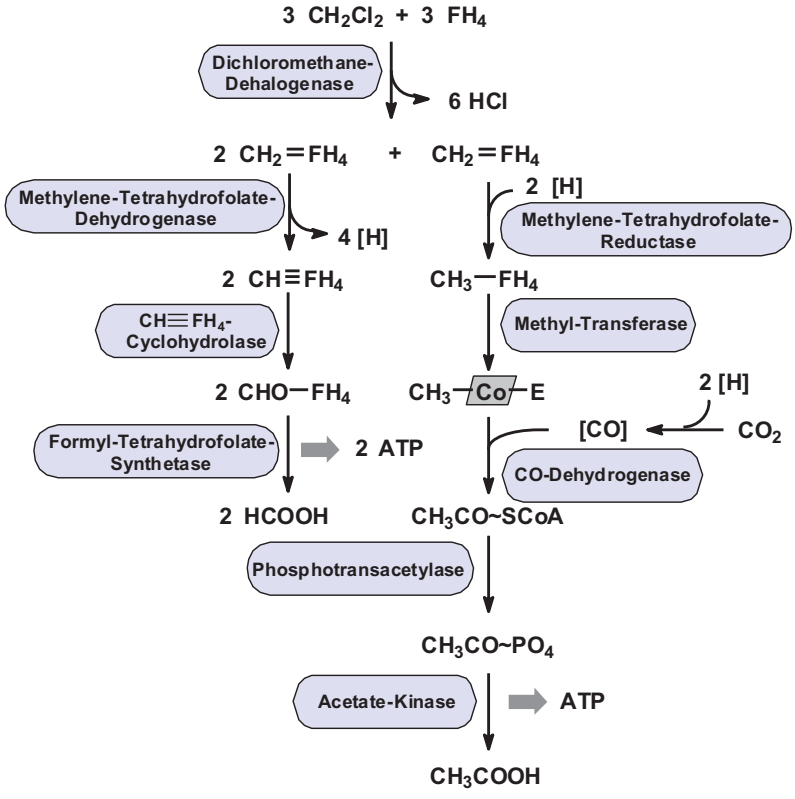
▪ Acetogenesis of Chloromethanes

Chloromethane and dichloromethane can serve as growth substrates for anaerobic

organisms such as *Dehalobacterium formicoaceticum* in an acetogenesis. The degradation sequence is shown for dichloromethane in ◘ Fig. 6.78. For every 3 moles of dichloromethane, 3 ATP and the products acetate and formate are formed in a molar ratio of 1:2. In the first reaction step, dichloromethane dehalogenase eliminates the two chlorine substituents, producing methylene tetrahydrofolate. Two-thirds is oxidized to formate, while 1-third of the methylene tetrahydrofolate is converted to acetate by incorporating CO_2 through the acetyl-CoA synthetase reaction. A balance in reduction equivalents results from oxidation in the acetyl-CoA pathway and consumption by methylene tetrahydrofolate reductase and CO dehydrogenase. 1 ATP is formed via substrate step phosphorylation by acetate kinase. Evidence for an ATP synthase also suggests a chemiosmotic mechanism for ATP formation (◘ Fig. 6.79).



◘ **Fig. 6.78** Successive dechlorination of tetrachloroethene



◘ **Fig. 6.79** Degradation sequence for dichloromethane by *Dehalobacterium formicoaceticum* (FH_4 = tetrahydrofolate)

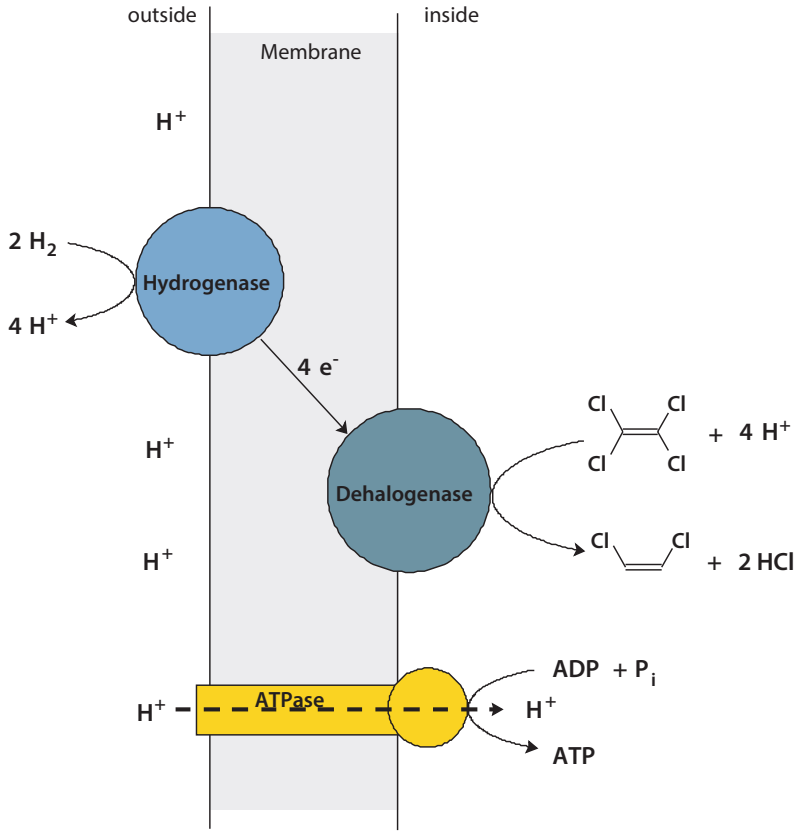


Fig. 6.80 Schematic of dehalorespiration of tetrachloroethene, scalar mechanism of proton gradient generation

Chloroethenes as Electron Acceptors

Polychlorinated ethenes such as PER and TRI can be used as electron acceptors under anaerobic conditions (Table 6.10). Reductive dechlorination takes place, usually terminating at the *cis*-dichloroethene stage. In *Dehalococcoides ethenogenes* 195, complete dechlorination and formation of ethene is observed.

Figure 6.80 shows a simplified scheme of the energy formation during the reductive dechlorination of tetrachloroethene to *c*DCE with hydrogen as electron donor. There is no H^+ transport across the membrane, but **consumption of H^+ inside** and **formation of H^+ outside**, leading to the formation of a proton gradient. This is then used by the ATP synthase for ATP generation.

6.2.5 Organohalogens from Nature/Natural Sources

Organohalogen compounds are prominent environmental pollutants from anthropogenic sources. For a long time it was assumed that there were hardly any natural sources of halogenated organic compounds. In recent years, efficient analytical methods have increasingly succeeded in detecting natural organohalogens.

The ubiquitous distribution of the four halides (Cl-, Br-, I-, F-) means that organohalogens are formed both abiotically and biogenically in all regions of our planet (oceans and terrestrial environment). Abiotic sources of organohalogens in the

environment include volcanic activities and biomass burning (Andreae & Merlet, 2001; Jordan et al., 2000; Rudolph et al., 1995).

More than 5000 naturally occurring compounds from the organohalogen group of substances have already been identified (Gribble, 1992; Gribble, 2015). Although most of the compounds discovered are chlorine compounds, bromides, iodides, and fluorides have also been found (◘ Fig. 6.81a). The tyrian purple, dibromindigo, is representative of the bromides. Thyroxine, secreted by the thyroid gland, is an iodide and the highly toxic fluoroacetate is one of the rare organofluorides. These three representatives, thyroxine from man, Tyrian purple from snails, and fluoroacetate from plants, also show that independent species use organohalogens for different purposes.

Evidence suggests that the majority of organohalogens arise from microbial activities. Many of these substances are of relevance to organisms as secondary metabolites. Halogenation is the strategy used to increase biological activity. Thus, halogenated natural products are not formed "by chance", but fulfil important tasks.

Several antibiotics isolated from fungi and bacteria are halogenated in nature, for example chloramphenicol, chlortetracycline, clindamycin and griseofulvin (◘ Fig. 6.81b). An impressive example of the effectiveness of chlorinated natural products is the antibiotic chloramphenicol, which is produced by *Streptomyces venezuelae*. It is a broad-spectrum antibiotic and has also been produced synthetically since the 1950s.

Of note is the finding that brominated and chlorinated dibenzodioxins and dibenzofurans are formed by natural systems such as algae, lichens, and sponges, and in peat and humic-rich soil layers (◘ Fig. 6.81c). Contrary to earlier claims, forest fires are not a major source of these compounds.

In addition to such complex biochemically active substances, various ecosystems such as hypersaline lakes (Ruecker et al., 2014; Weissflog et al., 2005), freshwater wetlands (Hardacre & Heal, 2013), marine ecosystems (Ballschmiter, 2003; Carpenter et al., 2003) and soils (Albers et al., 2011; Haselmann et al., 2000, 2002; Keppler et al., 2002, 2006) also released VOX: simple alkanes such as chloro-, dichloro-, trichloro- and tetrachloromethane, bromomethane, other simple bromoalkanes, mixed bromochloromethanes, iodomethanes, other simple iodoalkanes, simple organofluorines.

A major source is marine algae, which produce several chlorinated methane- and ethane-containing compounds. In addition, several million tons of methyl bromide are produced annually by marine organisms. According to initial mass balances, natural organohalogen compounds are not only ubiquitous in our environment, but for some compounds—for example chloromethane, trichloromethane or tetrachloromethane—natural formation exceeds anthropogenic synthesis by a factor of ten to one hundred (◘ Table 6.11).

Since halides are not particularly reactive, they must be activated for halogenation. So far, five different classes of enzymes have been found that carry out the halogenation reactions (◘ Table 6.12).

Furthermore, methyltransferases in plants, fungi and algae are involved in the formation of halomethanes (Wuosmaa and Hager, 1990).

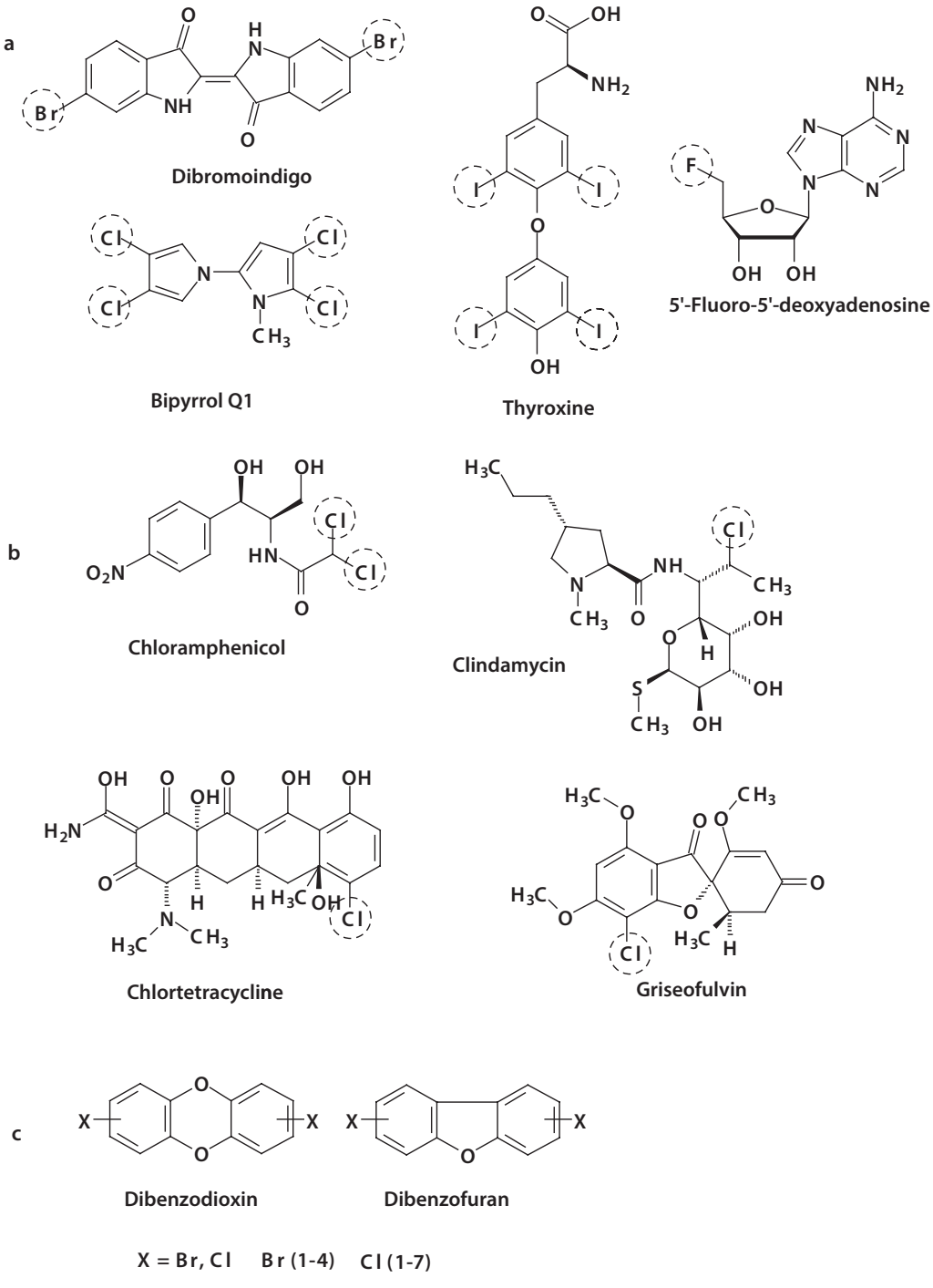


Fig. 6.81 Some examples of organohalogen compounds produced by natural systems. **a** complex compounds bearing chlorine, bromine, iodine or fluorine substituents, **b** compounds with antibiotic activity, **c** compounds with high environmental hazard potential: chlorinated and brominated dibenzodioxins or furans

Table 6.11 Sources and estimated quantities of dichloromethane and trichloromethane (Gg per year)

Sources	Dichloromethane	Trichloromethane (chloroform)	Literature
Oceans	190	360	Cox et al. (2003)
Soil/mushrooms		235	Cox et al. (2003), Trudinger et al. (2004)
Termites		100	Khalil et al. (1990)
Peat bogs		4.7	Dimmer et al. (2001)
Biomass combustion	60	2	Cox et al. (2003), Rudolph et al. (1995)
Rice farming		23	Laturnus et al. (2002)
Volcanoes	0.021 ± 0.013	0.095	Schwandner et al. (2004)
Microalgae		23	Laturnus et al. (2002)
Macroalgae (not seaweed)	0.32	10	Baker et al. (2001), Ekdahl et al. (1998)
Industry	650	66	Cox et al. (2003), Trudinger et al. (2004)

1 Gg (Gigagram) = 10^9 g ≈ 1000 t

Table 6.12 Five classes of halogenating enzymes

Halogenases	Halogenation mechanism	Proposed form of activated halogen	Needs for substrate	Necessary cofactor and cosubstrate	Literature
Heme iron-dependent haloperoxidases	Non-specific, electrophilic in plants, fungi, bacteria, mammals	X^+	Aromatic and rich in electrons	Haem, H_2O_2	Blasiak and Drennan (2003), Butler and Sandy (2009), van Pée and Unversucht (2003), Vaillancourt et al. (2006)
Vanadium-dependent haloperoxidases	Non-specific, electrophilic in fungi, bacteria, marine algae	X^+	Aromatic and rich in electrons	Vanadat, H_2O_2	
Flavin-dependent halogenases	Specific and regioselective *e.g.* antibiotics *P. fluorescens*	X^+	Aromatic and rich in electrons	$FADH_2$, O_2	van Pée and Patallo (2006)

(continued)

Table 6.12 (continued)

Halogenases	Halogenation mechanism	Proposed form of activated halogen	Needs for substrate	Necessary cofactor and cosubstrate	Literature
Non-haem iron-dependent halogenases	Radical	$X^{\bullet}$	Aliphatic, non-activated C-H centres	Fe(II), O_2, α-ketoglutarate	Vaillancourt et al. (2005)
Nucleophilic halogenases	*Streptomyces cattleya*	X^-	Electrophilic, good leaving group (S-adenosyl-L-methionine)		Dong et al. (2004)

6.3 Degradation and Humification of Nitroaromatics

6.3.1 Environmental Problem Caused by Nitroaromatics

Nitroaromatics are important building blocks for chemical syntheses of dyes, herbicides, pharmaceuticals, polyurethane foams, but also for widely used fragrances (musk xylene and musk ketone). Inputs to the environment via industrial wastewater are accordingly essentially limited to the water pathway. Di- and especially trinitro derivatives of benzene, toluene and phenol are important for the production of explosives, which is why contaminations of TNT (2,4,6-trinitrotoluene), trinitrobenzene, picric acid (2,4,6-trinitrophenol) are often found in the underground of former plants for the production and processing of explosives. The total production of TNT, the most important explosive of the 2nd World War, amounted to over 800'000t in the Third Empire. In the Third Empire, ammunition was produced and stored in twenty production facilities until the end of World War 2. The ammunition plants were spread all over the country. A study from 1996 lists 3240 suspected sites for abandoned munitions, of which at least 750 are potentially contaminated with explosives. It is estimated that the contaminated areas add up to over 10,000 km^2.

6.3.2 Possibility of Microbial Degradation of Nitroaromatics

Various initial reactions have been demonstrated for the elimination of nitro groups from nitroaromatics (Fig. 6.82):

1. Oxidative cleavage of nitrite.
2. Reduction of a nitro group and elimination of ammonium.
3. Reduction of the aromatic ring and elimination of nitrite.

The former degradation mechanism has been described under aerobic conditions for various mono- and dinitroaromatics (4-nitrophenol, nitrobenzene, 2,4-dinitrotoluene, 2,6-dinitrotoluene). The mentioned nitroaromatics are utilized as growth substrate. It proceeds in two steps: (1) A **monooxygenase**

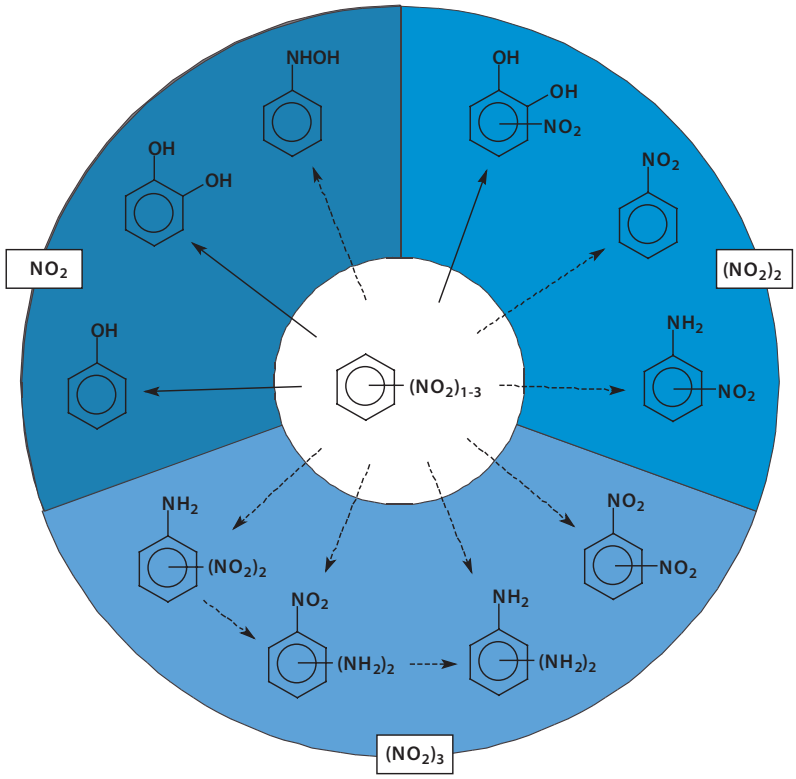


Fig. 6.82 Initial reactions in the microbial degradation of aromatic nitroaromatics: the three segments show the products formed in the degradation of mononitro- (dark grey/blue), dinitro- (medium grey) and trinitro-substituted compounds (light grey); full arrows stand for oxidative, interrupted arrows for reductive mechanisms

forms a labile *gem*-nitroalcohol from *p*-nitrophenol, from which nitrite is spontaneously eliminated (Fig. 6.83). The resulting quinone is converted by a reductase to the ring cleavage substrate hydroquinone. (2) A **dioxygenase** forms catechol by generating a labile dihydrodiol from, for example, nitrobenzene, which spontaneously rearomatizes, causing the elimination of nitrite. Further degradation occurs via known pathways.

The second alternative has been described for the aerobic degradation of 4-nitrotoluene and combines oxidative and reductive steps (Fig. 6.84): Three oxidative steps cause the conversion of 4-nitrotoluene to 4-nitrobenzoate. The subsequent reduction of the nitro group occurs via the intermediate nitroso- (R-NO) to the hydroxylamino compound (R-NHOH), from which ammonium is released by lyase. It is therefore a so-called partial reduction of the nitro group, since it ends at the stage of the hydroxylamino group and does not proceed to the amino group ($R\text{-}NH_2$).

Cometabolically, the initial reduction of a nitro group was observed, leading stepwise to the amino group via the nitroso and hydroxylamino intermediates, with frequent accumulation of products of partial degradation.

An unusual initial reaction of reduction of the aromatic ring has been described for polynitroaromatics such as TNT, 2,4-dinitro- and 2,4,6-trinitrophenol (picric acid). Enzymatically catalyzed hydrogenation of the ring leads to colored Hydride-

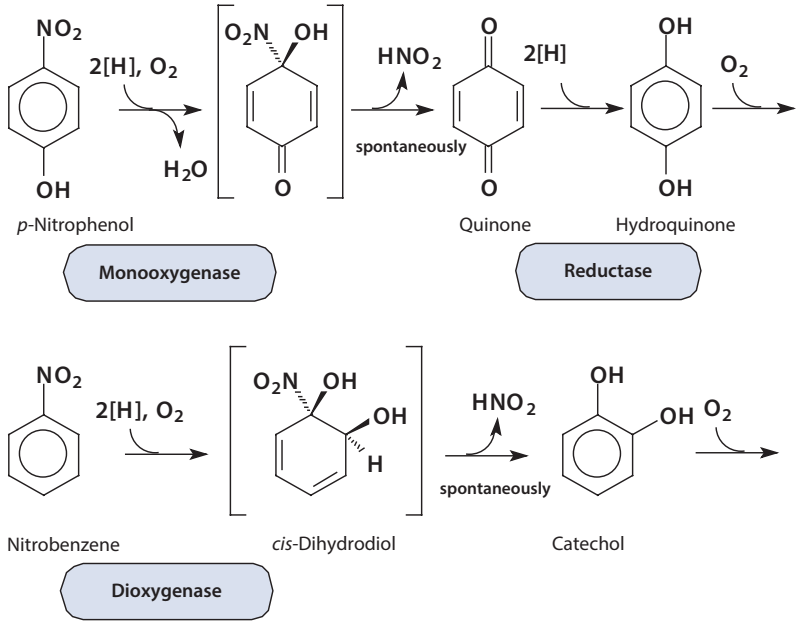


Fig. 6.83 Oxidative mechanisms in the degradation of *p*-nitrophenol and nitrobenzene

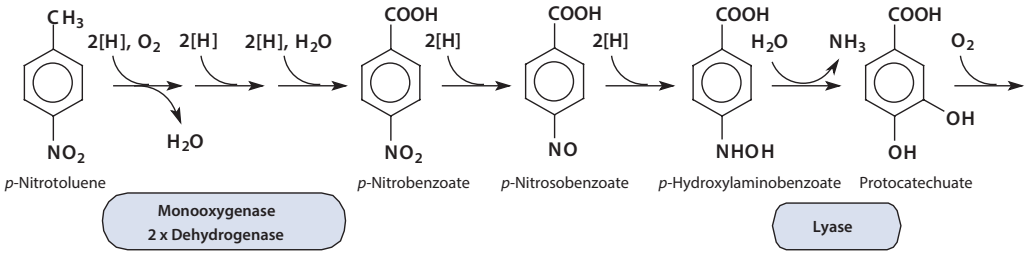


Fig. 6.84 Reaction sequence in the degradation of 4-nitrotoluene

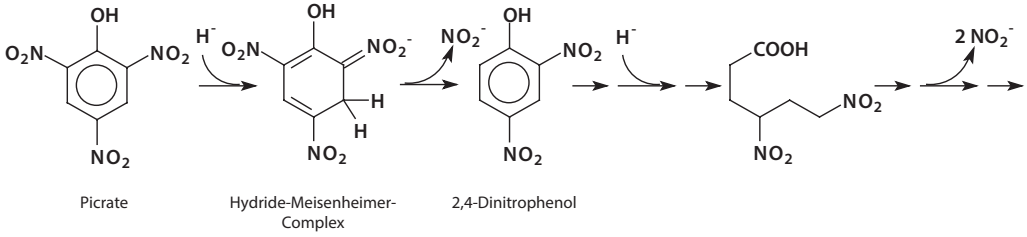


Fig. 6.85 Reaction sequence of the degradation of picric acid with formation of a Hydride-Meisenheimer complex

Meisenheimer complexes from which nitrite is eliminated (Fig. 6.85). Bacterial strains capable of denitrifying dinitrophenol and picric acid via Hydride-Meisenheimer complexes and using them as carbon, energy and nitrogen sources have been isolated. The

same strains are also able to reductively hydrogenate TNT, but nitrite is not split off.

Due to its electrophilic properties and chemical structure, TNT is difficult to mineralize biologically. Due to the triple substitution with nitro groups, TNT is highly oxidized and largely protected from oxidative attack by aerobic microorganisms. Therefore, only reductive initial reactions can be considered for microbial conversion. Obviously, the ability for anaerobic reduction of nitro groups to amino groups is ubiquitously distributed in anaerobic populations, as shown for TNT. In addition, reductive ring hydrogenation can also occur by aerobic bacteria. So far, only a partial reduction of nitro groups could be demonstrated by aerobic microorganisms, whereas under anaerobic conditions a complete reduction of nitro groups is possible. TNT is reduced to triaminotoluene (TAT) via aminodinitrotoluene (ADNT) (4-amino-2,6-dinitrotoluene or 2-amino-4,6-dinitrotoluene) and diaminonitrotoluene (DANT) (2,6-diamino-4-nitrotoluene or 2,4-diamino-6-nitrotoluene). Compared to the high conversion rate of TNT, the rates of conversion of aminodinitro- and diaminonitrotoluene decrease with the number of reduced nitro groups.

6.3.3 Elimination of Trinitrotoluene by Sequestration on Soil

Methods have been developed for the bioremediation of TNT-contaminated soils. They are based—since a complete degradation of the TNT in the soil does not take place—on a determination of the TNT metabolites in the form of non-extractable residues. Essentially, this involves the addition of readily degradable carbon sources aimed at cometabolic reduction of TNT and subsequent incorporation of these metabolites into the soil organic matrix. For TNT and its reduced metabolites, both sorption to clay minerals and sorption and binding to soil organic matter have been described (◘ Fig. 6.86).

A two-stage anaerobic/aerobic process in soil suspension (see ◘ Fig. 6.87) and various composting processes have been tested as methods for incorporating TNT into humic substances.

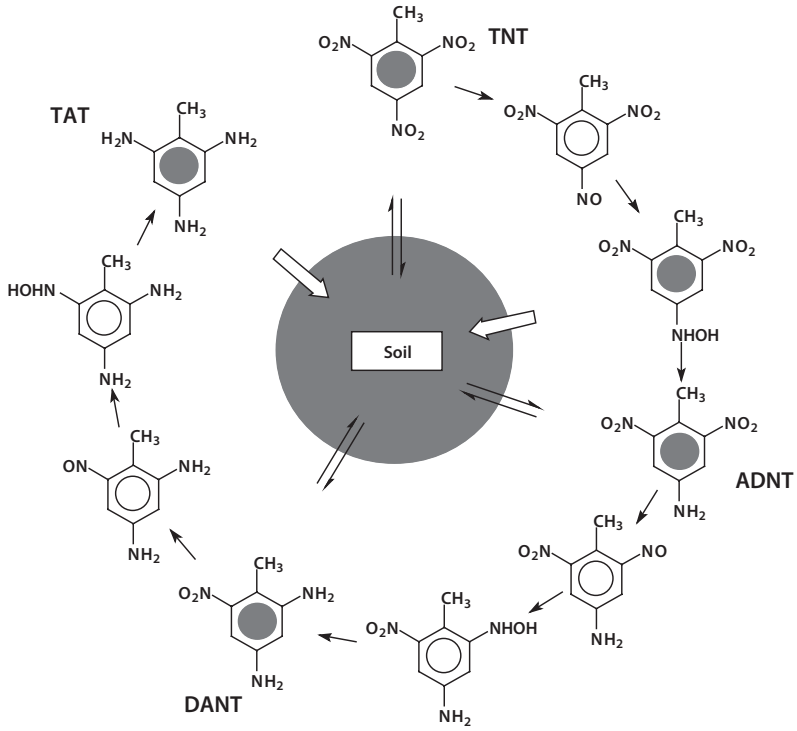


Fig. 6.86 Interaction of TNT and detected metabolites with soil. Irreversible sorptions are indicated by strong hollow arrows. Metabolites with a tendency to bind to soil components are indicated by colored rings

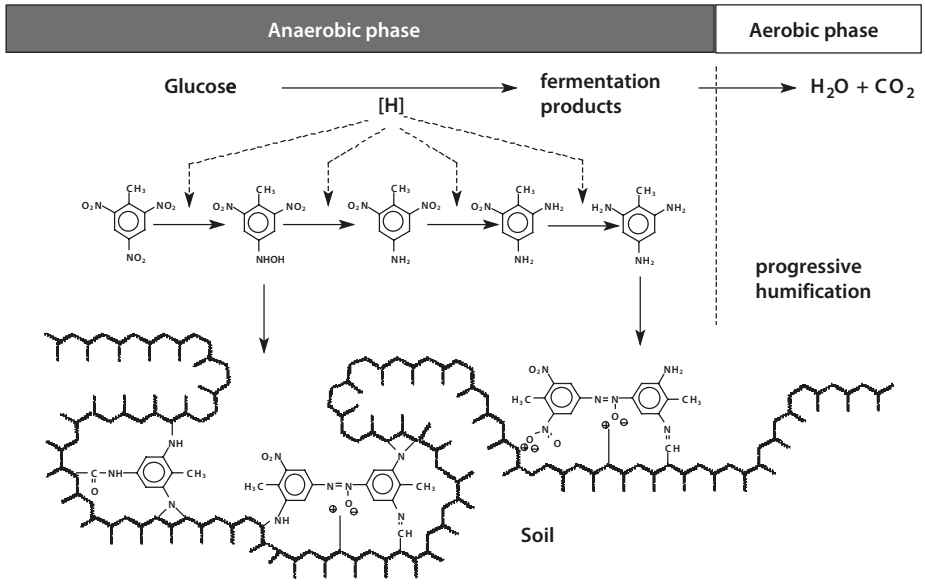


Fig. 6.87 Reduction and covalent incorporation of the transformation products of TNT into soil humic substances during anaerobic-aerobic treatment in soil suspension. (Adapted from Achtnich et al., 1999)

6.4 Degradation of Aromatic Sulfonic Acids and Azo Dyes

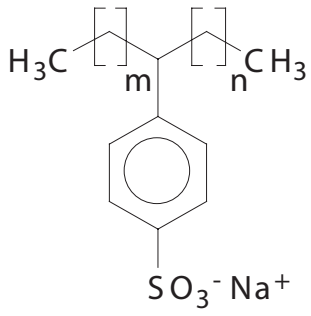
6.4.1 Aromatic Sulfonic Acids

6.4.1.1 Use and Environmental Relevance

Around 1.5 million tonnes of **linear alkyl benzene sulfonates (LAS)** are produced worldwide each year. They are used in powder and liquid detergents, rinsing agents and cleaning agents. Linear alkyl benzene sulfonates consist of aromatic sulfonic acids which are connected in the *para*-position with linear alkyl chains. Belonging to the group of anionic surfactants, LAS are a mixture of different isomers with chain lengths between 10 and 13 C atoms. Due to their polar (SO_3H group) as well as apolar structure (C chain), they are water-soluble on the one hand and also show an affinity for fats on the other, which gives them their surface-active properties (◘ Fig. 6.88).

LAS belong to the group of anionic surfactants which can be detected by means of the sum parameter methylene blue active substances **"MBAS".**

The aquatic toxicity of LAS is described as follows: The longer the C-chain of the hydrophobic part of the molecule, the higher the aquatic toxicity. A LD_{50} of 8.5 mg/L after 48 h exposure was found in *Daphnia magna*.



◘ **Fig. 6.88** Structure of linear alkylbenzene sulfonates

In addition to alkylbenzenesulfonates, aromatic aminosulfonic acids are of great importance as precursors and degradation products of azo dyes.

In 1978, the proportion of aromatic sulfonic acids in the Lower Rhine amounted to 7–15% of the total organic load, depending on the seasonal fluctuations of the water flow (Malle, 1978), and already at that time made clear its importance as an important contaminant.

6.4.1.2 Degradation of Aromatic Sulfonic Acids

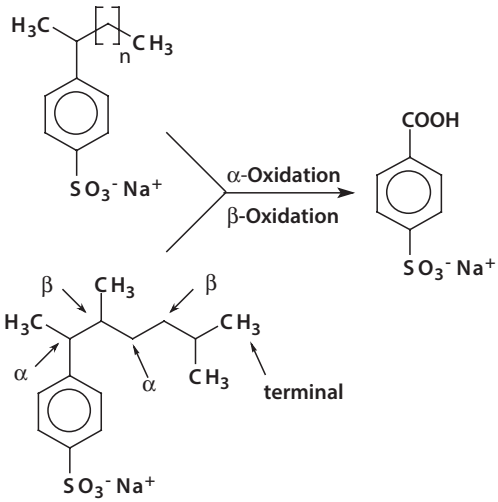
Aromatic sulfonic acids are highly polar substances and have a high foreign substance character, in particular due to the presence of the sulfonic acid group.

After use, most of the LAS used as detergents end up in waste water and subsequently in the sewage treatment plant. They are readily and rapidly biodegradable under aerobic conditions and non-degradable under anaerobic conditions. The overall elimination rate for LAS in a biological three-stage wastewater treatment plant (activated sludge process) is usually above 99%. Since LAS are not degraded anaerobically, they accumulate in the digestion tower and, along with nonylphenol (the main metabolite of alkylphenol ethoxylates), represent the most important xenobiotic organic substances in sewage sludge in terms of quantity.

Two strategies were used to isolate bacteria capable of using aromatic sulfonic acids: (1) the sulfonic acids were used as a carbon and energy source; (2) they served as a sulfur source. Both ways led to success, although the experiments with sulfonic acids as a source of sulfur were very difficult, since only 1/500 by weight of sulfur is needed compared to carbon. Minor impurities from other sulfur sources therefore had to be excluded.

In the following, the degradation sequences of organisms that use the substances as carbon and energy sources are

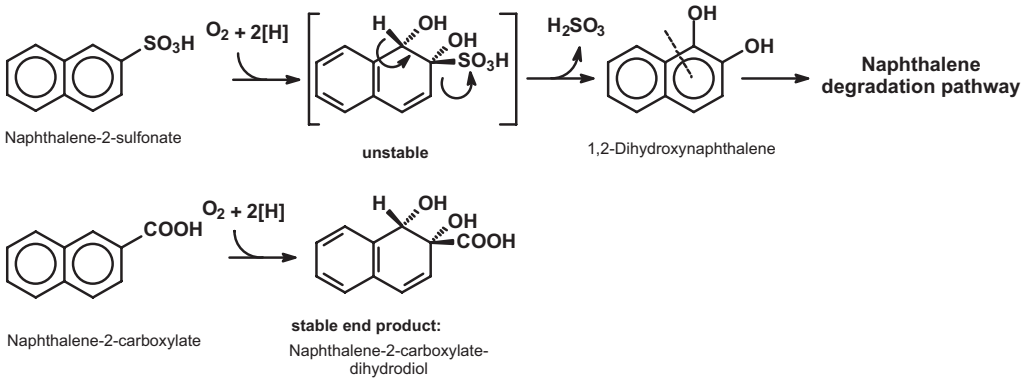
addressed. The degradation of LAS and also of branched-chain alkylbenzenesulfonates first takes place at the alkyl chain, whereby the enzyme sequence known from the degradation of alkanes, such as terminal monooxygenase and/or α-oxidation and subsequently β-oxidation cascade, takes place. Sulfobenzoate is thus formed (◘ Fig. 6.89).



◘ **Fig. 6.89** Degradation of LAS and branched-chain alkylbenzene sulfonates (possible oxidations in the side chain are marked by arrows) and formation of sulfobenzoate

Important results on the microbial degradation of aromatic sulfonic acids were obtained using naphthalene-2-sulfonic acid as a carbon and energy source. Desulfonation, the crucial step in degradation, is initiated by a **dioxygenase,** which produces an unstable product. The inert C-SO_3 H bond is destabilized (oxygen and SO_3H group are located on the same carbon atom) in such a way that the SO_3H group is spontaneously eliminated as sulfite to give 1,2-dihydroxynaphthalene, a metabolite of naphthalene degradation. Thus, no specific enzyme is responsible for the desulfonation, but a "normal" naphthalene 1,2-dioxygenase performs the step of activating the aromatic ring. This was shown as follows: If naphthalene-2-carboxylic acid was used instead of naphthalene-2-sulfonic acid, the cells produced a stable product which was excreted and could be isolated. The actual elimination of the SO_3H group thus proceeds spontaneously. In the degradation of naphthalene-2-sulfonic acid, the dehydrogenase following the ring-activating dioxygenase reaction in naphthalene degradation is skipped, since the diphenol stage is reached immediately by the release of sulfite (◘ Fig. 6.90).

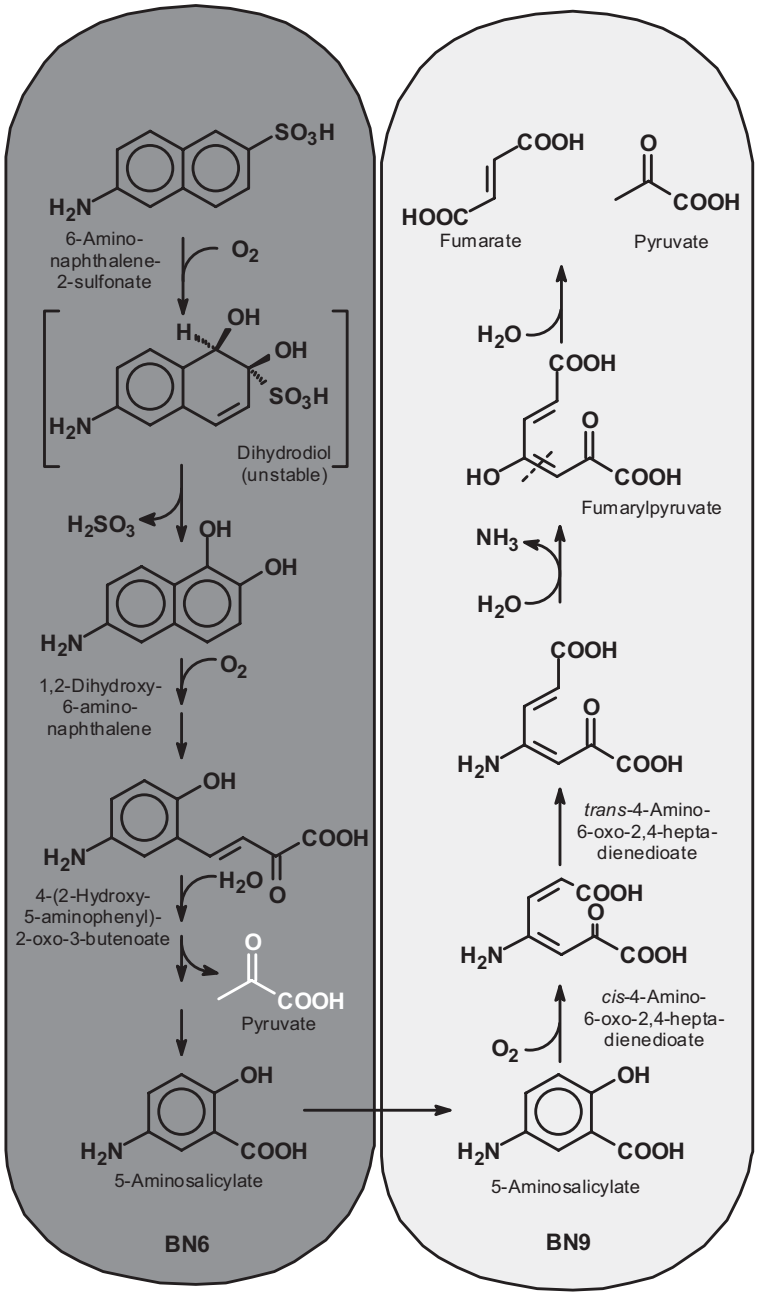
The example of 6-aminonaphthalene-2-sulfonate and *p*-toluenesulfonate is a good



◘ **Fig. 6.90** Initiating dioxygenation with elimination of the sulfonic acid group for naphthalene-2-sulfonic acid and analogous reaction with naphthalene-2-carboxylic acid to form the stable dihydrodiol. (According to Beckmann, 1976)

illustration of the complexity of the degradation of aromatic sulfonic acids.

The degradation of 6-aminonaphthalene-2-sulfonate illustrates that sometimes the cooperation of strains is necessary for the overall degradation. Crucial to the degradation of this xenobiotic is initially, as shown in ◘ Fig. 6.91, the double hydroxyl-



◘ **Fig. 6.91** Syntrophy of degradation using the example of 6-aminonaphthalene-2-sulfonate

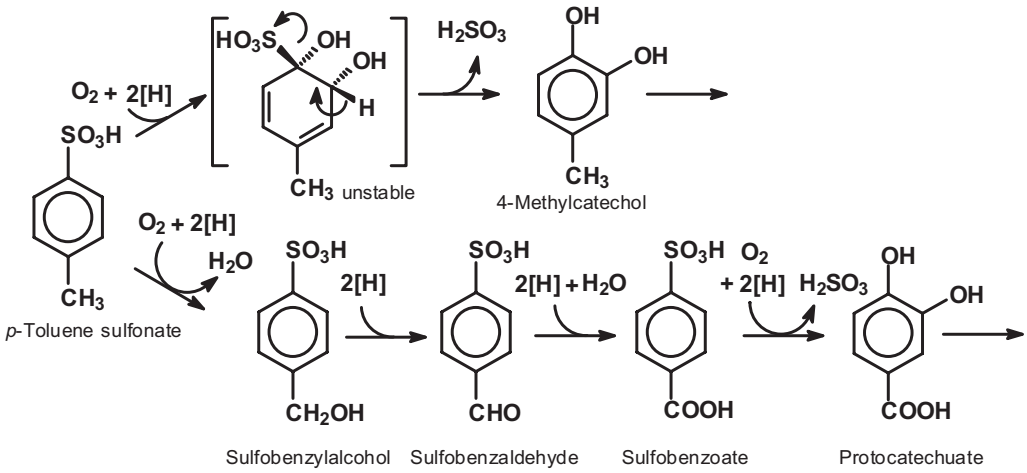


Fig. 6.92 Desulfonation by ring-activating dioxygenases in the degradation of toluenesulfonate: Initial and late elimination

ation by bacterial strain BN6, which results in the spontaneous (non-enzymatic) cleavage of the sulfonic acid group as sulfite. A limitation of the degradation exists at the step of 5-aminosalicylate, which is not further degraded by the desulfonating strain, but is released to the medium. By utilizing the three C-atoms (as pyruvate) of the sulfonated ring, the degradation profitable for the bacterial strain BN6 is conceivable, but a complete degradation of 6-aminonaphthalene-2-sulfonate is only achieved by the presence of a second bacterial strain BN9, which utilizes the 5-aminosalicylate as growth substrate very efficiently.

The example of the degradation of *p*-toluenesulfonate makes it clear that the elimination of the sulfonic acid group can occur both **directly** by initiating ring-activating dioxygenation and **at a later stage** by dioxygenation using sulfobenzoate-1,2-dioxygenase.

Analogous reaction sequences known from the degradation of chloroaromatics with chlorocatechols as central metabolites have also been described for the degradation of aromatic sulfonic acids. In the case of orthanilate, elimination of the sulfonic acid group is a consequence of *meta*-cleaving dioxygenase with 3-sulfocatechol, resulting in a normal metabolite of the *meta*-pathway 2-hydroxymuconate. In the case of 4-sulfocatechol, which is formed from 4-sulfoaniline, elimination occurs from the product of muconate isomerization and corresponds to the reaction in the degradation of 4-chlorocatechol (Figs. 6.92 and 6.93).

6.4.2 Degradation of Azo Dyes

Azo dyes are characterized by the presence of one or more azo groups (–N=N–) and sulfonic acid groups (–SO_3H). They are the largest and most versatile class of dyes. More than 2000 different azo dyes are in use today to color various materials such as textiles, leather, plastics, cosmetics and food.

The largest amount of azo dyes is used for the dyeing of textiles. It is estimated that about 10% of the dyes used do not bind to the fibres during the dyeing process, enter wastewater treatment plants and are then released into the environment.

There is only one example of an azo group in a natural product (4,4′-dihydroxyazobenzene) and therefore the azo dyes produced by industry are also to be addressed

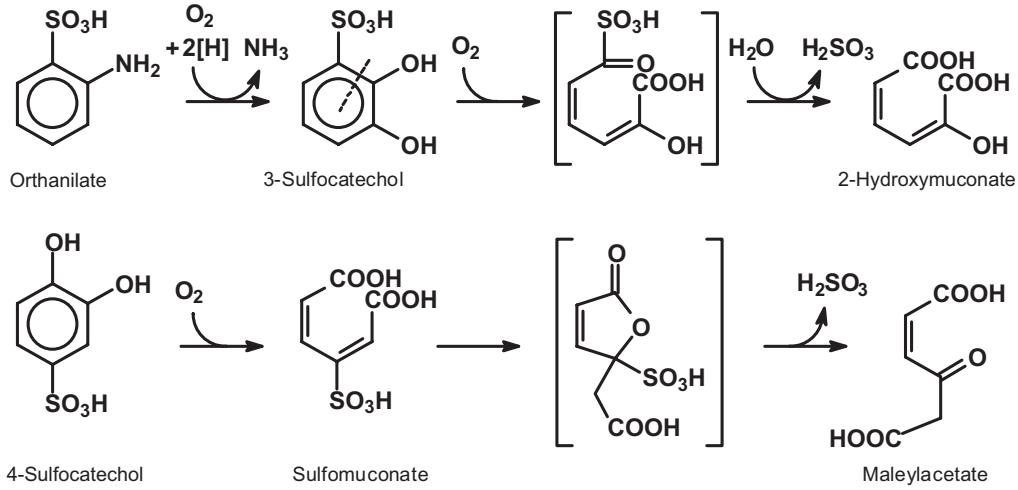
as xenobiotics. Consequently, it is not surprising that azo dyes turn out to be persistent in conventional wastewater treatment plants.

The current state of the art for the treatment of wastewater contaminated with azo dyes includes physicochemical techniques such as adsorption, precipitation, chemical oxidation, photodegradation or membrane filtration.

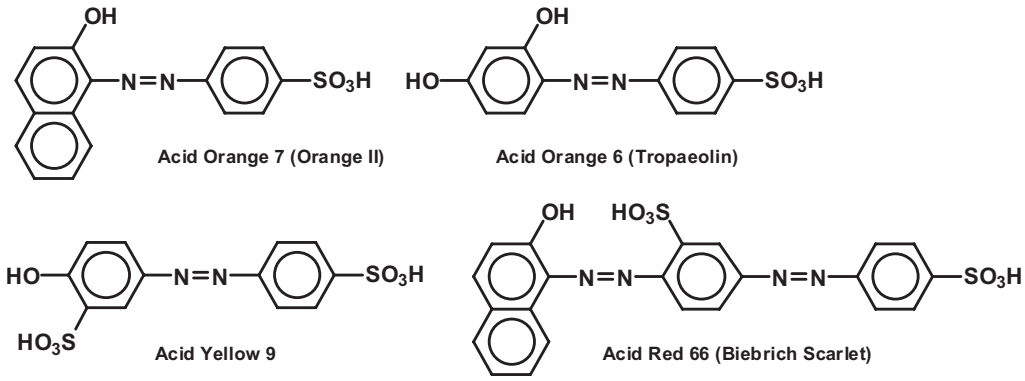
Although azo dyes are considered to be poorly biodegradable, it has been shown in recent years that under certain environmental conditions microorganisms are capable of transforming azo dyes to non-color products or even mineralizing them completely, and the following possibilities have been investigated:

1. Decolorization of azo dyes by lignin-degrading enzymes of white rot fungi.
2. Cometabolic reductive cleavage of azo dyes by aerobic bacteria.
3. Growth of aerobic bacteria using azo dyes as carbon and energy sources.
4. Anaerobic reduction of azo dyes by bacteria (non-specific reduction).

Lignolytic fungi can decolorize azo dyes with their ligninases, manganese-dependent peroxidases, or laccases (◘ Fig. 6.94).



◘ **Fig. 6.93** Examples of the late elimination of a sulfonic acid group: during or after ring cleavage



◘ **Fig. 6.94** Examples of azo dyes decolorized by white rot fungi

For some model azo dyes, the degradation pathways have been studied and mineralization to CO_2 has been shown.

In most cases, bacterial degradation begins with reductive cleavage of the azo group, resulting in the formation of colorless amines. These reductive processes have been described for some aerobic bacteria growing with very simple azo dyes. These specially adapted microorganisms form "real" azoreductases, which cleave the azo group reductively in the presence of molecular oxygen.

Much more common is the reductive cleavage of azo dyes under anaerobic conditions. This reaction usually occurs with very low specific activity, but it is carried out quite non-specifically by a variety of organisms. In these non-specific anaerobic processes, redox mediators of small molecular size (for example, flavins or quinones) are very often involved, which are enzymatically reduced by cells. These reduced mediator compounds can reduce the azo group in a purely chemical reaction. The sulfonated amines formed by these reactions can then be aerobically microbially degraded.

6.5 Plastics, Bioplastics

In 2005, the consumption of conventional plastics amounted to about 53 million tonnes in Europe and more than 9 million tonnes in Germany. In 2010, production is said to have amounted to 304 million tonnes worldwide and 75 million tonnes in Europe. The quantities are distributed among different types of plastics (see Table 6.13).

Table 6.13 Synthetic plastics

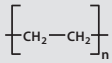
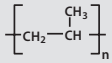
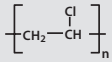
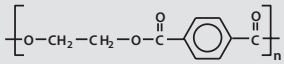
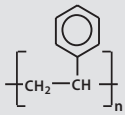
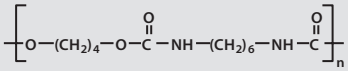
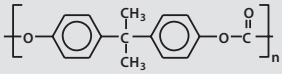
Type of plastic/basic unit	Application	Proportion (%)
Polyethylene (PE) 	Carrier bags	24
Polypropylene (PP) 	Pipes	14
Polyvinylchloride (PVC) 	Floor coverings	12
Polyethylene terephthalate (PET) 	Beverage bottles	14
Polystyrene (PS) 	Disposable packaging Foodstuffs	6
Technical plastics (incl. blends)	Automobile construction	7
Synthetic elastomers	Tires	4

Table 6.13 (continued)

Type of plastic/basic unit	Application	Proportion (%)
Polyurethane (PU) 	Mattresses	4
Polycarbonate 	CDs & DVDs	1.5
Others		~14

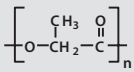
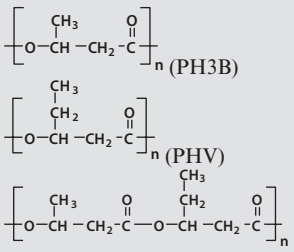
For about 20 years, there have been increasing efforts to produce plastics partly or completely from renewable raw materials. Problems in waste management, the recognition of the finite nature of fossil raw materials and the general discussion about greenhouse gases have contributed to the increased use of renewable raw materials for the production of plastics.

Plastics made from renewable raw materials are usually referred to as bioplastics or biopolymers. According to current usage, the prefix "bio" stands for the properties "biobased" and "biodegradable". Biobased refers to products that are partially or completely derived from renewable raw materials. These products can be both biodegradable and non-biodegradable.

The production and consumption volumes of bioplastics are still very low compared to conventional plastics. Statistics on the production and consumption of bioplastics do not yet exist. The consumption of bioplastics was estimated at 50,000 t in Europe and about 5000 t in Germany for the year 2005. The share of bioplastics in total plastics consumption was thus only about 0.1% in Europe and about 0.05% in Germany (Table 6.14).

The shape and order of the macromolecules are an important distinguishing feature of plastics:

Table 6.14 Biological plastics[a]

Type of plasic/basic unit	Application example
Polylactic acid (PLA) 	Packaging of short-lived goods, agricultural mulch films, catering items, medical technology
Polyhydroxyalkanoate (PHAs)  (PH3B) (PHV) (copolymer PHBV)	Biomaterial for medical applications

[a]Although a reduction in product prices has been recorded for biodegradable plastics in recent years due to new developments and the establishment of larger production capacities, the economic competitiveness of biodegradable plastics remains limited. Compared to polyethylene or polypropylene, for example, there are still price differences in the range of 2–5 times for biodegradable plastics

- **Thermoplastics** have a linear or branched structure and are not cross-linked. They become soft when heated and can be melted. Examples are polyethylene, polystyrene and polyvinyl chloride.
- In **thermosets,** the molecular chains are closely interlinked in three dimensions. The addition of heat therefore does not lead to melting, but to decomposition at a sufficiently high temperature. Examples are bakelite and certain polyurethanes.
- **Elastomers** are hard or soft elastic plastics in which the macromolecules are arranged three-dimensionally in a wide mesh. Soft-elastic elastomers can be deformed by stretching by a multiple of their length, returning to their original shape after unloading. The best known example is rubber.

The properties of the plastic therefore vary depending on its molecular structure. Today, suitable synthesis processes allow the production of almost tailor-made plastics, which is also a major reason for their widespread use and multiple applications.

For the production of plastics based on renewable raw materials, petrochemical components as well as other auxiliaries and additives—such as lubricants, stabilizers and antistatics—usually have to be added. Only in this way can the properties of the products come close to those of conventional plastics. The proportions of additives can be significant in terms of quantity. In starch plastics, for example, the proportion of fossil additives can be up to 50%.

The main applications of biodegradable plastics in Europe are in the packaging and catering sectors. There are also applications in agriculture and horticulture as well as in the pharmaceutical and medical sectors.

Already introduced to the market on a relatively wide scale are products such as:

- Waste bags,
- Carrier Bags,
- Disposable tableware (cups, plates, cutlery),
- Packaging films,
- Bottles,
- Fruit and vegetable peelings,
- Packaging aids (loose-fill chips),
- expandable foams,
- Mulch films,
- Flower pots.

In Asia, moreover, applications are increasingly appearing in the technical field—for example as mobile phone or PC housings.

6.5.1 Degradability of Plastics

Biodegradable plastics are not necessarily made from renewable raw materials; there are also plastics made from fossil, non-renewable resources that are biodegradable. Biodegradability is therefore not linked to the raw material base, but depends solely on the chemical structure of the material.

In order to be able to ensure the environmental compatibility of plastics, various organisations have been endeavouring since the end of the 1980s to develop a suitable basis for assessing degradable polymeric materials (Fig. 6.95).

Studies to evaluate biodegradability are usually conducted in a three-tier test hierarchy such as:

1. **Determination of the biodegradation potential of a substance:** Screening tests such as the CO_2 development test (OECD Guideline 301B) are carried out in the laboratory under defined conditions in synthetic environments (defined nutrient media).
2. **Investigation of biodegradation under realistic conditions:** Simulation tests carried out in the laboratory which simulate conditions, for example, during a composting process (DIN V 54900-2, Section 8) and enable the degradation behaviour of a substance to be balanced.

International Standardization Organisation (ISO)

TC 61 SC5 WG 22 „Biodegradability of Plastics“

Comité Européenne de Normalisation (CEN)

TC 261 SC4 WG2 „Degradability of Packaging Materials“

American Society for Testing and Materials (ASTM)

ASTM D-20.96.01

Deutsches Institut für Normung (DIN)

FNK 103.3 „Bioabbaubare Kunststoffe“

Biodegradable Plastics Society, Japan

Fig. 6.95 Different organizations and standard procedures they work on. (Adapted from Witt et al., 1997)

3. **Investigation of biodegradation under environmental conditions:** Field tests in water, soil or compost (complex systems, varying environmental conditions) are intended to meet the higher requirements for the representation of the actual environmental behaviour of a substance (for example DIN V 54900-3, section 7). According to DIN EN 13432, biodegradability means that a material must have degraded to more than 90% to water, carbon dioxide and biomass after a specified time under defined temperature, oxygen and humidity conditions in the presence of microorganisms.

The following rules concerning the degradability of plastics have been obtained:

- **High-molecular technically used polymers** are usually **not accessible to biological attack,** especially if their main chain consists of pure **C–C bonds**, as is the case with polyethylene, polypropylene and polystyrene, for example.
- Natural polymers such as proteins, cellulose, starch and lignin contain heteroatoms (oxygen, nitrogen) in the polymer chain, which provide targets for enzymatic hydrolysis and oxidation in biological systems. Thus, among the technically used polymers described as biodegradable, there are essentially those that contain **C–O** or **C–N bonds in the polymer chain.**
- The hydrolysability of bonds in the polymer chain follows the sequence
  – Ester > Ether > Amide > Urethane.
- One of the few polymers with pure C–C bonds in the main chain that is considered degradable is polyvinyl alcohol (PVOH). Here, degradation occurs via primary oxidation of the OH groups with subsequent cleavage of the main chain similar to fatty acid degradation.
- **Polymers with aromatic components or branched domains** tend to have greater resistance to microbial attack than **straight-chain aliphatic components.**
- For enzymatic hydrolysis, the polymer chain must be flexible enough to fit into the active site of the degrading enzyme. This is considered to explain the easy biodegradation of the **flexible aliphatic polyesters,** while the **rigid aromatic polyesters** resist biodegradation.
- An analogous effect is observed with polyamides. Here, the **restriction of chain flexibility** is caused by intermolecular

interactions in the form of hydrogen bonds.
- Copolymers of aliphatic and aromatic polyesters as well as of amides and esters are generally amenable to microbial attack. The degradation rates of these compounds increase with increasing aliphatic component content or with increasing ester content.
- **Cross-links** in polymers limit the mobility of the polymer chain and thus the accessibility of the polymer by the enzyme, which also leads to an impairment of the degradation rates.

While the chemical structure and composition of the polymers determine the basic biodegradability, the physical properties of the polymers essentially influence the speed of the biodegradation process. Some tendencies can be derived:
- Very low molar masses of the polymers favour degradation.
- In comparable degradation processes, polymers with low melting points are degraded better than those with high melting points.
- Amorphous areas in the polymer are attacked more quickly than crystalline areas.
- Polymers with hydrophilic surfaces degrade better than hydrophobic materials.
- The degradation rate increases with decreasing particle size (= larger surface area).

6.5.2 Bioplastics

Figure 6.96 shows a classification of bioplastics with regard to their material origin.

Natural fibre-reinforced plastics made of hemp and flax fibres with polypropylene, polyethylene, polyethylene terephthalate or phenolic resin and wood-plastic composites are among the most important **biobased,**

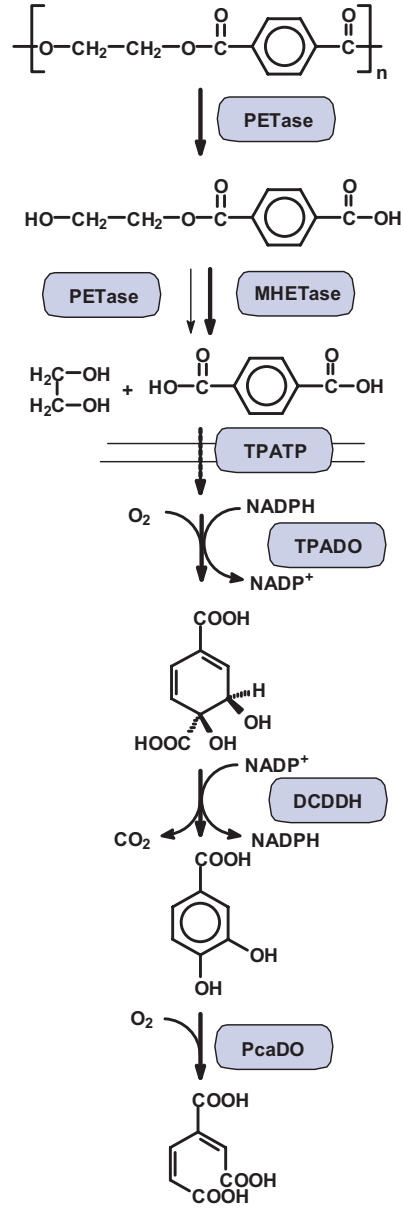


Fig. 6.96 Proposed PET degradation pathway. Extracellular PETase hydrolyzes PET to form MHET (mono (hydroxethyl) terephthalate) as the major product and TPA. MHETase, a lipoprotein, hydrolyzes MHET to form TPA and EG. TPA is introduced by the TPA transporter (TPATP) and degraded by TPA-1,2-dioxygenase (TPADO). This is followed by degradation by 1,2-dihydroxy-3,5-cyclohexadiene-1, 4-dicarboxylate dehydrogenase (DCDDH). Ring cleavage of the resulting PCA occurs by PCA-3,4-dioxygenase (PcaDO) (Yoshida et al., 2016)

non-degradable plastics. Wood-plastic composites are thermoplastically processable composites, for example made of polyethylene or polypropylene with up to 80% wood flour and additives such as adhesion promoters, UV protection agents and color pigments. In Europe, the construction and furniture industry alone now uses 12,000 t per year. The production volume in the European automotive industry has grown to 50,000 t annually.

Biodegradable plastics of plant origin represent by far the largest market segment of bioplastics and have the greatest economic importance.

Biodegradable plastics can be produced from a variety of plant-based raw materials. In addition to cellulose and sugar, starch in particular occupies a key position. In addition to its good availability, it offers a good price-performance ratio. The most important starch suppliers are corn, wheat and potatoes.

Of the biodegradable plastics developed in recent years, the biobased starch plastics, polylactide and polyhydroxy fatty acids, and the fossil-based polyester have been the most successful.

- Thermoplastic starch is currently the most important and most common bioplastic. Its share of the total bioplastics market is about 80%. Starch plastics are primarily processed into films, injection moulded articles or coatings.
- Polylactic acid (PLA) is a biodegradable polyester and is polymerized from the monomer lactic acid. Lactic acid is primarily produced using corn starch. PLA and PLA blends have been established for years as specialty polymers in the medical field and increasingly as packaging and fiber materials.
- Polyhydroxy fatty acids (PHF) are thermoplastic polyesters obtained by the action of bacteria or fungi on sugar or starch. The best known representatives are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). Microorganisms store PHF as a reserve substance. It is obtained by extraction from the cells. Depending on the bacterial species and the choice of substrate, a variety of plastics with varying properties can be produced.

6.5.2.1 Biopol: A Degradable Thermoplastic Resin

Polyhydroxybutyrate is a bacterial storage substance. It performs the function that fats do in higher organisms. When there is an excess of carbon sources such as sugars and a lack of nitrogen or phosphorus sources, up to 80% of the cell dry weight is accumulated intracellularly as poly-β-hydroxybutyrate in *Cupriavidus necator* (formerly: *Alcaligenes eutrophus*) and other bacteria. The sugars are metabolized to acetyl-CoA. Condensation then occurs to acetoacetyl-CoA, which is reduced to β-hydroxybutyryl-CoA and further polymerized to polybutyrate with about 25,000 units. When propionic acid is added to the nutrient medium, heteropolymers are formed that contain hydroxybutyrate as well as hydroxyvalerate building blocks. This improves the flexibility and elasticity of the polyhydroxyalkanoates (PHA). The properties of the polyhydroxyalkanoates can be influenced in many ways by further additions of aliphatic and aromatic compounds. The polyhydroxyalkanoates can be obtained by extraction. More elegant is the enzymatic degradation of the bacterial biomass, leaving behind the PHA granules, which are further processed.

Using genetic engineering methods, the enzymes necessary for polyhydroxybutyrate synthesis were transferred into plants and expressed. PHB granules are formed in the transgenic plants.

The name biopol stands for a group of thermoplastic polyesters based on hydroxybutyrate units with hydroxyvalerate units statistically distributed over the polymer chains (◘ Fig. 6.97).

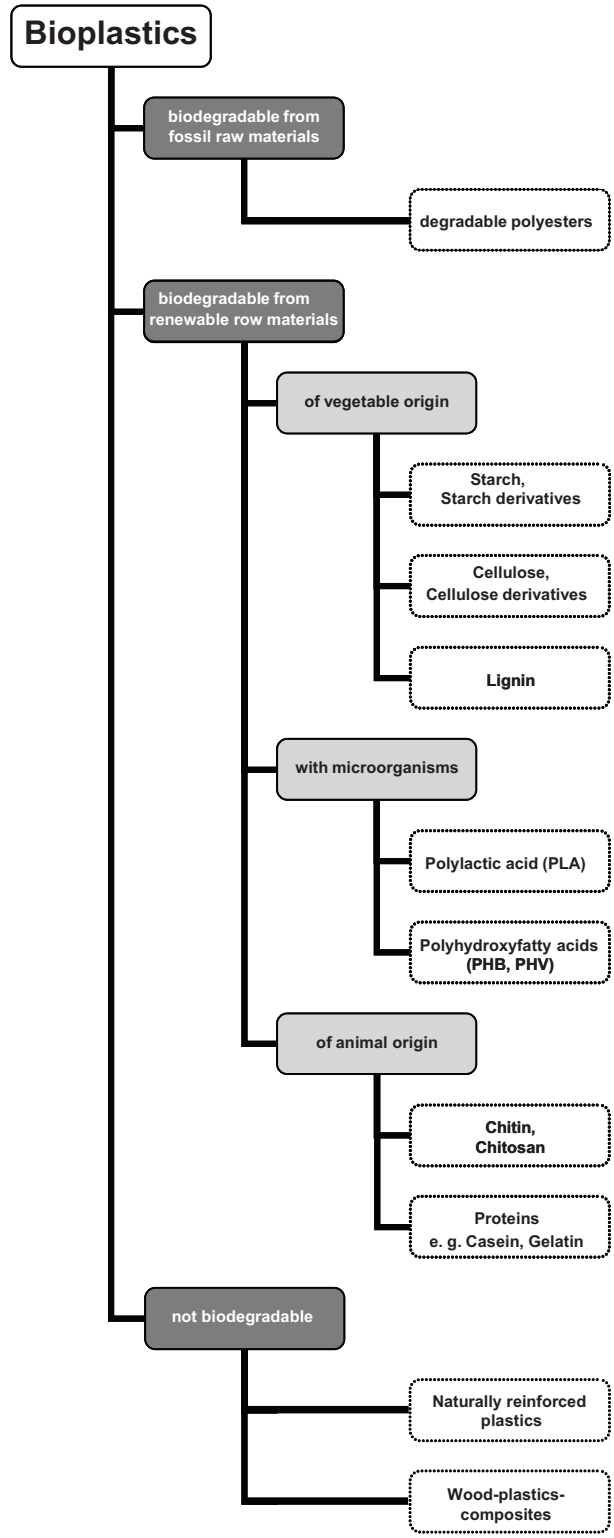


Fig. 6.97 Systematics of bioplastics

Biopol is thermoplastic, fully biodegradable and available in different versions with different physical properties. The plastic is used in the cosmetics industry for bottles and other containers. Likewise, closures for bottles, tubes and jars, Biopol-coated papers and cardboards for bio-waste bags and sacks as well as paper cups can be produced.

Since polymers cannot in principle be transported into a bacterial cell due to their size, the PHA molecules must first be hydrolysed outside the cell. This is done by extracellular PHA depolymerases. They hydrolyse the polyesters into oligomers, dimers and monomers, thereby converting the water-insoluble polyester into water-soluble low molecular weight cleavage products that can be transported into the cell. The degradation of poly(3HB) produces free 3-hydroxybutyrate, which is converted via acetoacetate and acetoacetyl-CoA into acetyl-CoA and thus reaches the central metabolism (◘ Fig. 6.98).

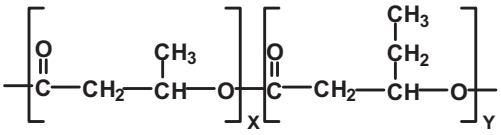
6.5.2.2 Degradable Plastics: Not Only from Renewable Raw Materials

Important biodegradable plastics, some of which have been introduced commercially, are based partly or entirely on fossil raw materials. In some cases, the raw material base can be shifted flexibly: depending on the price level, renewable or fossil feedstocks are used for synthesis.

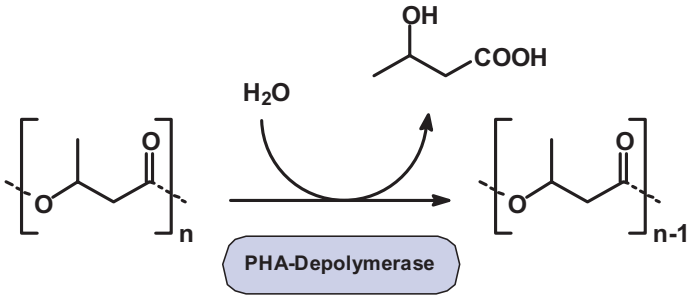
Polyesteramide "BAK 1095" (Bayer)

Starting materials are inexpensive monomers such as ε-caprolactam, adipic acid and diols (for example 1,4-butanediol). These are reacted with each other in a condensation vessel in a typical polycondensation reaction (◘ Fig. 6.99).

Depending on the process control, the properties of the polyester amides can be varied within certain limits with the same gross composition. The products have excellent rotting properties. After 50 days, a film of 200 µm thickness is degraded to over 95% in the compost.



◘ **Fig. 6.98** Structure of Biopol



◘ **Fig. 6.99** PHA depolymerase reaction, the cleavage of a marginal 3-hydroxybutyrate molecule from poly(3HB) is shown. Hydrolysis of the ester bond also occurs in the polyester

6.5.3 An Assessment of the Environmental Impact of Plastics and Bioplastics

Bio-based plastics are materials that have so far been used in niche applications and whose market share will continue to grow.

In particular, recyclable products made of fibre-reinforced plastics and wood-plastic composites have advantages over plastics made of fossil raw materials from the point of view of environmental protection due to the conservation of fossil resources and the reduction of CO_2 emissions.

Meaningful environmental assessments and thus statements about their sustainability are not yet available for the majority of products made from biobased biodegradable plastics.

In a recent life cycle assessment according to the international standard DIN EN ISO 14040 and 14044, disposable beverage cups made of PET, polystyrene, cardboard and PLA were compared with a reusable cup made of polypropylene. It was shown that the reusable cup system is clearly superior to all disposable solutions from an environmental protection point of view. This means that even biodegradable cups made of PLA do not represent a favourable alternative. The environmental impact of PLA cups is comparable to that of PET and is thus significantly higher than that of disposable cups made of cardboard.

The use of biodegradable plastics based on fossil raw materials is viewed particularly critically. This is because these plastics—unlike plastics based on renewable raw materials—are not advantageous in terms of resource conservation and CO_2 savings. At the same time, they do not have the potential for material recycling that conventional plastics do.

In a life cycle assessment that includes both biodegradable plastics made from fossil raw materials and those made from renewable raw materials, the fossil-based plastics perform significantly worse. The primary energy input here is higher by a factor of 5–9 and the contribution to the anthropogenic greenhouse effect is higher by a factor of 5–7 than for biologically based plastics or fossil-based conventional plastics such as polyethylene and polystyrene.

Plastic Craving

Over 300 million tonnes of plastic were produced worldwide in 2013, including around 56 million tonnes of polyethylene terephthalate, better known by its abbreviation PET. This type of plastic is mainly used for beverage bottles. Only a small proportion of it is actually recycled later. The very long shelf life is particularly problematic. According to the Federal Environment Agency, it takes up to 450 years for a plastic bottle to disintegrate. This leads to considerable environmental pollution in landfills, but especially in the world's oceans. Microplastics are formed over the years (through mechanical destruction), which in turn has very negative effects on a wide variety of living creatures.

Yoshida et al. (2016) describe the first known microorganism capable of degrading and completely recycling PET. They collected 250 samples near a recycling plant for PET bottles and, in a consortium of several microorganisms, came across the previously unknown bacterium *Ideonella sakaiensis,* which is capable of decomposing PET. They observed that the bacteria needed only six weeks at 30 °C to almost completely decompose a thin plastic film.

Ideonella sakaiensis can attach to PET surfaces and first excretes a highly specific enzyme (PETase) that breaks the chemical bonds in the plastic. The degradation products are then cleaved by a second selective enzyme (MHETase) into the monomers ethylene glycol (EG) and terephthalic acid (TPA). These basic building blocks of PET can be completely degraded by *Ideonella sakaiensis* and consequently serve as the sole source of growth.

The accessibility of the "smooth" plastic surface is particularly important for breaking up the polymer. Here, the *Ideonella sakaiensis strain* seems to have developed special mechanisms (Yoshida et al., 2016; Tanasupawat et al., 2016).

It has recently been discovered that there are many more and diverse plastic-eating bacteria than previously thought. PET hydrolases occur in the phylum of *Actinobacteria, Proteobacteria* and *Bacteroidetes.* Within the *Proteobacteria*, *Beta-, Delta-* and *Gammaproteobacteria* are the main hosts. Surprisingly, in marine environments bacteria within the phylum *Bacteroidetes* seem to be the main host for PET hydrolase genes, whereas in terrestrial metagenomes these were *Actinobacteria* or *Proteobacteria.* Overall, PET hydrolases are rare enzymes. The highest occurrence was detected in samples contaminated with crude oil (Danso et al., 2018) (◘ Fig. 6.100).

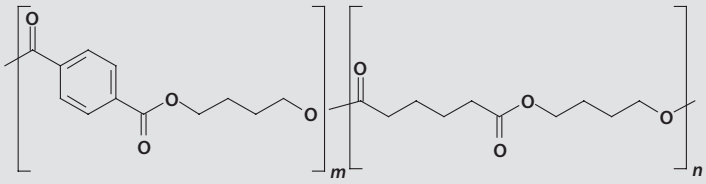
Microorganisms can decompose plastic in the soil

The microbiological degradation of an important plastic for agriculture in the soil could be directly demonstrated. By using state-of-the-art analytical methods, the flow of carbon from the biodegradable polymer into carbon dioxide and microbial biomass was tracked.

Since the 1960s, plastic films have been used in agriculture for so-called mulching (covering the soil). They have many benefits—for example, they allow better distribution and slower evaporation of moisture in the soil, ideal temperatures for plant roots and protection against weeds and insects. This improves conditions for plant growth, while reducing water consumption and the use of herbicides and fertilizers.

However, in addition to all the economic advantages that the use of mulch films achieves, there are also potentially major disadvantages for the environment, especially for soils. This is because mulch films are usually made of non-degradable polyethylene (PE). If these PE films are not completely removed from the soil after harvesting, residues remain and accumulate in the soil over time. It is estimated that six million tons of plastic were used in agriculture worldwide in 2016, two million tons of which were in the form of mulch films alone. Even if only a small part of the films gets into the soil, the residues impair plant growth and yield.

A promising way to circumvent the accumulation of plastics in agricultural soils is to use films made of polymers that can be degraded by soil microorganisms. A promising polymer used in mulch films in this respect is polybutylene adipate terephthalate (PBAT).



So far, biodegradation by respiration of the polymer carbon to CO_2 and simultaneous incorporation of polymer carbon into microbial biomass have not been directly demonstrated. Furthermore, it remained unclear whether all organic building blocks of the polymer could be degraded by microorganisms to ensure that none of them remained in the soil.

To study biodegradation in soils in more detail, a special PBAT was used whose building blocks contained an increased amount of the stable carbon isotope ^{13}C, instead of the conventional carbon ^{12}C. The polymer was then used as a building block for the biodegradation process. Since the ^{13}C isotope accounts for only about 1% of all carbon in the environment, it can be excellently used in enriched form in polymers to follow the flux of carbon atoms from the polymer during biodegradation in soils.

The degradation process in the soil takes place in two steps: First, the PBAT must be broken down (depolymerized) into its individual building blocks by microbial enzymes. Then the small building blocks can be taken up and utilized by the soil microorganisms.

In order to detect the incorporation of the ^{13}C-carbon into the biomass of soil microorganisms, the polymer samples from the soil experiments were investigated using high-resolution secondary ion mass spectrometry (NanoSIMS). The measurements showed that both fungi and unicellular microorganisms were involved in the degradation of the polymer. All three components of the polymer were used by microorganisms (Zumstein et al., 2018).

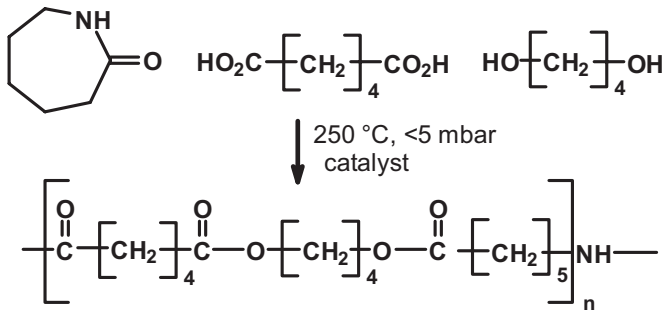


Fig. 6.100 Preparation of a polyesteramide from ε-caprolactam, adipic acid and 1,4-butanediol

Press Release No. 37/2012 Federal Environment Agency, Dessau-Roßlau Bioplastics are not better. Packaging made from biodegradable plastics is not superior to that made from conventional plastics.

The study by the Institute for Energy and Environmental Research Heidelberg (ifeu) commissioned by the UBA "Investigation of the environmental impacts of packaging made of biodegradable plastics" evaluated a total of 85 life cycle assessments, studies and specialist articles. All environmental advantages and disadvantages of the respective packaging were taken into account. In addition, the study analyses the use of packaging in the retail sector, taking into account the current situation as well as forecasts (Detzel et al., 2012).

Biodegradable plastics for packaging made from renewable raw materials have no overall ecological advantage. The cultivation and processing of plants for this packaging acidifies soils and eutrophies water bodies more than the production of conventional plastic packaging. In addition, higher fine dust emissions are produced. The increasingly available bioplastic bags therefore also have no environmental advantage.

This also makes it clear that the currently widely touted bioplastic bags offer no environmental advantages over conventional plastic bags. Only reusable bags, such as cloth bags and bags made of other durable materials, are truly environmentally friendly.

Packaging made of biodegradable plastics was also unable to establish itself in the retail sector. In the reference period of the 2009 study, bioplastic packaging had a maximum market share of 0.5%. In total, 2645 million tonnes of plastic packaging were consumed in Germany in 2009.

Packaging made of biodegradable plastics is currently subject to a special scheme under Section 16(2) of the Packaging Ordinance, which is intended to facilitate its introduction onto the market: Manufacturers and distributors of such packaging made of biodegradable plastics do not have to participate in the existing take-back systems for packaging. As beverage packaging, they are also not subject to the mandatory deposit if they are made from at least 75% renewable raw materials.

6.6 Complexing Agents: Aminopolycarboxylic Acids

Aminopolycarboxylic acids (aminopolycarboxylates, APCs) have the property of forming very stable, water-soluble complexes with di- and trivalent metal ions. They are therefore used for a variety of applications in household and industry, for example to control the solubility and precipitation of metals or to remove precipitates already formed in pipes, bottles and on membranes. The quantitatively most important areas of application worldwide are the photographic industry, electroplating, the paper industry, agriculture and use in the form of industrial and commercial cleaning agents. In 1998, APC was consumed in quantities of approximately 180,000 tonnes worldwide. Due to the application profile, APCs are discharged into the corresponding wastewater of the production or application and from there via the wastewater treatment plant into the aquatic environment.

The biodegradability of APCs is decisively influenced not only by the organic ligand itself, but also by the metal ion it binds. In principle, the degradability of metal-APC complexes decreases with increasing size and complexity of the ligand. In addition, the degradability of a given

APC decreases as a function of the bound metal ions with increasing complex stability.

One of the natural APCs produced by plants or microorganisms, for example to realize the uptake of metals, is ethylenediamine disuccinate (EDDS). This biodegradable complexing agent (actually more precisely: the S,S-stereoisomer) is used, among other things, in soil remediation, but cannot be used for many industrial purposes due to its relatively low complexing capacity. The technically most widely used synthetic aminopolycarboxylic acids are nitrilotriacetate (NTA) and ethylenediaminetetraacetate (EDTA). While the structurally simple and only comparatively "weak" metal complex-forming NTA is degraded by many bacteria under oxic or anoxic conditions, the more complex and relatively stable complex-forming EDTA is generally considered "biologically incapable" and is not biologically degraded in wastewater treatment plants or in nature. However, photodegradation is described for the Fe(III)-EDTA complex, which can be regarded as the main sink for partial degradation of EDTA in aquatic systems.

The two best characterized aerobic NTA-utilizing genera (*Chelatobacter* and *Chelatococcus*) belong to the *Alphaproteobacteria.* These organisms are apparently present in fairly high numbers in surface water, soils and sewage treatment plants.

The NTA monooxygenase has already been cloned and sequenced. Under nitrifying conditions, NTA degradation by an NTA dehydrogenase has been described. The first two steps of degradation by *Chelatobacter* and *Chelatococcus* are catalyzed by a monooxygenase (NTA-MO) and a membrane-bound iminodiacetate dehydrogenase (IDA-DH).

Various pure cultures could also be isolated for a complete mineralization of EDTA, whereby the degradability is more clearly dependent on the complexed metal ion compared to NTA. Thus, it was shown that bacteria which are able to degrade various EDTA complexes (for example Ca-, Mg- and Mn-EDTA) cannot grow with the more stable Fe(III)-EDTA complex (◘ Fig. 6.101).

Studies on the metabolism of EDTA revealed that EDTA enters cells by means of an inducible, very specific and energy-dependent transport system, whereby Ca^{2+} is transported with the EDTA. An EDTA monooxygenase (EDTA-MO), which shares many characteristics with the NTA-MO from strictly aerobic NTA-degrading bacteria, is responsible for the initial step of EDTA degradation. The EDTA-MO catalyzes the successive, oxidative cleavage of two acetyl residues from the EDTA molecule, ultimately releasing N,N′-ethylenediamine diacetate (N,N′-EDDA) and two molecules of glyoxylate via the metabolite ethylenediamine triacetate (ED3A).

The further degradation of N,N′-EDDA presumably occurs by various pathways, including again the cleavage of an acetyl residue in the form of glyoxylate and the formation of ethylenediamine monoacetate (EDMA). It is likely that this reaction is catalyzed by a dehydrogenase. However, the formation of ethylenediamine from N,N'-EDDA by an iminodiacetate oxygenase from strain BNC1 is also reported.

Although the type of metal-EDTA complex influences both the transport of the complexing agent and its enzymatic degradation by the EDTA-MO, a direct dependence on the stability of the complex is described only for the transport. Only complexes with relatively low stability (Mg-EDTA, Ba-EDTA, Ca-EDTA and Mn-EDTA, conditional complex formation constants $<10^{14}$) can be transported into the cells, whereas a comparable dependence for the activity of the EDTA-MO on the com-

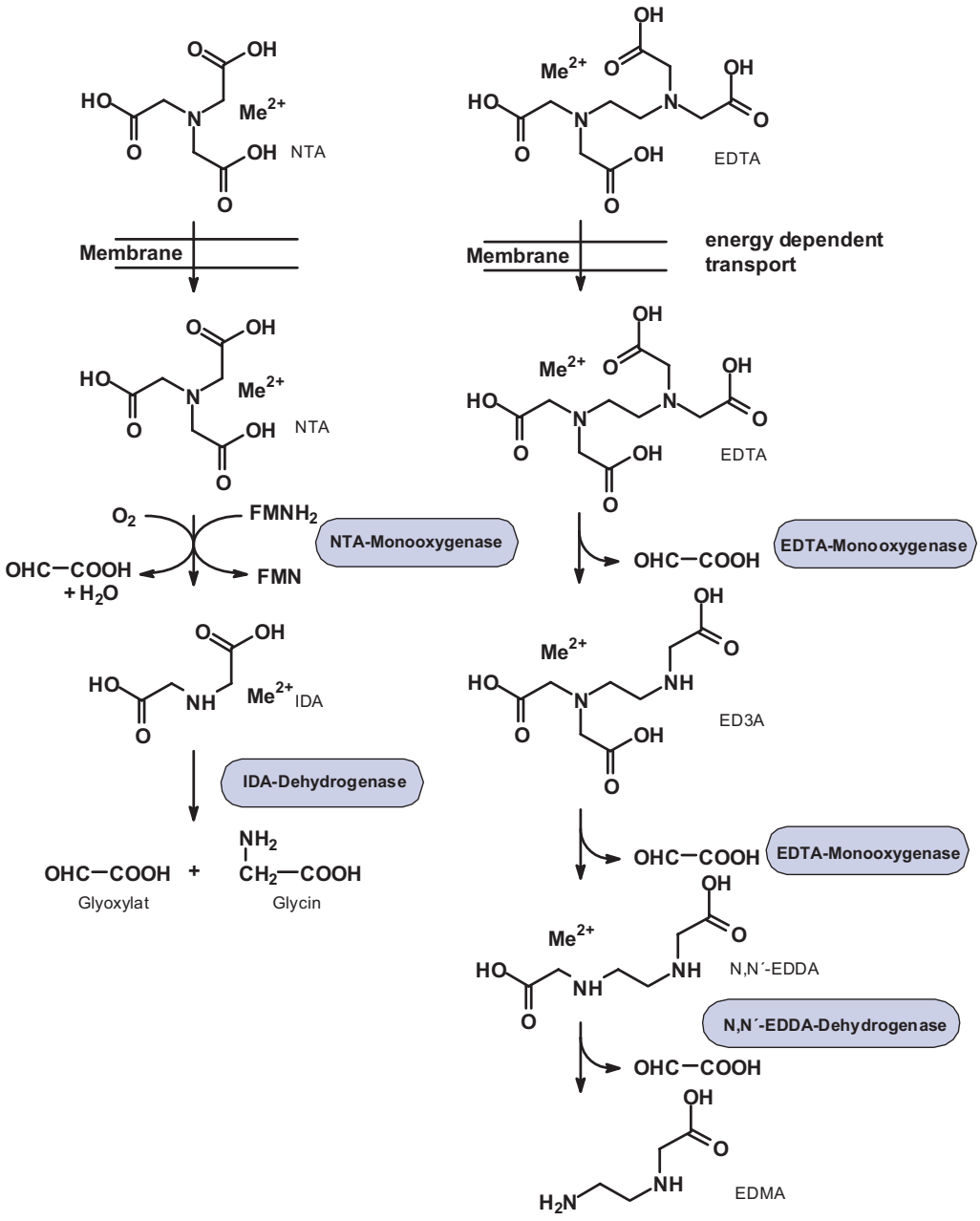


Fig. 6.101 Degradation of complexing agents

plex stability was not found. Preferred substrate is Mg-EDTA, followed by Zn-EDTA, Mn-EDTA and Co-EDTA, whereas free and calcium complexed EDTA are not converted.

6.7 Endocrine Active Compounds

Endocrine disrupting substances in the environment have been the subject of discussion for a number of years and concerns about

the effects of these substances have led to numerous investigation programmes in national and international frameworks. Endocrine disruptors, their effects and the risk they pose are politically explosive.

Endocrine disrupting substances are both natural and synthetically produced substances that act on the endocrine system. Highly effective are hormones, but other substances can also act like hormones or intervene in hormonal regulation by other means.

The input of natural and synthetic hormones into the environment occurs after their excretion with urine and faeces via municipal wastewater treatment plants. The estrogens occur only in very low concentrations (a few ng/L) in wastewater and can only be determined analytically with great effort. However, endocrine disrupting substances in wastewater can also originate from industrial and municipal sources. Relevant substances among many others are nonylphenols, which are mainly formed as degradation products of alkylphenol ethoxylates in the course of wastewater treatment, but also bisphenol A and organotin compounds.

6.7.1 Tributyltin Compounds

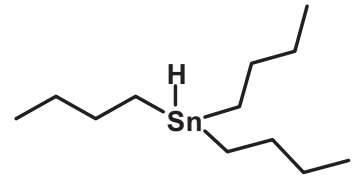
Organotin compounds do not occur in nature. They are exclusively produced synthetically. The production of these compounds began in 1936 and increased worldwide to 63,000 t in 1986. Current worldwide production is about 30,000–50,000 t per year (Umweltbundesamt Berlin, 2001). About 60% of organotin compounds are used as stabilizers in plastics (PVC, silicones, polyurethanes). The remainder is used as biocides in antifoulings, pesticides and wood preservatives and in industrial starting products for tri-, di- and monoderivatives or intermediates.

The organometallic tributyltin (TBT) was and is used in antifouling paints against fouling on ships because of its strong biocidal effect. In the process, TBT is inevitably released into the surrounding water body, where it accumulates in suspended matter and sediments.

Since 1990, the sale of antifouling paints containing tributyltin has been banned in most countries in Western Europe and in the USA and Canada. Even after the regulation of antifouling paints, high TBT concentrations are found in marine sediments worldwide. Due to slow degradation, contaminated sediments represent contaminated sites that are particularly problematic for harbour redevelopment.

TBT is so far the only known non-steroidal compound to which an androgenic effect is attributed. In the meantime, a clear cause-effect relationship between the introduction of TBT into the aquatic environment and pseudohermaphroditism (partial formation of male sex organs analogous to the effects of the addition of testosterone) in marine snails has been demonstrated.

The toxicity of the butyltin family to bacteria falls in the order tributyl- > dibutyl- > monobutyltin > tin. They show effect on cell membranes. Inhibitory concentrations are in the range of 0.04–50 µM. Little information is available on microbial and abiotic degradation in natural habitats. Degradation is thought to involve successive elimination of alkyl residues. A very low concentration of the environmental chemical appears to be important for biodegradation, otherwise the toxic effect on bacteria will occur (◘ Fig. 6.102).



◘ **Fig. 6.102** Tributyltin (TBT)

6.7.2 Alkylphenols

Alkylphenols and in particular their derivatives are produced worldwide on a scale of about 500,000 t/a (Europe 100,000 t/a) mainly for the production of detergents of the group of alkylphenol polyethoxylates (APnEO). Among these, 4-nonylphenol (4NP) is the leading product in Germany with a production capacity of about 35,000 t/a and a share of 70%. The technical product contains a large number of isomeric, **branched nonylphenols.** Worldwide, nonylphenol polyethoxylates (NPnEO) account for 82% of all APnEO. NPnEO were added to household cleaners and detergents as nonionic surfactants from the 1940s to the late 1980s. They are also used in many industries as antioxidants, wetting agents, emulsifiers for pesticides, auxiliaries in leather and paper processing, drilling, levelling and dyeing aids, and for the pretreatment of wool. By the year 2000, all NPNEOs should be replaced on a voluntary basis in the industrial sector in Europe as well.

NPnEO themselves are not particularly toxic and show no hormonal activity. However, the polyethoxylate chains are cleaved off during aerobic, microbial degradation in wastewater treatment plants, initially forming nonylphenol monoethoxylates (NP1EO) and nonylphenol diethoxylates (NP2EO). These degradation products are much more stable than the original NPnEO and show a high tendency to accumulate on suspended solids or in the sediment of rivers. Some of the NP1EO and NP2EO are oxidized to nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC), respectively. Under anaerobic conditions in sewage sludge or sediment, biodegradation of the unoxidized ethoxy groups occurs with the release of 4-NP (◘ Fig. 6.103).

In addition to its aquatic toxicity, 4-NP has another special significance. It was rather accidentally discovered its estrogenic effect in 1991. Other short-chain alkylphenols substituted in the 4-position such as 4-sec-butylphenol, 4-tert-butylphenol, 4-tert-pentylphenol and 4-iso-pentylphenol also show estrogenic effects. Alkylphenols with smaller side chains than four C atoms are inactive, as are all phenols substituted at positions 2 and 3. In addition to NPnEO, the degradation products of octylphenol polyethoxylates (OPnEO), primarily 4-octylphenol (4-OP), are also of interest.

The degradation products of NPnEO, NP1EC and NP2EO showed decreasing estrogenic potency in the order 4-OP, NP1EC, 4-NP and NP2EO. Alkylphenol ethoxylates with more than three ethoxylate groups were found to be inactive. However, they are mostly converted to the active derivatives NP1EO and NP2EO in the sediment.

6.7.3 Bisphenol A

Bisphenol A (BPA) is one of the most widely produced chemicals in the world, at 3.8 million tonnes per year.

For humans, bisphenol A has already been identified as a substance of very high concern (SVHC) due to its reproductive and hormonal effects (BPA is estrogenic). The hormonal effect on fish and amphibians has been proven. BPA has been included in the so-called candidate list under REACH.

Bisphenols and in particular bisphenol A is the starting material for polycarbonate plastics as well as epoxy resins. Its range of application is therefore very broad. The substance is still found in many everyday products such as drinking bottles, food cans, DVDs, sales slips made of thermal paper or food packaging. Due to their use in housings (electrical engineering, electronics), spectacle lenses, car headlight lenses as well as in paints and adhesives, they are widely found and are also deposited as waste in landfills.

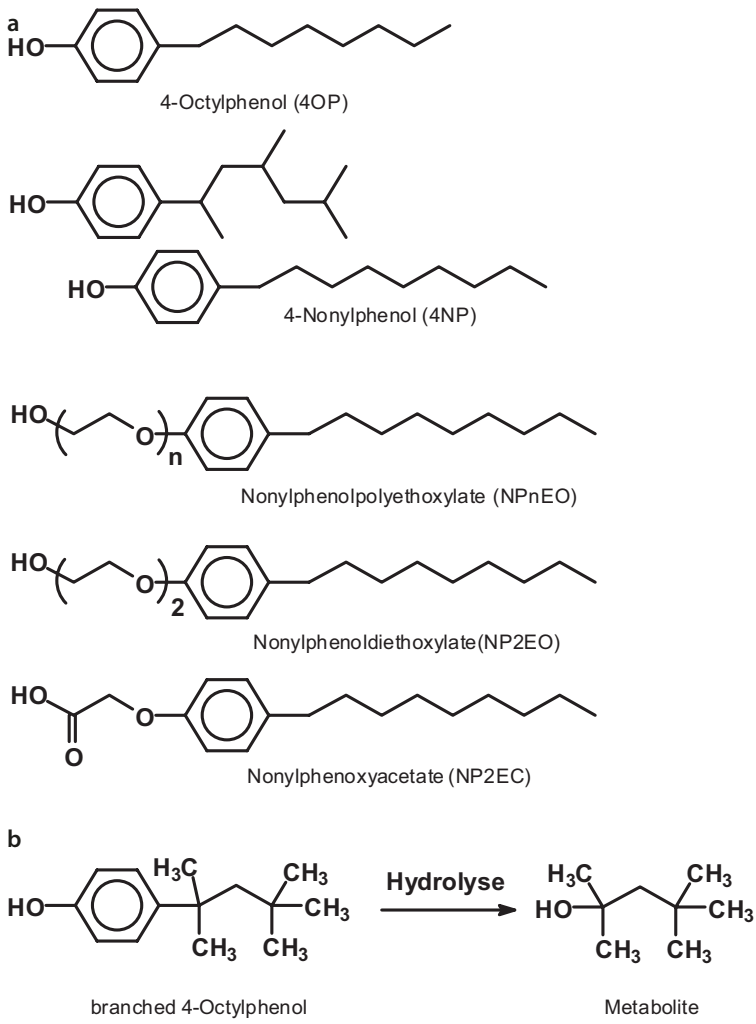


Fig. 6.103 Alkylphenols. (**a**) Examples of unbranched and branched alkylphenols and their derivatives, (**b**) hydrolysis eliminates estrogenic potency

Due to its ubiquitous use and distribution, BPA is regularly detected in municipal wastewater, sewage sludge, surface waters and sediments.

Due to their lipophilic character (log-K_{ow} = 3.18), these substances may bind to solid organic substances in wastewater treatment plants and, as a consequence, be discharged with the sewage sludge.

BPA undergoes relatively rapid aerobic degradation in water, sludge and soil, whereas anaerobic degradation has not yet been detected (Fig. 6.104).

Further degradation of 4-hydroxybenzoate proceeds via protocatechuate followed by *meta*-cleavage (see Fig. 6.16).

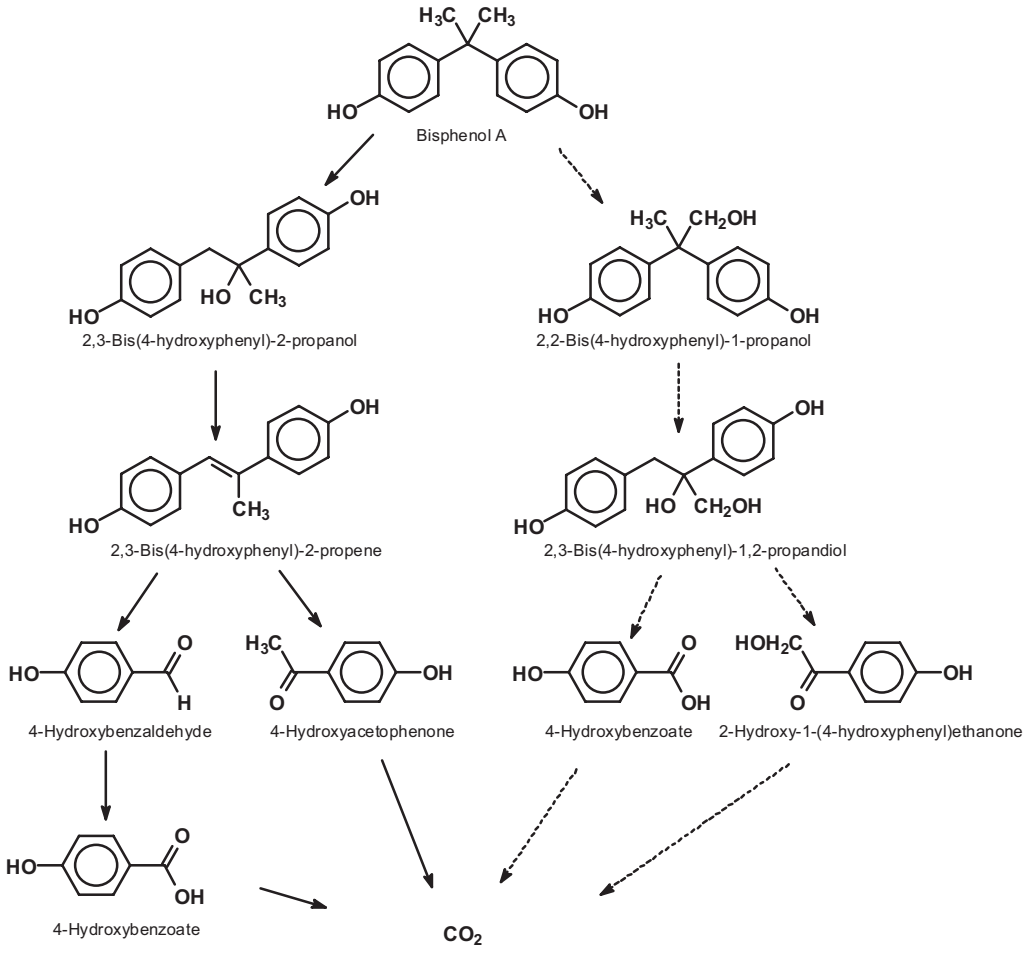


Fig. 6.104 Degradation pathway for bisphenol A: main pathway (*left*), secondary pathway (*right*)

6.8 Methyl Tert-Butyl Ether

The gasoline additive methyl tert-butyl ether (MTBE) is one of the most widely produced chemicals worldwide. World production capacity was approximately 25 million tonnes in 1999, with Germany producing 680,000 tonnes in 2001. Although MTBE is used worldwide, 61% is consumed in the USA, but only 44% is produced there. MTBE has been added to gasoline since the mid-1970s in the U.S. and since the early 1980s in Germany to improve anti-knock properties. Oxygen is required for the complete combustion of gasoline in the engine. This is introduced mechanically during the intake phase through the piston stroke into the gasoline engine and compressed there. However, since this amount of oxygen is not sufficient for complete combustion to CO_2, additional oxygen is introduced into the combustion chamber of the engine by chemical means using additives. Particularly in high-octane Super Plus gasoline (RON 98, research octane number), up to 15% MTBE is added to increase the octane number and ensure more complete combustion.

MTBE is problematic for groundwater mainly because of its physicochemical properties: highly volatile (with a Henry constant K_H = 0.54 atm- L/mol, comparatively less volatile from water than benzene K_H = 5 atm- L/mol), readily soluble in water (50 g/L at 25 °C, compared to other components of gasoline such as benzene of 1.79 g/L) and highly mobile in soil (very low partition coefficient logK_{ow} of 1.06). In addition, it has a low odor and taste threshold. Its use in the USA, for example, has led to considerable groundwater contamination in some cases and subsequently to fierce controversy, culminating in the ban on MTBE in California from 2003. The quantities used in Europe are predominantly lower.

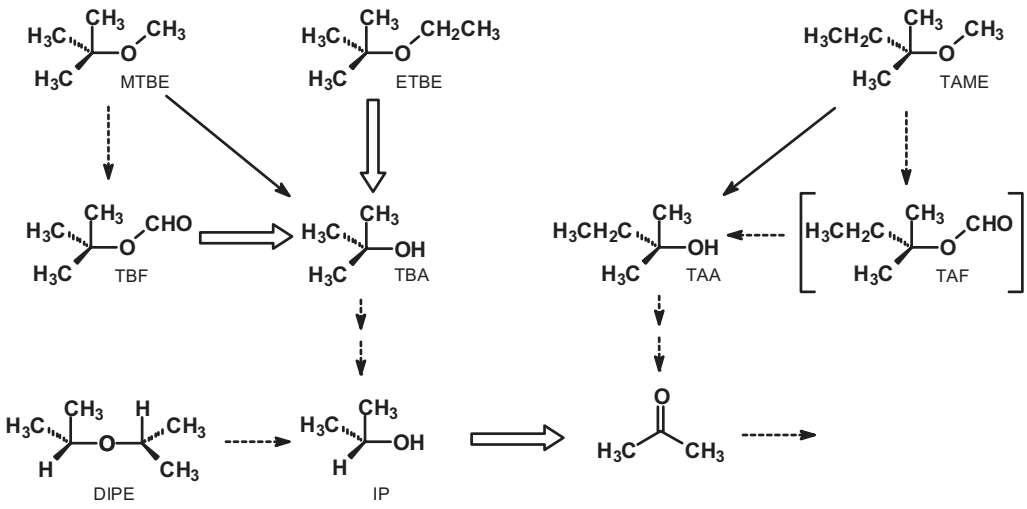
MTBE is diffusely released from the atmosphere, but is also released point source into groundwater through gasoline contamination. The input of MTBE with petrol with about 1000 releases per year occurs more frequently in the EU than previously assumed.

In Germany, MTBE can be detected in almost all surface waters investigated. Measurements in recent years have revealed mean concentrations of about 0.1–0.3 µg/L. Apparently, input via the air pathway plays a role. Groundwater investigations show MTBE loads in the range of 0.1–0.5 µg/L in urban areas, in rural areas these values are lower by a factor of 10.

In general, MTBE is considered to be poorly biodegradable. Nevertheless, degradation of MTBE under aerobic, anaerobic and cometabolic conditions is described.

Aerobic mineralization of MTBE at laboratory scale was demonstrated. The microorganisms were isolated from different sources, generally from wastewater treatment plants of the petroleum processing or chemical industry. Mixed cultures and also pure cultures were obtained, which can use MTBE as a carbon and energy source. It is noticeable that cell yields with MTBE (0.1–0.2 g cells/g MTBE) are generally lower than those with aromatics. Furthermore, it was observed that lower degradation rates resulted compared to that with aromatics.

With strain PM1, the degradation pathway was partially elucidated (◘ Fig. 6.105).



◘ **Fig. 6.105** Proposed degradation pathway for MTBE and other ethers by strain PM1 (Church et al., 2000). *tert*-butyl formate (TBF), diisopropyl ether (DIPE), ethyl *tert*-butyl ether (ETBE), *tert*-butyl alcohol (TBA), isopropanol (IP), *tert*-amyl alcohol (TAA), *tert*-amyl methyl ether (TAME), *tert*-amyl formate (TAF). The arrows indicate different speeds: hollow: fast; dashed: very slow

However, it still contains some only suspected steps. With a degradation rate comparable to that for MTBE, strain PM1 also converts other ethers such as *tert*-amyl methyl ether, ethyl *tert*-butyl ether and diisopropyl ether, as well as alcohols such as *tert*-butyl alcohol and *tert*-amyl alcohol.

Contradictory data are available on the anaerobic degradation of MTBE. In general, anaerobic degradation of MTBE and other oxygenated ethers under methanogenic conditions has rarely been observed. Studies on the degradation potential of different sediments with MTBE and TBA showed success when Fe(III)oxides and humic substances were added to the mixtures. TBA degrades much faster than MTBE under iron-reducing and methanogenic conditions. Anaerobically, degradation of TBA is relatively rapid and complete. The degradation rates are comparable to that of aerobic TBA degradation. In general, it is highlighted that MTBE must be classified as a substance whose degradation is very difficult under anaerobic conditions.

Furthermore, **cometabolic conversion of** MTBE by bacteria and fungi is possible. MTBE cometabolism is carried out especially by microorganisms that grow on short-chain alkanes ($<C_8$). Here, the ability of growth with *iso*-alkanes *is* of particular interest. There are a variety of co-substrates that support the degradation of MTBE, such as alkanes, aromatics and cyclic compounds. The initiating step in cometabolism is thought to be carried out by cytochrome P-450 monooxygenases. The formation of *tert*-butyl formate shall precede TBA accumulation.

6.9 Glyphosate

Glyphosate is a compound from the group of phosphonates. It is the biologically active main component of some broad-spectrum or total herbicides. Since the second half of the 1970s, it has been marketed by Monsanto as an active ingredient under the name *Roundup* for weed control. Worldwide, it has been the most important ingredient in herbicides by volume for years. Glyphosate products are now sold by more than 40 manufacturers. The quantity produced was estimated at 600,000 tonnes in 2008, 650,000 tonnes in 2011 and 720,000 tonnes in 2012. Glyphosate is used in agriculture, horticulture, industry and private households.

Glyphosate has a non-selective effect against plants. It is a competitive inhibitor of phosphoenolpyruvate of the plant 5-enolpyruvylshikimate-3-phosphate synthase and thus prevents the biosynthesis of the aromatic amino acids phenylalanine, tryptophan and tyrosine as well as some benzoic acid derivatives. This means that all plants treated with it die. Exceptions are crops that have been genetically modified to be herbicide resistant to glyphosate.

Compared to other herbicides, glyphosate usually has lower mobility, shorter lifetime and lower toxicity in animals. These are generally desirable properties for herbicides used in agriculture.

The degradation of glyphosate in soil is generally rapid. The half-life in different soils varies over a wide range. In addition to biological activity in the soil, there are factors that influence the sorption of glyphosate to the soil and thus affect the rate of degradation.

The occurrence and fate of glyphosate and its major metabolite AMPA were also studied in a lake, Greifensee, Switzerland. Monthly vertical concentration profiles in the lake showed an increase in glyphosate concentrations in the epilimnion from March to July, followed by a very sharp decrease in August. A similar pattern was observed for AMPA. Concentrations of glyphosate and AMPA in the two main tributaries were generally much higher than in the lake. A substantial amount of glyphosate and AMPA in the epilimnion was

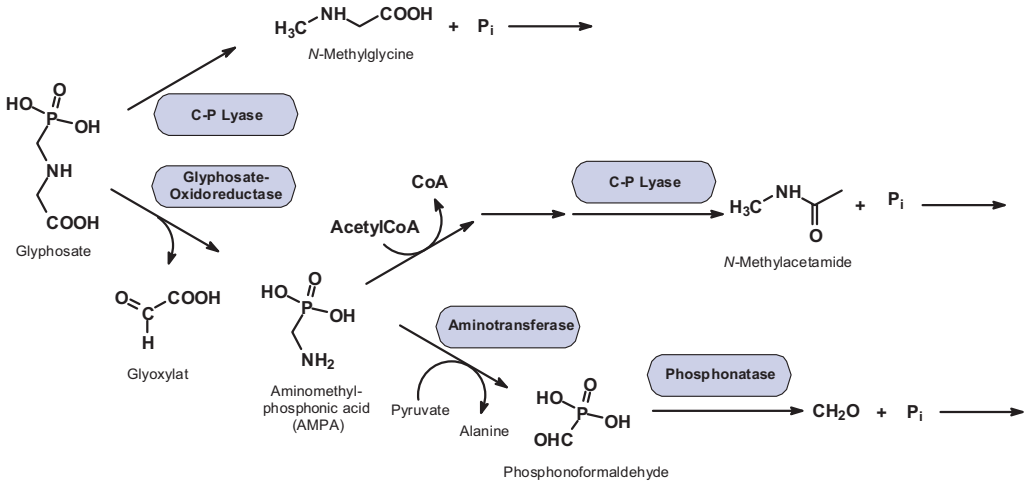


Fig. 6.106 Proposed degradation pathways for glyphosate

removed primarily in July and August, with half-lives of days. The rapid drop in concentrations coincided with high water temperatures and phytoplankton densities and low phosphate concentrations. This indicates that glyphosate was used as an alternative source of phosphorus by bacterio- and phytoplankton. This interpretation is consistent with the knowledge that glyphosate and AMPA utilization is regulated by Pi levels.

Metagenomic analysis of lake water revealed the presence of organisms known to degrade glyphosate and AMPA.

Various mechanisms exist for the degradation of phosphonic acids. Among the various pathways involving cleavage of C–P bonds, the C–P lyase pathway has the broadest substrate specificity and is the only known pathway for the direct release of phosphorus from glyphosate. The *phn* genes encoding the enzymes for this pathway are widely distributed among bacteria. A general pattern of gene organization of C–P lyase pathway-specifying genes, *phn,* has evolved among microorganisms. Despite this general gene organization, there is a marked difference in the specificity of glyphosate use among different organisms.

Further, initial cleavage of glyphosate may result in the formation of AMPA. This latter process appears to be a property of cells cultured in the presence of glyphosate, which over a prolonged period of time may have caused changes in one or more genes to enhance the catalytic property for glyphosate cleavage (Hove-Jensen et al., 2014; Huntscha et al., 2018; Sviridov et al., 2015) (Fig. 6.106).

Test Your Knowledge

- What is the main source of oil contamination in the sea?
- What is Phytan, Pristan, Hopan?
- What are "Tar Balls"?
- Roughly calculate the fate of oil after 3 years at sea.
- What is oxygen needed for in microbial alkane degradation?
- Describe the β-oxidation of fatty acids.
- How does alkane degradation take place when there is no oxygen? What do you think of the comment, "nitrate is added as an oxygen substitute"?
- Do side groups in the alkane hinder the process in the degradation

sequence? Are there ways to eliminate them?
- What is known about the degradation of cycloalkanes?
- Describe the introductory reaction steps in aerobic aromatic degradation by bacteria! What do the fungi do differently?
- How do mono- and dioxygenases differ?
- Which metabolites are suitable for ring cleavage?
- What is extra- and intradiol ring cleavage? Which terms are used synonymously?
- Name the difference between aerobic and anaerobic degradation using toluene as an example!
- What are the key metabolites in anaerobic aromatic degradation?
- What does the reaction sequence in benzoyl-CoA degradation remind you of when you think of alkane degradation?
- Why are PAHs poorly degradable?
- Lignin- and manganese-dependent peroxidase: Which substances are attacked by them? What is the role of H_2O_2? Where does it come from?
- Explain the problems caused by benz[*a*]pyrene in grilled products!
- Describe the degradation of carbazole and dibenzofuran compared to that of naphthalene!
- Distinguish the cleavage of an ester and an ether!
- What do biosurfactants do and how? What is a micelle?
- Sketch dehalorespiration using the example of 3-chlorobenzoate! How is the proton gradient generated?
- What do you mean by the *ortho*-pathway for chlorocatechols?
- What is a cluster, an operon, a plasmid?
- What is transformation, transduction, conjugation when it comes to transferring DNA?
- What is hidden behind the abbreviation HCH?
- Draw atrazine!
- How can trichloroethene be biologically removed from the environment? What are suitable co-substrates for this purpose?
- Compare the conversion of TRI by methane monooxygenase with that by an isoprene utilizer!
- Chlorethenes as electron acceptors: What intermediates occur? Which product is particularly problematic?
- What is the strategy for microbial elimination of a potential hazard from TNT in soil?
- Why do azo dyes contain SO_3H groups?
- Which way of cleaving an azo group is ubiquitously used?
- Describe a syntrophy based on the degradation of 6-aminonaphthalene-2-sulfonic acid!
- What properties should plastics have in order to degrade well?
- Say something about the degradability of complexing agents (see also ► Chap. 15).
- Which endocrine disrupting substances in the environment do you know? Which consequences occur in the ecosystem?
- Is the gasoline additive MTBE allowed in the US? What part of the molecule causes difficulty in degradation?

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The Microbial Nitrogen Cycle

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_7

Numerous important redox reactions of nitrogen in nature are carried out almost exclusively by microorganisms, which is why microbial participation in the nitrogen cycle is of great importance. Nitrogen exists in different oxidation states, ranging from −3 in ammonium to +5 in nitrate. Microbial conversions involve all oxidation states. Some nitrogen compounds are gaseous and can easily escape into the atmosphere (■ Table 7.1).

■ Table 7.2 and ■ Fig. 7.1 summarize the nitrogen compounds relevant to the

■ **Table 7.1** Oxidation states of important nitrogen compounds

Compound/function/process	Oxidation state	Present form under normal conditions
Organic-bound N ($R–NH_2$)	−3	Solid, ion in solution
Ammonia (NH_3)	−3	Gas, solid salt, ion in solution
Hydrazine ($NH_2–NH_2$), N_2-fixation	−2	
Diimine ($NH{=}NH$), N_2-fixation	−1	
Hydroxylamine (NH_2OH), ammonification	−1	
Molecular nitrogen (N_2)	0	Gas
Nitrous oxide (N_2O), denitrification	+1 (average per N)	Gas
Nitric oxide (NO), denitrification	+2	Gas
Nitrite (NO_2^-), electron acceptor	+3	Solid salt, ion in solution
Nitrogen dioxide (NO_2)	+4	Gas
Nitrate (NO_3^-), electron acceptor	+5	Solid salt, ion in solution

■ **Table 7.2** Processes in the nitrogen cycle

Process with function	Organisms
N_2-fixation ($N_2 \rightarrow NH_3$) Symbiotic Free-living (aerobic) Free-living (anaerobic)	*Bradyrhizobium, Frankia, Rhizobium* *Azotobacter, Cyanobacterium* *Clostridium,* sulfur purple bacteria, non-sulfur purple bacteria, green sulfur bacteria
Ammonification (organic N → NH_4^+) Carbon and energy source	Many organisms
Nitrification ($NH_4^+ \rightarrow NO_3^-$) (electron donor, carbon via CO_2-fixation)	*Nitrosomonas, Nitrobacter, Nitrospira*
Denitrification ($NO_3^- \rightarrow N_2$) (electron acceptor, nitrate respiration, energy and carbon from organic substrates)	*Bacillus, Paracoccus, Pseudomonas*
Dissimilatory nitrate reduction to ammonium (DNRA) ($NO_3^- \rightarrow NH_4^+$) (Fermentative, NADH regeneration)	*Wolinella, Desulfovibrio, Enterobacteriaceae, Pseudomonas* and others
Anaerobic ammonium oxidation (ANAMMOX) ($NH_4^+ + NO_2^- \rightarrow N_2$)	*Brocadia anammoxidans, Kuenenia stuttgartiensis*
Assimilatory nitrate reduction	Many organisms

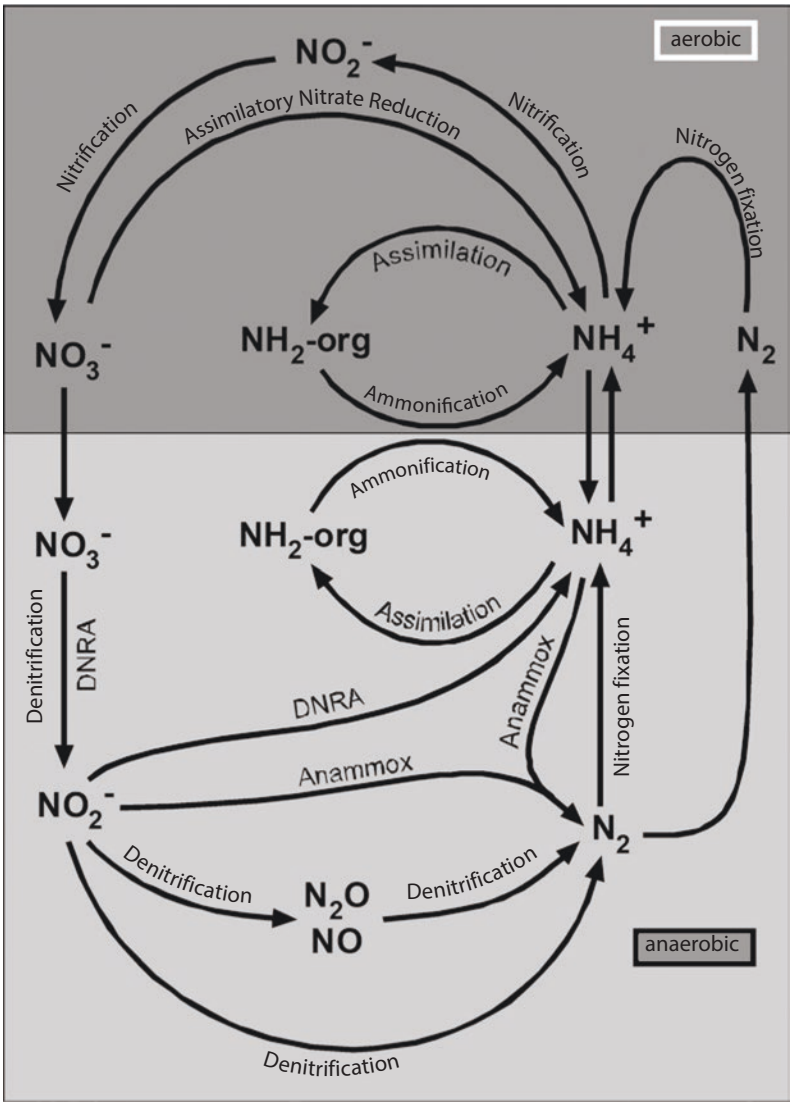


Fig. 7.1 Nitrogen cycle

nitrogen cycle and the metabolites that produce them, with their respective functions for the microorganisms. The individual processes are discussed below.

7.1 Nitrogen Fixation

The biological fixation of atmospheric nitrogen is of great ecological importance, as it provides bound nitrogen to terrestrial and aquatic ecosystems. All organisms are dependent on fixed nitrogen. Fixed nitrogen (ammonium or nitrate) is a limiting nutrient in many ecosystems.

Only prokaryotes are capable of biological nitrogen fixation. Nitrogen-fixing bacteria occur in soils as well as in waters and sediments. Both heterotrophic and autotrophic bacteria have acquired this ability. A number of bacteria perform fixation only in symbiosis with plants. The main groups of

nitrogen-fixing bacteria, free-living and symbiotic species respectively, are summarized in ◘ Tables 7.3 and 7.4. The fixation rates in relation to the soil area show that the nitrogen supply by symbiotic systems is much greater than by the free-living species. Associative symbiosis, in which bacteria are closely associated with plant roots, occupies an intermediate position in terms of fixation rates. However, the rates are subject to very large differences depending on the climatic zone and population density.

◘ **Table 7.3** Representatives of free-living nitrogen-fixing bacteria and some fixation rates for terrestrial systems

Main groups	Selected representatives respectively symbiotic systems	Fixation rate ($kg\ N \cdot ha^{-1} \cdot a^{-1}$)
Anaerobic bacteria	*Clostridium pasteurianum* *Desulfotomaculum nigrificans* *Desulfovibrio vulgaris*	0.5
Facultative anaerobic bacteria	*Klebsiella pneumonia* *Pantoea agglomerans*	
Aerobic bacteria	*Azotobacter vinelandii* *Beijerinckia indica* *Gluconacetobacter diazotrophicus* *Hydrogenophaga pseudoflava* *Methylomonas methanica*	0.3 0.3
Phototrophic anaerobes	*Allochromatium vinosum* *Chlorobium limicola* *Chloroflexus auranticus* *Heliobacterium chlorum* *Rhodospirillum rubrum*	
Cyanobacteria	*Anabaena* sp. *Nostoc* sp. *Synechococcus* sp.	10–30
Methanogenic archaea	*Methanobacterium formicicum* *Methanococcus vanniellii* *Methanothermus facilis*	
Halophilic archaea	*Halobacterium halobium*	

◘ **Table 7.4** Systems of nitrogen fixation between bacteria and plants

Type of symbiosis/bacteria	Plant	Fixation rate ($kg\ N \cdot ha^{-1} \cdot a^{-1}$)
Associative symbioses with grasses		
Azospirillum lipoferum *Azorhizophilus paspali* *Azoarcus* sp.	*Digitaria decumbens* *Paspalum notatum* *Leptochloa fusca* (Callary grass)	20–50 19–50
Endo-symbioses with legumes		

Table 7.4 (continued)

Type of symbiosis/bacteria	Plant	Fixation rate ($kg\ N \cdot ha^{-1} \cdot a^{-1}$)
Rhizobium leguminosarum bv. *viceae*	Pea	100–200
Rhizobium leguminosarum bv. *trifolii*	Clover	100–200
Bradyrhizobium japonicum	Soybean	50–200
Azorhizobium caulinodans	Sesbania (tropical tree)	100
Endo-symbioses of actinomycetes with trees and shrubs		
Frankia alni	*Alnus* (Alder) *Casuaria* (Australian tree) *Eleagnus* (Willow) *Hippophae* (Sea buckthorn)	
Cyanobacterial symbioses with cyanobacteria		
Anabaena azollae	*Azolla* (water fern)	80–250

Biochemistry of Nitrogen Fixation

Dinitrogen (N_2) is a very stable molecule whose reduction requires a high activation energy. The energy of the N–N bond is 930 kJ/mol. Bacteria capable of nitrogen fixation possess the **nitrogenase** enzyme complex. The enzyme consists of two protein components, a reductase that yields electrons with high reducing power, and the nitrogenase proper. Both proteins are composed of subunits. They have several iron-sulfur proteins and the nitrogenase also contains molybdenum (MoFe protein).

The provision of the reduction force is upstream of the fixation process. In electron transfer, ferredoxin, also an iron-sulfur protein, plays a crucial role as a carrier. The electrons are first transferred to nitrogenase reductase. In an ATP-dependent process, the redox potential is lowered to such an extent that further electron transfer to nitrogenase results in a highly reduced reaction system. At this system, the so-called iron-molybdenum cofactor (FeMo-Co), N_2 binding and stepwise reduction to ammonium takes place. The reaction is very energy-consuming; for example, 16 molecules of ATP are required to reduce one N_2. Thus, about 1 mol of glucose is required to reduce 1 mol of N_2. The overall reaction is:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

The reaction shows that the nitrogenase complex not only reduces N_2 to NH_3, but also simultaneously reduces protons to H_2. This is a partial step inherent to the process. In many cases, even more H_2 is formed. The increased H_2 formation is then a competitive reaction for the N_2 reduction and up to 50% of the electrons can be transferred to protons (Fig. 7.2).

The following regulatory mechanisms are in place to maintain the economy of cellular metabolism:

- The nitrogenase complex is only formed when there is no bound nitrogen source.
- The synthesis is **repressed** by **ammonium.**
- In the presence of energy deficiency associated with high ADP levels, ADP inhibits nitrogenase activity.

Nitrogenase is irreversibly inactivated by oxygen. At the same time, however, oxygen is required as the final acceptor of the respiratory chain for the production of sufficient ATP. The free-living nitrogen fixers achieve **the protection of nitrogenase from oxygen** by different mechanisms:

- This problem does not exist for **anaerobes** such as *Clostridium pasteurianum* and *Desulfovibrio species.*
- Facultative anaerobes such as *Klebsiella pneumoniae* and purple bacteria bind N_2 only under **anaerobic conditions.**
- The aerobic *Azotobacter* and *Beijerinckia* species perform **respiratory protection.** Oxygen is intercepted by very intensive respiration, which is expressed in a high substrate consumption and a high respiration rate.
- *Azotobacter* additionally possesses a **conformational protection by** which the nitrogenase changes the conformation upon oxygen access in such a way that the sensitive enzyme areas are protected. In this reversible state, it is not active, but is also not inactivated.
- Many filamentous cyanobacteria possess **heterocysts** in which nitrogen fixation takes place. In addition to nitrogenase and an H_2-regenerating hydrogenase, these larger thick-walled cells contain only photosystem I, which provides ATP. Photosystem II, by which oxygen is formed during photolysis of water, is localized in the adjacent vegetative cells along with the other components of the oxygenic photosynthetic system. It provides reduction equivalents and assimilates to the heterocysts.
- Unicellular cyanobacteria such as *Gloeocapsa* sp. achieve oxygen protection not by spatial but by **temporal separation.** They fix N_2 at night, when there is only a very low oxygen partial pressure in the cell due to photosynthesis not taking place.

Symbiotic systems show high fixation rates. This is due to the effective supply of organic substrates to the bacteria by the host. Bacteria (rhizobia and others) reside as irregularly shaped **bacteroids** within the cytoplasmic membrane of plant cells. The bacterial cells that have become meristematic thus form the **root nodules.** Nitrogenase, which accounts for up to 10% of the soluble protein, is synthesized by the bacteroids, while the plant supplies the bacteroids with assimilates (mainly organic acids). The bacteroids release the ammonium formed into the surrounding plant cytoplasm, where amino acid synthesis occurs. The plant contributes to the symbiosis not only by supplying the organic carbon sources, but also by synthesizing leghemoglobin. Leghemoglobin is a compound similar to hemoglobin that acts as an oxygen carrier. It ensures a low oxygen partial pressure, which on the one hand still allows respiration and thus ATP formation, but on the other hand protects nitrogenase from oxygen. Leghemoglobin is a symbiotic product, so the protein part is formed by the plant, the heme part by the bacteroid.

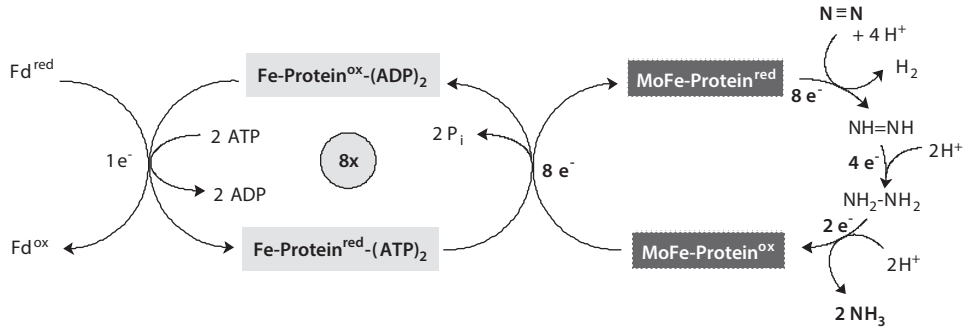


Fig. 7.2 Model of the nitrogenase reaction. Fd, ferredoxin

7.2 Ammonification

Organically bound nitrogen is released as ammonium when organisms die and from animal excreta through proteolysis, nucleic acid degradation and subsequent ammonification.

Ammonium is the nitrogen compound formed during the aerobic and anaerobic decomposition of organic substances in soils and waters.

In soils, much of the ammonia released by aerobic decomposition is rapidly recycled and converted to amino acids in plants and microorganisms. Some of the nitrogen compounds produced by protein and nucleic acid degradation enter humus formation (see ► Sect. 4.4), and humus provides a store of fixed nitrogen that becomes bioavailable again during humus degradation.

Under anoxic conditions, ammonia is stable, and this is the main form in which nitrogen occurs in most anoxic sediments.

Ammonium is present as NH_4^+ at neutral pH, and even under weakly alkaline conditions it can escape into the atmosphere as volatile ammonia. It is released in particularly high concentrations from factory farms and in areas with dense animal populations (for example, cattle pastures). Globally, ammonia accounts for only about 15% of the nitrogen released to the atmosphere. Most of the rest enters the atmosphere in the form of N_2 or N_2O (from denitrification).

7.3 Nitrification

Nitrification, the oxidation of NH_3 to NO_3^-, is an important process in nature and occurs readily in well-drained soils at neutral pH through the activity of nitrifying bacteria. Under aerobic conditions, ammonium is also oxidized to nitrate via nitrite by nitrification in water bodies.

The nitrifying bacteria live chemolithotrophically, they gain their energy through the oxidation of ammonium or nitrite to nitrate.

Two aerobic groups of bacteria are involved in nitrification, which carry out the following overall reaction:

$$NH_3 + 2O_2 \rightarrow NO_3^- + H_2O + H^+$$

The ***Nitrosobacteria*** oxidize ammonia to nitrite. The biochemistry of oxidation has been studied in detail for *Nitrosomonas europae*.

The reaction proceeds via hydroxylamine and a (NOH) intermediate, yielding nitrite as the final product:

$$NH_3 \rightarrow NH_2OH \rightarrow (NOH) \rightarrow NO_2^-$$

The first step is catalyzed by ammonium monooxygenase. That is, the oxygen in the hydroxylamine comes from molecular oxygen. The reaction consumes reducing power:

$$NH_3 + 2e^- + 2H^+ + O_2 \rightarrow NH_2OH + H_2O$$

The physiological donor appears to be a compound of the respiratory chain. Hydroxylamine dehydrogenase, a periplasmic enzyme that reduces periplasmic cytochrome c, catalyzes the reaction:

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-$$

Since the electrons of hydroxylamine oxidation are probably provided at the level of ubiquinones, one suspects that the reduced ubiquinones provide the reducing power for the monooxygenase reaction *in vivo*.

Thus, two electrons flow into the ammonium monooxygenase and the remaining two into the respiratory chain:

$$½O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

Thus, the overall reaction:

$$NH_3 + 1½O_2 \rightarrow HNO_2 + H_2O$$

Consequently, for the oxidation from ammonium to hydroxylamine, the cell must use two energy-rich electrons, which, on the other hand, would be available for energy generation. The provision of electrons from the hydroxylamine at the level of ubiquinones also makes it clear that an energy-dependent, retrograde electron transport is needed to generate NAD(P)H for CO_2 fixation (◘ Fig. 7.3). If one takes the low energy yield of ammonium oxidation and the fact of electron transport as membrane-bound, then the formation of extensive membrane systems can be explained as an adaptation to allow the complex reactions to proceed at a sufficiently high rate.

Nitrobacteria oxidize nitrite to nitrate. Oxidation by *Nitrobacter species* proceeds according to the following half-reactions:

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

$$½O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

Nitrite/nitrate oxidoreductase is localized on the inner side of the cytoplasmic membrane. A proton motor force is established by a proton-pumping cytochrome oxidase. Of course, *Nitrobacter* must also have a complete electron transport system to accomplish the formation of NAD(P)H by retrograde electron transport.

The end product of the first process is therefore the output product of the second process.

Nitrifiers are slow-growing bacteria that have a high metabolic rate due to their low energy yield.

Nitrifying bacteria are widespread, preferring a neutral to alkaline pH range.

Environmental problems related to nitrification result from the increased input of nitrogen compounds into the biosphere. Natural nitrification causes the breakdown of minerals, for example the release of potassium, calcium and phosphate, through the formation of nitric acid. Nitric efflorescence in stables and cellars are often end products of nitrifying bacteria.

However, the process, which contributes to soil formation and soil fertility, also causes corrosion of structures made of sandstone and cement. Some nitrifiers colonize porous sandstones to a depth of several centimeters. This endolithic lifestyle is made possible by ammonium emissions, mainly from factory farming of pigs and poultry. Acid formation thus leads to the destruction of the sandstone structure of historic buildings (see ► Chap. 13).

The widespread overfertilization of agricultural land with ammonium compounds, urea and liquid manure leads to strong nitrification. Ammonia is cationic and is strongly absorbed by negatively charged clay minerals and humus. On the other hand, the nitrate formed, although readily assimilated by plants, is leached into groundwater horizons

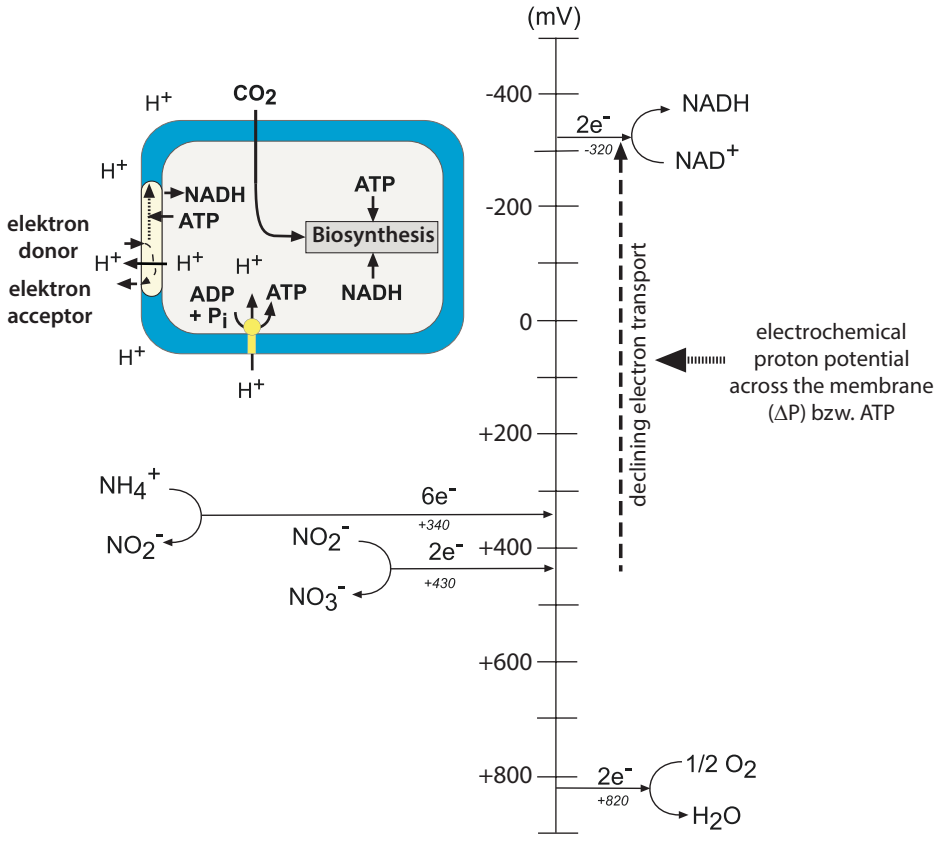


Fig. 7.3 Different energy levels during nitrification

due to its very high water solubility. The consequences of the leaching of nitrite and nitrate are a loss of nitrogen for plant nutrition, therefore nitrification is not beneficial in agricultural practice. Furthermore, an alarming accumulation of nitrate in groundwater leads to a questioning of its use as drinking water.

Factory farming as a source of ammonium contributes to another environmental problem, the over-fertilisation of forests, which is reflected in the recent damage to forests.

Nitrification Without Division of Labour

For decades, it has been assumed that ammonia and nitrite oxidation, **nitrification** are catalyzed as a concerted activity by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and by nitrite-oxidizing bacteria (NOB).

Recently, complete ammonia oxidizers ("Comammox"), which oxidize ammonia to nitrate alone without division of labor with another microorganism, have been identified in the bacterial genus *Nitrospira*, which was previously thought to contain only canonical nitrite oxidizers.

The question arises as to ecological niches in which Comammox *Nitrospira* successfully compete with canonical nitrifiers.

A pure culture of a comammox bacterium, *Nitrospira inopinata, has* now been isolated and characterized. This isolate has been shown to be adapted to growth in oligotrophic and dynamic habitats based on a

high affinity for ammonia. It has a low maximum rate of ammonia oxidation but high growth yield compared to canonical nitrifiers. In comparison, four AOA isolated from soil and hot springs showed the following nitrification kinetics: surprisingly poor substrate affinities and lower growth yields. Thus, contrary to previous assumptions, AOAs are not necessarily the most competitive ammonia oxidizers in highly oligotrophic environments. *Nitrospira inopinata* has the highest substrate affinity of all ammonia oxidizer isolates analyzed, with the exception of the marine AOA *Nitrosopumilus maritimus.* The results suggest an important role of Comammox organisms in nitrification under oligotrophic and dynamic conditions.

Nitrospira is widely distributed in nature, but cultivation-independent tools to distinguish Comammox with strictly nitrite-oxidizing *Nitrospira* are required for assessing the distribution and functional significance of Comammox *Nitrospira* in ecosystems.

New PCR primer sets have been developed that specifically target the *amoA genes* encoding subunit A of the various ammonia monooxygenases of Comammox *Nitrospira.* The new primer set covers up to 95% of the comammox amoA clade A and 92% of the clade B sequences in a reference database of 326 comammox amoA genes with sequence information at the primer binding sites.

Application of the primers to 13 samples from engineered systems (groundwater wells, drinking water treatment and wastewater treatment plants) and other habitats (rice and forest soils, rice rhizosphere, brackish lake sediment and freshwater biofilm) detected comammox *Nitrospira* in all samples and revealed considerable diversity of comammox in most habitats.

Quantitative PCR with equimolar primer mixtures was highly sensitive and specific and allowed efficient quantification of Clade A and Clade B comammox *amoA* gene copy numbers in environmental samples.

The relative abundance of Comammox *nitrospira,* compared to canonical ammonia oxidizers, was highly variable in different environments.

(Daims et al., 2015; Kits et al., 2017; Martens-Habbena et al., 2009; Pjevac et al., 2017; van Kessel et al., 2015).

7.4 ANAMMOX

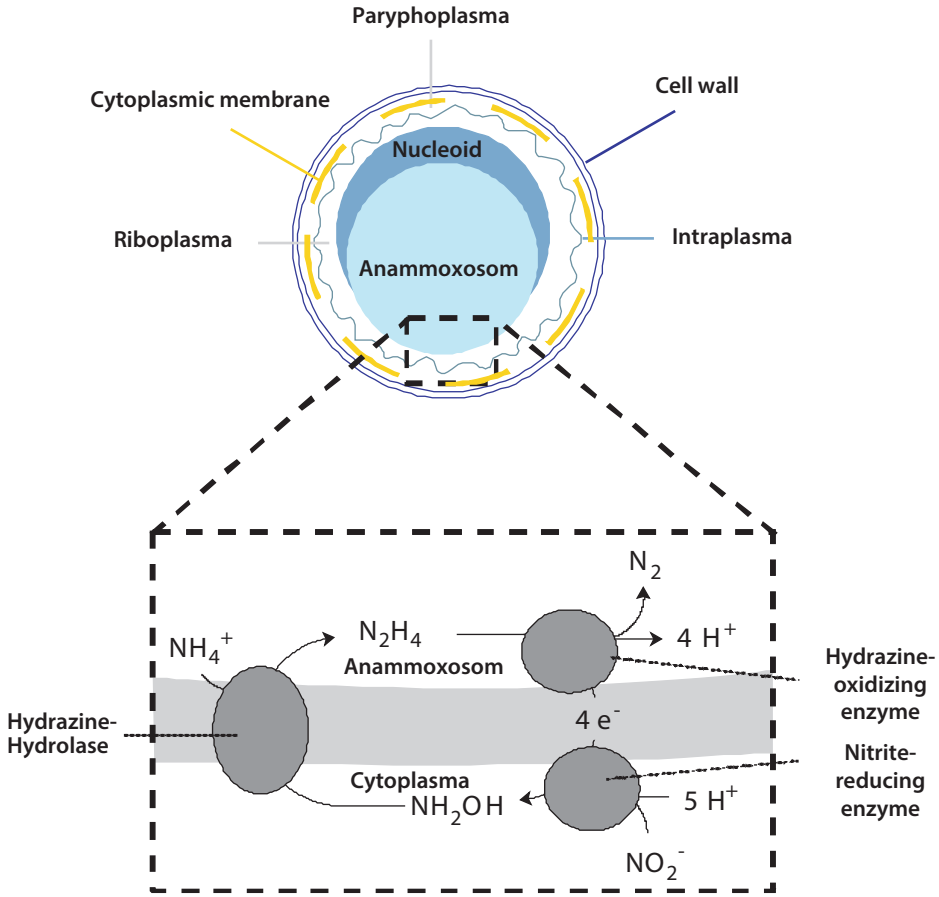
In addition to aerobic oxidation of ammonium by nitrifiers, oxidation in anaerobic environments has recently been found. **Anaerobic ammonium oxidation (ANAMMOX)** is carried out by very slow-growing organisms. It requires nitrite as an oxidizing agent in addition to ammonium as an inorganic electron donor. It takes place in wastewater treatment plants with a high carbon load. This process is discussed in the area of the transition from oxic to anoxic ecosystems, where nitrite and ammonia occur in the absence of oxygen.

Anaerobic ammonium oxidation is the microbial oxidation of ammonium with nitrite to N_2 under strictly anoxic conditions.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \Delta G^{0'} = -335\text{kJ / Ammonium}$$

It is carried out by planctomycete-like bacteria. Anammox is not only important for the oceanic nitrogen cycle, but is also substantially involved in nitrogen removal from municipal and industrial wastewater.

The process occurs in a wide range of environments, including marine sediments, ice at sea and other anaerobic water columns. It is thought to be responsible for up to 50% of the global removal of fixed nitrogen from the oceans (◘ Fig. 7.4).



◘ **Fig. 7.4** Morphology of the anammox cell and proposed model of the anammox process

7.5 Nitrate Reduction

Nitrate can be reduced to ammonium by **assimilatory nitrate reduction** and thus enter into organic nitrogen compounds. It thus serves the microorganisms and plants to build up amino acids and other N-containing cell building blocks.

In contrast, the function of **dissimilatory nitrate reduction** is energy production. It is also called **nitrate respiration,** since nitrate serves as an electron acceptor for a form of respiration under anaerobic conditions.

Two pathways of nitrate and nitrite respiration are known: **Denitrification** and **ammonification** (◘ Fig. 7.5).

7.5.1 Denitrification

Denitrification produces elemental nitrogen and, in small quantities, also N_2O. In the global context, this process is the main pathway by which gaseous N_2 is produced biologically.

The following reduction steps are involved in denitrification:

$$NO_3^- + 2e^- + 2H^+ \rightarrow$$
$$NO_2^- + H_2O \ (\text{Nitrate reductase})$$
$$NO_2^- + e^- + 2H^+ \rightarrow$$
$$NO + H_2O \ (\text{Nitrate reductase,NO forming})$$
$$2NO + 2e^- + 2H^+ \rightarrow$$
$$N_2O + H_2O \ (\text{Nitrogen oxide reductase})$$
$$N_2O + 2e^- + 2H^+ \rightarrow$$
$$N_2 + H_2O \ (\text{Dinitroge oxide reductase})$$

Nitrate and NO reductase are localized in the cytoplasmic membrane, whereas nitrite and N_2O reductase are periplasmic.

At the onset of anaerobiosis, N_2O is initially formed preferentially, as the enzyme following in the metabolic pathway, N_2O reductase, is formed more slowly. Increased N_2O formation occurs primarily in the presence of excess nitrate and a lack of electron donors.

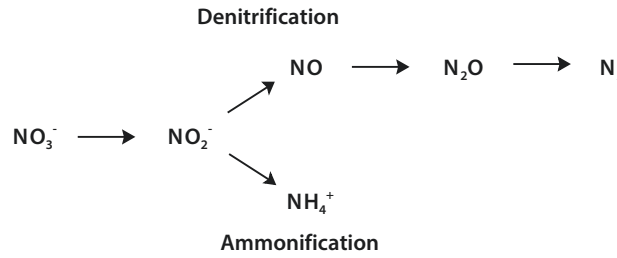
In this way, the denitrifying bacteria can completely degrade organic substrates in the absence of atmospheric oxygen; the energy gain achieved in this process is approximately equal to that of respiration.

The ability to denitrify is widespread among soil and water bacteria, important representatives are Pseudomonads, *Bacillus licheniformis, Paracoccus denitrificans.* Denitrificans are **facultatively anaerobic;** in the absence of oxygen**,** enzymes are induced to gradually reduce nitrate to N_2. In soil, this condition occurs during waterlogging when nitrate is present. This results in **nitrogen loss from the soil.**

Some bacteria, for example *Escherichia coli*, perform a variant of nitrate respiration. They reduce the nitrate only to the level of nitrite (nitrate/nitrite respiration).

Because microorganisms are much less able to use N_2 as a nitrogen source, denitrification is a harmful process because it removes fixed nitrogen from the environment.

In wastewater treatment, denitrification is a useful process because it removes nitrate from the water, minimizing algae growth when the water is released into lakes and rivers.



◘ **Fig. 7.5** Ways of nitrate/nitrite reduction

7.5.2 Dissimilatory Nitrate Reduction to Ammonium

Also starting from the NO_3 pool, **dissimilatory nitrate reduction to ammonium (DNRA)** reduces nitrate directly to NH_4^+.

The first step in this reaction, the reduction of nitrate to nitrite, is the energy-producing step. It is identical to that of denitrification:

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$$
$$(\text{Nitrate reductase})$$

The further reduction of nitrite to ammonium is catalyzed by an NADH-dependent reductase.

$$NO_2^- + 3NAD(P)H + 5H^+ \rightarrow$$
$$NH_4^+ + 3NAD(P)^+ + 2H_2O$$
$$(\text{Nitrite reductase, ammonia forming})$$

This second step does not provide additional energy, but it leads to fixed nitrogen and makes reduction equivalents available again through the reoxidation of NADH to NAD^+. These reduction equivalents are then needed for the oxidation of carbohydrates, for example.

Indeed, it has been observed that under carbon limiting conditions, nitrite accumulates (denitrification prevails), while ammonium is the main product in the presence of large amounts of carbon (DNRA prevails).

The conversion is carried out by facultative or obligate fermenting bacteria. In addition to a lack of oxygen, a high supply of organic carbon and available NO_3^- as well as a low redox potential must be present as conditions for the DNRA process. The importance of DNRA in terrestrial ecosystems is underestimated. This thesis is confirmed by Silver et al. (2001), who demonstrated DNRA rates of up to 0.9 mg N/kg dry weight of soil · day for tropical forests. This value was higher by a factor of three than the N turnover via denitrification (up to 0.3 mg N/kg dry weight of soil · day).

A second environmental factor that selects for DNRA is a low level of available electron acceptor. It is therefore not surprising that the process takes place mainly in saturated, carbon-rich environments such as stagnant waters, sewage sludge, some sediments with a high content of organic material, and the ruminant stomach.

Table 7.5 lists various bacteria that carry out DNRA. It is noticeable that most bacteria have a fermentative and hardly any oxidative metabolism.

Table 7.5 Bacteria using dissimilatory nitrate or nitrite reduction to ammonium (DNRA)

Behaviour towards oxygen/ genus	Typical habitats
Obligate Anaerobic	
Clostridium	Soil, sediments
Desulfovibrio	Sediments
Selenomonas	Rumen
Veillonella	Intestinal tract
Wolinella	Rumen
Optional Anaerobic	
Citrobacter	Soil, waste water
Enterobacter	Soil, waste water
Erwinia	Soil
Escherichia	Soil, waste water
Klebsiella	Soil, waste water
Photobacterium	Seawater
Salmonella	Waste water
Serratia	Intestinal tract
Vibrio	Sediment
Microaerophilic	
Campylobacter	Oral cavity
Aerobic	
Bacillus	Soil, food
Neisseria	Mucous membranes
Pseudomonas	Soil, water

Test Your Knowledge

- What is the important function of denitrifiers in the nitrogen cycle in nature?
- What would happen if there was no denitrification?
- Why do denitrifiers play an essential role in wastewater treatment in sewage plants?
- Why is denitrification undesirable in agriculture?
- How do assimilatory and dissimilatory nitrate reduction differ in function?
- What gases can be released during denitrification?
- What is the function of nitrifiers in the nitrogen cycle?
- What carbon source do nitrifiers use and how do they convert it into cellular components?
- Why do nitrifiers have to do retrograde electron transport?
- Say something about the growth rate of nitrifiers.
- Using the oxidation states of nitrogen, state the total number of electrons released in the oxidation of ammonium to nitrate.
- What does chemolithotrophy mean?
- What is ANAMMOX?
- Where is a problem with nitrogen fixation when you think about the consumption of a lot of ATP and the oxygen sensitivity of nitrogenase? What solutions to this contradiction have organisms found?
- Who operates the "COMAMMOX" process?

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Cycles of Sulfur, Iron and Manganese

Contents

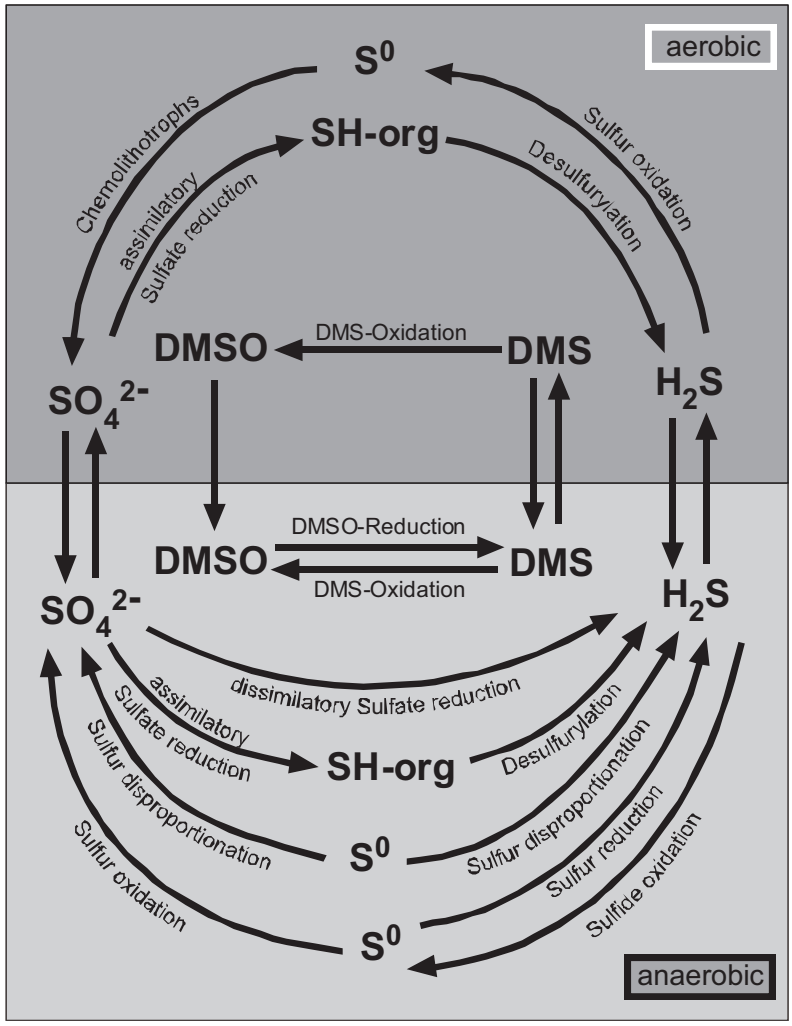
W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_8

8.1 Sulfur Cycle

Conversions of sulfur are even more complex than those of nitrogen because of the different oxidation states of sulfur and the fact that some conversions occur at considerable rates, both chemically and biologically. The redox cycle for sulfur and the involvement of microorganisms in sulfur transformations are shown in ◘ Fig. 8.1. Although a large number of oxidation states are possible, only the following form significant amounts in nature: −2 (sulfhydryl, R-SH, and sulfide, HS^-), 0 (elemental sulfur, S^0), and +6 (sulfate, SO_4^{2-}) (◘ Tables 8.1 and 8.2).

8.1.1 Sulfate Reduction

A common volatile sulfur gas is hydrogen sulfide (H_2S). It is formed by bacterial reduction of sulfate (◘ Fig. 8.1) or originates from geochemical sources, such as sulfide springs or volcanoes. The form in which sulfide occurs in the environment is pH-



◘ **Fig. 8.1** Sulfur cycle

Table 8.1 Naturally occurring sulfur compounds and their oxidation states

Oxidation state	Gas	Aerosol	Water	Soil	Mineral	Biological
−2	H_2S, RSH, DMS, OCS		H_2S, HS^-, S^{2-}, RS^-	HS^-, S^{2-}	S^{2-}	Methionine, Cysteine, Glutathione
−1					FeS_2	
0				S_8	S^0	
+2 (average per S)			$S_2O_3^{2-}$			
+4	SO_2	HSO_3^-	HSO_3^-, SO_3^{2-}	SO_3^{2-}		
+6	SO_3	HSO_4^-, SO_4^{2-}	HSO_4^-, SO_4^{2-}	$CaSO_4$	$CaSO_4$	

DMS Dimethyl sulfide, *OCS* Carbonyl sulfide (in the troposphere)

Table 8.2 Processes in the sulfur cycle

Process with function	Organisms
Sulfate reduction ($SO_4^{2-} \rightarrow H_2S$) Electron acceptor in anaerobic respiration	*Desulfobacter, Desulfovibrio*
Sulfur reduction ($S^0 \rightarrow H_2S$) Electron acceptor in anaerobic respiration	*Desulfuromonas,* many hyperthermophilic archaea
Sulfide/sulfur oxidation ($H_2S \rightarrow S^0 \rightarrow SO_4^{2-}$) Aerobic: sulfur chemolithotroph, electron donor Anaerobic: electron donor	*Beggiatoa, Paracoccus, Thiobacillus* Sulfur purple bacteria, green sulfur bacteria, some chemolithotrophs.
Sulfur disproportionation ($S_2O_3^{2-} \rightarrow H_2S + SO_4^{2-}$)	*Desulfovibrio,* and others
Reduction of organic sulfur compounds (DMSO → DMS) Electron acceptor in anaerobic respiration	*Campylobacter, Escherichia, Wolinella succinogenes*
Oxidation of organic sulfur compounds ($CH_3SH \rightarrow CO_2 + H_2S$)	Phototrophic or chemotrophic sulfur-oxidizing bacteria
Desulfurylation (organic-S → H_2S)	Many organisms

CH_3SH Mercaptan, *DMSO* Dimethyl sulfoxide

dependent: below pH 7, H_2S predominates; HS^- and S^{2-} occur above pH 7.

Sulfate-reducing bacteria (sulfate or sulfur as electron acceptor, sulfate/sulfur respiration) are widespread in nature, but their activities are limited in many anoxic biotopes such as freshwater and many soils by the low concentrations of sulfate there. This limitation is not present in the ocean (~28 mM SO_4^{2-}).

Sulfate-reducing bacteria are a large, phylogenetically very heterogeneous group

Table 8.3 Pylogenetic groups of sulfate reducers

Gram-negative, mesophilic *Deltaproteobacteria*
Desulfobacter postgatei
Desulfovibrio desulfuricans
Gram-positive endospore formers
Desulfotomaculum acetoxidans
Desulfosporosinus orientis
Thermophilic bacteria
Thermodesulfobacterium commune
Thermodesulforhabdus norvegica
Thermophilic archaea
Archaeoglobus fulgidus
Archaeglobus veneficus

of anaerobic bacteria. Many of them are strictly anaerobic; however, some survive exposure to oxygen, and a few can even use oxygen.

Based on analyses of the 16S rRNA gene sequence, sulfate-reducing bacteria are classified into four phylogenetic groups (see Table 8.3). Sulfate-reducing bacteria are found among both Gram-negative and Gram-positive bacteria, and some of them belong to the Archaea. Even endospore-forming representatives are known.

Because of the need for organic electron donors (or molecular hydrogen, which is a product of the fermentation of organic compounds) to operate sulfate reduction, sulfide is produced only when significant amounts of organic material are present. In many marine sediments, the rate of sulfate reduction is limited by carbon and can be greatly increased by the addition of organic material. This is of considerable importance for marine pollution because the discharge of sewage, sewage sludge and garbage into the sea can lead to significant increases in organic matter in sediments. Since HS^- is a toxic substance for many organisms, the formation of HS^- by sulfate reduction is potentially harmful. A common detoxification mechanism for sulfide in the environment is its association with iron, resulting in the formation of insoluble FeS. The black colour of many sediments where sulfate reduction occurs is due to the accumulation of FeS.

The reaction sequence of **dissimilatory sulfate reduction (sulfate respiration)** is shown in Fig. 8.2.

The reduction of SO_4^{2-} to H_2S proceeds through several intermediate steps. Sulfate, after being transported into the cell, is activated by ATP, forming adenosine-5′-phosphosulfate, an anhydride. Then sulfite is formed from APS by APS reductase. Further reduction by sulfite reductase produces H_2S. A total of 8 electrons are taken from the respiratory chain by the two enzymes, which are cytoplasmic enzymes. Thus, the process of dissimilatory sulfate reduction is part of the electron transport process leading to the formation of a proton motor force and then ATP synthesis by ATPase.

Sulfate-reducing bacteria are not only phylogenetically but also metabolically very heterogeneous. A distinction is made between the following groups:

- **Representatives of one group are able to utilize acetate.** A complete oxidation to CO_2 and H_2 takes place. The conversion is carried out by enzymes that are known from the reductive acetyl-CoA pathway of CO_2 fixation. In such bacteria, this also provides the prerequisite for degrading acetyl-CoA, which results from the degradation of long-chain fatty acids by β-oxidation, to CO_2. Representatives of the group are *Desulfobacter, Desulfobacter postgatei, Desulfobacterium, Desulfococcus, Desulfonema* and *Desulfotomaculum acetoxidans.*
- **Representatives of another group can not oxidize acetate.** The incomplete oxidation of lactate, propionate and primary alcohols such as ethanol leads to acetate and CO_2. These bacteria almost always also use molecular hydrogen (H_2) as an electron donor, and some may even use benzoate. As mentioned, lactate is a common substrate of sul-

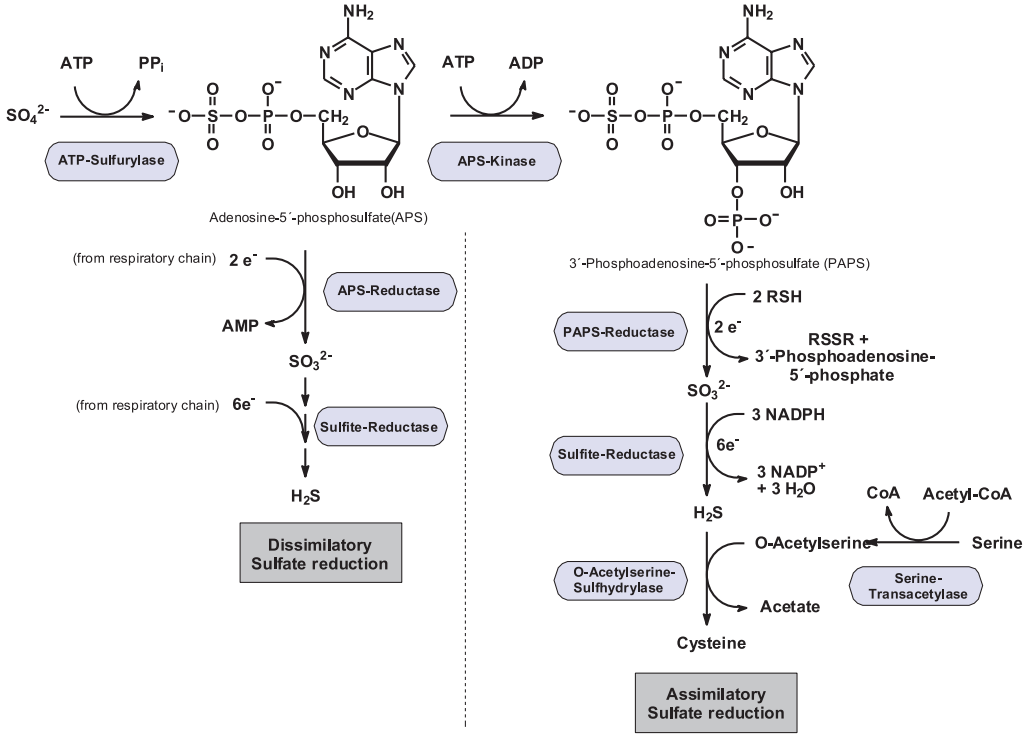


Fig. 8.2 Reactions of sulfate reduction. dissimilatory (left), assimilatory (right)

fate-reducing bacteria. In *Desulfovibrio desulfuricans,* lactate is oxidized to pyruvate by a lactate dehydrogenase. This is converted to CO_2 and acetyl-CoA by pyruvate:ferredoxin oxidoreductase, which is then converted to acetate via acetyl-phosphate by phosphotransacetylase and acetate kinase. In the last step, energy is obtained by substrate step phosphorylation. In total, four reduction equivalents are released per molecule of lactate; thus, the oxidation of two molecules of lactate is required to reduce one molecule of sulfate to sulfide. The group includes representatives of the genera *Desulfobulbus, Desulfomicrobium, Desulfotomaculum* and *Desulfovibrio*.

- **Other sulfate-reducing bacteria use H_2 as facultative chemolithotrophic species.** These include, for example, *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis.*
- **Few sulfate reducers can grow chemolithotrophically, that is, using H_2 as their sole energy source and fixing CO_2.** CO_2 is assimilated via the reductive acetyl-CoA pathway or the reductive tricarboxylic acid cycle. Autotrophic representatives include *Desulfobacterium species*, *Desulfobacter hydrogenophilus, Desulfonema limicola* and *Desulfotomaculum orientis.*

Another expression of the metabolic diversity of sulfate reducers is the ability of many representatives to bind N_2.

Assimilatory sulfate reduction uses sulfate to synthesize sulfur-containing cellular components, for example methionine and cysteine. Microorganisms and plants carry out this assimilatory sulfate reduction, animals depend on organic sulfur compounds.

In the assimilatory reduction, another phosphate residue is attached to the APS, forming 3′-phosphoadenosine-5′-

phosphosulfate. In this intermediate, the sulfate residue is then reduced and sulfite is formed. This is followed by further reduction by sulfite reductase and formation of cysteine as the first sulfur-containing amino acid.

8.1.2 Reduction of Elemental Sulfur

Dissimilatory sulfate reduction to H_2S as a form of anaerobic respiration is an important ecological process, especially among hyperthermophilic archaea. Although sulfate-reducing bacteria can also carry out the reduction of sulfur, in nature most S^0 reduction is probably carried out by S^0 reducers phylogenetically distinct from them, which are incapable of reducing SO_4^{2-} to H_2S . However, the habitats of S^0 reducers are generally also those of sulfate reducers, so the two groups coexist from an ecological standpoint.

8.1.3 Sulfur Disproportionation

Certain sulfate-reducing bacteria can use sulfur compounds of an intermediate oxidation state and perform a special form of energy metabolism, disproportionation. The process refers to the cleavage of, for example, thiosulfate ($S_2O_3^{2-}$) into a more oxidized (sulfate) and a more reduced form (hydrogen sulfide) than the original compound:

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S \qquad \Delta G^{0'} = -22\text{kJ / Reaction}$$

Thus, one sulfur atom of $S_2O_3^{2-}$ is more highly oxidized while the other is further reduced.

8.1.4 Oxidation of Sulfide and Elemental Sulfur

Under oxic conditions, sulfide (HS^-) oxidizes rapidly and spontaneously at neutral pH. Sulfur-oxidizing bacteria are also capable of catalyzing the oxidation of sulfide, but because of the rapid spontaneous reaction, bacterial oxidation of sulfide occurs only in areas where H_2S rising from anaerobic areas meets O_2 sinking from aerobic areas (see also ► Fig. 10.22 Stripe watt). In the aerobic epilimnion, hydrogen sulfide or sulfur is converted to sulfate by **chemolithoautotrophic sulfur oxidizers.** *Thiobacillus* species and the filamentous *Beggiatoa* species occurring in nutrient-rich waters use H_2S as an energy source. The energy is used, among other things, for CO_2 assimilation.

The sulfur compounds most commonly used as electron donors by *Acidithiobacillus* species in their chemolithotrophic metabolism are H_2S, S^0, and $S_2O_3^{2-}$, and the energy-producing reactions are as follows:

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad \Delta G^{0'} = -798\text{kJ / Reaction}$$
$$2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \qquad \Delta G^{0'} = -587\text{kJ / Reaction}$$
$$S_2O_3^{2-} + H_2O + 2O_2 \rightarrow 2SO_4^{2-} + 2H^+ \qquad \Delta G^{0'} = -818\text{kJ / Reaction}$$

If light is available, anaerobic oxidation of H_2S can also take place, catalyzed by **phototrophic sulfur bacteria**. But this only occurs in restricted areas, mostly in lakes, where sufficient light can penetrate the anoxic zones.

So instead of oxidizing water to oxygen, these phototrophs use an analogous oxidation of sulfide to sulfur.

$$2H_2S + CO_2 \rightarrow 2S^0 + (CH_2O) + H_2O$$

The sulfur purple bacteria use H_2S or $S_2O_3^{2-}$ as an external electron donor, sulfur remains and is deposited in the cell as sulfur granules.

Elemental sulfur, S^0, is chemically stable in most environments in the presence of oxygen, but is readily oxidized by sulfur-oxidizing bacteria. Although a variety of sulfur-oxidizing bacteria are known, representatives of the genus *Acidithiobacillus* are most commonly involved in the oxidation of elemental sulfur.

Elemental sulfur is very insoluble, and the bacteria that oxidize it attach themselves firmly to the sulfur crystals. The oxidation of elemental sulfur leads to the formation of sulfate and hydrogen ions. Sulfur oxidation characteristically results in a decrease in pH. Elemental sulfur is occasionally added to alkaline soils to bring about a lowering of pH, relying on the ubiquitous *Thiobacilli* to carry out the acidification process.

8.1.5 Organic Sulfur Compounds

The degradation of sulfur-containing organic matter occurs aerobically to sulfate, and anaerobically to H_2S. Thus, anaerobic protein degradation is a source of H_2S occurring in aquatic sediments. About 5–10% of H_2S comes from this source, 90–95% from dissimilatory sulfate reduction.

In addition to inorganic forms of sulfur, living organisms also synthesize an enormous variety of organic sulfur compounds that also enter the biogeochemical sulfur cycle. Many of these malodorous compounds are highly volatile and can therefore enter the atmosphere.

The most abundant organic sulfur compound in nature is dimethyl sulfide (DSM). It is produced mainly in marine environments as a degradation product of dimethylsulfonium propionate (DMSP).

The primary sources of DMSP in marine surface waters are micro- and macro-algae, although some halophytic plants also form DMSP.

DMSP is produced by phytoplankton, whereby it fulfils various functions. The best known is the osmotic property to regulate cell volume. In some organisms it also functions as an antioxidant, predator repellent and cryoprotectant.

Consistent with its function as an organic osmolyte, DMSP accumulates to very high and osmotically significant concentrations in marine phytoplankton up to an order of magnitude of 0.1–1 M.

DMSP is released from phytoplankton by cellular lysis due to zooplankton grazing, senescence and viral infection.

The importance of DMSP lies not only in its usefulness as a source of reduced sulfur and carbon for marine microorganisms, but especially in the fact that DMSP is the precursor of the climate-relevant gas dimethyl sulfide.

There are two main degradation pathways for DMSP (◘ Fig. 8.3):

(a) The **cleavage pathway** by a DMSP lyase leads to DMS and acrylate and is carried out by eukaryotic algae and many bacteria.

    The **demethylation/demethiolation pathway,** which has only been found in bacteria, leads to the formation of 3-methylmercaptopropionate (MMPA) and a methyl compound. MMPA can be degraded either by the "double demethylation pathway" to 3-mercaptopropio-

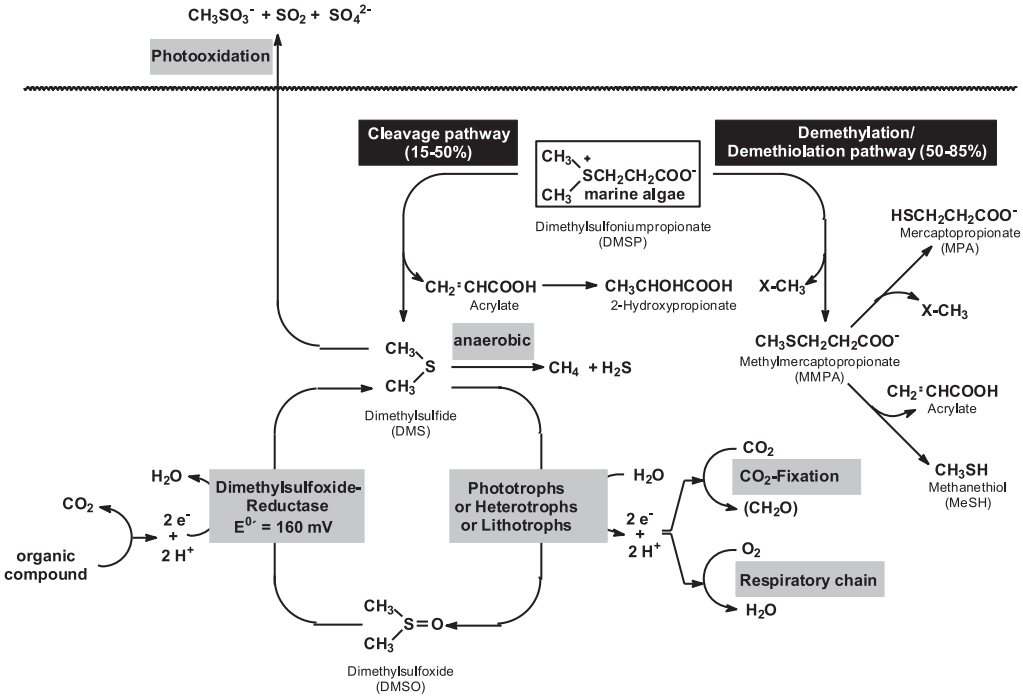


Fig. 8.3 Possibilities of degradation or conversion of dimethylsulfonium propionate, dimethyl sulfide and dimethyl sulfoxide. X-CH_3 : unknown metabolite with a terminal methyl group. It is likely that some metabolites in the degradation of dimethylsulfonium propionate are present as CoA esters (Reisch et al., 2011)

nate (MPA) and a methyl compound or by demethiolation, which leads to methanthiol (MeSH) and acrylate. MeSH can be further metabolized to sulfide and sulfate, or it can serve as a precursor in the synthesis of the sulfur-containing amino acid methionine, thus completing the cycle, since methionine is the precursor in the formation of DMSP (Fig. 8.3).

(b) These two different pathways of DMSP degradation have fundamentally different biogeochemical consequences. The fission pathway leads to the release of DMS, which causes the formation of cloud-forming nuclei and thus counteracts the process of global warming. The demethylation/demethiolation pathway leads to the uptake of the carbon and sulfur components of DSMPs into the microbial cells, thereby increasing the residence time in the biomass.

Roseobacter strains were among the first aerobic bacterial isolates capable of growing with DMSP. They are the only known bacteria that possess both pathways, the cleavage and the demethylation/demethiolation pathway, sometimes in the same organism. They possess a positive chemotactic response to DMSP degradation products. Consequently, *Roseobacter* strains are optimally adapted to algal blooms where DMSP is released locally in large and varying amounts. *Roseobacter* strains are also involved in the transformations of DMS, methanethiol (CH_3SH), methanesulfonate ($CH_3SO_3^-$) and dimethyl sulfoxide (DMSO).

95% of the dimethyl sulfide in the atmosphere comes from the splitting of dimethyl sulphonium propionate and is formed mainly over the ocean.

Dimethyl sulfide released into the atmosphere undergoes photochemical oxidation to methanesulfonate, SO_2 and SO_4^{2-}.

Dimethyl sulfide produced in anoxic biotopes is used microbially as a substrate for methanogenesis, producing CH_4 and H_2S. It is also used as an electron donor for photosynthetic CO_2 fixation in phototrophic purple bacteria, forming DMSO. It further serves as an electron donor for photosynthetic CO_2 fixation in phototrophic purple bacteria, DMSO is thus formed. It is also an electron donor in the energy metabolism of certain chemoorganotrophs and chemolithotrophs, where DMSO is also produced.

Many bacteria, including *Campylobacter, Escherichia, Wolinella succinogenes,* are able to use pyruvate as an electron donor and DMSO as an electron acceptor in the production of energy for anaerobic respiration. Similarly, many purple bacteria in which DMSO can also perform this function. In turn, dimethyl sulfide is formed. DMS has a strong, pungent odor, and the bacterial reduction of DMSO to DMS is indicated by the characteristic odor of DMS.

DMSO is a common natural product and is found in both marine and freshwater environments.

Many other organic sulfur compounds are involved in the sulfur cycle, including methanethiol, dimethyl disulfide (H_3CS-SCH_3), and carbon disulfide (CS_2), yet in global terms the production and consumption of dimethyl sulfide is quantitatively the most important.

Details on Microbial Sulfur Oxidation

Dissimilatory oxidation of reduced inorganic sulfur compounds and elemental sulfur to sulfate is one of the major reactions in the global sulfur cycle and is restricted to prokaryotes. Many lithotrophic microorganisms derive their energy for growth from these reactions.

The sulfur-oxidizing lithotrophic prokaryotes are phylogenetically diverse and belong to the Archaea and Bacteria.

Within the Archaea, sulfur oxidation is restricted to the thermoacidophilic order *Sulfolobales.* In contrast, a large number of lithotrophic, mostly mesophilic bacterial species are known such as *Acidithiobacillus, Aquaspirillum, Aquifex, Bacillus, Beggiatoa, Methylobacterium, Paracoccus, Pseudomonas, Starkeya, Thermithiobacillus, Thiobacillus* and *Xanthobacter.*

In addition, many phototrophic bacteria use reduced sulfur compounds as electron donors for anoxygenic photosynthesis. They include the genera *Allochromatium, Chlorobium, Rhodobacter, Rhodopseudomonas, Rhodovulum* and *Thiocapsa,* which are mainly mesophilic.

Various sulfur compounds such as sulfide, elemental sulfur, sulfite, thiosulfate, and polythionates can serve as substrates. Organisms differ in their ability to use these different sulfur compounds. Oxygen-dependent and -independent pathways of sulfur oxidation carried out by enzyme complexes in bacteria and archaea are described.

Sulfur Oxidation Pathways in Bacteria

The SOX system

The best studied system in terms of proteins and reactions involved is the oxygen-independent SOX sequence (sulfur oxidizing pathway) used in mesophilic bacteria when growing at neutral pH, such as in the *Alphaproteobacterium Paracoccus pantotrophus.*

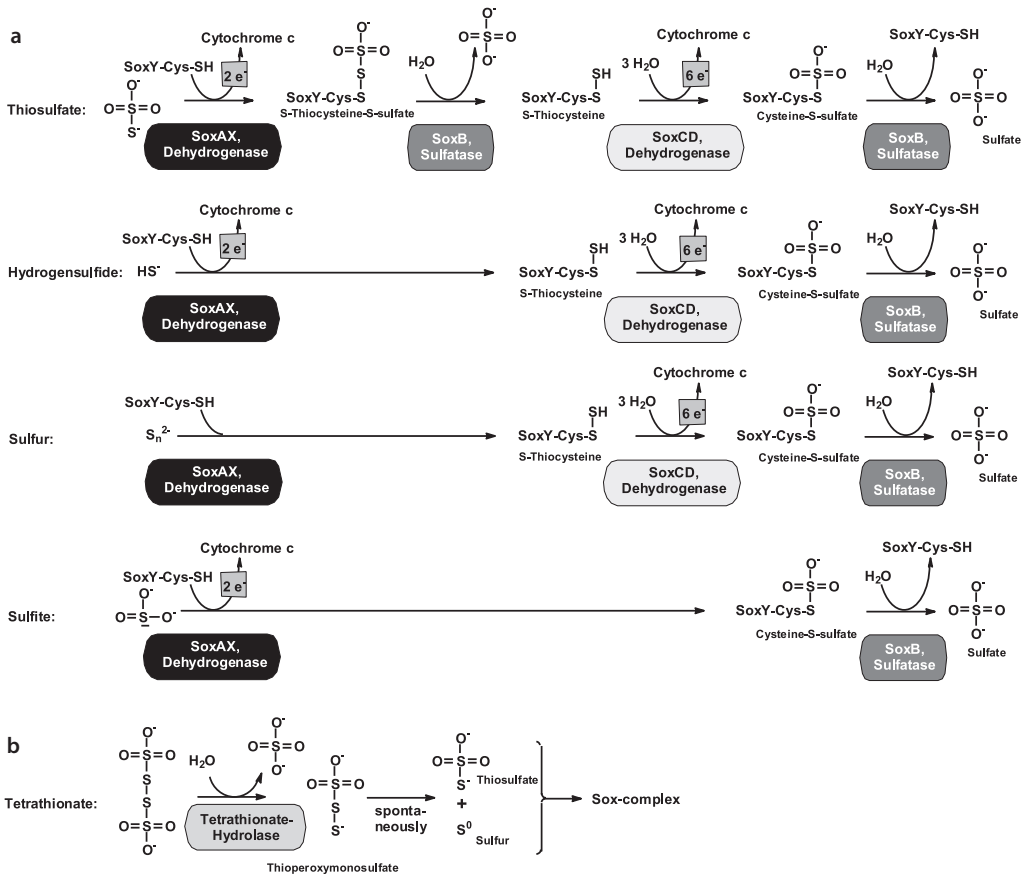
Thiosulfate oxidation to sulfate takes place at the subunits of the periplasmic thiosulfate oxidizing multienzyme complex (TOMES). The oxidation to sulfate proceeds without free intermediates. Each

subunit has a single cysteine whose thiol can form disulfide bonds.

The SoxAX subunit appears to initiatively oxidize and transfer thiosulfate to the substrate carrier protein SoxYZ, forming SoxY-thiocysteine S-sulfate. The SoxB subunit hydrolyzes sulfate from the thiocysteine S-sulfate residue to generate S-thiocysteine. The enzyme subunit SoxCD can then oxidize the outer sulfur atom, producing SoxY-cysteine-S-sulfate. Finally, SoxB hydrolyzes sulfate again, thus completing the cycle by forming back the cysteine residue of SoxY. The sequence is summarized in ◘ Fig. 8.4a.

The reaction sequence of sulfite oxidation is shorter, SoxCD is not required. Sulfite is attached to SoxY by SoxAX to form SoxY-cysteine S-sulfate, which subsequently hydrolytically cleaves sulfate by SoxB.

Sulfide is initially oxidized by SoxXA, an S-thiocysteine residue of SoxY is formed. This is further converted by oxidation and hydrolysis.



◘ **Fig. 8.4** Proposed sulfur oxidation in *Paracoccus pantotrophus*. (After Friedrich et al., 2001). **a** Reaction sequences of the Sox system. **b** Formation of the Sox substrates from tetrathionate. The protons released in the reactions are not shown

Sulfur probably exists as polysulfide (S_n^{2-}) and is bound to SoxY to be released as sulfate after oxidation and hydrolysis like hydrogen sulfide.

A tetrathionate hydrolase hydrolyzes tetrathionate to sulfate and thioperoxymonosulfate (S-S-SO_3^{2-}). The spontaneous decomposition to sulfur and thiosulfate then provides the substrates for the Sox system.

The electrons released in the course of the oxidations are transferred to cytochrome *c* and then reach the terminal oxidase.

The protein subunits SoxYZAB appear to be critical for sulfur oxidation and are present in all sulfur-oxidizing bacteria. *Beta-* and *Gammaproteobacteria* and Chlorobia possess *sox* gene clusters without *soxCD.* The involvement of only a partial *sox cluster* in sulfur oxidation is thus clear.

Sulfur oxidation in phototrophic bacteria

Chlorobiaceae are anoxygenic phototrophic green sulfur bacteria that oxidize hydrogen sulfide to sulfuric acid and deposit sulfur globularly outside the cell in the meantime. The genome of *Chlorobium tepidum,* a moderate thermophile, has a cluster of 13 genes, of which *soxFXYZAB* are homologous to the corresponding genes of *P. pantotrophus.* The *soxCD* genes are absent from the *C. tepidum* genome. The incomplete Sox enzyme system functions for thiosulfate oxidation and results in the release of sulfur (or polysulfide) by the Sox system.

The anoxygenic phototrophic purple bacterium *Allochromatium vinosum* is a *Gammaproteobacterium.* The *soxAXB* and *soxYZ* genes have been detected in it. *A. vinosum* deposits transitional protein-coated sulfur granules in the periplasm as an obligate intermediate during sulfide and thiosulfate oxidation to sulfate. The deposited sulfur is in the form of sulfur chains, possibly as organyl sulfanes (RS_n–R or R–S_n–H with $n \geq 4$).

A common characteristic of *C. tepidum* and *A. vinosum* is the formation of globular sulfur, the absence of the *soxCD* genes *of* the *sox* cluster, and the presence of *dsr* genes. *dsr* gene clusters encode a dissimilatory siroheme sulfite reductase and other proteins. In *A. vinosum,* they are involved in the mobilization of intracellular sulfur deposition during anaerobic sulfur oxidation. The complete *dsr* gene cluster of *A. vinosum* comprises 15 genes, *dsrABEFHCMKLJOPNRS.* The ubiquitous presence of *dsr* genes in anoxygenic phototrophic sulfur bacteria highlights their importance in sulfur oxidation.

Sulfur oxidation in meso-acidophilic bacteria

The best known bacteria that catalyze the oxidation of inorganic sulfur compounds under acidic conditions (pH 1–3) and at elevated temperature (up to 45 °C) are *Acidiphilium* and *Acidithiobacillus* spp.

A general scheme for the oxidation of elemental sulfur in these Gram-negative species is the following (◘ Fig. 8.5): Extracellular elemental sulfur (S_8) is mobilized by thiol groups of special outer-membrane proteins and transported to the periplasmic space as persulfide sulfur. The persulfide sulfur is oxidized to sulfite by periplasmic sulfur dioxygenase (SOR) and further to sulfate by sulfite:acceptor oxidoreductase (SAOR). The latter enzyme most likely uses cytochromes as electron acceptors. Only two of the six electrons transferred from sulfur to sulfate in the redox reactions enter the respiratory chain and can thus be used for ATP synthesis.

The glutathione-dependent sulfur dioxygenase oxidizes exclusively the sulfane sulfur of monoorganyl polysulfanes (RS_nH, $n > 1$), especially persulfide ($n = 2$), but not H_2S.

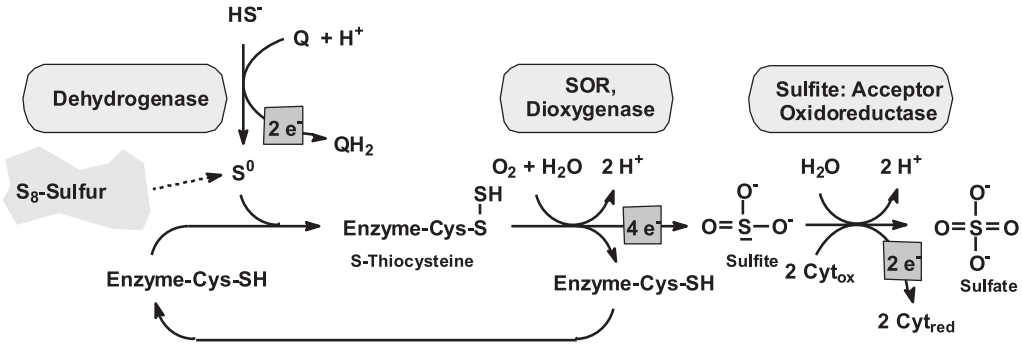


Fig. 8.5 Oxygen-dependent sulfur oxidation in *Acidithiobacillus* sp. (According to Rohwerder & Sand, 2003)

8

Free sulfide is therefore first oxidized to elemental sulfur. This is done by a dehydrogenase (SQR), which uses quinones (Q) as electron acceptors.

Sulfur Oxidation Pathways in Archaea

Members of the *Sulfolobales* colonize terrestrial hydrothermal outcrop channels, solfataras from which H_2S flows, hot springs, and other habitats of volcanic origin and are well adapted to extreme and hostile growing conditions. Inorganic sulfur compounds and elemental sulfur are among the main energy sources in these habitats and are utilized by sulfur-dependent chemolithoautotrophic organisms that form the basis of food chains in these light-independent ecosystems. *Acidianus ambivalens,* a thermoacidophilic and chemolithoautotrophic member of the *Sulfolobales,* within the archaeal kingdom of *Crenarchaeota,* was studied as a model organism for these sulfur-dependent hyperthermophilic Archaea.

A. ambivalens oxidizes sulfur to sulfuric acid for energy production during aerobic growth. The initiating step is catalyzed by the soluble cytoplasmic enzyme, sulfur oxygenase reductase (SOR), which appears to be the only sulfur-oxidizing enzyme in the archaea. Sulfur must be transported from the medium into the cytoplasm across the cytoplasmic membrane. The *A. ambivalens* SOR is active only with air, but not under H_2 or N_2 atmosphere, showing that it is indeed an oxygenase.

The SOR catalyzes the oxygen-dependent formation of sulfite, thiosulfate, and hydrogen sulfide from elemental sulfur. Since no energy is formed during this step, the function of the SOR is most likely to be the generation of soluble sulfur species from the more or less insoluble substrate of elemental sulfur.

The products of the SOR reaction are substrates for membrane-bound enzymes such as thiosulfate:quinone oxidoreductase (TQO) and a sulfite:acceptor oxidoreductase (SAOR), in which substrate oxidation is coupled to energy production.

Understanding on the mechanism of sulfur oxidation in the thermoacidophilic archaeon *A. ambivalens* has made great progress with the discovery of TQO activity and its genes, as well as its identification in *A. ferrooxidans.* TQO catalyzes the formation of tetrathionate from two molecules of thiosulfate, directing electrons into the quinone pool of the cytoplasmic membrane. The further pathway of the tetrathionate is not known (Fig. 8.6).

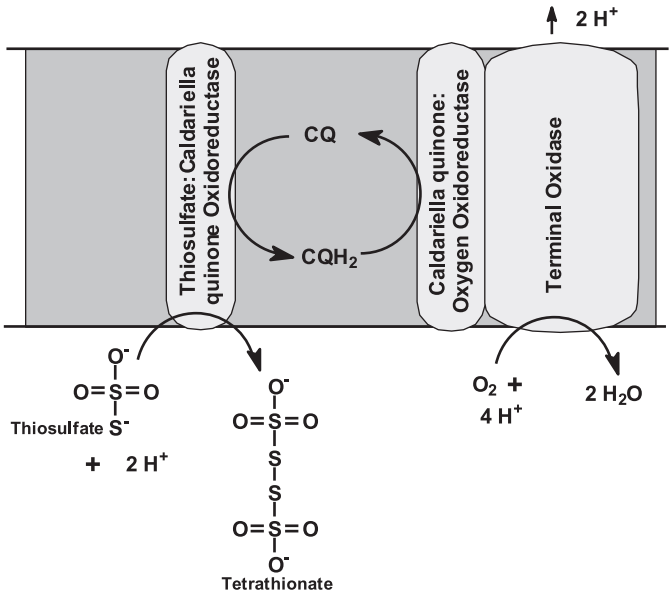


Fig. 8.6 Thiosulfate:quinone oxidoreductase in *Acidianus ambivalens*. (According to Müller et al., 2004)

8.2 The Iron Cycle

Iron is one of the most abundant elements in the Earth's crust and occurs in a variety of minerals, such as oxides/hydroxides (like hematite α-Fe_2O_3, magnetite Fe_3O_4, goethite α-FeOOH), carbonates (like siderite $FeCO_3$) or sulfides (like pyrite FeS_2). Iron occurs in nature mainly in two oxidation states, divalent (Fe^{2+}) and trivalent (Fe^{3+}). Fe^0, on the other hand, is usually the product of human activity and is formed by smelting divalent or trivalent iron ores to produce cast iron. The transformation between the different forms of iron is of great importance biochemically, geologically and ecologically.

Crucial for the environmental behaviour of iron is the relationship between oxidation state, solubility and pH as expressed in Fig. 8.7. At pH 1–2, divalent and trivalent iron exist in water as dissolved ions, i.e. as Fe^{2+} and Fe^{3+}. The reduction potential of the redox couple Fe^{3+}/Fe^{2+} is +770 mV at pH values below 2.5. Towards higher pH values, Fe^{3+} increasingly precipitates in the form of insoluble hydroxides ($Fe(OH)_3$ or FeOOH), free Fe^{2+} dominates over free Fe^{3+}, the tendency to reduce decreases and the reduction potential is in the negative range at pH 7 for example at $E^{0'} = -236$ mV for the $Fe(OH)_3/Fe^{2+}$ pair or $E^{0'} = -274$ mV for the α-$FeOOH/Fe^{2+}$ pair. In the presence of higher carbonate concentrations, little free Fe^{2+} is also present, so that at many sites at pH 7 the $Fe(OH)_3 + HCO_3^-/FeCO_3$ pair with an $E^{0'}$ of about +100 mV is more relevant. It is not surprising, given the diversity of forms of iron, that iron reduction and oxidation at acidic and neutral pH are carried out by different organisms using fundamentally different chemical species as redox substrates.

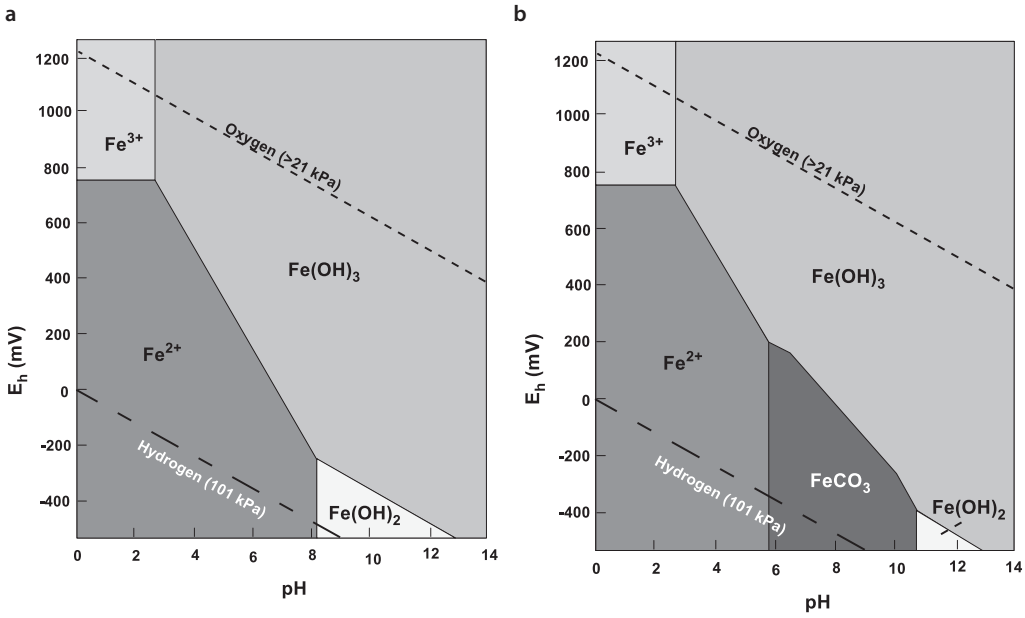


Fig. 8.7 Iron species as a function of pH and the prevailing redox potential at 25 °C. (After Widdel et al., 1993). **a** without inorganic carbon, **b** in the presence of inorganic carbon Dashed lines: (top) Potential of the redox couple O_2/H_2O at atmospheric concentration of oxygen as a function of pH. (bottom) Potential of the redox couple H^+/H_2

8.2.1 Oxidation of Divalent Iron

An oxidation of bivalent iron can be caused by very different physiological groups of microorganisms. According to the importance of Fe^{2+} for the metabolism, according to pH-preference and requirement of oxygen for energy production, 5 groups are to be distinguished.

Among the best known Fe^{2+} oxidizers are the aerobic, **organoheterotrophic** bacteria with a preference for neutral pH values, such as *Sphaerotilus natans.* These filamentous, sheath-forming *Betaproteobacteria,* deposit iron oxides in the sheath, love eutrophic sites and can contribute to bulking sludge problems in wastewater treatment plants (cf. ► Chap. 14).

In contrast to these bacteria, others use divalent iron as an electron donor for energy metabolism, i.e. they are chemolithotrophic (cf. ► Chap. 3). First of all, the **aerobic, neutrophilic, chemolithothrophic** iron oxidizers such as *Gallionella ferruginea* or *Leptothrix ochracea*, which have also been known for a long time, should be mentioned. They use the very high potential difference at neutral pH between the Fe^{3+}/Fe^{2+} pair (see above) and the O_2/H_2O pair for energy production. However, this potential difference is so large that the Fe^{2+} in the presence of higher oxygen concentrations already oxidizes chemically very quickly to trivalent iron and is thus no longer available as an electron donor for the bacteria. Therefore, these organisms occur especially at sites where formed Fe^{2+} just comes into contact with oxygen or where low oxygen concentrations, possibly even microaerobic conditions, prevail. Water bodies or drainage pipes in bogs, ferruginous groundwater or moist soils are such habitats. The reaction carried out by aerobic, neutrophilic bacteria can be summarized as follows:

$$4Fe^{2+} + O_2 + 10H_2O \rightarrow 4Fe(OH)_3 + 8H^+$$

To prevent the product, an insoluble iron hydroxide, from accumulating in the cytoplasm, oxidation must occur on the outside of the cell membrane (see below). The neutrophil iron oxidizers accumulate iron hydroxides or oxides, for example, in sheaths such as *L. ochracea* or helical stalks such as *G. ferruginea.*

Of considerable importance are also the **aerobic, acidophilic, chemolithotrophic** microorganisms and the iron oxidation they cause at low pH. Firstly, Fe^{2+} is stable to oxygen at a pH below 4 and thus available to the microorganisms. Second, where pyrite (FeS_2) or other sulfide iron-containing rocks are exposed to oxidation, leaching of these minerals results in low pH and the formation of high concentrations of dissolved Fe^{2+}. *Acidithiobacillus ferrooxidans* (a *Gammaproteobacterium,* formerly called *Thiobacillus ferrooxidans*) and *Leptospirillum ferrooxidans* from the *Nitrospira* phylum have been isolated particularly frequently from such sites. However, it is now clear that the ability to oxidize Fe^{2+} in acidic conditions also occurs in other phyla, such as among the *Firmicutes* in *Sulfobacillus* spp. and among the *Actinobacteria* in *Acidimicrobium ferroxidans* and *Ferrimicrobium acidophilum.* Among the *Archaea* there are also a number of acidophilic species with the ability to oxidize Fe^{2+}, such as *Sulfolobus metallicus* and *Ferroplasma* spp.. The majority of these acidophilic microorganisms grow autotrophically with Fe^{2+} as electron donor, some are also capable of heterotrophic or mixotrophic growth.

Since the Fe^{3+} is in principle soluble under acidic conditions, the reaction can be described by the following reaction equation:

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

However, the Fe^{3+} is then often precipitated under the pH and concentration conditions given at leach sites, initially mostly as metastable schwertmannite ($Fe_8O_8(OH)_6SO_4$), which changes to goethite (α-FeOOH) at weakly acidic pH and to the iron hydroxysulfate mineral jarosite (for example $KFe_3(SO_4)_2(OH)_6$) at lower pH in the presence of monovalent cations.

Due to the very positive reduction potential of +770 mV, practically only oxygen can be considered as an electron acceptor for Fe^{2+} oxidation at low pH. How do these acidophilic, chemolithotrophic organisms succeed at all in obtaining energy in the form of ATP by oxidation of Fe^{2+} at extremely low pH? While their cytoplasm has an approximately neutral pH of 6–7, the pH of the external medium is, for example, 2. This pH difference across the membrane represents a natural proton gradient that can be used by ATP synthase (see ► Sect. 3.1.1) to produce ATP. To keep the pH of the cytoplasm neutral despite the influx of protons, the oxidation of Fe^{2+} occurs on the outside and the reduction of O_2 on the inside of the membrane (◘ Fig. 8.8). Since the reaction taking place on the inside consumes protons, the natural proton gradient is maintained as long as Fe^{2+} is present and supplying electrons. The proton gradient of bacteria living in an acidic medium is thus achieved by iron oxidation, which leads to proton consumption in the cell interior by the formation of water.

Since the reduction potential of the Fe^{3+}/Fe^{2+} pair is very positive at pH 2, the potential difference of iron oxidation and thus the energy yield is very low (~30 kJ/mol). Moreover, much of the energy gained must be invested in retrograde electron transport to provide NADH for CO_2 assimilation. Consequently, these bacteria must oxidize large amounts of iron to grow. It has been estimated that the autotrophic acidophilic *Acidithiobacillus ferrooxidans* must oxidize approximately 71 mol of Fe^{2+} to be able to fix 1 mol of CO_2 (Hedrich et al., 2011). Thus, even a small number of cells can be responsible for the oxidation of a large amount of iron.

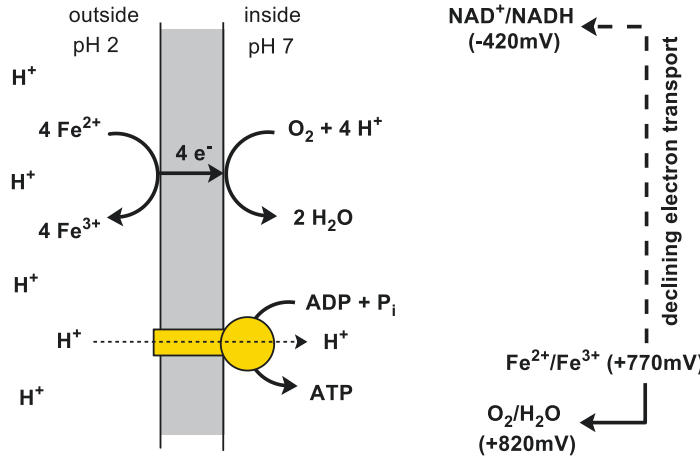


Fig. 8.8 Iron oxidation: electron and proton flow

At neutral pH, the low reduction potential of the $Fe(OH)_3/Fe^{2+}$ pair of $E^{0'} = -236$ mV also allows oxidation of Fe^{2+} by **anaerobic, neutrophilic, denitrifying** microorganisms. Thus, the reduction potentials of all redox pairs of the nitrate reduction pathway at pH 7.0 ($E^{0'}$ values: NO_3^-/NO_2^-, +430 mV; NO_2^-/NO, +350 mV; NO/N_2O, +1180 mV; N_2O/N_2, +1350 mV) are much more positive than those of the redox pairs of divalent and trivalent iron. Thus, they are favorable electron acceptors for Fe^{2+} oxidation by denitrification according to the following reaction equation:

$$10Fe^{2+} + 2NO_3^- + 24H_2O \rightarrow 10Fe(OH)_3 + N_2 + 18H^+$$

Although this type of metabolism has only been known for about 20 years, it appears to be widespread. It has been found in new isolates from the *Beta-* and *Gammaproteobacteria* groups as well as in longer known denitrifying bacteria and in the thermophilic archaeon *Ferroglobus placidus.* In this respect, this type of iron oxidation is probably of considerable importance at anoxic sites. Interestingly, some of the denitrifying iron oxidizers seem to require organic compounds as a carbon source, i.e. to have a chemolithoheterotrophic metabolism.

Finally, Fe^{2+} is also oxidized by certain **anaerobic phototrophic bacteria**, purple bacteria such as strains of the genera *Rhodomicrobium* or *Rhodovulum* and green sulfur bacteria of the species *Chlorobium ferrooxidans,* under anoxic conditions. In this case, however, it is not used as an electron donor in energy metabolism, but serves as an electron donor for autotrophic CO_2 reduction according to the following reaction equation:

$$4Fe^{2+} + HCO_3^- + 10H_2O \rightarrow 4Fe(OH)_3 + (CH_2O) + 7H^+$$

It is assumed that anoxygenic, phototrophic microorganisms led to the formation of the so-called *banded iron formations* (see ► Chap. 4, alternative hypothesis of BIFs). These represent the most important iron ore deposits and were partly formed 2.7–3.8 billion years ago, when gaseous oxygen was not yet present on Earth, or only in very low concentrations.

8.2.1.1 Oxidative Leaching of Pyrite and Other Sulfides at Low pH

One of the most common manifestations of iron in nature is **pyrite,** FeS_2. Pyrite has a highly insoluble crystalline structure and is formed from the reaction of sulfur with iron(II) sulfide (FeS). It is very common in coal and lignite and in many ores. Bacterial oxidation of pyrite is of critical importance in the development of acidic conditions and corresponding environmental stresses associated with mining. In addition, the oxidation of sulfides by bacteria also plays an important role as a production process of **microbial ore leaching**.

The **attack** on the pyrite takes place predominantly **oxidatively by hydrated Fe^{3+}**. Electrons are removed from the pyrite, whereby the sulfur component is finally also oxidised and released as **thiosulfate** ($S_2O_3^{2-}$).

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+$$

The thiosulfate can further react to other partially oxidized sulfur compounds such as tetrathionate ($-O_3S-S-S-SO_3^-$), trithionate ($-O_3S-S-SO_3^-$) or elemental sulfur and finally to sulfate. In the initial phase of a pyrite leaching at still neutral pH, the sulfur compounds are relatively stable, accumulate to some extent and are ultimately oxidized to sulfate by neutrophilic or moderately acidophilic thiobacilli. If the pH is already low, oxidation to sulfate may be purely chemical or involve acidophilic bacteria such as *Acidithiobacillus thiooxidans* or *A. ferrooxidans*. These reactions of thiosulfate oxidation can be summarized in the following reaction equations, depending on the electron acceptor:

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+$$
$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$$

Together with the attack on the pyrite, the following reaction equations for pyrite oxidation are obtained:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$
$$FeS_2 + 6Fe^{3+} + 2O_2 + 4H_2O \rightarrow 7Fe^{2+} + 2SO_4^{2-} + 8H^+$$

The Fe^{3+} attacking the pyrite is reduced to Fe^{2+} during the reaction. In this respect, the progress of the reaction is dependent on the Fe^{2+} being oxidised back to Fe^{3+}. As discussed above, this can occur purely chemically at relatively high, for example neutral, pH values. With ongoing leaching and acidic pH values, aerobic acidophilic Fe^{2+} oxidizers such as *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans* and others are responsible for Fe^{2+} oxidation. This results in an iron cycle as shown in ◘ Fig. 8.9a.

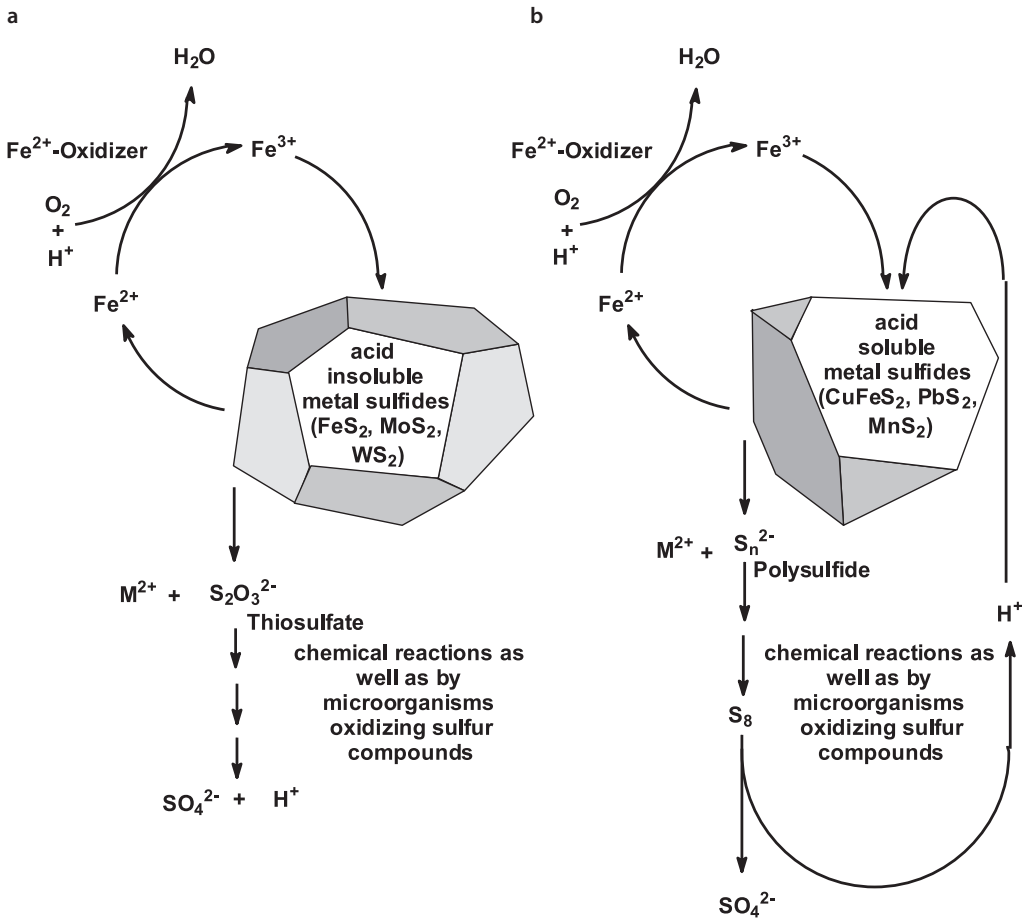


Fig. 8.9 **a** Thiosulfate, **b** Polysulfide mechanism of sulfide leaching. (After Rohwerder et al., 2003). Bioleaching of metal sulfides, means that it is the function of bacteria to biologically form sulfuric acid to provide protons for hydrolytic attack, and/or to hold the iron ions in the oxidized form (ferric ions) for oxidative attack

Taking into account the oxygen consumption for Fe^{2+} reoxidation, the different reaction equations given for pyrite oxidation lead to the same result:

$$FeS_2 + 3\,1/2\,O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$$

It should be emphasized that this equation is the sum of the series of individual reactions shown above. The equation is not intended to express that molecular oxygen as a reagent attacks the pyrite. Rather, it serves as an electron acceptor for iron and sulfur compound oxidizing microorganisms. The oxygen atoms of the sulfate appear to be generally derived from water.

A. ferrooxidans can attack pyrite relatively quickly at moderately positive potentials and was, among other reasons, frequently found at sites with sulfide oxidation. However, *L. ferrooxidans* tolerates even lower pH values (growth still at pH 1.2), higher Fe^{3+} and lower Fe^{2+} concentrations, and more positive potentials than *A. ferrooxidans,* and in this respect the *Leptospirillum* is often superior to *Acidithiobacillus* in commercial leaching plants.

The described microbial attack on the pyrite is based on an indirect mechanism in that the bacteria do not attack the sulfide directly with their membrane or enzymes, but by providing Fe^{3+}. On the other hand, the leaching organisms do attach to the pyrite in the form of a biofilm and create a reaction space with suitable Fe^{3+} concentrations with their "extracellular polymeric substances" (EPS) (◘ Fig. 8.9a). Since the EPS-mediated contact with the sulfide accelerates leaching, it is also referred to as **"contact leaching".** Meanwhile, it has been reported that pyrite can be attacked by *A. ferrooxidans* via carrier molecules containing cysteine-derived SH groups in addition to the mechanism described. From the resulting polysulfide, sulfur is released in colloidal form and possibly stored in the EPS layer as an energy reserve.

Some other sulphide minerals such as sphalerite (ZnS), chalcopyrite ($CuFeS_2$), galenite (PbS), realgar (As_4S_4) or auripigment (As_4S_6) are not as stable as pyrite and, apart from Fe^{3+}, can already be effectively attacked by protons, formally first producing H_2S. In the presence of Fe^{3+} this oxidizes to **polysulfides** and further to elemental sulfur (S_8) (◘ Fig. 8.9b). The elemental sulfur can be oxidized to sulfuric acid by sulfur-oxidizing bacteria such as *A. ferrooxidans* and *A. thiooxidans*, providing new protons for dissolution of the sulfides. Since polysulfides are the characteristic sulfur-containing intermediates of this type of leaching, this is referred to as the polysulfide mechanism.

Bacterial oxidation of sulfide minerals is the most important factor in the emergence of acid waters, a common environmental problem in areas with coal or ore mining. The mixing of acidic waters from mining operations with natural waters in rivers or lakes also seriously degrades their quality in some cases. The high loads of sulfate and bivalent iron resulting from the mining of pyrite, as well as the low pH values, lead to problems for the organisms living in the waters, but also with regard to their further use as cooling or even drinking water. In addition, the acid formed attacks other minerals associated with the coal and pyrite. Thus, as explained, other sulfides can be dissolved and from these, for example, zinc, lead or arsenic can be released. But aluminium ions are also soluble at low pH and can be dissolved out of the aluminium-bearing minerals common in nature. Since mine water often contains high concentrations of Al^{3+} as well as heavy metals or metalloids, it can be highly toxic to aquatic life.

Part of the environmental problems mentioned above is due to the fact that pyrite oxidation is often incomplete, resulting only in divalent iron. Under acidic conditions, as mentioned above, the complete oxidation of iron allows the precipitation of the iron hydroxysulfate jarosite (for example, $KFe_3(SO_4)_2(OH)_6$), which can lower the iron and sulfate load. However, acidification tends to be increased due to the hydroxide ions that are also bound.

8.2.2 Reduction of Trivalent Iron

Trivalent iron is one of the most abundant metals in soil and rocks. It is an electron acceptor for a wide variety of both chemoorganotrophic and chemolithotrophic bacteria, and since Fe^{3+} is widely distributed in nature, its reduction is a major form of anaerobic respiration. Trivalent iron reduction is common in wet soils, swamps, and anoxic lake sediments and results in the production of divalent iron, a more soluble form of iron. Bacterial iron reduction can thus lead to the solubilization of iron, an important geochemical process.

The energetics of Fe^{3+}-reduction was investigated using the Gram-negative bacterium *Shewanella putrefaciens*, in which Fe^{3+}-dependent anaerobic growth occurs with various organic electron donors. Other important Fe^{3+} reducers belong to the family *Geobacteraceae* within the *Deltaproteobacteria* such as *Geobacter, Geospirillum* and *Geovibrio. Geobacter metallireducens* as a model for studying the physiology of Fe^{3+} reduction can oxidize acetate with Fe^{3+} as an acceptor as follows:

$$\text{Acetate}^- + 8\text{Fe}^{3+} + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 8\text{Fe}^{2+} + 7\text{H}^+ \qquad \Delta G^{0'} = -815\text{kJ / mol Acetate.}$$

That is, during the dissimilatory oxidation of 1 mol of acetate with Fe^{3+}, 11 mol of ATP could theoretically be generated. However, since $Fe(OH)_3$ has an extremely low solubility product ($[Fe^{3+}][OH^-]^3 = 10^{-39}$), the concentration of the Fe^{3+} ion at pH 7 is 10^{-18}. Only below pH 4 does the concentration of Fe^{3+} reach the micromolar range. Consequently, the free energy of the reaction ($\Delta G^{0'} = -34$ kJ/mol acetate) is not even sufficient to allow the synthesis of 1 ATP. Since the naturally occurring Fe^{3+} minerals in the soil (see above) have even lower solubility products, nothing is yet known about the exact amount of energy that can be conserved during the reaction.

Geobacter may also use H_2 or other organic electron donors.

However, archaea can also obtain energy from Fe^{3+} reduction, such as the hyperthermophilic Fe^{3+} reducer *Ferroglobus placidus,* with acetate as donor.

Trivalent iron can form complexes with various organic compounds, making it soluble and more accessible to iron(III)-reducing bacteria.

Fe^{2+} in the Ocean

Oceans are generally considered to be oxygenated. Almost everywhere, however, Fe^{2+} is absent. Significant exceptions to oceanic areas devoid of Fe^{2+} are hydrothermal outcrop channels, either at seamounts or at points of crustal spreading. There, anoxic fluids loaded with Fe^{2+} come into contact with cold, oxygenated ocean water.

It has been estimated that the current flux of Fe^{2+} from hydrothermal outcrop channels is approximately 3×10^{11} mol/yr (Emerson et al., 2007; Holland, 2006).

Microbial Ore Leaching for Metal Extraction: Paths to Riches?

Microbial leaching of metal sulfhides not only takes place as an undesirable process leading to environmental problems in mining regions, but is also used on a large scale for the extraction of metals. **Copper** is the main focus here. Microbial leaching of copper sulfides has basically been used for centuries, but without first understanding the basics. There are now a number of plants processing more than 10,000 tonnes of ore per day, and in some cases even more than 20,000 tonnes of ore per day. The largest plants use chalcocite (Cu_2S)-containing ore. It is leached with acid or with Fe^{3+} ions by the following reactions:

$$2\text{Cu}_2\text{S} + 4\text{H}^+ + \text{O}_2 \rightarrow 2\text{CuS} + 2\text{Cu}^{2+} + 2\text{H}_2\text{O}$$
$$\text{Cu}_2\text{S} + 4\text{Fe}^{3+} \rightarrow 2\text{Cu}^{2+} + 4\text{Fe}^{2+} + \text{S}$$

Intermediately formed covelline (CuS) is also oxidized by Fe^{3+}:

$$\text{CuS} + 2\text{Fe}^{3+} \rightarrow \text{Cu}^{2+} + 2\text{Fe}^{2+} + \text{S}$$

Technically, copper leaching is usually carried out in aerated heaps with crushed ore, over which the leaching solution is trickled. The metallic copper is recovered by solvent extraction-electrowinning (SXEW). The Cu^{2+}-containing solution produced during leaching is passed through a solvent extraction circuit in which the metal ions in solution are exposed to organic solvents such as turpentine or other petrochemicals, binding to the solvent by chelation. The metal ion is then extracted from the solvent by changing the pH in a second acid circuit. The metal in this second circuit is pumped into an electrical cell and deposited at the anode by electrolysis.

The most important copper ore, chalcopyrite ($CuFeS_2$), is not leached satisfactorily by the usual heap processes. Plants with stirred tank reactors are available here and investigations are underway into the use of thermophilic microorganisms.

The use of microbial leaching in the extraction of **gold** is now also of considerable commercial importance. In deposits, this precious metal is often enclosed by sulfides such as pyrite (FeS_2) or arsenopyrite (FeAsS). Leaching processes are used to make the gold more amenable to the conventional cyanide leaching that follows and to increase recovery. Large aerated stirred tank reactors are common, but so are windrow processes.

8.3 The Manganese Cycle

The metal manganese has several oxidation states, of which Mn^{4+} and Mn^{2+} are the most stable and biologically important. Both forms differ considerably with respect to their solubility, while Mn^{2+} is soluble, the oxidized form, manganese dioxide, is present as an insoluble solid in water. Mn^{2+} is stable under aerobic conditions at pH values below 5.5, in the absence of oxygen also at higher pH values.

8.3.1 Oxidation of Divalent Manganese

Manganese oxidizing bacteria can be found among a variety of genera, such as *Arthrobacter, Bacillus, Leptothrix,* and *Streptomyces.* The Gibbs free energy of oxidation of Mn^{2+} to MnO_2 is negative.

$$2Mn^{2+} + O_2 + 2H_2O \rightarrow 2MnO_2 + 4H^+ \qquad \Delta G^{0'} = -29.3\text{kJ / mol}$$

Due to the insolubility of brownstone, its size is high. The product is found in hydrothermal outcrop channels, swamps, and it is a significant part of the dark-colored surface layer of rocks exposed to weathering.

8.3.2 Reduction of Tetravalent Manganese (Mn^{4+}): Anaerobic Respiration

Anaerobic reduction of Mn^{4+} to Mn^{2+} is carried out by various, mostly chemoorganotrophic microorganisms such as *Shewanella* (facultative anaerobe) and *Geobacter* (anaerobe). In *Shewanella putrefaciens,* anaerobic growth occurs on acetate with Mn^{4+} as electron acceptor. The redox potential of the Mn^{4+}/Mn^{2+} pair is extremely high; thus, several compounds should be able to donate electrons for Mn^{4+} reduction.

$$MnO_2 + 4H^+ + 2e^- \rightarrow Mn^{2+} + 2H_2O \qquad E^{0'} = +615\text{mV}$$

The bacteria that use oxidized metal ions such as Fe^{3+} or Mn^{4+} as terminal electron acceptors must leave them outside the cell because of the insolubility of the salts or metal oxides. Thus, they must transfer reduction equivalents from the cytoplasm to the extracellular electron acceptors. It is still unclear how electron transfer works and how the process is coupled to proton excretion. In some iron-reducing bacteria, special pilus-like surface structures have recently been discovered that may be possible electron conductors from the cell to the iron mineral (see ► Sect. 18.5). However, soluble and diffusible redox mediators such as thiols or phenols or quinones may also be involved in the process. Their oxidized form could be reduced by membrane-bound reductase, diffuse to the mineral, reduce it chemically, and then start the cycle again as an oxidized form.

Manganese reduction seems to be of minor importance in freshwater, but to play an important role where manganese is accumulated, such as marine sediments of the Baltic Sea or the rock material mentioned above.

Test Your Knowledge

- Where do sulfate-reducing bacteria occur in nature?
- Name locations where you can smell or see with the naked eye the activity of sulfate-reducing bacteria.
- Draw the sulfur cycle.
- Aerobic organisms also have to reduce sulfate. How do the processes that take place there differ from those in sulfate-reducing bacteria?
- Name the oxidation states of sulfur in the reduction of sulfate to sulfide.
- Which organisms produce sulfate from hydrogen sulfide? Which electron acceptor functions in these microorganisms?
- What form (bivalent or trivalent) does iron have in the mineral $Fe(OH)_3$? FeS?
- How is $Fe(OH)_3$ formed?
- Questions about the occurrence of iron ions at pH 2 and at neutral!
- Is H_2S a substrate or product of sulfate-reducing bacteria? What is it in the chemolithotrophic bacteria?
- Why does bacterial oxidation of sulfur cause a pH drop?
- Which organic sulfur is most common in nature?
- How can CuS be oxidized under anoxic conditions?
- Why is it important to keep the leaching liquid acidic when copper leaching?

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Heavy Metals and Other Toxic Inorganic Ions

Contents

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9.1 Toxicity

The term **heavy metals** is used arbitrarily to summarize a group of metals, whereby a clear, scientifically accepted definition is lacking. The density of >5 g/cm^3 or toxicity are often associated with the term heavy metal in public. Lists of heavy metals often include semimetals such as arsenic and thallium.

The use of the term in the sense of toxicity is extremely problematic, since many of the elements designated by it, such as iron, chromium, cobalt, copper, manganese, molybdenum, nickel, vanadium, zinc and tin, are essential for the metabolism of humans, animals, plants and microorganisms. Other elements such as lead, cadmium, gold, mercury, silver, strontium and thallium, on the other hand, have no physiological significance.

With regard to contamination of drinking water and soils, heavy metals are of particular importance, as some are toxic even at low concentrations. In higher concentrations, however, essential metals such as cobalt, copper, nickel and zinc are also toxic and not only non-essential ones such as lead, cadmium and mercury. At elevated concentrations, both groups can cause growth inhibition in microorganisms and metabolic disorders in humans.

The following mechanisms determine the toxicity of heavy metals:

1. Inactivation of enzymes. In this context, the divalent transition metals are particularly effective. They react readily with the amino and sulfhydryl groups of proteins; heavy metal cations, especially those with high atomic numbers such as Ag^+, Cd^{2+} and Hg^{2+}, tend to bind to SH groups. The minimum inhibitory concentration of these metal ions is a function of the complex dissociation constant of the respective sulfide. By binding to SH groups, the metals can inhibit the activity of sensitive enzymes.
2. Metal cations interact with physiological cations and thus hinder their function in metallo-enzymes:
   - Cd^{2+} and Hg^{2+} with Zn^{2+}
   - Cd^{2+} with Ca^{2+}
   - Ni^{2+} and Co^{2+} with Fe^{2+}
   - Zn^{2+} with Mg^{2+}
3. Metals can also change the permeability of cell membranes and thus influence the transport of substances.
4. Metals can alter genetic material and have a carcinogenic effect.

Environmental damage caused by heavy metals with serious effects on humans has become known:

1. Cadmium-containing wastewater was discharged onto rice fields in Japan. **Itai-itai disease** was the result.
2. Wastewater containing mercury flowed into **Minamata Bay,** so that the very toxic methylmercury compounds appeared in fish due to an accumulation. According to current estimates, about 17,000 residents of this bay were more or less severely damaged by the mercury compounds, and about 3000 may have died from the poisoning (**Minamata disease**).
3. Thousands of people in **Iraq** had consumed grain treated with mercury-containing fungicides. At least 450 people died as a result. Damage to brain cells with extensive destruction in many regions of the brain and spinal cord were the consequences of the poisoning.
4. In the **Amazon,** mercury illegally entered the river water and food during gold panning. Minamata disease was also found among the Indians.
5. A study in the Philippines showed that over 70% of workers in gold mines or other mineral-processing areas showed signs of mercury intoxication (in Selin, 2011).

Many environmental factors influence the **bioavailability of** metals in ecosystems and thus toxicity. For the bioavailability of heavy metals, the free metal ions are decisive. The

chemical form and mobility of heavy metals are strongly influenced by pH and redox potential (Eh value). Water hardness also has a major effect, with higher toxicity in soft water. In contrast, salinity hardly changes toxicity. Due to complexation, humic substances in the water and sulfide formation in the sediment strongly lower the concentrations of toxic ions. Overall, temperature has only a minor influence, although higher bioaccumulation/toxicity is often observed with an increase in temperature.

9.2 Environmental Quality Standards

The cases of poisoning have led to the analysis of heavy metals in water bodies, sediments and fish. Quality targets for flowing waters and limit values for wastewater discharges have been set. In western industrialized countries, the heavy metal problem has therefore decreased, but significant contaminated sites are still present in soils and waters. The problem of metal pollution of ecosystems has increasingly shifted to emerging and developing countries.

An overview of the quality standards in water bodies developed by LAWA (Working Group of the Federal States on Water Issues), ICPE (International Commission for the Protection of the Elbe) and ICPR (International Commission for the Protection of the Rhine) is shown in ◘ Table 9.1.

On the basis of toxicological/ecotoxicological investigations, graded precautionary values were defined depending on the soil type in accordance with the Federal Soil Protection and Contaminated Sites Ordinance (◘ Table 9.2).

◘ **Table 9.1** Heavy metal quality standards for water bodies[a]

Regulation according to (unit)[b]	Protected property	Arsenic	Lead	Cadmium	Chromium	Copper	Nickel	Mercury	Zinc
LAWA (mg/kg)	Aquatic communities		100	1.2	320	80	120	0.8	400
	Suspended particles and sediments		100	1.5	100	60	50	1	200
LAWA (µg/L)	Drinking water supply		50	1	50	20	50	0.5	500
	Fishing		5	1	–	–	–	0,1	–
	Irrigation of farmland		50	5	50	50	50	1	1000
ICPE (mg/kg)	Aquatic communities	40	100	1.2	320	80	120	0.8	400
	Suspended particles and sediments	30	100	1.5	150	80	60	0.8	200
ICPR (mg/kg)			100	1	100	50	50	0.5	200

[a]► http://www.umweltbundesamt.de/wasser-e/themen/oberflaechengewaesser/ow_s2_2.htm
[b]Suspended particles and sediments (in mg/kg) and water phase (in µg/L)

Table 9.2 Precautionary values for heavy metals in soil[a]

	Clay	Loam/silt	Sand
Metal	(mg/kg dry matter)		
Lead	100	70	40
Cadmium	1.5	1	0.4
Chromium	100	60	30
Copper	60	40	20
Nickel	70	50	15
Mercury	1	0.5	0.1
Zinc	200	150	60

[a]► http://www.umweltbundesamt.de/boden-und-altlasten/boden/bodenschutz/vorsorge.htm

This took into account the different sorption capacities of the soils. Below the precautionary values, the concern of a harmful soil change can be excluded according to the current state of knowledge.

9.3 Natural and Anthropogenic Occurrences

Heavy metals are natural constituents of minerals (Table 9.3). Lead, cadmium, chromium, cobalt, nickel, thallium and zinc are present in isomorphous positions of the main elements silicon and aluminium as trace constituents. Heavy metal contents of rocks are normally in the mg/kg range. Significantly higher contents are found in ultrabasic effusive and serpentinite rocks. Ores (arsenopyrite, galena, zinc blende) consist of up to 100% heavy metals (e.g. digested copper and mercury). Heavy metals naturally enter soils through weathering of rocks and ores. Other sources of heavy metals are continental dust immissions and outgassing from seawater and volcanoes.

The loads originating from the above-mentioned natural sources are mostly insignificant compared to those from anthropogenic sources. Anthropogenic sources of heavy metals are emissions from industrial and incineration plants and motor vehicles (Table 9.4). Furthermore, the recycling of metal-bearing wastes, wastewater treatment, and the use of fertilizers and pesticides contribute to the input of heavy metals into soils.

The input of anthropogenic heavy metals into soils occurs via point and diffuse sources. Industrial plants and chimneys of large combustion plants (power and cement plants, waste incineration plants) are point sources. The highest metal accumulations are in the immediate vicinity of emitters due to input via atmospheric deposition. Heavy metals are transported in particulate, vapor and gaseous form. Volatile elements such as arsenic and mercury evaporate at relatively low temperatures and condense highly enriched in small particles and aerosols.

Heavy metal contamination of soils in the immediate vicinity of roads (up to 10 m next to main roads) is the result of motor vehicle traffic through tyre abrasion and combustion residues.

Table 9.3 Occurrence of heavy metals on earth, ordered by concentration in the earth's crust

Metal	Average concentration found in the earth's crust (ppm)	Rock with the highest concentration	Sources
Chromium	100		Does not occur freely in nature. Chrome iron ore (chromite) [Fe, $Mg(CrO_4)$] is the most important mineral
Nickel	80	Lava rock	Mainly found in iron nickel gravel (pentlandite) [$(Ni, Fe)_9S_8$] and garnierite ores
Zinc	75	Shale	Found in the minerals zincblende (sphalerite) [ZnS], calamine, franklinite, smithsonite [$ZnCO_3$], willemite, and zincite [ZnO]
Copper	50	Basalt	Pure copper is rarely found in nature. Usually copper is found in minerals such as azurite, malachite and colored copper pyrites (bornite) and in sulfides such as in chalcopyrite [$CuFeS_2$], covelline [CuS], chalcocite [Cu_2S] or oxides such as cuprite [Cu_2O]
Lead	14	Granite	Commonly found in ores such as galena (galenite). But also in lead apatite (pyromorphite), antimony lead lead (boulangerite) and cerussite ores
Arsenic	1.8		Found in arsenopyrite. Mainly formed as a by-product in the refining of some sulfide ores
Thallium	0.6		Found in iron pyrite, crookesite [$Cu_7(Tl, Ag)Se_4$], hutchinsonite [$(Tl, Pb)_2As_5S_9$], and lorandite [$TlAsS]_2$
Cadmium	0.11	Shale	Obtained as a by-product of zinc refining
Mercury	0.05	Sandstone	Most mercury comes from ores such as cinnabar or mercury sulfide

Table 9.4 Anthropogenic sources of heavy metals

Anthropogenic sources		Mainly emitted heavy metals
Metalworking industries	Copper and nickel production	Arsenic
	Non-ferrous metal processing plants	Lead and cadmium
	Leather, steel, building materials, paint production, corrosion protection	Chromium
Power plants and large combustion plants		Lead and cadmium
Cement plants		Thallium
Motor traffic	Until the introduction of unleaded petrol	Lead
	Tyre abrasion, fuel combustion residues and galvanised car bodies	Cadmium, zinc (one quarter of atmospheric emission)
Waste water trickling on trickle fields		Lead, cadmium, chromium, copper and zinc
Sewage sludge, biowaste and dredging sludge		All
Farm manure (slurry, dung, poultry droppings)		Copper and zinc
Mineral fertilizer		Cadmium and chromium
Pesticides and wood preservatives	The use of metal compounds in crop protection (with the exception of copper) has long been prohibited in Germany. Soils in hops and viticulture in particular have high copper contents	Arsenic, copper and mercury

9.4 Resistance of Microorganisms

Heavy metals are neither microbially nor chemically degradable. Figure 9.1 clearly shows the great difference in the toxicity of heavy metals to bacteria, using the example of *Escherichia coli.* Mercury is the metal that is toxic even at low concentrations.

Microorganisms have evolved different mechanisms to protect themselves from toxic heavy metal concentrations. In fact, all bacteria have genes for resistance to toxic metal ions such as Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^+ and Zn^{2+}.

Broadly speaking, the adaptation of microoganisms to the presence of heavy metal ions can occur in two ways:

1. They can survive due to inherent properties associated with their cell wall struc-

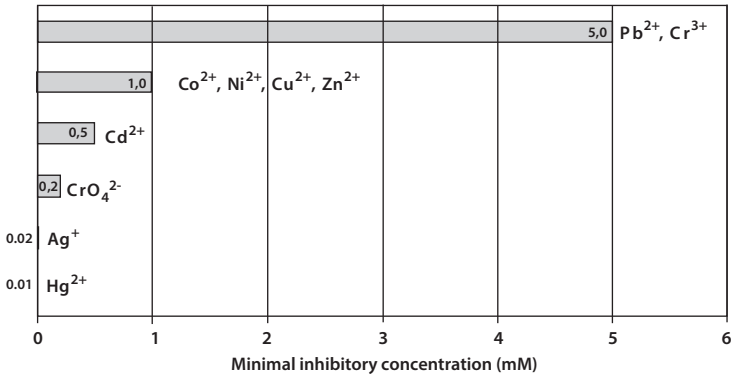

Fig. 9.1 Minimum inhibitory concentration of heavy metals against *Escherichia coli.* (Values from Nies, 1999)

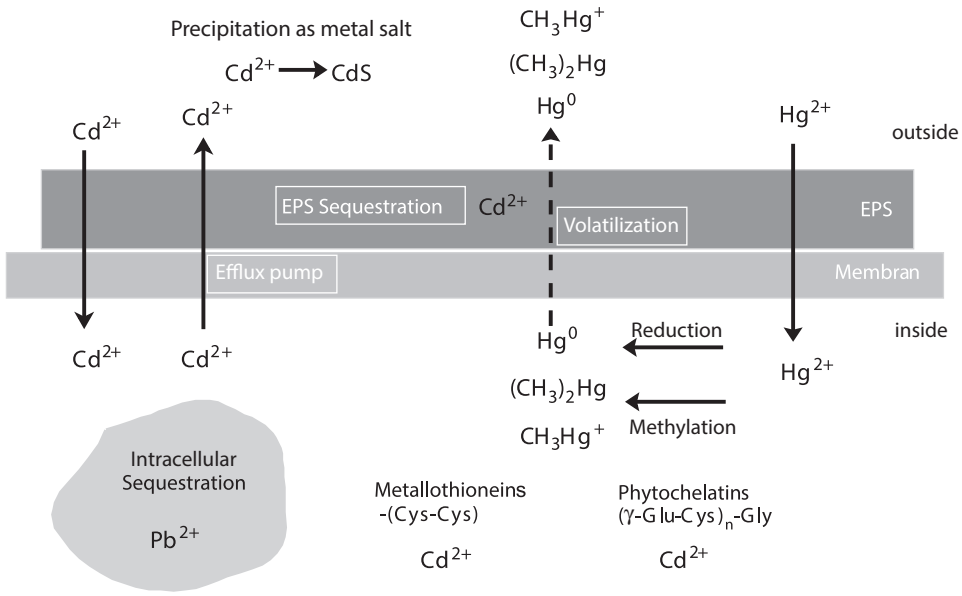

Fig. 9.2 Schematic representation of the mechanisms of heavy metal resistance of microorganisms

ture, extracellular polymeric substances (EPS), and binding/precipitation of metals inside or outside the cell.

2. Alternatively, they develop specific mechanisms of detoxification when confronted with the metals. These latter mechanisms include **active efflux mechanisms** as well as **reduction/oxidation, alkylation/dealkylation,** and **intracellular compartmentation/sequestration** [production of metallothioneins (small cysteine-rich proteins with a molecular weight of 6000–10,000 that lack aromatic amino acids) and phytochelatins (metal-binding peptides with a structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, with $n = 3–8$)].

The two types of mechanisms (Fig. 9.2) often overlap because extracellular precipitation and crystallization due to initial metal efflux alters the physico-chemical environment of the cells.

Microbial adaptations to heavy metal stress must be viewed as a dynamic process involving **general cellular responses** such as slime formation in biofilms, **specific resis-**

tance mechanisms, and changes in the chemical form of metals and **toxicity that** are influenced by both the microorganisms and environmental components.

The largest group of resistance systems functions by means of energy-dependent efflux of toxic ions. Numerically fewer resistance mechanisms involve enzymatic transformations (oxidation, reduction, methylation and demethylation) or metal-binding proteins (such as metallothionein SmtA, chaperone CopZ and periplasmic silver-binding protein SilE). Some of the efflux resistance systems are ATPases and others are chemiosmotic ion/proton exchangers.

These resistance determinants have been on Earth for billions of years. They have therefore not only evolved due to the anthropogenic release of heavy metals. This could be shown using the example of arsenic resistance: the DNA and amino acid sequences and also the structures of the genes and proteins involved in arsenic resistance indicate a primeval origin (Lebrun et al., 2003).

Arsenic resistance and metabolism systems occur in three forms. The most common has been found to be the ***ars*** operon, which is encoded in most bacteria on the genome or many plasmids. ***arr*** genes for periplasmic arsenate reductase, which functions as a terminal electron acceptor in anaerobic respiration, have only recently been discovered. Then there are ***aso*** genes for periplasmic arsenite oxidase, which functions as an electron donor in aerobic resistance of arsenite (◘ Table 9.5).

Cd^{2+}-Efflux pumps of bacteria are either P-type ATPases of the inner membrane or consist of three polypeptides (RND, Resistance-Nodulation-Cell Division Superfamily) a chemiosmotic complex composed of an inner membrane pump, a periplasm-bridging protein and a channel in the outer membrane. In addition to the best studied three-polypeptide chemiosmotic system, **Czc** (Cd^{2+}, Zn^{2+}, and Co^{2+}), others are known to efflux Ag^{+}, Cu^{+}, Ni^{2+} and Zn^{2+}.

Resistance to inorganic mercury, Hg^{2+}, and organomercury, such as CH_3Hg^{+} and phenylmercury involve a number of metal-binding and membrane-transporting proteins as well as mercury reductase and organomercury lyase, which generally convert the more toxic to the less toxic forms.

If detoxification of a heavy metal is to be by reduction, the redox potential should be between that of the hydrogen/proton and that of the oxygen/water pair, which is the physiological range for most aerobic microorganisms. Consequently, Hg^{2+}, chromate, arsenate and Cu^{2+} can be reduced by cells, but not Zn^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+} (◘ Table 9.6).

Furthermore, a heavy metal that has been reduced should diffuse out of the cell or it would reoxidize itself again. Most reduction products are quite insoluble (Cr^{3+}) or even more toxic ($H_2AsO_3^{-}$) than the parent compound. Thus, if a cell is to use reduction as a detoxification mechanism, there must be an efflux system for export of the reduced product. Only in the case of mercury do the properties of "reducibility" and a "high vapor pressure of the metallic reduction product" fit together. Mercury is therefore detoxified by reduction of Hg^{2+} to Hg^{0}, by diffusible efflux of the Hg^{0}.

What option is available or takes place for non-reducible heavy metals when reduction is not desirable? Complexation is an efficient way when cells are exposed to low concentrations of heavy metals. Aerobic cells, for example, can detoxify Cd^{2+} by forming CdS; to do this, the sulfate reduction process requiring high energy must proceed with PAPS (phosphoadenosine-5′-phosphosulfate). Heavy metal metabolism is generally transport metabolism.

Table 9.5 Main mechanisms of heavy metal resistance in bacteria

Gene code	Toxic ions	Mechanism
arr	AsO_4^{3-}	Respiratory arsenate reductase
ars	AsO_4^{3-}, $As(OH)_3$ AsO_2^{-}	Reduction of arsenate (arsenate reductase) with subsequent ATPase-mediated efflux of arsenite
aso	$As(OH)_3$	Arsenite oxidase and transport
cad	Cd^{2+}	P-type ATPase-mediated efflux of cations
chr	CrO_4^{2-}	Chromate Efflux Permease
cop	Cu^{2+}, Cu^{+}	Copper resistance and transport, sequestration of Cu^{2+} outside the cytoplasmic membrane
czc	Cd^{2+}, Zn^{2+}, Co^{2+}	Cation/proton antiporter chemiosmotic efflux (CBA[a] efflux permease)
cnr	Co^{2+}, Ni^{2+}	Cation/proton antiporter chemiosmotic efflux (CBA efflux permease)
mer	Hg^{2+} and organomercury	Mercury reductase: reduction of Hg^{2+} to Hg^0 by *merRTPBAD* or of organomercury by *merRTPBAD* and transport.
ncc	Ni^{2+}, Co^{2+}, Cd^{2+}	CBA Efflux Permease
nre	Ni^{2+}	CBA Efflux Permease
pbr	Pb^{2+}	Lead resistance and efflux
pco	Cu^{2+}, Cu^{+}	Copper resistance and efflux
sil	Ag^{+}	Silver resistance and binding
tel, teh, kil	TeO_3^{2-}	Tellurite resistance

[a]The designation CBA was chosen for an order of genes on the chromosome to distinguish them from ABC ATPase systems

Table 9.6 Comparison of redox potentials for estimating the microbial reducibility of heavy metals

Redox couple		Number of electrons	$E^{0'}$ (mV)
Oxidized form	Reduced form		
$2H^+$	H_2	2	−420
O_2	$2H_2O$	4	+820
Hg^{2+}	Hg^0	2	+440
CrO_4^{2-}	Cr^{3+}	3	+929
SeO_4^{2-} [Selenate]	SeO_3^{2-} [Selenite]	2	+475
$HAsO_4^{2-}$ and $H_2AsO_4^-$; As(V) [arsenate].	H_3AsO_3 and $H_2AsO_3^-$; As(III) [arsenite]	2	+139
Cu^{2+}	Cu^+	1	−262
Zn^{2+}	Zn	2	−1180
Cd^{2+}	Cd	2	−820
Co^{2+}	Co	2	−700
Ni^{2+}	Ni	2	−650

9

9.5 Mercury

Mercury is a relatively rare element in the earth's crust, which is only increasingly released into the environment through anthropogenic influences. Through natural weathering and volatilization as well as through volcanism, about 50–100 · 10^3 t of mercury enter the environment annually. It is estimated that this value has increased about fivefold as a result of civilization processes. anthropogenic sources include mining and smelting of sulfide ores, and industrial processes (electrolysis in the chloralkali industry) and products (lamps, amalgams in dentistry) (Table 9.7). Apart from intentional uses, there is also the unintentional release of mercury into the air through coal and petroleum combustion.

Emissions of volatile mercury have resulted in its presence in soils of industrial agglomerations at concentrations of 0.1–1 mg/kg. Discharges of industrial and

Table 9.7 Mercury consumption in the EU[a]

Use	Estimated quantities 2007 (t)
Chloralkali electrolysis	160–190
Batteries	7–25
Dentistry amalgam	90–110
Measuring and control equipment	7–17
Switches and electrical control	0–1
Lamps	11–15
Chemicals	28–59
Other use	15–114
Total	**320–530**

[a]► http://ec.europa.eu/environment/chemicals/mercury/index.htm

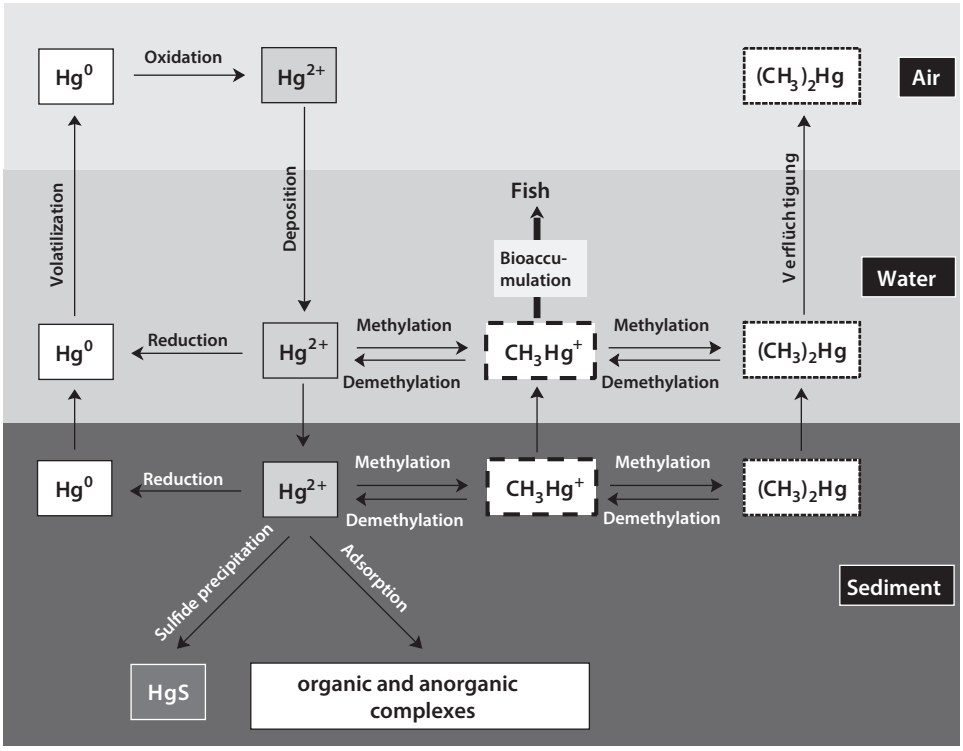

Fig. 9.3 Mercury cycle. The microbial reactions serve for detoxification, for higher organisms they lead to toxic metabolites

municipal waste water cause considerable contamination of river sediments and sea bays. Levels of 10–100 mg/kg have been detected in harbour silt in the Elbe and Rhine rivers. In Minamata Bay in Japan, the concentration was 2000 mg/kg.

Mercury, like other metals, is not degraded but oxidized or reduced and converted to organic compounds.

The various mercury compounds enter a biogeochemical cycle in which microorganisms play a major role (Fig. 9.3).

In the atmosphere, mercury occurs as a relatively non-toxic metal that is oxidized to Hg^{2+} by photochemical processes. In this form it returns to soils and waters. The mercury cation (Hg^{2+}) causes poisoning because it reacts with the SH groups of proteins. Microorganisms have developed various **resistance mechanisms** to mercury. One mechanism is reduction to metallic mercury. Many microorganisms possess a mercury reductase (MerCA protein, related to glutathione reductase) that catalyzes the following reaction:

$$Hg^{2+} + NADPH \rightarrow Hg^{0} + NADP^{+} + H^{+}$$

The volatile metallic mercury escapes from the aqueous environment and undergoes the reaction steps described above.

Methylation to methyl and dimethyl mercury is another bacterial detoxification reaction. The transfer of the methyl group takes place through vitamin B_{12} (cobalamin):

$$Hg^{2+} + CH_3 - B_{12} \rightarrow CH_3 - Hg^{+} + B_{12}{}^{+}$$
$$CH_3 - Hg^{+} + CH_3 - B_{12} \rightarrow (CH_3)_2 - Hg + B_{12}{}^{+}$$

Especially the dimethyl mercury is very volatile.

The bacterial detoxification process results in products that are about 100 times more toxic to higher organisms than Hg^{2+}. As lipophilic compounds, they are well absorbed and accumulate in aquatic food chains by several orders of magnitude. In fish, 100 mg Hg/kg body weight have thus been detected.

Two further bacterial reactions are of importance for the mercury cycle, the **demethylation of** the methylmercury compounds to Hg^0 and the **precipitation** as HgS, which is caused by the H_2S formation of the sulfate reducers. Mercury sulfide is very insoluble under anaerobic conditions. However, when HgS-containing sludge and sediments or sewage sludge are exposed to aerobic conditions by applying them to the soil surface, **remobilization** occurs. Thus, irreversible elimination of mercury is difficult due to the cycling processes outlined.

9.6 Arsenic

Arsenic (As) is only the 20th most abundant element in the earth's crust at about 1.8 ppm. Arsenic is highly toxic and enters drinking water in many regions of the world due to geological conditions or mining activities. More than 100 million people are at risk, especially in Southeast Asia, where deeper groundwater sources have had to be tapped in recent decades. Chronic arsenic poisoning leads to severe skin damage, kidney and liver dysfunction, and even cancer. In Southeast Asia, arsenic contamination varies so much locally that each individual well would have to be tested. Thousands of cases of arsenic poisoning due to contaminated drinking water are diagnosed every year in Bangladesh.

An example that has recently been the subject of public discussion with regard to interesting microorganisms is Mono Lake in eastern California. It is not a particularly comfortable body of water, with a pH of 10, a salt concentration 3 times higher than that in the ocean, and a high arsenic content of 200 µmol/L.

Arsenic is also used selectively in metallurgy, wood preservation, paints, medicine, pest control, and as a feed additive in poultry where it accelerates growth. Although these anthropogenic sources have also contributed to some cases of arsenic contamination, volcanic eruptions, low temperature volatility and natural weathering of arsenic-containing minerals are the largest sources in the environment.

Arsenic can exist in the form of arsine, arsenosugar, or other arsenic-containing organics such as arsenobetaine, but the most common species found in natural waters are arsenate [$HAsO_4^{2-}$ and $H_2AsO_4^-$; As(V)] and arsenite [$HAsO_{33}$ and $H_2AsO_3^-$; As(III)]. These two oxyanions readily convert into each other. Their different chemical properties determine whether they precipitate in solid form or are mobile in the water phase. The predominant form of inorganic arsenic in the aqueous aerobic environment is arsenate, while arsenite is more prevalent in anoxic environments. Arsenate is firmly adsorbed on the surface of quite a few widely distributed minerals. Arsenite adsorbs less firmly and to fewer minerals, making it the more mobile oxyanion.

Arsenate is an analogue of phosphate and thus inhibits oxidative phosphorylation. As(III) is much more toxic than As(V) due to its high affinity for sulfhydryl groups in proteins and the associated inactivation.

The microbial metabolism that causes the conversion of arsenic between As(V) and As(III) largely determines which As species is found in the environment. Since both As(III) and As(V) adsorb to $Fe(OH)_3$, the fate of arsenic in the environment is often coupled to that of iron. In sediments

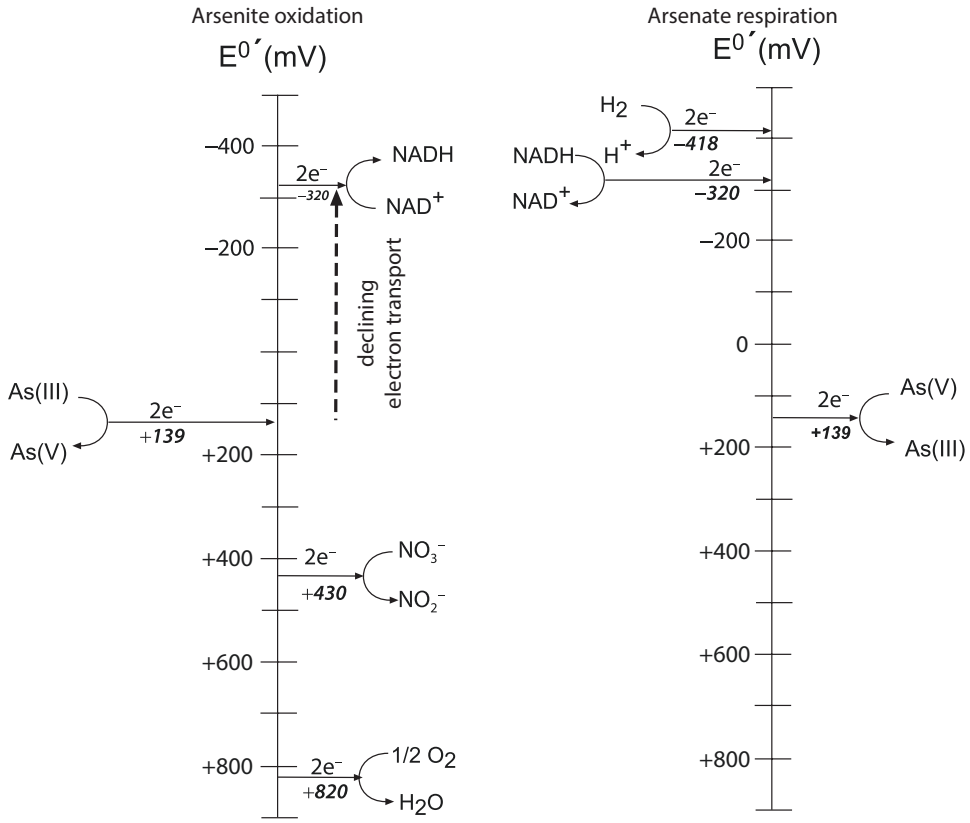

Fig. 9.4 Function of arsenic species as electron donor and acceptor respectively

not dominated by iron, As(III) is generally more mobile than As(V).

Microorganisms that perform the conversions of arsenic between As(III) and As(V) are diverse in their phylogeny and overall physiology. Microorganisms that use arsenic for energy production fall into two classes, the **chemolithoautotrophic As(III) oxidizers** and the **heterotrophic As(V) reducers**.

9.6.1 Arsenite Oxidation

Chemolithoautotrophs obtain energy by coupling the oxidation of As(III) to the reduction of oxygen or nitrate (Fig. 9.4). Members of the *Alphaproteobacteria (Rhizobium),* also *Pseudomonas arsenitoxidans* and a *Hydrogenophaga* sp. operate metabolism with oxygen with the following reaction equation:

$$2H_2AsO_3^- + O_2 \rightarrow 2HAsO_4^{2-} + 2H^+$$
$$\Delta G^{0'} = -131.4 \text{ kJ / mol}$$

Ectothiorhodospira performs nitrate respiration with As(III) as electron donor:

$$H_2AsO_3^- + NO_3^- \rightarrow HAsO_4^{2-} + NO_2^- + H^+$$
$$\Delta G^{0'} = -56.5 \text{ kJ / mol}$$

Not all As(III)-oxidizing bacteria, such as *Alcaligenes faecalis,* obtain energy from As(III) oxidation. These As(III)-oxidizing heterotrophs probably do not use As(III) as an electron donor for respiration, but rather As(III) oxidation is incidental or a form of detoxification for these strains.

9.6.2 Arsenate Reduction

A diverse group of heterotrophic bacteria can use As(V) as a terminal electron acceptor for respiration (◘ Fig. 9.4). These organisms include members of the *Gamma, Delta, Epsilonproteobacteria,* Gram-positive bacteria, thermophilic Eubacteria, and *Crenarchaeota*. Most As(V)-breathing strains couple the oxidation of lactate to acetate to As(V) reduction to As(III). Some isolates can mineralize acetate to CO_2 and/or use H_2 as an electron donor. Most known As(V) breathers are obligate anaerobes; a few are also facultative aerobes.

9.6.3 Arsenate Methylation

Microbial methylation has also been demonstrated for **arsenic.** Whether this reaction also serves detoxification is unclear. The methylated compounds are usually more volatile. For higher organisms and humans, dimethyl- and trimethylarsine are always more toxic.

9.7 Selenium

The toxic effects of selenium on biological systems are concentration-dependent and vary from organism to organism. Selenium, on the other hand, is an essential element for prokaryotes and also eukaryotes and is therefore readily assimilated. Selenium deficiency is thought to be related to reduced activity of selenium-containing enzymes such as glutathione peroxidase. Selenium is found in selenocysteine-containing selenoproteins, selenium-containing t-RNAs as well as selenoenzymes (Heider & Böck, 1994). To prevent deficiency, selenium is often added to cow feed. This in turn may be another anthropogenic source of selenium in the environment.

The soluble oxyanions selenate [Se(VI) SeO_4^{2-}] and selenite [Se(IV)SeO_3^{-}] are the primary forms of selenium in the oxic environment. They disappear in the oxic/anoxic transition zone and are replaced by elemental selenium [Se^0], which is the dominant species in anoxic sediments. The reduction of Se(VI) and Se(IV) to insoluble Se^0 occurs in the presence of high concentrations of sulfate (>300 mM), as observed in hot brines in the San Joaquin Valley (California, USA). The transformation of selenium in nature occurs mainly by biological processes. Se(VI) is not subject to rapid chemical reduction under physiological conditions of pH and temperature. Therefore, abiotic reduction of selenate is unlikely to play a role in the natural environment.

Selenium compounds can also act as electron acceptors for anaerobic respiration (SeO_4^{2-}/SeO_3^{-}, $E^{0'}$-value: +475 mV). Although not normally found in large quantities in natural systems, selenium compounds are occasionally found as contaminants and can support the anaerobic growth of various bacteria.

The reduction of SeO_4^{2-} to SeO_3^{-} and finally to Se^0 is an important method for selenium removal from water and is used as a clean-up method of selenium-contaminated soils (bioremediation). Most bacteria capable of reducing selenium compounds can also use other electron acceptors, such as Fe^{3+}, Mn^{4+} and organic compounds, and in most cases exhibit facultative aerobic metabolism.

Microbial methylation has also been demonstrated for selenium. Thus, both bacteria (e.g. *Flavobacterium* and *Pseudomonas* species) and fungi (e.g. *Aspergillus, Cephalosporium, Fusarium* species) form dimethylselenide.

Process for Eliminating Heavy Metals from the Environment

The central difference to the organic pollutants discussed in ► Chap. 6 should be noted: In the case of toxic heavy metals or metalloids, combustion or **microbial degradation** to unproblematic compounds is fundamentally **not possible** due to their elemental nature. Of importance, however, are the aspects of conversion into less toxic forms of bonding or oxidation states and, in particular, mobilization or immobilization.

Basically, different **principles of interaction** play a possible role with regard to microbiological processes for the purification of contaminated soils or waters, which are subdivided here according to the underlying mechanism as well as the effect produced.

Three mechanisms are of particular importance for the **elimination of** metals from the environment:

- Biotransformation,
- Biosorption and bioaccumulation
- Sulfide precipitation.

Biotransformations include reductions and oxidations as well as the formation of organometallic compounds. They require metabolically active cells.

A number of bacteria can oxidize arsenite [As(III)] to arsenate [As(V)] in **wastewater containing arsenic.** Arsenate can then be precipitated from wastewater with Fe(III) salts more effectively than arsenite.

Chromium enters wastewater as a hexavalent compound from the leather and metal industries. The toxic chromate (CrO_4^{2-}) and dichromate ($C_2O_7^{2-}$) are reduced by bacteria (e.g. *Alcaligenes, Enterobacter* and *Pseudomonas* species) to the less toxic trivalent chromium. The reaction takes place at the cell surface, forming poorly soluble chromium hydroxides that precipitate.

Biovolatilization for the environment means the shift from a medium to the gas phase. Mechanism of Hg^{2+} resistance involves as a crucial step the reduction of the metal ion to the volatile Hg^0 and has been exploited to develop a purification process, a **reductive biovolatilization.**

Further, the methylation of metals should be mentioned. Arsenic, selenium and mercury can be methylated and thus **volatilized** by various fungi and bacteria, a process that could be called **alkylating biovolatilization.** Methylations probably fulfil detoxification functions for the microorganisms, but for the environment they mean a shift from a medium to the gas phase, which is also associated with the formation of even more toxic compounds for humans and animals.

Bioaccumulation. The **silver** produced in the film industry can be accumulated by silver-resistant bacterial strains (*Pseudomonas maltophila,* Coryneform bacteria) up to concentrations of 200 mg silver/g cell dry matter.

Cadmium accumulation has also been demonstrated by resistant *Klebsiella, Pseudomonas* and *Staphylococcus* strains. *In* addition to cadmium, the binding of copper and zinc to **metallothioneins** was observed in bacteria. Metallothioneins are low molecular weight cysteine-rich polypeptides to whose SH groups heavy metals are bound.

Biosorption, in contrast to bioaccumulation, is not bound to living metabolically active cells. In **biosorption**, the heavy metals are adsorbed on various exopolymers of the cell surface with a negative charge. Processes based on this can be used to remove metals from water, which is being studied, for example, on UO_2^{2+}-contaminated waters. Using cells of *Bacillus,* preparations were made that sorbed Cd, Cr, Cu, Hg, Ni and Zn nonselectively up to 10% of dry weight. Sorption occurs from highly dilute solu-

tions. The accumulated heavy metals can be stripped with acids and the biomass reactivated by alkali treatment.

Heavy metal sulfide precipitation Due to H_2S formation by sulfate-reducing bacteria, cadmium, copper and zinc can be precipitated as CdS, CuS or ZnS. For wastewater treatment, this process is used in constructed wetlands and in anaerobic biogas reactors.

Recently, attempts have been made to clean soils contaminated with heavy metals by the combined use of metal-leaching sulfur oxidizers and precipitating sulfate reducers. In this process, a sulfide-containing solution is applied to the upper aerobic soil zone, which leads to the development of *Acidithiobacillus.* Due to the sulfuric acid formation, heavy metals go into solution, which are precipitated as sulfides in deeper anaerobic soil layers. A prerequisite is that organic nutrients are introduced in the anaerobic zone for the sulfate reducers. Some of the anaerobically formed H_2S is recycled to supply sulfide to the topsoil. Metal elimination of the upper soil layers was achieved in this way.

9.8 Uranium

Uranium (U) is a common groundwater contaminant at sites where nuclear testing was conducted and weapons material was produced during the Cold War era. Because of its prevalence and potential toxicity, remediation strategies are of interest to stabilize the U(VI) and minimize the risk of transport out of the contaminated areas.

Remediation of uranium contaminated sites can be accomplished by changing the oxidation state from the soluble U(VI) to the less soluble U(IV) using biological reductive metal precipitation to limit transport out of contaminated areas. Reduction of U(VI) is facilitated by a large number of different microorganisms such as sulfate (SRB) and Fe(III)-reducing (FeRB) bacteria as well as other microorganisms such as *Clostridium* sp. and *Deinococcus radiodurans.*

Background

Deinococcus radiodurans shows exceptional resistance to ionizing radiation and desiccation. Relative to most other organisms, *Deinococcus* has a survival rate many orders of magnitude higher. An exposure to 5 kGy of ionizing radiation causes fragmentation of the genome into hundreds of fragments in any bacterium. *Deinococcus* is no exception to this. But *Deinococcus* seems to handle this catastrophe loosely. In a period of 3–4 h, overlapping fragments are assembled into a complete chromosome, allowing normal cell growth to occur. There is no measurable lethality. Attempts on the molecular basis to explain the phoenix-like property led to a variety of hypotheses. Noteworthy among them are suggestions the condensed appearance of the *Deinococcus* genome or the unusual power to avoid protein oxidation may be key to radiation resistance.

Regardless of which physiological or metabolic adaptation helps *Deinococcus* survive, it is hard to imagine extreme genome restoration without the aid of DNA repair.

Test Your Knowledge

- Which forms of mercury are most toxic to living things?
- How is mercury detoxified by bacteria? How does resistance to inorganic and organic mercury compounds occur in bacteria?
- Name at least three different mechanisms that can lead to resistance of

bacteria to heavy metals or metalloids!

- What can microorganisms use arsenate for, what can they use arsenite for?
- Compare the mechanisms of removal of organic compounds and heavy metals from environmental media. What distinguishes the purification of soils or waters when contaminated with inorganic pollutants from when contaminated with organic pollutants?
- Compare "natural" and anthropogenic sources of arsenic contamination.
- What processes are possible for the elimination of heavy metals from the environment?
- Give examples of the possibility of immobilization of metals and metalloids under oxidizing conditions. By what microbially induced mechanisms can inorganic pollutants be immobilized under reducing conditions?
- Can bacteria detoxify Cd^{2+} and Ni^{2+} via reduction?
- Name three different ions of inorganic pollutants that can be used for energy production by electron transport phosphorylation.

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Microorganisms at Different Sites: Living Conditions and Adaptation Strategies

Contents

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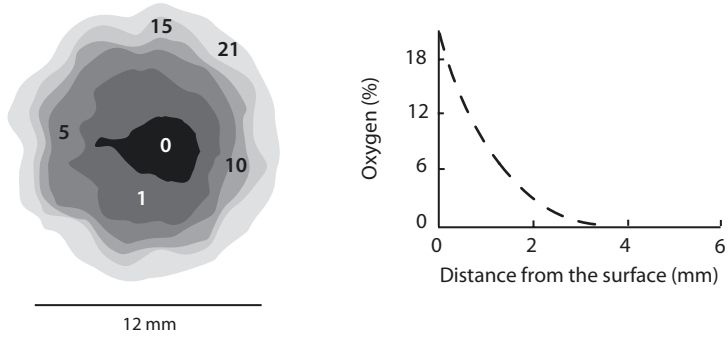
The natural habitats of microorganisms are extraordinarily diverse. Every biotope allows the growth of microorganisms, even when extreme physical or chemical conditions prevail. Habitats include, for example, the free water of oceans or lakes. Other organisms, however, live on or in solid matrices such as sediments or soil. Microorganisms also colonize the surface of higher organisms and in some cases even live within plants and animals. High population densities are achieved in such habitats.

The growth of microorganisms in nature depends on the **growth conditions** and the available **resources (nutrients)**. Differences in physicochemical conditions (temperature, pH, water, light, oxygen) and in the type and amount of different resources of a biotope define the niche for a particular microorganism. Countless niches exist on Earth, which are responsible for the great metabolic diversity of today's microorganisms.

The small size of the microorganisms allows habitats in the smallest space. **Physical** and **chemical gradients** can exist within a few millimetres in habitats that strongly influence the microorganisms. This can be illustrated by the distribution of oxygen in a soil particle. Soil particles are not homogeneous in terms of their oxygen content. The outer zones of a small soil particle may be completely aerobic, while completely anaerobic conditions may exist in the centre (◘ Fig. 10.1). This shows that different niches can exist within very small spatial dimensions. It explains how different physiological types of microorganisms can coexist in such a soil particle. Anaerobic microorganisms can be active in the center of the represented soil particle, microaerophiles further outside. Obligate aerobic microorganisms may carry out their metabolism in the outer 2–3 mm of the particle, while facultative anaerobic bacteria may be distributed throughout the particle. A similar situation is found in biofilms, which will be addressed later.

The physico-chemical conditions in the microworld can also change rapidly. For example, a more pronounced oxygen gradient may result due to microbial respiration in the soil particle or after an increase in soil water content in the microenvironment. Thus, the microenvironments are **heterogeneous** and can **change very rapidly.**



◘ **Fig. 10.1** Soil particles and oxygen concentration

Physico-Chemical Boundary Conditions for Survival and Optimal Growth, Respectively

In their entirety, the microorganisms cover an astonishingly broad range of physico-chemical conditions, whereas individual species are always very restricted (see ◘ Table 10.1).

Growth of microorganisms is possible over a wide temperature range. *Polaromonas* still grows at 0 °C, while *Pyrodictium* is known as an example of growth at the other extreme of 121 °C. For individual species, the temperature range of optimal growth is related to their habitat. To characterize this range, we speak of psychrophilic, mesophilic, thermophilic, hyperthermophilic, or extremely hyperthermophilic organisms, depending on whether they are adapted to low, medium, high, or even very high growth temperatures (◘ Fig. 10.2b). Colonization of deep-sea hydrothermal vents by prokaryotes in the range of 125 to about 140 °C has been documented. An upper limit of survival of about 150 °C is assumed.

Pressure can also determine whether an organism can grow in a habitat. This is particularly evident in barophilic organisms of the deep sea. Deep-sea microorganisms must be able to withstand the enormous hydrostatic pressure that prevails at great depths. The pressure increases by 1 atm every 10 m. Thus, a microorganism growing at a depth of 5000 m must be able to withstand a pressure of 500 atm. Some organisms tolerate high pressure, they are **barotolerant**, others need the high pressure, they are **barophilic** (◘ Fig. 10.3).

The distribution of barotolerant and barophilic bacteria is a function of depth. Organisms isolated from depths up to about 3000 m are barotolerant. Barotolerant isolates do not grow at pressures above 500 atm. In contrast, cultures obtained from greater depths, 4000–6000 m, are barophilic, growing optimally at pressures of about 400 atm. Samples from even greater water depths (10,000 m) reveal extremely (obligate) barophiles. They grow fastest at pressures of 700–800 atm and almost as well at 1035 atm, the pressure in their natural habitat. They do not grow at pressures below 400 atm.

With regard to the chemical boundary conditions, the **pH value of** the habitat plays a special role. While many of the known organisms grow preferentially in the neutral range (neutrophilic microorganisms), acidophilic microorganisms grow preferentially or exclusively at low to very low pH values (up to pH 0), while alkaliphilic microorganisms are adapted to higher pH values. Habitat pH is often strongly influenced by microbial activity. This is also reflected in the fact that acid-forming organisms are usually more or less acidophilic.

High **salinity levels** in a habitat can also limit the growth of microorganisms via the available water. Organisms that tolerate relatively high salt concentrations are referred to as halotolerant microorganisms. If they grow at all at elevated salt concentrations, such as some archaea that grow only at salt concentrations above 0.2 M (e.g. *Halobacterium*), they are referred to as halophilic microorganisms (see ► Sect. 10.1.2.1).

Table 10.1 Microbial response to environmental influences

Influencing factors with their microorganisms	Description/definition of the behaviour	Representative microorganisms
Water activity		
Osmotolerant	Is able to grow over a wide range of water activity or osmotic concentration	*Staphylococcus aureus*
Halophilic	Requires higher concentrations of NaCl, usually above 0.2 M for growth	*Halobacterium salinarum*
pH		
Acidophilic	Optimum growth between pH 0 and 5.5	*Sulfolobus, Picrophilus oshimae*
Neutrophils	Optimum growth between pH 5.5 and 8.0	*Escherichia, Euglena*
Alkalophilic	Optimum growth between pH 8.0 and 11.5	*Bacillus alcalophilus, Natrobacterium gregoryi*
Temperature		
Psychrophilic	Grows well at 0 °C and has an optimum growth temperature at 15 °C or lower	*Bacillus psychrophilus, Polaromonas vacuolata*
Psychrotrophs	Can grow at 0–7 °C; has an optimum growth temperature between 20 and 30 °C and a maximum around 35 °C	*Listeria monocytogenes, Pseudomonas fluorescens*
Mesophilic	Has an optimum around 20–45 °C	*Escherichia coli*
Thermophilic	Can grow at 55 °C or higher; optimum often between 55 and 65 °C	*Bacillus stearothermophilus, Thermus aquaticus*
Hyperthermophilic	Has an optimum between 80 and even 113 °C	*Sulfolobus, Pyrococcus, Pyrodictium, Pyrolobus fumarii*
Oxygen concentration		
Obligate aerobes	Is entirely dependent on atmospheric O_2 concentration for growth	*Micrococcus luteus, Pseudomonas*
Facultative anaerobes	Does not need O_2 to grow, but grows better in the presence of O_2	*Escherichia, Enterococcus*
Aerotolerant anaerobes	Grows equally well in the presence and absence of O_2	*Streptococcus pyogenes*
Obligate anaerobes	Does not tolerate O_2 and dies in the presence of O_2	*Clostridium*
Microaerophilic	Requires O_2 concentrations below 2–10% for growth and is destroyed by atmospheric concentration (20%)	*Campylobacter*
Pressure		
Barophile	Grows much faster at high hydrostatic pressure	*Photobacterium profundum, Methanococcus jannaschii*

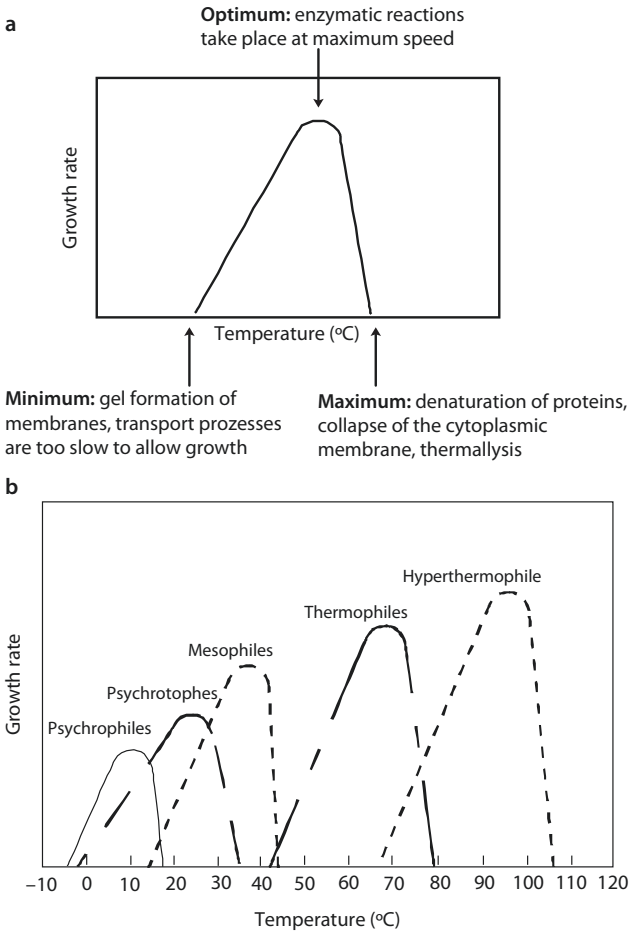


Fig. 10.2 **a** Influence of temperature on metabolism. **b** Temperature ranges of growth of different organisms

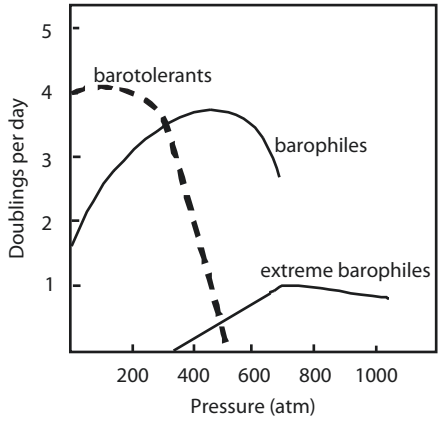


Fig. 10.3 Dependence of growth rate on pressure in barotolerant, barophilic and extremely barophilic bacteria

10.1 Microbial Competition and Cooperation

Nutrients often enter an ecosystem in varying amounts. A large accumulation of nutrients—for example, leaves or a carcass—may be followed by a period of severe nutrient deficiency. That is, microorganisms lead a "banquet or starvation" existence in nature. Many microorganisms have evolved biochemical systems that produce storage polymers as reserve materials to store excess nutrients available under favorable growing conditions for use during periods of nutrient scarcity. In nature, however, extended periods of exponential

growth of microorganisms are rare. Growth more often occurs in spurts that are closely linked to the presence of nutrients. Because physicochemical conditions in nature are rarely all optimal at the same time, growth rates of microorganisms outdoors are generally far below the maximum growth rates determined in the laboratory. Estimates of the growth rate of soil bacteria have shown that they grow in nature at less than 1% of the maximum rate measured in the laboratory. These low growth rates reflect the fact that

- nutrients are often present in small quantities,
- the distribution of nutrients in the microbial habitat is not uniform, and
- microorganisms usually compete with other microorganisms in natural environments.

Competition among microorganisms for available resources can be intense. The following points are of importance:

- Due to the slow hydrolysis of particulate organic matter, the growth of heterotrophic microorganisms in most ecosystems is controlled by the availability of carbon and energy substrates.
- In nature, microorganisms grow largely with mixtures of substrates, so that growth is controlled not by a single substrate but by two or more substrates simultaneously.
- The kinetic properties of a cell can change by adaptation.

10.1.1 Growth Rates and Nutrient Concentrations

Growth or degradation phenomena can be satisfactorily described for the most part by the following four parameters, the **kinetic parameters:**

1. Themaximum specific growth rate (μ_{max}, h^{-1}).
2. The affinity constant (K_s, µg/L), as used in the Monod growth model, denoting the substrate concentration at $\mu = 0.5\ \mu_{max}$.
3. The stoichiometry parameter, yield coefficient ($Y_{X/s}$, g cells per g substrate converted), which describes the efficiency of conversion of the growth substrate into cellular material.
4. A limiting substrate concentration (s_{min}, µg/L) at which bacterial growth is zero, i.e. the substrate concentration necessary for maintenance metabolism.

In an ecosystem, fast and slow growing species can live side by side and with each other. In the simplest case, the outcome of a competition between different microorganisms depends on rates of nutrient uptake, inherent metabolic rates and ultimately growth rates. Growth rate is one of the central selection criteria.

In ◘ Fig. 10.4 substrate saturation curves for two different types of microorganisms are shown for comparison. **Organism I** has a high substrate affinity, that is, a low K_s value and a low maximum specific growth rate. **Organism II** has a high K_s and a high μ_{max}.

At a high substrate supply, organism **II** will grow much faster than organism **I**, and it will likely outgrow organism **I** with it. At low supply below the intersection of the two saturation curves, organism **I** has a higher growth rate than organism **II** and will outcompete it because of this substrate. Organism **I** is better adapted to low but steady substrate influx, while organism **II** prefers to have the sudden substrate surge as an advantage. Obviously, organism **I** represents the **autochthonous** type of microorganism that grows slowly but steadily and takes advantage of low but steady substrate influx. Organism **II** represents the **zymogenic** type, which grows rapidly at times but is unable to sustain itself under low substrate influx.

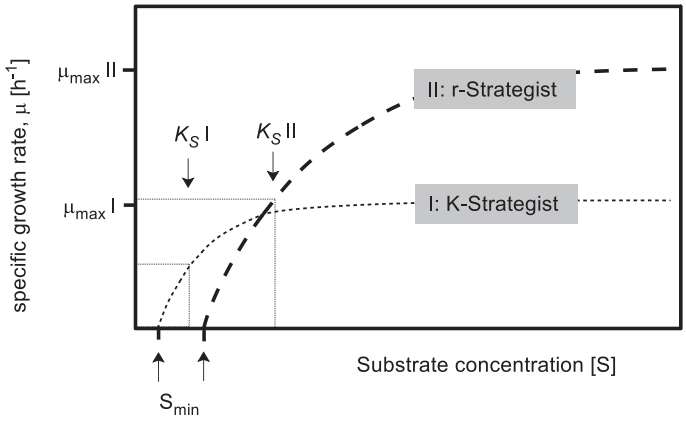


Fig. 10.4 Microbial adaptation strategies to substrate concentration

As early as 1925, Winogradsky used the term **autochthonous** for microorganisms that are adapted to the conditions prevailing in the ecosystem (Waksman, 1953, Dworkin, 2012).

In general ecology, we speak of representatives with **r-** and **K-strategy**. The symbol **r** goes back to rate and indicates species with a high growth rate, but which are easily lost from an ecosystem, this depending on the changes in the system **(r-strategist). K** denotes capacity for diverse metabolic services that allow organisms to exist without rapid reproduction, making constant and optimal use of all the system's resources **(K-strategist).**

Microbial Growth

Natural habitats of bacteria can be characterized by strong fluctuations in nutrient supply or by great constancy.

In the laboratory, the two ideal types of nutrient supply are realized by two different cultivation forms of microorganisms:

- When a nutrient medium is inoculated with bacteria in a closed vessel, the bacteria can develop until some nutrient becomes limiting or some inhibitory metabolite accumulates (Fig. 10.5). The concentration of nutrients here decreases as the cultivation progresses and is referred to as **static culture** or batch culture, although "limited culture" might be a more accurate term.
- If, on the other hand, fresh nutrient medium is constantly supplied to a bacterial culture and the corresponding culture fluid is removed, constant conditions are established in which the bacterial culture continues to grow (Fig. 10.6). This form of cultivation is referred to as **continuous culture.**

Chemostat data: bacterium with parameters: μ_{max} = 1 h^{-1}, $Y_{X/s}$, = 0.5 g cells per g substrate reacted, K_s = 0.2 g/L; substrate concentration in feed: 10 g/L; D_m: Dilution rate with maximum productivity.

The stability of the flow equilibrium in the chemostat is based on the limitation of the growth rate by a substrate. If one sets a constant inflow rate over a longer period of time, the system regulates itself.

Characterization of microbial growth or ecological strategies requires parameters that are most easily derived under the assumption of static culture conditions:

If we first assume that a nutrient medium is inoculated with a certain number N_0 of appropriately adapted bacteria and that these initially divide unhindered and simultaneously, then their number will double with each division cycle. The bacterial number N after n synchronous division cycles would then be:

$$N = N_0 \times 2^n$$

The time that elapses between two successive divisions is called the generation time g, its reciprocal the division rate ν.

In fact, however, the bacteria of a culture do not divide simultaneously and their number N as well as, in particular, their mass X will initially increase continuously and exponentially in a static culture. The increase in bacterial mass per unit time dX/dt depends at this stage on the mass already present and on a factor μ called the **growth rate**:

$$dX / dt = X \times \mu$$

By conversion and integration between the times t_0 and t one obtains from this:

$$\ln(X / X_0) = \mu(t - t_0) \quad \text{oder or}$$

$$X = X_0 \times e^{\mu \times (t - t_0)}$$

If we consider the doubling time ***t*d**, that is, the time that elapses until $X = 2X_0$, then:

$$\ln 2 = \mu \times t_d \quad \text{or} \quad \mu = \ln 2 / t_d$$

However, the phase of exponential growth is very limited in a static culture. At some point, the dwindling concentration of a nutrient or the accumulation of inhibitory metabolites will limit further growth. Bacterial mass approaches the maximum **capacity *K*** of the system. The increase in bacterial mass per unit time dX/dt can therefore be described more correctly as follows, taking into account the capacity of the system, instead of using the above equation:

$$dX / dt = X \times \mu \times (K - X) / K$$

(after Lengeler et al.,1999)

The additional term is approximately equal to 1 as long as the bacterial mass X is small in comparison to the capacity K. As X approaches K, the term approaches 0 and growth eventually comes to a halt.

Example: Thoughts on capacity limits

How long does it take for a cell of the following microorganism species to grow to such an extent that the cells formed cover the entire surface of the earth, including the oceans (510 million km^2), with a layer one meter thick (without gaps between the cells; no change in density)?

Accepted:

1. The microorganism had a volume of 1.6 μm^3 (rods of 2 µm length and 1 µm diameter).
2. The microorganism divides every 20 min (like *Escherichia coli* under optimal conditions).
3. The system has an unlimited capacity, which means that nutrients do not become scarce.

It would theoretically only take about 36 h.

The example shows that capacity limits and in particular nutrient limitations can take effect very quickly in the case of exponential growth and can significantly slow down or even stop growth.

In numerous cases, it is observed that microorganisms grow faster at high substrate concentrations in the exponential

phase than at low ones. Thus, the growth rate μ is not a constant, but approaches a maximum value μ_{max} with increasing substrate concentration (◘ Fig. 10.7), and the growth rate achieved at a given substrate concentration depends critically on the affinity of the microorganism for the substrate. Due to the formal similarity to the Michaelis-Menten equation described in enzyme kinetics, the **Monod equation** was formulated as follows:

$$\mu = \mu_{max} \times [S] / (K_S + [S])$$

[*S*]—Substrate Concentration; μ_{max}—Maximum growth rate; K_S—Substrate affinity constant

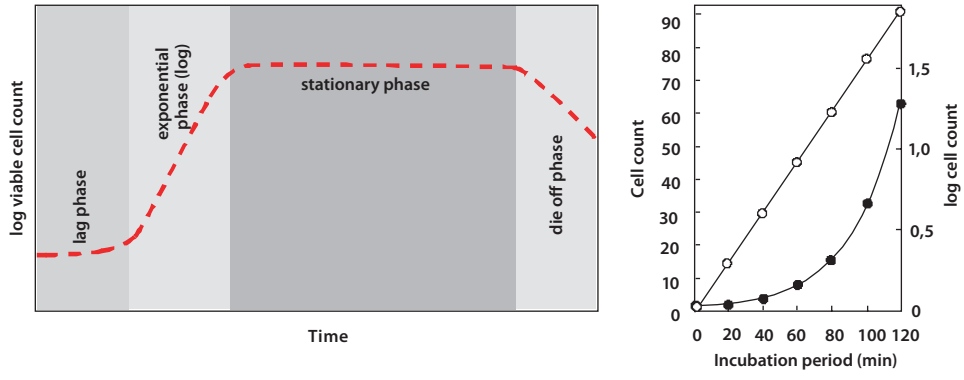
10.1.2 Adaptation

To survive and compete successfully in nature, most microorganisms are able to meet environmental challenges by adapting their cellular constitution in terms of structure and metabolic function. Whether the adaptive changes occur at the phenotypic level, the genotypic level, or both, it is clear that they affect growth and/or the kinetic degradation properties that the cell possesses.

During their life cycles, many heterotrophic microorganisms encounter habitats that differ significantly in terms of spectrum and concentration of available nutrients. As an example, when *Escherichia coli* leaves its primary habitat, the nutrient-rich **(copiotrophic)** anaerobic gut of warm-blooded animals and humans with its abundant influx of carbonaceous compounds, it must adapt to a nutrient-poor **(oligotrophic)** secondary habitat (water, soil or sediment). There, concentrations of nutrients are typically in the low micromolar or even nanomolar range and the availability of the carbon and energy source limits the growth of the heterotrophic microorganisms.

Microorganisms can adapt to growth at different, extracellular substrate concentrations by considerably adjusting their kinetic properties (in Monod terms: μ_{max} and K_s). The following strategies have been described for both Gram-negative and Gram-positive microorganisms:

- A single uptake system is used, which has different kinetic properties with respect to concentration for its substrate (so-called multiphasic kinetics).



◘ **Fig. 10.5** Illustration of static culture and phase of a growth curve

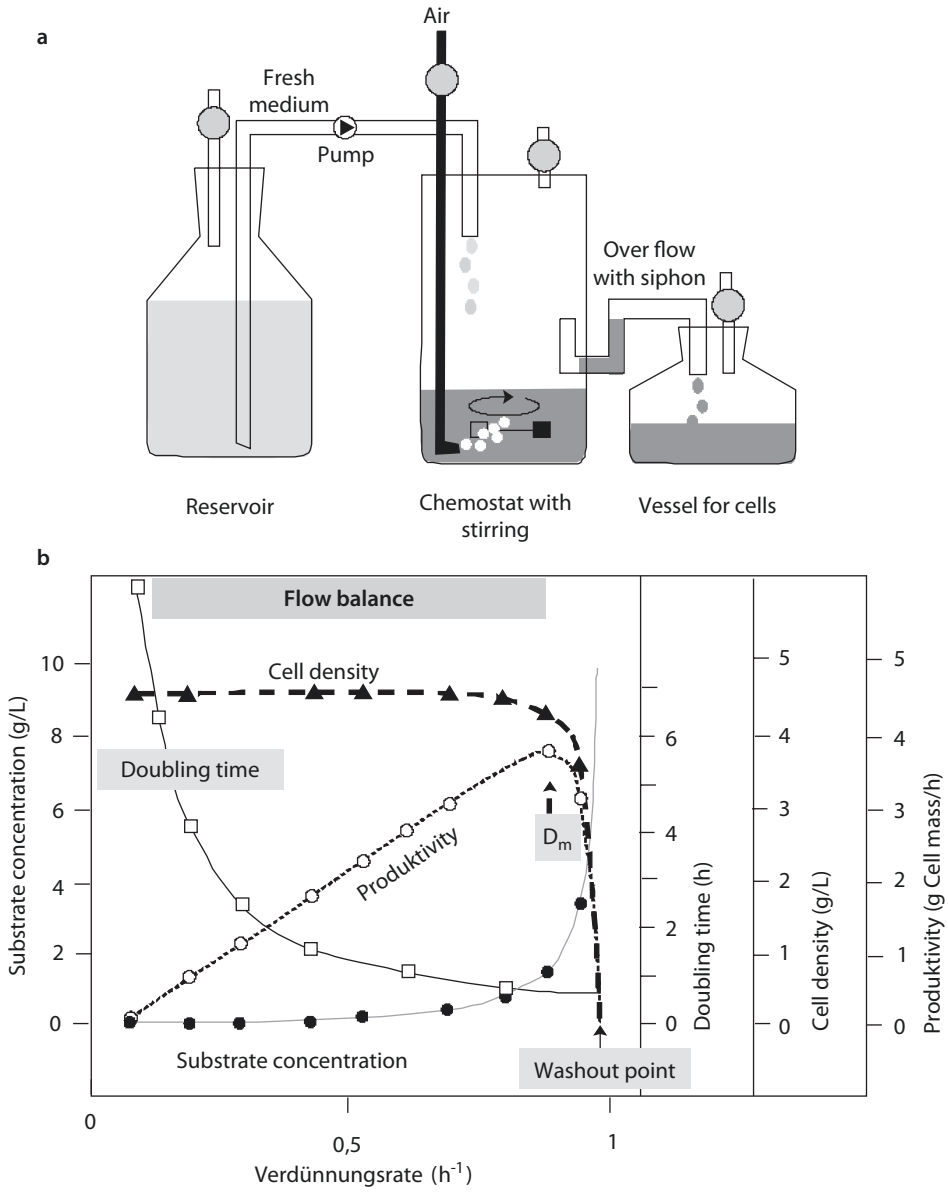


Fig. 10.6 (**a**) Necessary details of building a chemostat and (**b**) substrate concentration in the chemostat

- The microorganisms switch between two or more transport systems with different affinities. This has been observed for different sugars or for glycerol and ammonia. Such changes may involve the modification of outer membrane components.
- Other, less well-defined changes are used, such as varying catabolic and/or anabolic performance, for example, to minimize cell-internal energy consumption.
- Another strategy is to increase the cell surface area in the presence of deficiency.

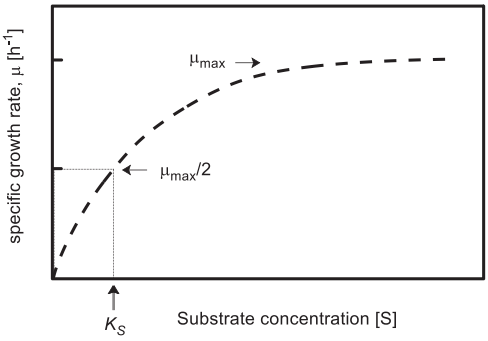


Fig. 10.7 Monod kinetics with K_s

These types of adaptation differ considerably in terms of the length of time it takes to make the change.

- A multiphasic system reacts immediately.
- Switching between different transport systems can take place relatively quickly, i.e. within a few minutes to hours.
- Adaptation at the population level (evolution and enrichment of competitive strains) is attributed to long-term changes.

The process of adaptation was found to be reproducible and faster at low than at high growth rates.

It was further shown that an affinity constant (K_s) for glucose drops from a few milligrams per liter when grown under batch conditions to 30 μg/L under steady-state conditions in the chemostat.

During growth at low glucose concentration, glucose is transported into the cell mainly by the high-affinity galactose-binding protein/maltose system rather than by the glucose phosphotransferase system, which was previously considered to be the only relevant glucose transport system in *Escherichia coli* under these conditions.

In contrast to the repressive role exerted by glucose when present at millimolar concentrations, expression of transport systems for other sugars has been observed in cells growing at levels of nano- to micromolar of glucose. This leads to an expansion of the utilization potential of the bacterium for other substrates.

All this makes it clear that the classification of bacteria according to nutrient concentrations as typically **oligotrophic** or **copio(eu)tropic** is arbitrary. In a similar vein, a long-known observation is that the ability of a microorganism to utilize nutrients at high or low concentrations depends on the substance being studied. Again, this makes oligotrophy and copiotrophy fuzzy concepts.

10.1.2.1 Adaptation to the Presence of High Salt Concentration

All organisms need water. The availability of free water is an important factor influencing the growth of microorganisms in nature. Generally, the presence of water is expressed in terms of water activity, a_w. The a_w value is the ratio of the vapor pressure of a solution to that of pure water. Consequently, the values vary between 0 and 1.0.

Water diffuses from a region of high water concentration to a region of high solute concentration by the process of osmosis. In most cases, the cytoplasm of a cell has a higher solute concentration than the environment, so water tends to diffuse into the cell. However, if a cell is in an environment of low water activity, then the water will have a tendency to flow out of the cell. This can lead to serious problems if the cell has no way to counter-react.

In nature, osmotic influences are of interest especially in habitats with high salt concentrations. Salt ions carry a more or less large hydrate shell in aqueous solution. At elevated salt concentrations, proteins and nucleic acids, which also depend on an adequate hydrate shell to maintain their structure, compete with such ions for water. The problem of increased salt concentrations is thus primarily a competition for free water (Fig. 10.8).

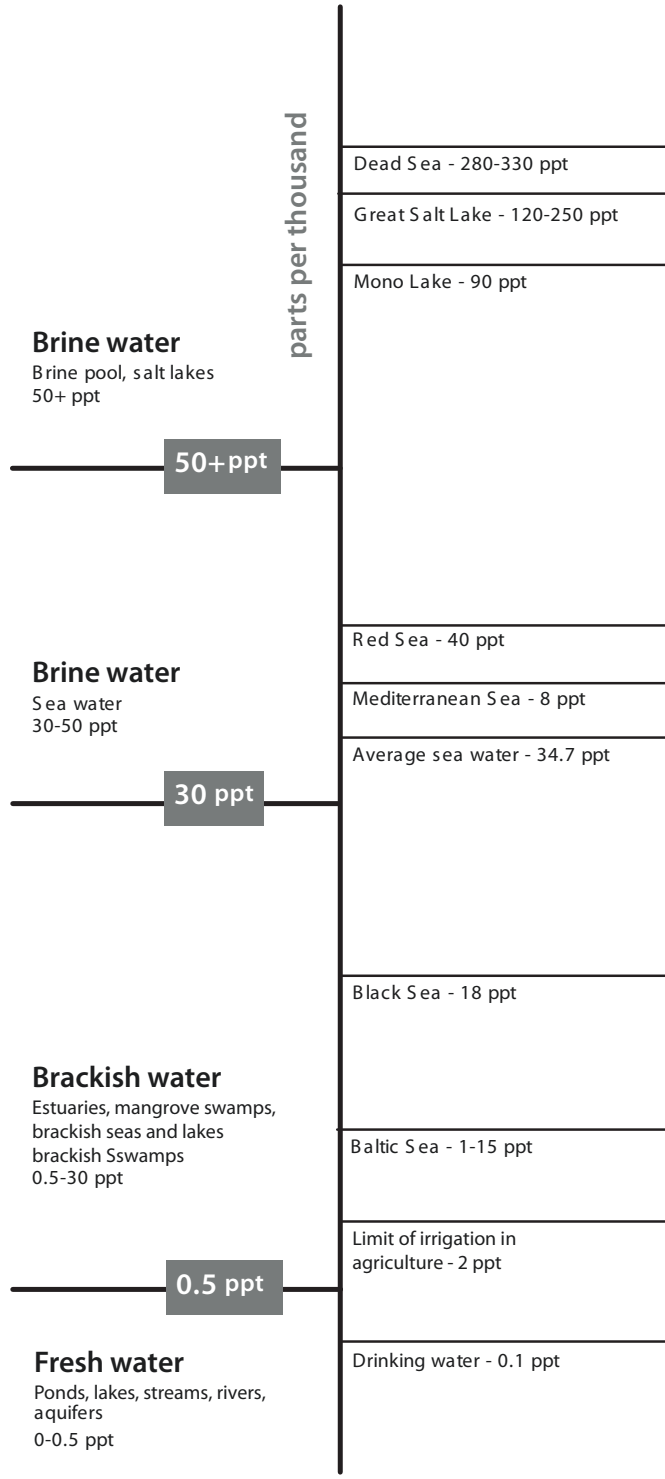


Fig. 10.8 The Practical Salinity Scale (PSS-78) used today is based on the proportionality of salinity and electrolytic conductivity of seawater samples relative to a standard KCl solution and is dimensionless. However, a PSU, which stands for *Practical Salinity Unit*, is often found to indicate salinity. A salinity of 35 does not correspond exactly to 35 g of salt per litre of solution

Water activities in agricultural soils generally vary between 0.9 and 1.0. Typical soil bacteria can adapt to such regular changes in water activity.

Seawater contains about 3% NaCl and small amounts of many other minerals and elements. The water activity of 0.98 is not yet a major physiological challenge.

The diversity of properties of saline and hypersaline habitats is reflected in the great diversity of microorganisms adapted to life under these particular conditions:

1. **Extreme halophiles** are generally defined as organisms that grow optimally in media containing a concentration of 150–300 g/L (2.5–5.2 M) NaCl.
2. **Moderate halophiles** are those that grow optimally in media that have a concentration of 30–150 g/L (0.5–2.5 M) NaCl.
3. Some non-halophilic microorganisms can tolerate high salt concentrations and they are referred to as **halotolerant organisms** or
4. **extremely halotolerant organisms**.

Halophilic and **highly halotolerant** species have been discovered in each of the three domains of life: Archaea, Bacteria, and Eukarya. Within the **Bacteria**, we know halophiles in the phyla *Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria,* and *Spirochaetes.* Within the **Archaea**, the microorganisms that are particularly salt-requiring have been found in the class *Halobacteria.*

When an organism grows in a medium with low water activity, it can only obtain water from its environment by increasing the internal solute concentration. Two fundamentally different strategies have been identified that allow halophilic microorganisms to osmotically match their cytoplasm to that of the medium and cope with the multimolar salinity of their environment.

The most commonly found form of osmotic adaptation is the biosynthesis of organic osmotic solutes ('compatible solutes') or their accumulation from the medium. Intracellular salt concentrations are thereby maintained at levels far below the medium outside the cell. No extensive adjustment of the intracellular machinery is necessary to allow cells to function in high osmolarity environments.

The intracellular concentrations of solutes that do not interfere with enzymatic activity can be rapidly adapted, so that these microorganisms using the **'organic-substances-in strategy'** have often adapted to an amazingly wide range of salt concentrations. This **'salt-out' method** allows the internal mechanism of the cell to remain in its normal state, but requires a high energy input to produce the organic molecules.

All eukaryotic species, most halophilic bacteria, and also the halophilic methanogenic archaea use such organic solutes (osmolytes) to balance osmotic pressure. A variety of such solutes is known:

1. Yeasts and other eukaryotes such as halophilic green algae accumulate glycerol in the cell interior.
2. Halotolerant *Staphylococcus* use proline as a compatible solute.
3. Especially in the halophilic bacteria and the cyanobacteria, glycine betaine is the widely used compatible solute. It is a zwitterionic derivative of glycerol in which the hydrogens of the amino group are replaced by methyl groups. This leaves a permanent positive charge on the N atom, increasing solubility.
4. Some extremely halophilic bacteria form ectoine, a cyclic zwitterionic compound formed from aspartate semialdehyde.
5. A variety of glycosides and dimethylsufonium propionate are produced by marine algae, but very rarely accumulate even in small amounts (◘ Fig. 10.9).

The second, **'high-salt-in strategy'** involves the accumulation of molar concentrations of KCl in the cytoplasm. Sodium ions are pumped out of the cell in exchange for protons or potassium ions, whose hydrate shells are smaller than those of sodium ions. This

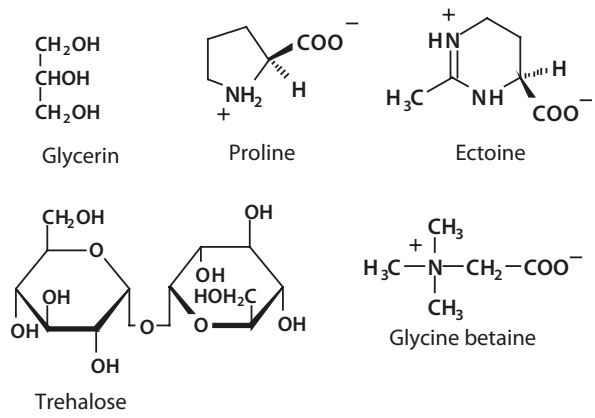


Fig. 10.9 Structures of some highly soluble compatible substances commonly found in halophilic and halotolerant microorganisms

'salt-in' method is more energetically efficient, but needs adaptation of the intracellular enzymatic machinery, since proteins must maintain their proper conformation and activity at near-saturated salt concentrations. The proteome of such organisms is highly acidic. In general, such halophilic proteins need salt for their structural stability and activity, so the microorganisms that use this type of osmotic adaptation need high salt concentrations and have limited ability to cope with low salt concentration media.

The 'salt-in' strategy has so far been found in a few groups of prokaryotes: the aerobic extremely halophilic Archaea of the family *Halobacteriaceae*, the red aerobic *Salinibacter (Bacteroidetes)* recently isolated from saline gardens, and a phylogenetically contiguous group of obligately anaerobic bacteria of the order *Halanaerobiales* affiliated to the low-G + C branch of Gram-positive bacteria, the *Firmicutes.*

The adaptation of proteins to extreme salinity is associated with a general increase in the amount of acidic amino acids, which simultaneously means a decrease in basic amino acids. This trend has been demonstrated using the genome of hyperhalophilic **salt-in** archaea such as *Halobacterium* NRC-1. In **salt-out** halophiles, on the other hand, the proteins only have an excess of acidic amino acids that are exposed to the hypersaline medium.

The proteins of the *Halanaerobiales,* which are active in the presence of high intracellular KCl concentrations, do not exhibit the typical acidic signature of the 'halophilic' proteins of the archaea of the order *Halobacteriales* or of the extremely halophilic bacterium *Salinibacter.*

The fact that extreme biotopes are often species-poor but extremely rich in individuals can be impressively documented by the following examples. The salt concentration reaches approx. 12% in the larger part of the Great Salt Lake in Utah, USA. A mass development of the red pigmented green alga *Dunaliella salina* can be observed, which is grazed by the brine shrimp *Artemia salina.* Representatives of the genera *Halomonas, Marinomonas*, *Pseudomonas* and others dominate among the microbial consumers. The salt concentration reaches the saturation limit in the northern arm of the lake at about 25%. Eukaryotes are not found here. Here, too, the water shows a reddish colouration due to extremely halophilic aerobic archaea of the genera *Halobacterium, Haloferax* and *Halomonas*, which are subject to practically no grazing at this site.

The water of the present Dead Sea contains 348 g/L salts (2 M Mg^{2+}, 0.5 M Ca^{2+}, 1.5 M Na^{+}, 0.2 M K^{+}, 6.5 M Cl^{-}, 0.1 M Br^{-}) and thus represents a particularly inhospitable environment of extreme hypersalinity. The pH is about 6.0. Contrary to its name, the Dead Sea is not biologically

dead. However, life is largely restricted to various extremophiles. After rainy winters, the water surface became so diluted that it triggered the development of microbial blooms. A red coloration of the Dead Sea was thus caused in 1980 and 1992 by the unicellular green alga *Dunaliella* and red archaea.

Lake Vida, Life in a Brine Under Antarctic Ice

Lake Vida in eastern Antarctica is covered by a layer of ice about 20 m thick and sits on several hundred meters of thick permafrost. The permanent ice layer encapsulates an extreme ecosystem of low temperature and high salinity (–13 °C; salinity, 200). This aphotic ecosystem (no light) is anoxic and consists of a NaCl-dominated slightly acidic (pH 6.2) brine. It had been noticed during ice core drilling that a yellowish brine collected in the boreholes, which took on a brown-red discoloration upon contact with air. Geochemical and microbiological analyses showed that, in addition to large amounts of salt, the water contained a great deal of dissolved highly saturated nitrous oxide (N_2O, ~60 µmol/L) and other nitrogen compounds such as ammonia (NH_4^+, ~3.8 mmol/L), nitrite (NO_2^-, ~25 µmol/L), and nitrate (NO_3^-, ~1 mmol/L), as well as high concentrations of sulfate ~ 60 mmol/L. Molecular hydrogen (H_2, 10.5 µmol/L) is also present in high concentrations. Also strongly represented are dissolved reduced metals, especially iron (Fe^{2+}, 256 µmol/L), and both inorganic (CO_2, 8.9 mmol/L) and organic carbon compounds.

The geochemistry of the brine suggests that abiotic brine-rock reactions occur in this system and that abundant sources of dissolved electron acceptors prevent sulfate reduction and methanogenesis from proving energetically beneficial.

The large amount of dissolved and gaseous nitrogen compounds, Fe^{2+} and H_2 indicate that serpentinization-like reactions occur in Lake Vida brine (weathering of olivine, an important component of basalts). In Lake Vida brine, the content of H_2 is so high that it may have resulted from both abiotic and biogenic production. The H_2 could eventually provide readily usable energy to allow depurination and racemization processes to occur in this harsh ecosystem. Microbial life in Lake Vida brine is reasonably diverse, maintains cellular rRNA, and ensures the ability to synthesize proteins at a very low level.

Microscopy, after staining with fluorescent dyes for nucleic acids (DAPI and SYBRGold; reference to ► Sect. 11.1), showed that particles the size of aquatic bacteria (>0.2–1 µm diameter) were present at 0.1–0.6 × 10^6/mL, and those <0.2 µm diameter were present at 50–60 × 10^6/mL.

Using detection of small subunit rRNA genes (SSU rRNA), it was observed in the brine that phylogenetically diverse and metabolically active *Bacteria* dominate the microbial community:

Eight bacterial phyla (% of SSU rRNA gene library)

- Proteobacteria (Gamma 39%, Epsilon 16%)
- Lentisphaera (15%)
- Firmicutes (11%)
- Bacteroidetes (9%)
- Spirochaeta (7%)
- Verrucomicrobia
- Candidate Division TM7*
- Actinobacteria

Murray et al. (2012), *Rheims et al. (1996).

10.1.2.2 Adaptation to the Presence of Solvents

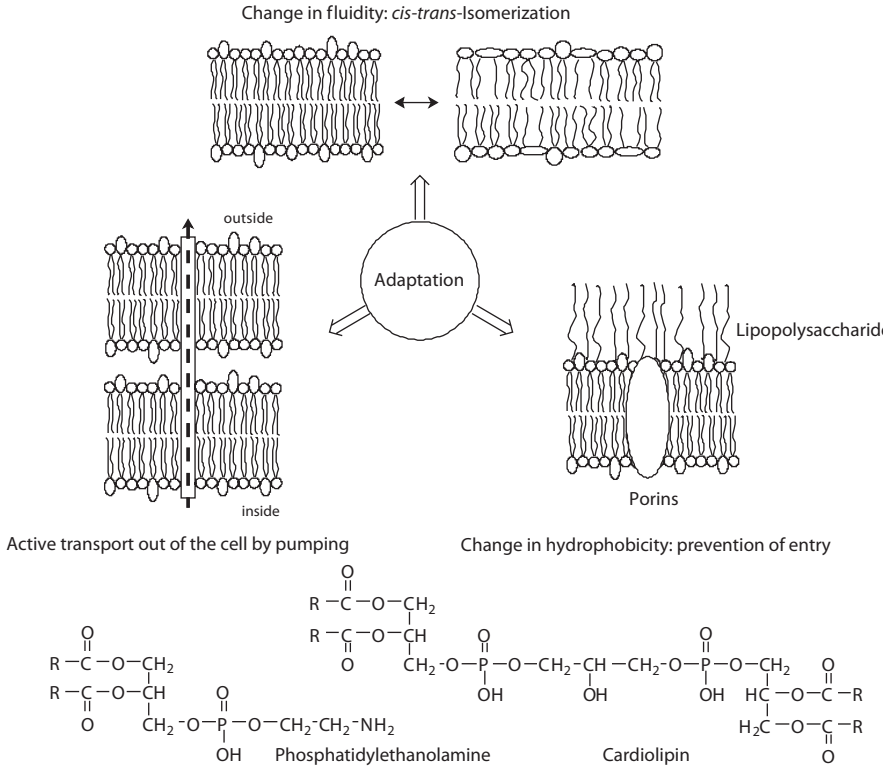
Solvents such as toluene are normally toxic to microorganisms. A correlation is seen between the toxicity of a solvent and its log-P_{ow} value. Thus, solvents with logP_{ow} values in the range 1.5 and 3 are extremely toxic to microorganisms.

In general, bacteria react to such stress by forming biofilms.

The many observations of toxic effects of aromatic and aliphatic hydrocarbons can largely be explained by the interaction of the compounds with the membrane and the membrane components. As a result of the accumulation of the solvents in the membrane, the membrane loses integrity. The permeability to protons, ions, metabolites, lipids and proteins increases. This phenomenon leads to collapse of cell membrane potential and inhibition of membrane protein functions. These negative effects on the structural and functional properties of membranes generally cannot be attributed to any specific reactive group in a molecule.

Pseudomonads have been isolated that have tolerance to solvents and can thus grow, for example, in the two-phase system toluene-water. Examples of such solvents and their logP_{ow} values tolerated by "new" organisms are toluene (2.5), octanol (2.8), styrene (3.0), xylene (3.0), cyclohexane (3.2) and hexane (3.5). The different mechanisms responsible for resistance in solvent-tolerant bacteria are aimed at readjustment of membrane fluidity after solvent exposure and the establishment of a physico-chemical barrier against intercalation of hydrocarbons into the membrane, respectively.

The following processes of solvent tolerance have been observed (◘ Fig. 10.10):



◘ **Fig. 10.10** Schematic representation of the mechanisms of adaptation to the presence of solvents. below: structures of phospholipids

- Isomerization of membrane unsaturated fatty acids from *cis* to *trans* isomers (leading to greater membrane order/rigidity).
- Decreasing the amount of unsaturated fatty acids in the membrane.
- Adaptation in phospholipid heads (decrease in the proportion of phosphatidylethanolamine in the heads and increase in the proportion of cardiolipin).
- The establishment of a lower surface hydrophobicity, which leads to the avoidance of the accumulation of the organic solvent molecules in the membrane (modification of the carbohydrate chains in the lipopolysaccharide).
- The establishment and induction of effective processes to extrude solvents from the cytoplasm or membranes. Solvent extrusion is mainly performed by energy-dependent efflux systems. An efflux process requires a cytoplasmic membrane export system that acts as an energy-dependent efflux pump, and an outer membrane protein that expands the periplasm and forms a continuous channel so that the toxic compound can be eliminated by cell membranes. In addition, an anchored lipoprotein facilitates the formation of the channel.

Adaptation through modification of the membrane is also evident in the example of psychrophilic bacteria. If one compares the cell membrane of psychrophilic and mesophilic bacteria, it is noticeable that the cell membranes of psychrophilic bacteria have a higher proportion of unsaturated fatty acids and thus maintain semifluidity in cold conditions.

10.1.3 Mixed Substrates

In contrast to the laboratory, growth in ecosystems proceeds under more complex conditions, because bacteria are confronted with mixtures of compounds that perform a specific function as nutrients.

In more or less all ecosystems, the availability of carbon and energy sources is severely constrained and carbon is available in the form of myriad, diverse compounds, all present at concentrations of a few micrograms per liter or less. These compounds are mainly derived from the hydrolysis of particulate organic matter or are excretory products of higher organisms. Recently, xenobiotic chemicals have been added due to increasing use.

Heterotrophic microorganisms are therefore not limited to the utilization of a single carbon source, but simultaneously utilize many of the compounds present in the environment. This even applies to mixtures of such carbon sources, which normally cause diauxic growth under the condition of high concentration (the growth behaviour is called "mixed-substrate growth"). That is, in addition to improved substrate affinity, the potential for different substrates to be used simultaneously should be considered when considering microbial competition at low environmental concentrations.

The growth rate during cultivation with two or three sugars is not controlled in the same way as by the individual concentrations of the sugars. It is controlled by either the total sugar concentration or a sum parameter such as total Disolved Organic Carbon (DOC) (available to the cell) in the culture medium.

In all ecosystems, even the oligotrophic ones, the concentration of non-characterized and accessible DOC is high enough to sustain bacterial growth. Since DOC is thought to be simultaneously recycled with contaminants, it is important to consider that this affects the rate and extent of contaminant degradation.

For most contaminants that are biodegraded, it is still uncertain whether their degradation in the environment occurs due to the accumulation of competent cells or due to induction of the relevant enzymes in potent degraders of the soil-borne population. This question is of high practical importance because the time scale at which the two variables change is very different. While

changes in a population due to enrichment of degrading strains usually require days to weeks (or even longer), induction of degrading enzymes occurs within minutes or hours.

Generalists do not compete successfully against **specialists** in their respective field, but hold their own in competition by using the diversity of substrates. In other words, strains that are versatile in terms of their nutrients outgrow the specialists under the condition of growth with mixed substrates.

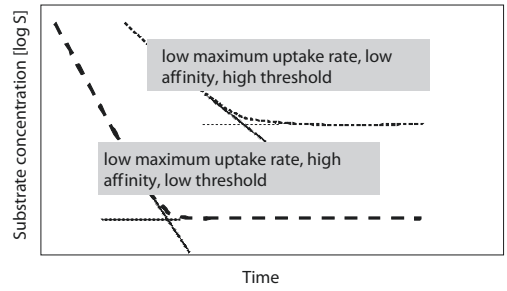


Fig. 10.11 Kinetics of substrate uptake by microbial communities

10.1.4 Limit Concentrations

A possible existence of a threshold has been postulated because relatively constant levels of dissolved organic carbon are present in the oceans. This carbon, probably due to the low concentration, is not available for microbial proliferation and consequently degradation (Jannasch, 1967). The level of dissolved organic carbon is in the range of 1 mg/L in the ocean and usually lower than 5 mg/L in oligotrophic freshwaters. Residual concentrations in the water phase (that is, the concentrations that remain after initial degradation) have been observed for both xenobiotics and natural substrates.

- In methanogenic systems, the observed limiting concentration can be explained by the limitation of the Gibbs free energy of the reaction (see Box ► Syntrophy).
- Aerobic degradation reactions are usually sufficiently exergonic and therefore other explanations for the observed limiting concentrations are necessary.
- Limit concentrations should be based on constraints on diffusion kinetics or correspond to the energy content of the maintenance metabolism.
- Limit concentrations should correspond to the minimum concentration of enzyme induction.
- Limit concentrations should be based on external factors such as the presence of other substrates (Fig. 10.11).

10.1.5 Microbial Cooperation

Instead of competing for the same nutrient, some microorganisms work together to perform a particular transformation that none of them could do alone. This type of microbial interaction, called **syntrophy**, is crucial for the competitive success of certain anaerobic bacteria, as explained in the section on methane (Sect. ► 4.5.1). Syntrophic relationships generally require that the two or more microorganisms involved in the process share the same microworld. The metabolic product of one microorganism must be readily available to the other.

Metabolic cooperation is also found in the activities of groups of microorganisms that carry out **complementary metabolism.** For example, in Sect. ► 7.3 we discussed metabolic transformations involving two separate groups of microorganisms, such as **nitrosifying** and **nitrifying** bacteria. Both oxidize NH_3 together to NO_3^-, although neither group is able to accomplish this alone. Or consider the activities of sulfate-reducing and sulfide-oxidizing bacteria: In this example, the product of one microorganism (H_2S from the reduction of SO_4^{2-}) provides the substrate for the other ($H_2S + O_2 \rightarrow S° + H_2O$ or $H_2S + 2O_2 \rightarrow H_2SO_4$). Such types of cooperative interactions occur frequently in microbial habitats.

Syntrophy

Syntrophy is essential (a) for global carbon cycling from natural polymers such as polysaccharides, proteins, nucleic acids and lipids to CO_2 and CH_4, (b) for waste decomposition and (c) for biofuel formation. Syntrophic microbial processes are ubiquitous in natural and man-made habitats that are devoid of oxygen and/or other external electron acceptors needed for the oxidation of reduced organic compounds.

Syntrophy is a tight-knit mutualistic interaction and generally involves two complementary microbial cell types whose combined metabolism enables reactions that are not possible by either organism. These metabolically distinct microorganisms are tightly bound together by the need to maintain the exchanged metabolites at very low concentrations.

A classic example of a syntrophic relationship is "Methanobacillus omelianskii". It took about 30 years to show that this culture consists of two microbial partners in close metabolic community. The "S organism" ferments ethanol to acetate and H_2, while *Methanobacterium bryantii* strain M.o.H. uses the H_2 produced to reduce CO_2. During the fermentation of ethanol

$$\text{Ethanol} + H_2O \rightarrow \text{Acetate}^- + H^+ + 2\,H_2$$

$\Delta G^{0'}$ at 1 atm H_2 is +9.68 kJ. In contrast, at 10^{-4} atm of H_2, the $\Delta G'$ value is -36.03 kJ. Thus, the presence of the H_2-consuming strain makes the ethanol fermentation exergonic.

Among the first examples of syntrophy was the community of phototrophic green sulfur bacteria (for example, *Chlorobium*) and chemolithotrophic sulfur-reducing bacteria (for example, *Desulfuromonas*) that exchange sulfur compounds such as elemental sulfur and sulfide between partners. The activity of both partners keeps the sulfur compounds at a non-inhibitory concentration, which allows the co-culture to thrive.

Furthermore, tightly coupled syntrophic communities between fermentative bacteria and methanogenic archaea were discovered, which involve an exchange of hydrogen or formate between the partners. The fermentative syntrophic bacteria form hydrogen and formate from their growth substrate (for example, propionate, butyrate and benzoate). However, the degradation of butyrate to acetate and H_2 at pH7,1 atm H_2 (101 kPa) and 1 M acetate and butyrate is thermodynamically unfavorable.

$$\text{Butyrate}^- + 2H_2O \rightarrow 2\,\text{Acetate}^- + H^+ + 2H_2 \quad \Delta G^{0'} = +48.6\,\text{kJ / mol Butyrate}$$

However, since the hydrogen and formate concentrations are kept low by the methanogens or other hydrogen/formate users, the decomposition of butyrate is thermodynamically favorable. At a partial pressure of 1 Pa H_2 and a concentration of 0.1 mM each for butyrate and acetate, the $\Delta G'$ value is -39.2 kJ per mole of butyrate.

Transfer of carbon, nitrogen and other compounds is another example of a form of syntrophy. Microbial consortia are able to perform anaerobic methane oxidation in combination with sulfate reduction. In this process, ANME archaea carry out the methane oxidation process, which would not take place without the always occurring withdrawal of electrons by the sulfate-reducing activity of the bacteria involved. To date, the transfer compound is not known.

Syntrophic metabolism thus requires very close physical contact and metabolic synchronization of the syntrophic part-

ners. In general, syntrophy operates close to thermodynamic equilibrium, with both partners having to share the limited energy resulting from the overall reaction. The thermodynamic limitation of anaerobic syntrophy is probably also a reason why it is difficult to cultivate such microorganisms, as enrichments destroy consortia.

Knittel and Boetius (2009), McInerney et al. (2009, 2011), Morris et al. (2013), Schink 2002, Schink and Stams (2006), Sieber et al. (2012), Stams and Plugge (2009).

10.2 Attachment to Surfaces and Biofilms

10.2.1 Surfaces

Surfaces are of great importance as microbial habitats. In the environment, there is practically no interface that is not colonized or can be colonized by microorganisms. In a surface, nutrient concentrations can sometimes be much higher than in solution. This can greatly affect the rate of microbial metabolism. At surfaces, the number and activity of microorganisms is usually much higher than in free water due to absorption effects. A surface can also be a nutrient itself, for example a particle of organic matter, where attached microorganisms degrade organic or inorganic nutrients directly from the surface.

10.2.2 Biofilms

The vast majority of microorganisms in nature live in the form of biofilms. The conditions for the formation of biofilms are practically ubiquitous. Biofilms are therefore found widely in nature and in/on technical systems.

Biofilms consist of a thin layer of slime in which microorganisms (for example bacteria, algae, fungi, protozoa) are embedded. They form predominantly in aqueous systems, either on the water surface or on an interface with a solid phase: between gas and liquid phases (for example, free water level), liquid and solid phases (for example, gravel at the bottom of a body of water) or also between different liquid phases (for example, oil droplets in water). The interface on which the biofilm forms is called the **substratum.** Apart from microorganisms, a biofilm mainly contains water. **Extracellular polymeric substances** (EPS) secreted by microorganisms form gels in combination with water, creating a matrix in which nutrients and other substances are dissolved. Often, the matrix also traps inorganic particles or gas bubbles. The EPS consist of biopolymers which, as gels, give the biofilm a stable form. These are a wide range of polysaccharides, proteins, lipids and nucleic acids. Various microorganisms live together in biofilms.

Aerobic and anaerobic zones can occur in the biofilm at a distance of a few hundred micrometers, which allows aerobic and anaerobic microorganisms to live closely side by side. Inside biofilms, solutes are transported mainly by diffusion. Convective mass transport processes occur at most in caverns and passages when water flows through them. In the area of the biofilm surface, convective mixing processes can additionally be triggered by movement of the outgrowths projecting into the flow.

The formation of a biofilm can be divided into three phases (◘ Fig. 10.12): the **induction phase**, the **accumulation phase** and the **existence phase**. Biofilm formation on

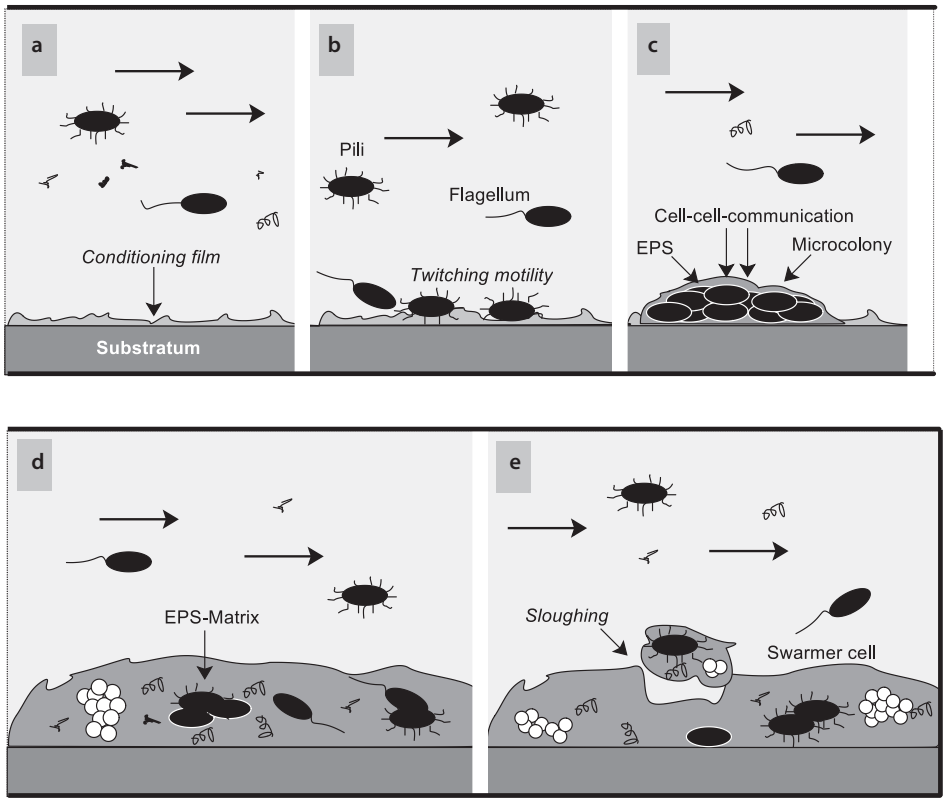


Fig. 10.12 Development and spread of a biofim in a water system. **a** *Conditioning film*, **b** reversible and irreversible adhesion, **c** EPS production and formation of microcolonies, **d** mature biofilm, **e** detachment of individual components: Biofilm fragments and active detachment of individual organisms by degradation of matrix polymers

solid surfaces usually begins with an **induction phase;** other biofilms often form without this. In the induction phase, a thin, viscous layer of organic matter accumulates on a water-wetted surface. This enables the microorganisms to attach themselves to the surface more easily. These biopolymers originate from the mucus coat that forms around bacterial cells, occasionally detaches completely or partially, and is adsorptively bound upon contact with interfaces. The organic layer is then colonized by germs in the **accumulation phase,** which use the organic substances as nutrients. The microorganisms communicate with each other via an intercellular communication system known as "quorum sensing". As a result, the individual organisms come together in large numbers. Microcolonies are formed. As a result of the proliferation of cells attached to a surface, the organisms spread. The interface is first colonized in the form of a film (biofilm). At the same time or later, the biofilms grow up in several layers and finally form three-dimensional structures. The **existence phase is** said to be when an equilibrium between growth and degradation of the biofilm has been established. The depth of the biofilm is limited, as whole parts of the biofilm regularly detach (*sloughing*).

Quorum Sensing

The ability of bacteria to communicate with each other is called **quorum sensing.** It allows the cells of a suspension to measure the cell density of a population and to react to it. Quorum sensing thus coordinates the behavior of bacteria of a species in a confined space. Bacteria that use quorum sensing produce and secrete signalling molecules such as *N*-acyl homoserine lactones, which act as autoinducers and pheromones, respectively. When their concentration exceeds a threshold value, autoinduction starts via a specific receptor and different genes are activated.

The life processes of bacteria in biofilm differ significantly from those in free suspension. Motile bacteria separate from their flagella and other EPS are formed in the biofilm.

Biofilms allow seemingly hostile biotopes to be inhabited. The matrix provides mechanical stability and allows organisms to establish long-term, synergistic interactions. The biofilm provides excellent protection for the individual microorganism and allows it to adapt to changing environmental conditions: Thus, tolerance to extreme pH fluctuations, pollutants or antibiotics as well as nutritional deficiencies increases.

Overview

Occurrence of biofilms. Biofilms occur everywhere—in all soils and sediments, on rocks, on plants and animals, even on and within ourselves. Even extreme, hostile environments such as in the ice of glaciers, in sulfurous hot springs, in aviation fuel and in oil tanks, even highly contaminated areas are colonised by biofilms. Biofilms colonize rocks, even rocks in the desert, they form microbial mats in wetlands.

Biofilms as a disturbance and safety risk. Almost everywhere biofilms appear, they can have undesirable or even harmful effects. Biofilms thus have considerable significance for human medicine and the economy. In the human body, bacterial cells in a biofilm are inaccessible to the immune system. This complicates the use of artificial surfaces, such as medical implants or catheters, as they can serve as sites for the development of biofilms containing pathogenic microorganisms. Biofilms are also important in oral hygiene. Dental plaque, a typical biofilm, contains acid-producing bacteria that are responsible for caries. In technical plants, biofilms can slow the flow of water or petroleum through pipelines, and clog membranes of water treatment plants (biofouling). They can accelerate the corrosion of pipes and initiate the decomposition of objects located under water, such as oil rigs, boats and coastal installations (biocorrosion) (reference to ► Chap. 13).

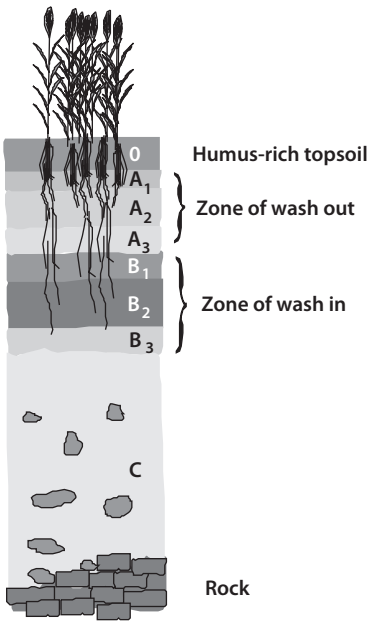
10.3 Soil as Microbial Habitat

Many key processes in the soil that affect ecosystem functioning occur near plants.

The process of soil development involves complex interactions between the parent material (rock, sand, glacial till and so on), topography, climate and living organisms. Soils can be divided into two broad groups—**mineral soils** and **organic soils**—depending on whether they originally came from the weathering of rocks and other inorganic materials or from sedimentation in swamps and bogs.

Soil is formed by weathering processes and microbial activities involving mainly lichens, cyanobacteria and chemoautotrophic bacteria. Soil formation is therefore a

combination of physical, chemical and biological processes. Due to downward movement of materials, layers are formed over time, and a typical **soil profile** is formed (◘ Fig. 10.13). It has the following basic structure: The layer consisting of plant materials (litter) that have not yet decomposed is called the O horizon. Below this is the A horizon of topsoil (about 1–30 cm deep), which is rich in organisms and organic matter. It is the horizon used for agriculture, the topsoil. The subsequent B horizon is the subsoil, into which dissolved minerals and organic acids are washed. It is poor in organic matter and lighter in color. In acid soils of cool humid zones, humic acids dissolve iron compounds from this layer (bleaching earth or podsols). In the lower part of this horizon, precipitation of rust-coloured iron-humus compounds occurs, which can lead to consolidation (local stone). The lower C horizon consists of bedrock material and washed-in materials. The living zone of the soil is the O and A horizons, where there is more or less active organism activity depending on the nutrient supply. It is the area of rhizosphere effects. The rate of development of a typical soil profile is normally a very slow process, extending over hundreds of years.



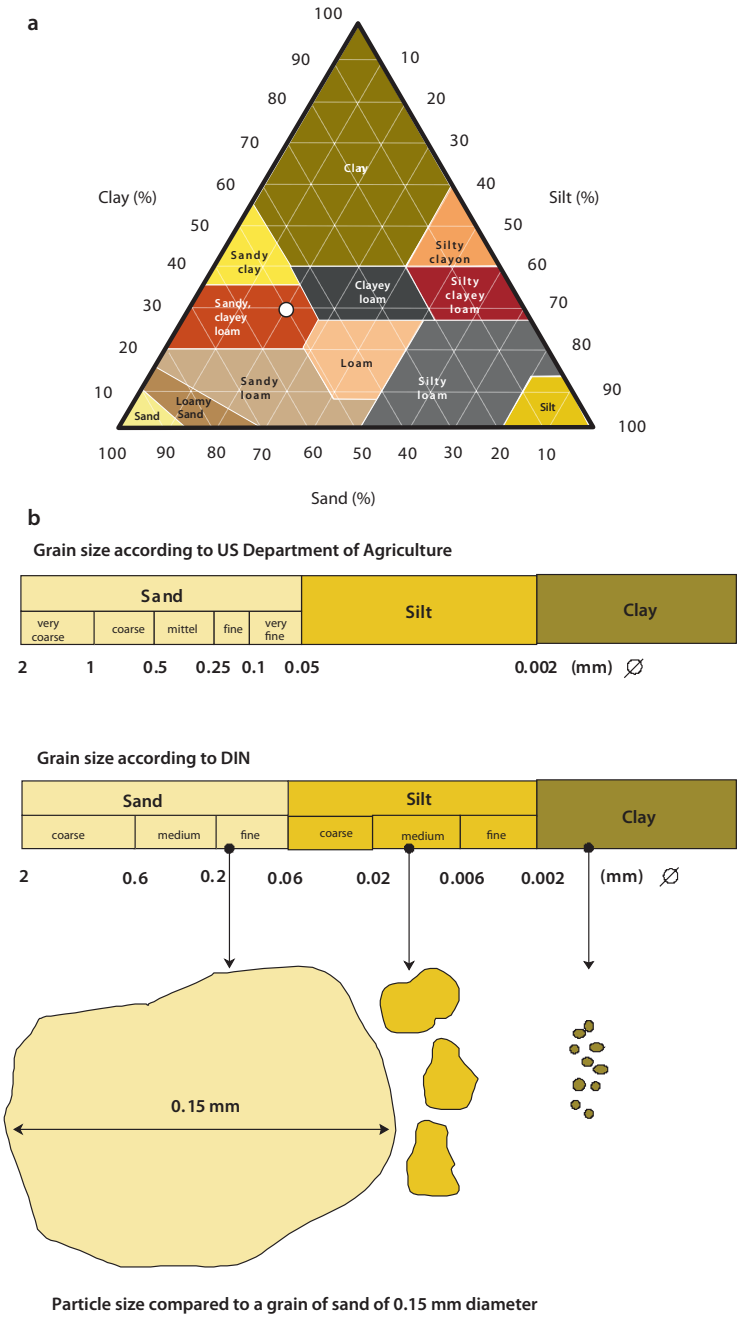
◘ **Fig. 10.13** Soil profile/horizons (schematic), height approx. 150 cm

The mass composition of the soil matrix by grain size is referred to as grain size or texture. Soil texture is an important descriptive aspect of a microbial habitat because it describes, in part, the interaction of the resident microorganisms. The relative proportions of the three main fractions (clay, silt, and sand) describes the soil type, which is most easily represented in the form of triangular diagrams called grain size diagrams (◘ Fig. 10.14).

Soil is a complex system of abiotic and biotic components. Abiotic components are inorganic and inanimate organic components, water and air. In soil, there is an intensive interpenetration of the three phases: soil matrix (solid phase), soil solution (liquid phase) and soil air (gaseous phase). The soil volume constitutes about 50% matrix and 50% pore volume, which is filled with soil solution (20–50%) and soil air (0–30%). The soil matrix is divided into a mineral fraction (90–98%) and an organic fraction (2–10%).

There is a **pore space** between the mineral particles of a porous medium. The pores allow the movement of air, water, chemicals and microorganisms.

The pore size represents a continuum that is conventionally divided into three ranges (◘ Fig. 10.15). The boundaries between the pore size ranges (coarse, medium and fine pores) are based on characteristic values of the water balance. The classification shows that the water in the **fine pores** is generally not available to plants. In the **middle pores,** however, it is available to plants. In terrestrial soils, the **coarse pores** are usually free of water, and their proportion is therefore decisive for the extent of aeration of the soil. This is particularly true for pores >50 µm in diameter, which are also referred to as **wide coarse pores.**



Particle size compared to a grain of sand of 0.15 mm diameter

Fig. 10.14 **a** Grain size triangle to represent the soil type of a fine soil according to the US-Soil Taxonomy. The point o corresponds to proportions of 50% sand, 20% silt and 30% clay. **b** Classification of soil particles by size according to the US and DIN nomenclature

The pore size is important for root growth and microbial activity, because root hairs (diameter >10 µm) are only able to penetrate coarse pores, while fungal mycelia (diameter approx. 3–6 µm) and bacteria (diameter 0.2–1 µm) can still live in medium pores. The fine pores, however, are not accessible to microorganisms.

The size of clay particles is approximately equal to that of a bacterial cell. Clay

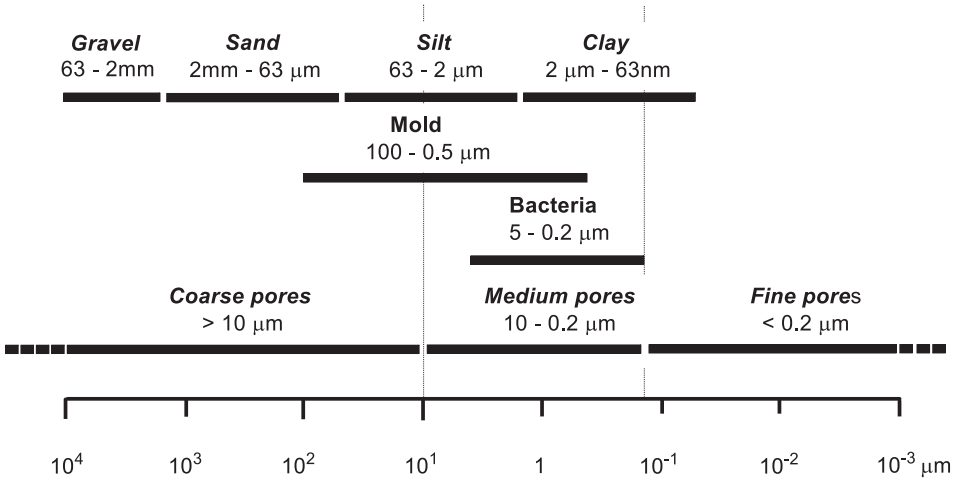


Fig. 10.15 Size comparison of particles, microorganisms and soil pores. (Modified after Schachtschabel & Hartge, 1958; Matthess & Pekdeger, 1981; Matthess et al., 1988)

affects the average of the pore size. Although the average pore size in clay soil is smaller than in a sandy soil, there are more pores there. Consequently, the total pore space in a fine-textured clay soil is larger than in coarse-textured sandy soil. However, since small pores do not transport water as quickly as large ones, a fine-textured medium will generally slow down the movement of air, water, chemicals, and microorganisms.

The proportion of coarse pores is generally greater the more coarse-grained the soils are, i.e. the more sandy or gravelly they are (Fig. 10.16). The proportion of fine pores, on the other hand, is greater the finer-grained the soils. In sandy soils, the proportion of coarse pores dominates at 30%, while it decreases with increasing clay content. In clayey soils it is sometimes only 2–3%.

There is a close relationship between the proportion of fine pores and the clay content, which is reflected, albeit less strongly, in the pore volume.

The proportion of central pores reaches a maximum of 15% in the silty soils (loess soils).

Anemic soils are mineral soils that have a very high proportion of undecomposed organic matter (over 7% by volume of raw humus). Anemic soils are essentially formed by the weathering of rock. Anemic bog is not a mire (bogs are formed from plant material (peat mosses)). The admixed raw humus is very similar in structure to the peat of true bogs, hence the name.

One of the main factors affecting microbial activity in soil is the availability of **water.** Water is a highly variable soil component as its presence depends on soil composition, rainfall, drainage and plant cover. Water is held in the soil in two ways, by adsorption to surfaces or as free water found in thin layers or films between soil particles. The water present in the soil contains various substances dissolved in it, and the whole mixture is called **soil solution.** In sandy soils, the proportion of plant available water to total stored water is 20–30%, that of unavailable

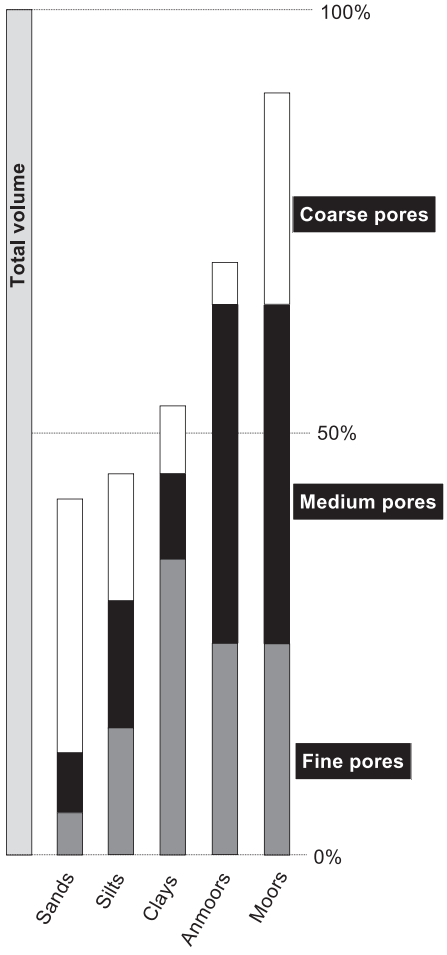


Fig. 10.16 Proportion of pore size ranges in the total volume of mineral soils (C content up to 2%) and organic soils. (Modified after Blume et al., 2010)

water is 10–20% and that of gravity water is 60–70%, while in clay soils about 50–70% is unavailable, 20–40% is available and only 5–15% is gravity water.

In well-drained soils, **air** penetrates easily and the oxygen concentration can be high. However, in fully soaked soils, oxygen dissolved in water is the only oxygen present, and it is rapidly consumed by microorganisms. Such soils quickly become anoxic and show profound changes in their biological properties.

The **nutrient situation of** a soil is the other significant factor affecting microbial activity. The greatest microbial activity takes place in the surface layers rich in organic matter, especially in and around the rhizosphere. The number and activity of soil microorganisms depends primarily on the balance of nutrients present. In some soils, carbon is not the limiting nutrient, but microbial productivity is limited by the presence of inorganic nutrients such as phosphorus and nitrogen.

If one wants to describe soil particles as habitats of microorganisms, it is important to point out the respective differently charged surface. An example: clay minerals have a permanent negative charge on their layer surfaces, which is saturated by exchangeable cations. This cation exchange capacity also depends on the size and thus the surface area. Small pieces have a larger surface area per mass than large ones. The different surface areas of the various clay particles clearly influence how many and which types of microorganisms colonise a particular soil habitat.

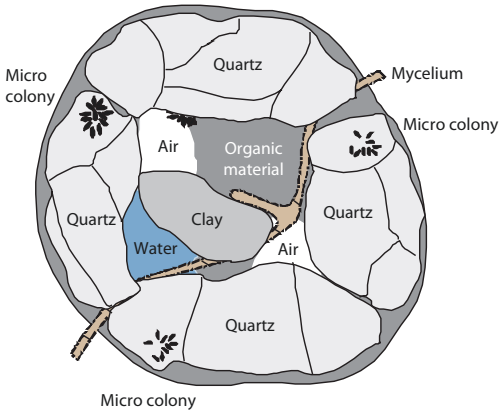
The biotic components of soil include plant roots, soil animals, algae, fungi and bacteria. In terms of volume, meadow soil is composed of about 45% inorganic and 5% organic substances. The remaining part consists of cavities, which are filled with air and water in equal parts. The organic matter consists of 85% humus, 10% roots, 3–4% microorganisms and 1–2% soil animals. The composition is subject to very large fluctuations depending on soil type, climate zone, season and environmental conditions.

A forest soil of our latitudes with a leaf fall of about 1000–2000 kg dry matter per ha contains in terms of size (in dry matter):

40 kg bacteria, 400 kg fungal mycelium, 5 kg insects and 10 kg earthworms. More descriptive are the germ numbers for 1 g air-dry arable soil: 10^8 to 10^9 bacteria and 10–100 m mycelium. However, germ counts and weights do not tell us anything about activity. The microorganisms are subject to turnover, they grow and serve as nutrients for other organisms. The generation times occurring in the soil are much longer than in the laboratory, they are of the order of days. Nutrient limitation prevails under natural conditions. Nutrient solutions used in the laboratory have a carbohydrate content around 10 g/L, but in nature the concentration is around 10 mg/L.

The soil is a heterogeneous medium. A higher substrate concentration occurs near the roots or dead organic matter. Clay minerals have sorptive properties, they bind nutrients and act as ion exchangers. Bacteria prefer to live in capillary pores, for the most part bound to surfaces by slimes (◘ Fig. 10.17). The formation of mucilage and the interweaving of soil particles by mycelia lead to a crumb structure (living obstruction), which is of great importance for soil aeration as well as for water drainage and thus for soil fertility. Overall, the soil consists of a large number of different microhabitats.

The deep layers of the soil, which extend several hundred meters below the surface, are also colonized by a variety of microorganisms, mainly bacteria. Samples from 300 m below the surface were found to contain a wide diversity of bacteria, including anaerobes such as sulfate-reducing bacteria, methanogens and acetogens, as well as numerous aerobic and facultative anaerobic bacteria. The microorganisms in the deep layers probably have access to nutrients because groundwater flows through their habitats. Measurements of their activity



◘ **Fig. 10.17** Schematic representation of a soil aggregate consisting of organic and mineral components. Only very few microorganisms occur freely in the soil solution, most of them adhere to the soil particles as microcolonies

indicate that the metabolic rates of bacteria in their natural habitats are relatively low. Therefore, compared to microorganisms in the upper soil layers, the biogeochemical importance of microorganisms in the deep layers may be minimal. However, there are indications that the metabolic activities of these subsurface microorganisms may be responsible for the mineralization of organic compounds and the release of products into groundwater over long periods of time.

Advances since the late 1990 in the analysis of microbial communities based on DNA extraction and analysis of 16S rRNA genes as opposed to culturing have revealed that there are bacterial phyla that are capable of growth but difficult to culture. The information in ◘ Table 10.2 is extracted from the work of Janssen (2006). They show the most prevalent bacterial phyla in 21 soil samples analyzed by community DNA analysis rather than culture-based techniques.

Archaea have also been detected in soils, although their abundance is generally low.

Table 10.2 Typical composition of bacterial soil communities

Bacterial phylum	Subphylum	Proportion of total soil population (%)[a]	Assessment of the relative ease or difficulty of isolation and cultivation[b]
Proteobacteria	*Alpha* *Beta* *Gamma* *Delta* *Epsilon*	**39**[c] 10–77[d]	Easy
Acidobacteria	Subdivision 1–7	**20** 5–46	Difficult
Actinobacteria	*Acidimicrobidae* *Actinobacteridae* *Rubrobacteridae*	**13** 0–34	Easy
Verrucomicrobia	*Verrucomicrobiae* *Spartobacteria* Subdivision 3 + 4	**7** 0–21	Difficult
Bacteriodetes	*Flavobacteria* *Sphingobacteria*	**5** 0–18	Easy
Chloroflexi		**3** 0–16	Difficult
Planctomycetes		**2** 0–8	Difficult
Gemmatimonadetes		**2** 0–4	Difficult
Firmicutes	*Bacilli* *Clostridia*	**2** 0–8	Easy
Other		**5**	
Unknown		**2**	

Modified after Janssen (2006)

[a]Based on analysis of 2920 clones from 21 different soil samples. Members of the 9 phyla account for 93% of the soil libraries on average

[b]Those phyla labeled "easy" have members that can be isolated from environmental samples using R2A agar within a relatively short period of time (a few days to 2 weeks). Those phyla labeled "difficult" require specialized media, usually with very low amounts of organic carbon, and often take weeks or months to grow. In many cases, only very few members of the difficult phyla have been isolated as a culture

[c]Mean value

[d]Value range

10.4 Aquatic Biotopes

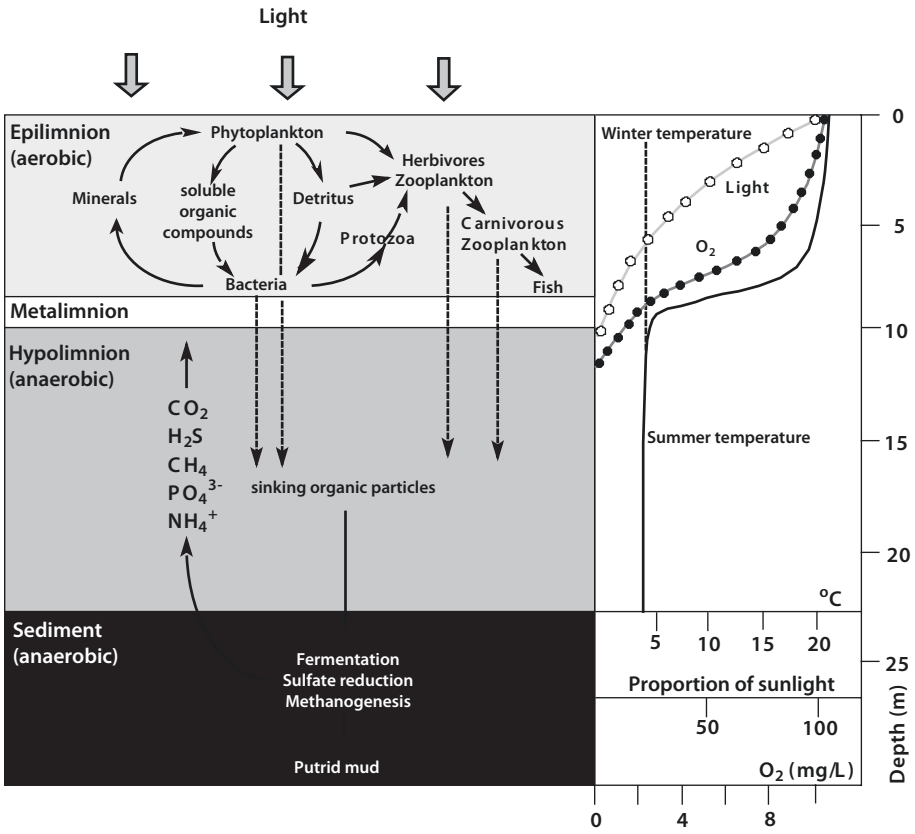
Due to their considerable differences in physical and chemical properties (oceans, salt marshes: salt water; estuaries: Brackish water; lakes, ponds, rivers: freshwater), aquatic biotopes have a diverse composition of their microbial species.

10.4.1 Freshwater Environment

10.4.1.1 The Free Water

Lakes are ecosystems that provide a good illustration of habitat organization. Most lakes in temperate climates are stratified in summer (◘ Fig. 10.18).

The stratification is due to the property of water to have the highest density at +4 °C. Cooler and warmer water is lighter and therefore has a buoyancy compared to water at +4 °C. In spring, after the ice cover melts, the wind causes the water to mix (spring circulation). The temperature is +4 °C in the entire water body. The upper layer, the **epilimnion**, warms up due to the effect of the sun. It floats on the colder water layers due to its lower density. In the layer below the epilimnion, the water temperature drops abruptly (**thermocline** or **metalimnion**). The subsequent deep layer, the **hypolimnion,** has a temperature of around 4 °C, as there is no heat transport through the thermocline into the depths. This results in a stable stratification over the summer (sum-



◘ **Fig. 10.18** The lake as a stratified ecosystem during summer time with food web and profiles of temperature, oxygen and light

mer stagnation). In late autumn and early winter, the surface layers become colder and therefore denser than the deep layers, and the water masses are circulated again by the wind. In winter, an ice cover forms and with it renewed stratification.

The annual cycle shown has an influence on the distribution of oxygen and nutrients in the lake. Oxygen has a limited solubility in water (8.9 g/L at 24 °C, 12.8 g/L at 5 °C). In a large water mass, its exchange with the atmosphere is slow. Significant photosynthetic oxygen production occurs only in the surface layers of a lake where light is available. A typical reduction of the content up to the thermocline results.

In lakes and other bodies of water, inorganic nutrients accumulate due to inflows, enabling an increasing production of organic matter through photosynthesis. Nutrient-poor or **oligotrophic** waters thus gradually change into nutrient-rich or **eutrophic** waters. **Eutrophication** is a naturally occurring aging process of water bodies that is accelerated by anthropogenic influences such as wastewater and fertilizer runoff.

In the water bodies, organic matter is built up and decomposed. Algae, higher plants and cyanobacteria are the primary producers at the beginning of the food chain. Phototrophic microorganisms play a considerable role as primary producers. Ultimately, the biological activity of an aquatic ecosystem depends on the rate of primary production by phototrophic microorganisms. In the case of nitrogen deficiency but sufficient phosphate supply, mass development of cyanobacteria occurs because many species are capable of N_2 fixation. The microalgae and cyanobacteria suspended in the water are referred to as phytoplankton.

A special feature of aquatic food chains, which represent a networked system with short-circuited cycles, is the phenomenon of the "microbial loop". Heterotrophic bacteria in the free water zone use the organic matter excreted by phytoplankton or formed by autolysis, mainly as soluble organic matter. In addition to these autochthonous substances formed in the lake, there are inflows of allochthonous substances. The total dissolved organic matter (DOM) is around 0.5–3 mg C/L in oligotrophic waters and around 10–20 mg C/L in eutrophic waters. The low substrate concentrations around 1 mg C/L present in oligotrophic waters are utilized by bacteria adapted to low substrate concentrations. These **oligotrophic** or **oligocarbophilic** bacteria include *Hyphomicrobium*, *Nevskia*, and *Vibrio* species. The concentration range around 10 mg C/L is utilized by representatives of the genera *Chromobacterium, Flavobacterium* and *Pseudomonas.*

Unlike the eukaryotes, which depend on particulate food, the bacteria absorb the dissolved organic compounds. Thus, the prokaryotes have a specific and quite different function than the higher organisms in the food chain. Thus, dissolved organic carbon not accessible to the higher organisms is partly oxidized to CO_2 and partly converted back into particulate matter, the bacterial cell mass. The bacteria serve as food for protozoa and other microorganisms of the zooplankton, so that the dissolved organic matter is returned to the food chain by the bacterial activity. Thus, bacteria are not at the end of a food chain as destructives, but are sandwiched between primary producers and consumers. About 50% of the organic matter formed during primary production may pass through the bacterial loop. Bacteria are also significantly involved in the degradation of particulate detritus.

Phytoplankton and detritus are the food source for herbivorous consumers, for example rotifers and daphnia. These serve as food for the carnivorous zooplankton. Other links in the food chain are insect larvae, worms and fish.

Organic matter that is not consumed in the surface layers sinks to the depths and is degraded by facultative anaerobic microorganisms that use the oxygen dissolved in the water. In lakes, the deep layers become anaerobic as soon as the oxygen is consumed; strictly

aerobic organisms cannot grow here. The lower layers are limited in species composition to anaerobic bacteria and a few species of microaerophilic animals. There, the transition from respiration to fermentation metabolism takes place, with important consequences for cycles of carbon and other nutrients.

Cyanotoxins as a Drinking Water Prob lem

Cyanobacteria are of great importance in marine, limnic and terrestrial habitats. Cyanobacteria cause problems in drinking water due to their usually unpleasant odour (geosmin and 2-methylisoborneol) and undesirable coloration of surface water. In addition, toxic secondary metabolites produced by them, cyanotoxins, pose a risk to the health of people supplied with polluted drinking water.

The toxins released during lysis of bacteria are very diverse in terms of chemical structure and include alkaloids such as cylindrospermopsin, which is produced by *Cylindrospermopsis raciborskii*, and cyclopeptides such as microcystins (MC) and nodularins, which are synthesized by *Microcystis aeruginosa* and *Nodularia* sp. Some cyanotoxins show toxic effects for example protein synthesis inhibitory activity or strong inhibition of a protein phosphatase, but also tumor promoting activity has been found. Cylindrospermopsin is known as a neurotoxin.

All cyanotoxins are water-soluble and have negative effects on various organ systems in humans. To minimize the risk to humans, appropriate treatment of drinking water is necessary (◘ Fig. 10.19).

Degradation of cyanobacterial toxins

If the cyanobacteria have not been removed by flocculation, by backwashing of rapid filters or by changing the filter material, the cells and the toxins remain in the drinking water and must be degraded into non-toxic compounds. The toxins themselves cannot be removed by flocculation or sand filtration. Activated carbon has the ability to bind the toxins, but the adsorption capacity is limited. The hydrophilicity/adsorption tendency of microcystins is variable but can be estimated by the Kow value.

Cylindrospermopsin is highly water soluble and therefore adsorbs unsatisfactorily to activated carbon or can be poorly removed by other filtration steps and flocculation.

Oxidation steps (ozonation and subsequent filtration) or bacterial degradation are necessary during drinking water treatment to remove the cyclopeptides. Such a "biofilm" exists on activated carbon and slow sand filters.

Once released into the water body, microcystins (MC) can persist for weeks before being degraded by bacteria of the genus *Sphingomonas*. MC-degrading bacteria are common in surface waters, regardless of whether contamination by cyanobacteria or microcystins has already been present in the water. Seventeen strains of Gram-negative bacteria with the property of MC degradation have been described. *Sphingomonas* sp. is not the only bacterial genus responsible for microcystin degradation. At least three hydrolytic enzymes are involved in the degradation of MC-LR by *Sphingomonas* sp.: the metalloprotease microcystinase catalyzes ring opening at the adda-arginine peptide bond, a possible serine peptidase cuts the linear peptide to form a tetrapeptide, and a possible metallopeptidase breaks the tetrapeptide into smaller peptides and amino acids (◘ Fig. 10.20).

Microcystin-degrading bacteria can utilize MC-LR, MC-YR and MC-RR (L: leucine; R: arginine, Y: tyrosine) as sole carbon and energy sources. The alkaline protease of *Pseudomonas aeruginosa* is another example of how a bacterium can cleave the peptide bonds of microcystins.

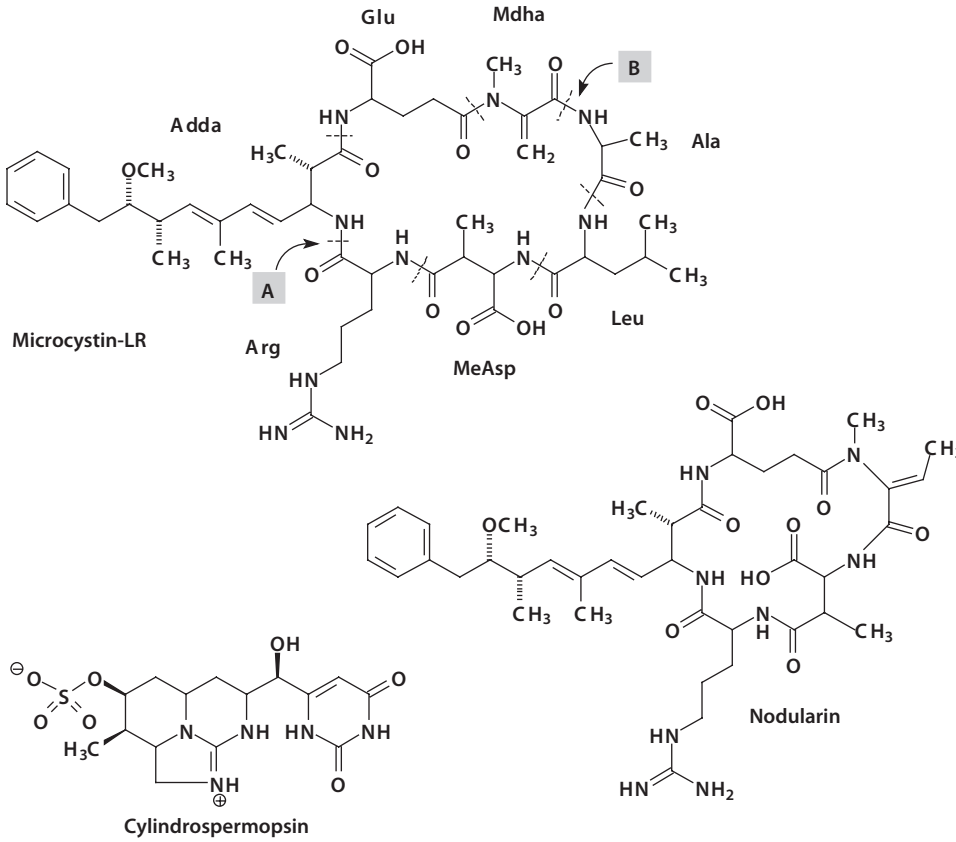


Fig. 10.19 Structures of microcystin, nodularin, and cylindrospermopsin. Adda: (2S,3S,8S,9S)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoat. D-MeAsp: D-erythro-β-methylaspartate. Mdha: *N-methyldehydroalanine*. Microcystin-LR (L: *L*-leucine; R: *L*-arginine). The two capital letters denote the two variable amino acids in the microcystin congeners. The initial sites of attack for degradation (A and B) are shown. Adda is specific for microcystin and allows an unequivocal detection by ELISA

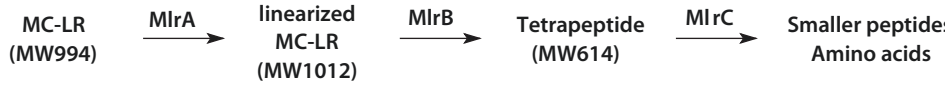


Fig. 10.20 MC-LR degradation pathway by *Sphingomonas* sp. (Bourne et al., 2001). MlrA-C: Microcystinases A-C

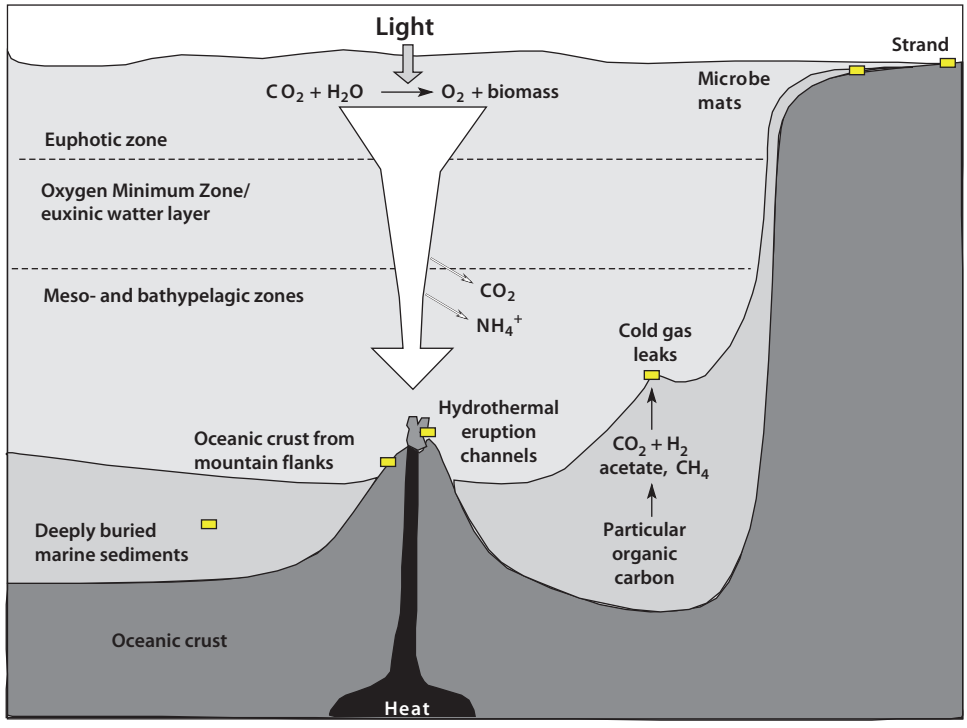
10.4.1.2 The Sediment

The dead particulate matter from phyto- and zooplankton (detritus) slowly sinks to the bottom and is partially mineralized in the sediment. Furthermore, insoluble organic particles from tributaries and leaf fall are washed into the sediment. Major components of the sediment are plant biomass and remains of zooplankton and insects. Oxygen is largely consumed by the aerobic degradation processes of the upper water layers during the summer months and does not diffuse back. The sediments of deeper waters are therefore generally anoxic. The main anaerobic degradation processes are methanogenesis and sulfate reduction. The proportion of both processes depends on the sulfate content. Since the sulphate content is low in freshwater lakes, most anaerobic degradation proceeds via methanogenesis. This methanogenic food chain (see Sect. ► 4.5.1) involves polysaccharide-degrading and fermenting bacteria as well as acetogenic and methanogenic bacteria. The end product methane rises and is a substrate for the aerobic methylotrophic bacteria of the epilimnion and thermocline (◘ Fig. 10.21).

10.4.2 Marine Environments

10.4.2.1 Coastal and Intertidal Areas

Coastal areas of the oceans are typically nutrient-rich and therefore allow for denser populations of phytoplankton. These in turn support higher-order bacteria and aquatic animals. Ocean bays and inlets that

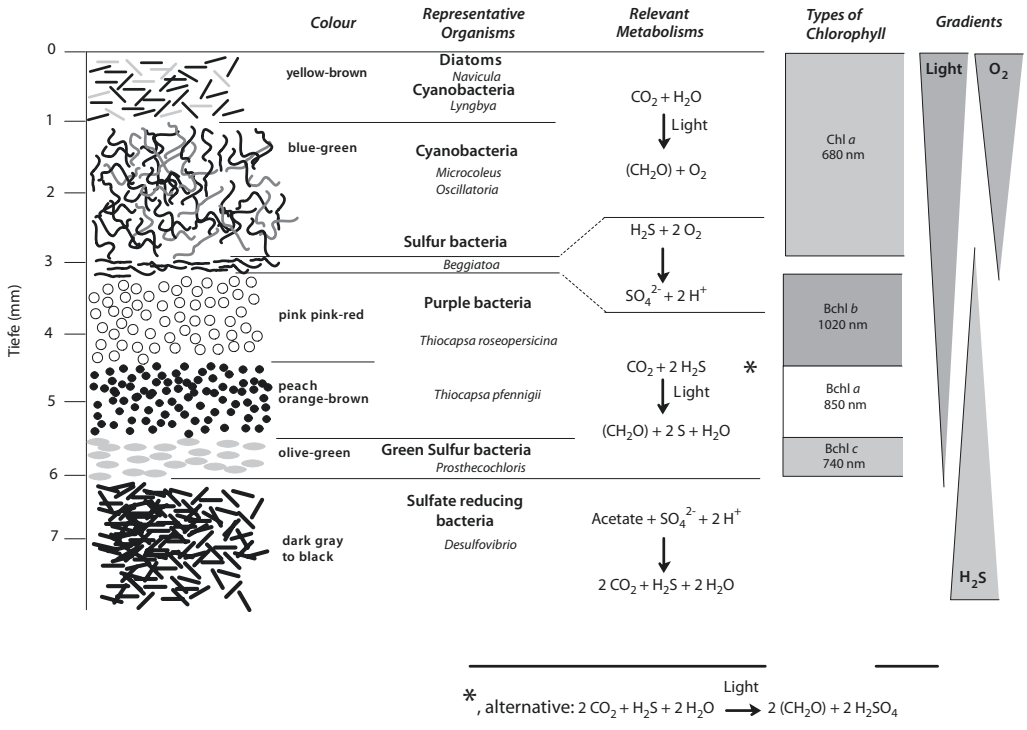


◘ **Fig. 10.21** Schematic representation of areas in the ocean that have been surveyed for microbial activity. Rectangles mark areas discussed in the text

receive high concentrations of nutrients from sewage and industrial wastes can have very high populations of bacteria and phytoplankton. Heavy pollution causes shallow marine waters to become anoxic due to O_2 consumption by heterotrophic bacteria. The production of H_2S by sulfate-reducing bacteria causes seawater to become toxic to marine higher organisms.

Intertidal zones are marine areas where the formation of stratified bacterial communities, **microbial mats,** which grow on solid substrates such as marine sediment, occurs. Characteristically, mats are exposed to sunlight and thus provide conditions for phototrophic microorganisms to exist. Microbial mats contain cyanobacteria, purple bacteria, green sulfur bacteria, colorless sulfur-oxidizing and sulfate-reducing bacteria. They form vertically stratified communities that can be visible as color stripes (color stripe mudflats). Microbial mats have a layer thickness of only a few millimetres. The typical groups of organisms and the sequence of layers are shown in ◘ Fig. 10.22.

The upper layer, which may consist of sand or washed-up algae, often contains diatoms. This is followed by a green layer of various cyanobacteria species. Below this is often a layer of colorless sulfur-oxidizing bacteria such as *Beggiatoa.* This is followed by one or more red layers of purple bacteria and then one of green sulfur bacteria. Below this is a black layer that extends very far downwards and is home to the sulfate-reducing bacteria. The black coloration is due to iron sulfide precipitated by H_2S. The H_2S odor is typical of this habitat. The color striped mudflat is a special form of sulphuretum, which refers to bacterial habitats where sulfate reduction forms H_2S. This compound, which is toxic to many organisms, is



◘ **Fig. 10.22** Schematic structure of a bacterial mat with participating microorganisms, their respective metabolites and their photopigments

used as an energy source and electron donor by sulfur-oxidizing bacteria and phototrophic purple and green sulfur bacteria.

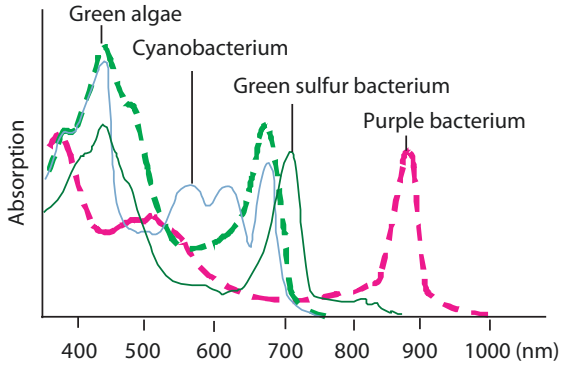
The bacterial mat of the color striped mudflat is a habitat with high primary production and high turnover rates of the metabolic cycles. Due to the **syntrophy,** the interaction of the bacterial community, a very high bacterial density and metabolic activity of the mat are possible.

The two groups of primary producers, cyanobacteria and purple bacteria, are low-light organisms. The sand layer with the diatoms absorbs part of the light. The cyanobacteria, because of their endowment with the photosynthetic pigments chlorophyll a and the phycobilins, absorb mainly light in the range 400–700 nm, while for the underlying layer of purple bacteria, because of the absorption of bacteriochlorophyll around 850 nm, radiant energy is absorbed that was not absorbed by the organisms living above (◘ Fig. 10.23).

The products of photosynthesis, after transformation by fermentation processes, provide the substrates for sulfate-reducing bacteria. These obligate anaerobes use sulfate, which is the end product of sulfur oxidation by *Beggiatoa* and anoxygenic photosynthesis by purple bacteria, as an electron acceptor. The end product of sulfidogenic metabolism, H_2S, rises to the top of the microbial mat and is taken up as a substrate by the purple bacteria and the sulfur-oxidizing bacteria and is thereby scavenged. This interaction structure results in substrate gradients.

A clear day-night change occurs. During the day, oxygen reaches deeper layers through the photosynthesis of cyanobacteria. At midday, *Oscillatoria* is the dominant organism. Through their ability to bind N_2, the cyanobacteria make a further contribution to the productivity of the system. At night, no oxygen is produced, but it is quickly consumed by *Beggiatoa.* The hydrogen sulfide reaches closer to the surface because the phototrophic bacteria do not consume it. At the same time, *Beggiatoa* has a higher concentration available as an energy source as long as there is a high enough concentration of the end-acceptor oxygen.

The color striped mudflat is a very good example to illustrate selection processes. The various bacteria accumulate where they find conditions that meet their requirements. There is a gradient of different nutrient and energy sources in which the bacteria nestle as a layer. The developing gradients around nutrients, H_2S, oxygen and light lead to the layered development of different phototrophic green sulfur bacteria, purple bacteria and cyanobacteria as well as sulfur-oxidizing bacteria.



◘ **Fig. 10.23** Absorption curves of algae, cyanobacteria, purple bacteria and green sulfur bacteria. Due to their different endowments of photosynthetic pigments, they can use different spectral components that have not been absorbed by the respective organisms living above them

Biogeochemical Processes and Redox Zones on the Beach of the Island Spiekeroog

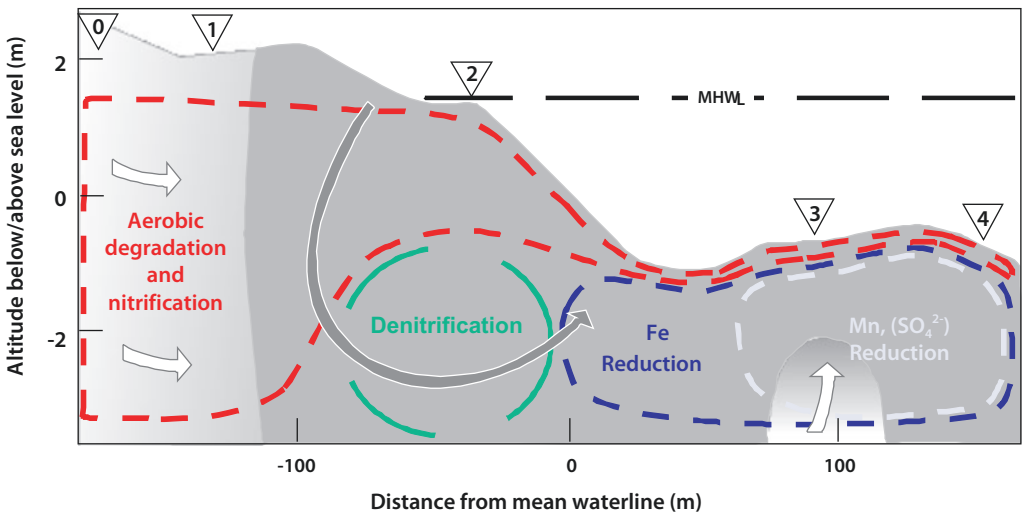
An interdisciplinary approach was used to investigate key processes in beach systems on the island of Spiekeroog, southern North Sea. Spatial variations in subsurface residence times of pore water, salinity, organic matter (OM) and redox conditions and their effects on nutrient cycling and microbial community patterns and microphytobenthos growth were analysed. Sediment and pore water samples were collected from the dunes to the low water line with sediment depths up to 5 m below the sediment surface (◘ Fig. 10.24).

In summary, the beach consists of three zones:

1. the freshwater-dominated oxic zone, which is located behind a backshore berm and is only irregularly flooded and has limited OM availability,
2. the seawater-influenced, oxic to suboxic USP zone, where pore water circulation can lead to periodic oxygen, nutrient and OM availability changes, and
3. the seawater-influenced, mainly anoxic zone in the ridge sediments. (Beck et al., 2017)

10.4.2.2 The Pelagic Zone

In the sea, pelagic refers to the high seas far from land, i.e. the open sea. It is the open water area above the bottom zone, far from the shore.



◘ **Fig. 10.24** Biogeochemical processes and redox zones on the beach studied on the island of Spiekeroog. At sites 0 and 1, which are only irregularly flooded by seawater, for example during storm surges or exceptional high water levels, oxic conditions and aerobic degradation of organic matter dominate. Site 2, located near the mean high water level (MHWL), exhibits oxic to suboxic conditions. Three biogeochemical zones can be distinguished: aerobic degradation, nitrification, and denitrification. In contrast, the oxide sediment layer at Sites 3 and 4 extends to only a few centimeters, while evidence of anoxic conditions and reduction of particulate Mn/Fe and SO_4^{2-} is found below. The color scale indicates the color gradient from fresh groundwater (white) to saline (gray). The arrows show the main groundwater flow paths as well as the upper saltwater plume (USP, dark grey arrow) and the terrestrial freshwater input of the island freshwater lens and the freshwater discharge tube (white arrows)

In the open oceans, primary production is rather low because inorganic nutrients, especially nitrogen and iron, limit the growth of phytoplankton. One consequence of the relatively low primary production in the oceans is relatively low heterotrophic microbial activity. This in turn ensures that even at great depths most ocean water is oxic.

10.4.2.2.1 Epipelagic Zone/Euphotic Zone

Visible light penetrates no further than about 300 m deep into the water of the open oceans. This upper region is called the **euphotic zone**, where sunlight provides enough energy for photosynthesis. The main primary production in the open ocean takes place in this **epipelagic zone.**

Below this zone, to a depth of about 1000 m, there is still considerable biological activity due to the activity of animals and chemoorganotrophic microorganisms. Water above 1000 m depth is comparatively biologically inactive.

Consequently, the biology of the euphotic zone differs considerably from that of the waters of the dark deep sea, the **meso-** and **bathypelagic zones.**

Cyanobacteria represent an important proportion of primary producers. They perform oxygenic photosynthesis and use the Calvin-Benson-Bassham cycle for carbon fixation. In addition to chlorophyll-using organisms (phototrophic eukaryotes and cyanobacteria), **aerobic anoxygenic photosynthetic bacteria** possessing bacteriochlorophyll-a are ubiquitous in the euphotic zone. Initially, members of the genus *Erythrobacter* were thought to be the major types of the latter-named bacteria in the ocean. However, members of the *Roseobacter* group *(Alphaproteobacteria)* and also *Gammaproteobacteria* were shown to be more important members of this microbial metabolic type.

Traditionally, **aerobic anoxygenic photosynthetic bacteria** are considered heterotrophs that supplement their energy requirements from light, making them unusually efficient in oligotrophic environments. However, various publications show that the organisms also occur in large numbers in both eutrophic and oligotrophic environments. It is assumed that nutrient concentrations, attachment to particles or light intensity influence the occurrence of aerobic anoxygenic photosynthetic bacteria. It is now known that at least some strains have the property of growing autotrophically or possess a mixotrophic carbon metabolism (◘ Table 10.3).

10.4.2.2.2 The Deep Sea

Organisms colonizing the deep sea are generally confronted with three important environmental extremes: **low temperature, high pressure** and **low nutrient concentration**. However, on the seafloor there are also ecosystems with high temperature and high concentration of nutrients.

◘ **Table 10.3** Microbial metabolism in the euphotic zone

Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway
Oxygen photosynthesis	H_2O	$NADP^+$	*Cyanobacteria, Algae*	Calvin Benson Bassham Cycle
Aerobic anoxygenic photosynthesis	H_2S or H_2O	O_2, $NADP^+$	*Roseobacter, Gammaproteobacteria*	3-Hydroxypropionate/4-hydroxybutyrate cycle

From 100 m depth, ocean water has a constant temperature of 2–3 °C. Bacteria isolated from depths greater than 100 m are psychrophilic. Some are extremely psychrophilic and grow only in a narrow range near the *in-situ* temperature.

Deep-sea microorganisms growing at a depth of 5000 m must be able to withstand the enormous hydrostatic pressure of 500 atm. Organisms isolated from depths up to about 3000 m are barotolerant and do not grow at pressures above 500 atm. In contrast, cultures obtained from greater depths, 4000–6000 m, are barophilic, growing optimally at pressures of about 400 atm. Samples from even greater water depths (10,000 m) reveal extremely (obligate) barophiles. They grow fastest at pressures of 700–800 atm and almost as well at 1035 atm, the pressure in their natural habitat. They do not grow at pressures below 400 atm.

10.4.2.2.2.1 Meso- and Bathypelagic Zones

The **meso-** and **bathypelagic areas** in the ocean represent the largest continuous habitat on Earth. Nevertheless, very little is known about the type of microorganisms that live there and their influence on the global environment. The **mesopelagic** extends from 200–1000 m depth and thus lies between the light and dark depth zones, which is where the name comes from. It marks the beginning of the deep sea proper, the aphotic zone. The **bathypelagial** ranges from 1000 to 4000 m depth.

Traditionally, the role of microorganisms in these zones has been considered as degraders of organic matter, to which the release of CO_2 is coupled. However, planktonic *Crenarchaeota,* which belong to "marine group 1" (MG1), dominate the prokaryotic cell number in this environment. Since they are known to be autotrophs, the original assessment must be questioned. These results have been supported by the isolation of the first pure cultures that possess a chemolithoautotrophic metabolism, with the oxidation of ammonium with oxygen acting as a source of energy and for reduction equivalents.

Crenarchaeota have been identified as the predominant **ammonium oxidizers** in the ocean. The ability to incorporate bicarbonate into their biomass has been confirmed in natural environments. Further, some *Crenarchaeota* engage in **heterotrophic** and/or **mixotrophic metabolism**.

Overall, the *Crenarchaeota* appear to be the main drivers of biogeochemical cycles in the ocean, with important implications for nitrogen and carbon cycling.

Over a period of thousands of years, a general circulation of the ocean takes place. However, the CO_2 released from the sinking biomass in the meso- and bathypelagic areas does not return to the surface due to autotrophy and is thus decoupled from the carbon cycle over a much longer period of time.

The likely carbon fixation pathway used by the autotrophic ammonium-oxidizing *Crenarchaeota* is the 3-Hydroxypropionate/4-hydroxybutyrate cycle. Frequent detection of the pathway's key genes in metagenomic databases confirms its presence even in previously uncultured microbial representatives in the ocean.

It has been estimated that ammonium-oxidizing Archaea fix **about 400 Tg of carbon per year,** making this cycle as significant for the pelagic region of the ocean.

To date, it is unclear which organisms carry out the oxidation of nitrite to nitrate in the ocean. Possible candidates are *Deltaproteobacteria* of the genus *Nitrospina,* which were found with MG1-Archaea in the Pacific Ocean. *Nitrospina* spp. likely uses the Wood-Ljungdahl pathway or the reductive tricarboxylic acid cycle, as both pathways have been identified in *Deltaproteobacteria,* rather than the Calvin-Benson-Bassham cycle.

The reductive tricarboxylic acid cycle also appears to be used by *Nitrospira* spp. another group of nitrite oxidizers found in the oceanic environment (◘ Table 10.4).

Table 10.4 Microbial metabolism in the meso- and bathypelagic zones

Metabolism	Electronics donor	Electron acceptor	Groups	Carbon fixation pathway(s)
Ammonium oxidation	NH_4^+	O_2	*Crenarchaeota, Proteobacteria*	3-Hydroxypropionate/4-hydroxybutyrate cycle, Calvin-Benson-Bassham cycle
Nitrite oxidation	NO_2^-	O_2	*Proteobacteria, Nitrospira, Nitrospina*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle

10.4.2.2.2.2 Oxygen Minimum Zones and Oxic-Anoxic Interfaces

In general, a distinction must be made between Oxygen Minimum Zones (OMZs) in the open ocean and euxinic waters. OMZs are oxygen-deficient waters that are layered between oxygen-containing water layers. Euxinic waters are characterized by transitions from oxic to anoxic as well as sulfidic waters leading to the formation of oxic-anoxic interfaces or "redoxclines". This means that they are comparatively thin typical horizontal layers in which the properties of the fluids vary very strongly over relatively short vertical distances.

One of the main differences between these two types of stratification is the presence of sulfide and other reduced sulfur compounds in euxinic waters and the general absence of these substances in OMZs. This has associated consequences for microbial communities and the processes that occur within them. Traditionally, heterotrophic processes are assumed to dominate in OMZs, i.e. aerobic respiration and denitrification are coupled to the oxidation of organic matter.

Anaerobic oxidation of ammonium (anammox) by a specific group of *Planctomycetes* is now known to be the major cause of nitrogen loss in OMZs. Since anammox is an autotrophic process, it is clear that a significant amount of inorganic carbon is fixed rather than released. Genome and enzyme studies of anammox bacteria provide the first evidence that the Wood-Ljungdahl pathway is used for carbon fixation. This makes the pathway quite significant in this pelagic area with up to **3.5 Tg of carbon per year**.

The anammox process is inhibited by oxygen. This means that organisms only continue their activity once anaerobic conditions have been established. It has therefore been observed that anammox bacteria live closely associated with aerobic, ammonium-oxidizing *Crenarchaeota* or bacteria. Although these organisms compete for ammonium, their activity in close proximity to anammox cells leads to a decrease in oxygen concentration and is therefore beneficial.

It is interesting to note that anammox has so far been found mainly in those areas where conditions in the water column are more reducing due to the periodic occurrence of reduced sulfur compounds. Such areas are found in the Black Sea, offshore the coast of Namibia, the Gulfo Dulce offshore Costa Rica and the Peruvian upwelling region of deep water.

At oxic-anoxic interfaces or thin layers of **permanently euxinic water columns** (for example, Black Sea, Cariaco Basin: northern, central coast of Venezuela) and intermittent **euxinic water columns (**for example, Baltic Sea, fjords), as well as coastal upwelling regions with **periodic influx of sulfidic water** (for example, coast of Namibia), sulfur-oxidizing microorganisms, including anaerobic phototrophs (especially *Chlorobiales* use the reductive tricarboxylic acid cycle), play a significant role.

Table 10.5 Microbial metabolism in the oxygen minimum zone/Euxinic water column

Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway(s)
Ammonium oxidation	NH_4^+	O_2	*Crenarchaeota, Proteobacteria*	3-Hydroxypropionate/4-hydroxybutyrate cycle, Calvin-Benson-Bassham cycle
Nitrite oxidation	NO_2^-	O_2	*Proteobacteria, Nitrospira, Nitrospina*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
Anammox	NH_4^+	NO_2^-	*Planctomycetes*	Wood-Ljungdahl Way
S-Oxidation	S_{red}	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
Anoxygenic aerobic photosynthesis	S_{red}	Ferredoxin	*Chlorobia*	Reductive tricarboxylic acid cycle

In some cases, nitrate is used as an alternative electron acceptor, which leads to further nitrogen loss due to autotrophic processes.

Epsilonproteobacteria have been identified as important members of the microbial community in the chemical stratification of the Black Sea, Cariaco Basin and Baltic Sea, where they fix inorganic carbon probably via the reductive tricarboxylic acid cycle, which is coupled to the oxidation of reduced sulfur compounds with O_2 or nitrate.

Even in an area of open ocean, such as that off the coast of Namibia, intermittent sulfide intrusion can cause mass growth of sulfur-oxidizing *Epsilon* and *Gammaproteobacteria.*

In particular, a group of *Gammaproteobacteria* closely related to non-cultured endosymbiotic sulfur-oxidizing bacteria has been identified as an important member of microbial communities in such waters that have low oxygen concentrations. The chemolithoautotrophic potential of this group has recently been confirmed (Table 10.5).

10.4.2.2.2.3 Hydrothermal Vents of the Deep Sea

Outcrop channels, first studied on the Galapagos reef, remain the showcase for explaining the importance of chemoautotrophic processes.

Hydrothermal eruption channels are generally located along mid-ocean ridges, where oceanic plates are forced apart by underlying magma chambers. However, fluids with distinct differences also occur at other locations such as back-arc spreading centers, hotspot volcanoes and deep-sea mountains, which then result in different biological communities.

- Back-arc basins are lake basins that can be formed by the subduction of one oceanic plate beneath another.
- Where the subducted plate dips below the overlying plate, there is a deep-sea channel. A forearc basin can develop between the deep-sea channel and the island arc, and a backarc basin on the

other side of the island arc, as the continental plate is stretched there and a spreading zone develops.
- Examples of backarc basins are: The Japanese basin between the Asian mainland and Japan (the South China Sea), as well as the Sulu Sea and the Celebes Sea.
- Examples of active backarc spreading zones are: the Mariana Arc east of the Philippines and the Kermadec-Tonga Ridge opposite the Kermadec-Tonga Trench, near the Fiji Islands northeast of New Zealand.
- Hotspots are centres of volcanic activity that are not directly caused by plate tectonic processes and are therefore not bound to plate margins. An example is Hawai.

The enormous depths of the deep sea create a huge hydrostatic pressure. Water therefore only boils at a depth of 2600 m at about 450 °C. Superheated (but not boiling) hydrothermal fluid with temperatures of 270–350 °C is released at certain sources. The hydrothermal fluids formed by seawater-rock interaction within the hydrothermal system are highly enriched in reduced chemical species such as H_2, H_2S, Fe^{2+} or methane, the composition and concentration depending on the geological environment.

The mineral-rich hydrothermal fluid ejected from black smokers cools rapidly as it enters cold seawater, forming a dark cloud of precipitated material (large amounts of metal sulfides, especially iron sulfides). The precipitated metal sulfides form a tower known as a "vent" around the source (See Fig. ► 4.33; note on anaerobic methane degradation, Sect. ► 4.5.1).

The mixing of these so-called "**geofuels**" (geological fuels) with cold, oxygenated deep-sea water either above or below the seafloor creates a chemical imbalance that can be harnessed by a large variety of metabolically versatile chemolithoautotrophic microorganisms. The biomass formed forms the basis for food chains in these highly productive ecosystems.

That is, microorganisms transfer energy from geothermal sources to higher trophic levels.

Hydrothermal systems have prevailed throughout the evolutionary history of the Earth, and they resemble places where life began. Even today, there are parts of the hydrothermal system that are quite independent of photosynthesis, similar to conditions on the early Earth. Processes that fall into this category are hydrogenotrophic methanogenesis, sulfur reduction, and sulfur disproportionation.

However, it is important to note that today many chemolithoautotrophic processes occurring at the springs and generally in the ocean are dependent on oxygen or other oxidized electron acceptors such as nitrate and sulfate. Ultimately, a significant portion, if not all, of biomass production at the springs and elsewhere in the ocean is associated with oxygenic photosynthesis.

Interestingly, chemoautotrophs couple the consumption of oxygen to the formation of new biomass rather than the decomposition of organic material. This has implications for the overall redox state of the Earth as well as the balance between O_2 and CO_2.

Based on thermodynamic and bioenergetic calculations, biomass production at deep-sea sources is estimated to be in the order of **5 Tg carbon per year.**

Currently available information suggests that the Calvin-Benson-Bassham cycle and the reductive tricarboxylic acid cycle are the main carbon fixation pathways at deep-sea outcrops.

The Reductive tricarboxylic acid cycle seems to be the dominant pathway in habitats characterized by temperatures between

20 and 90 °C. In contrast, the Calvin-Benson-Bassham cycle is thought to be the main pathway at a temperature <20 °C, while other carbon fixation pathways, such as the Wood-Ljungdahl pathway or the Dicarboxylate/4-hydroxybutyrate cycle operate in the >90 °C range.

The Reductive tricarboxylic acid cycle functions in *Aquificales* and *Epsilonproteobacteria,* both of which are considered important, if not the dominant, members of the community at deep-sea springs. The main growth temperature for *Aquificales*, which is divided into the families *Aquificaceae, Hydrogenothermaceae* and *Desulfurobacteriaceae,* is between 60 and 90 °C, with a few members growing above 90 °C. While *Aquificaceae* and *Hydrogenothermaceae* host mainly microaerophilic chemolithoautotrophs that generate energy through the oxidation of molecular hydrogen or reduced sulfur compounds, all members of *Desulfurobacteriaceae* are strictly anaerobes. They obtain energy by coupling hydrogen oxidation to the reduction of elemental sulfur or nitrate.

Aquificales appear to be primarily associated with sulfidic structures and prefer to colonize the surface areas of springs.

Epsilonproteobacteria have been identified as important members of the deep-sea microbial community in hydrothermal vents, with free-living bacterial populations in source fluids, black smoker chimney walls, and surfaces washed by hydrothermal fluids, as well as the fractured surface.

Epsilonproteobacteria possess metabolisms similar to those of *Aquificales*: (1) the oxidation of reduced sulfur compounds and hydrogen with oxygen and nitrate, or (2) the oxidation of hydrogen and elemental sulfur coupled to the fixation of inorganic carbon. Consequently, a similar ecological niche is occupied, but here at a lower temperature between 20 and 70 °C. Different groups of *Epsilonproteobacteria* can inhabit different niches.

Members of the *Nautilia*/*Caminibacter* group grow at temperatures between 40 and 70 °C. They rely on the oxidation of hydrogen coupled to the reduction of nitrate or elemental sulfur (S°) as an energy source.

In contrast, members of the *Sulfurimonas* and *Sulfurovum* groups grow at low temperature between 10 and 40 °C. They use reduced sulfur compounds and/or molecular hydrogen as electrondonors and oxygen or nitrate as electron acceptors. Compared to the other *Epsilonproteobacteria,* members of these groups can tolerate a relatively high oxygen concentration. The metabolic property may be advantageous in such ecosystems where intense mixing of hydrothermal fluids and seawater occurs as in rugged surfaces. Indeed, *Epsilonproteobacteria* related to *Sulfurovum* and *Sulfurimonas* spp. are often found as dominant populations in such places.

Arcobacter and related *Epsilonproteobacteria,* which have also often been found in deep-sea spring ecosystems, require higher sulfide concentrations.

Ammonium oxidation is another process likely involved in chemoautotrophic production at sources. *Crenarchaeota* carry this out under aerobic conditions using the 3-Hydroxypropionate/4-hydroxybutyrate cycle, while *Planctomycetes* are responsible for carbon fixation in the anaerobic environment using the Wood-Ljungdahl pathway.

The possibility of a complete nitrogen cycle may thus exist at the sources, carried out solely by autotrophic organisms: the reduction of nitrate to N_2 or ammonium is catalyzed by *Epsilonproteobacteria* and *Aquificales*, and methanogens then fix N_2.

Since mainly hyperthermophilic *archaea* grow at temperatures above 90 °C, most if not all biomass production at these temperatures is carried out by *Archaea* using alternative carbon fixation pathways. These include autotrophic methanogens such as *Methanopyrus kandleri*, *Methanocaldococcus jannaschii*, which are

involved in primary production on the seafloor, and sulfate-reducing *Euryarchaeota* of the genus *Archaeoglobus*. Both groups use the Wood-Ljungdahl pathway for carbon fixation.

Members of the *Crenarchaeota* such as *Ignicoccus* spp. and *Pyrolobus fumarii* also grow at temperatures above 90 °C and use the Dicarboxylate/4-hydroxybutyrate cycle for carbon fixation.

Very interestingly, *Ignicoccus* spp. derive their energy from the reduction of elemental sulfur with hydrogen, a very simple and very ancient type of metabolism matching the probably very ancient carbon fixation pathway.

The use of the 3-Hydroxypropionate/4-hydroxybutyrate cycle by hyperthermophilic *Crenarchaeota* has only been demonstrated in acidophilic members of the *Sulfolobales.* Members of this group have not yet been found at deep-sea springs, even in springs of the TOTO caldera in the Mariana volcanic arc with a pH of 1.6, although it is suspected that a previously unculturable group of organisms at deep-sea hydrothermal vents plays this role.

It remains to add that the Wood-Ljungdahl pathway is also significantly involved in chemoautotrophic production at low temperatures. It functions in mesophilic methanogens, sulfate reducers and anammox catalyzing *Planctomycetes* (◘ Table 10.6).

10.4.2.2.2.4 Cold Gas Leaks/Cold Seeps

There are obviously other oceanic areas where chemolithoautotrophic processes are important. Cold gas seeps and mud volcanoes should be addressed, where highly reduced fluids of non-hydrothermal origin occur from the subsurface, often containing methane and other hydrocarbons (◘ Table 10.7).

In these habitats, the anaerobic oxidation of methane and also that of other hydrocarbons is coupled to the reduction of sulfate. The H_2S formed drives chemoautotrophic production, which is mainly carried out by *Epsilon-* and *Gammaproteobacteria* either as free-living organisms or in symbiosis.

At methane seeps, anaerobic methane oxidizers fix large amounts of inorganic carbon, most likely via the Wood-Ljungdahl pathway.

On the other hand, methane-producing *Archaea* are important partners in autotrophic carbon fixation in deep-sea sediments, as are autotrophic acetogens. Similar to the meso- and bathypelagic domains, both groups process organic material originally formed by oxygenic phototrophs by utilizing the products of fermentative degradation of organic material, namely hydrogen and CO_2.

10.4.2.2.2.5 Sediment

Oxygen does not penetrate very deeply into the sediment because of its low solubility in water. Already in the upper millimetres it is completely consumed by aerobic organisms. This means that the deposited organic matter must be oxidized with other electron acceptors. In the sea, sulphate plays a central role in microbial degradation as an alternative to oxygen. In coastal sediments, for example, more than half of the organic input is mineralized via sulfate reduction.

At a concentration of 2.68 g of sulfate per liter of seawater (~28 mM), about 180 times more organic matter can be oxidized with dissolved sulfate as an electron acceptor than with dissolved oxygen (~0.2 mM).

Sulfate-reducing bacteria (for example, *Desulfotomaculum, Desulfomonas, Desulfovibrio species*) successfully compete for the fatty acids, acetate and hydrogen formed by fermentation and acetogenesis. In deeper sediment layers depleted of sulfate, methanogenesis may dominate. However, a considerable portion of sediment organic matter is not mineralized. Aromatic compounds are slowly degraded. Therefore, the accumulation of digested sludge, blackened by FeS, occurs, which can form deposits several metres thick (◘ Table 10.8).

Table 10.6 Microbial metabolism in hydrothermal vents

Temperature (°C)	Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway(s)
~4–20	Fe^{2+} oxidation	Fe^{2+}	O_2, NO_3^-	*Zetaproteobacteria*	Calvin Benson Bassham Cycle
	S-Oxidation	S_{red}	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
~20–70	S-Oxidation	S_{red}	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
	H_2-Oxidation	H_2	O_2, NO_3^-, S^0	*Epsilonproteobacteria, Aquificales*	Reductive tricarboxylic acid cycle
	Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl pathway
	Sulfate reduction	H_2	SO_4^{2-}	*Deltaproteobacteria*	Wood-Ljungdahl pathway, (Reductive tricarboxylic acid cycle)
~70–90	S-Oxidation	S_{red}	O_2, NO_3^-	*Aquificales*	Reductive tricarboxylic acid cycle
	H_2-Oxidation	H_2	O_2, NO_3^-, S^0	*Aquificales*	Reductive tricarboxylic acid cycle
	Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl pathway
	Sulfate reduction	H_2	SO_4^{2-}	*Archaeoglobal*	Wood-Ljungdahl pathway
>90	H_2-Oxidation	H_2	S^0, $S_2O_3^{2-}$, O_2, Fe^{3+}	*Crenarchaeota*	Dicarboxylate/4-hydroxybutyrate cycle, 3-Hydroxypropionate/4-hydroxybutyrate cycle
	Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl pathway
	Sulfate reduction	H_2	SO_4^{2-}	*Archaeoglobal*	Wood-Ljungdahl pathway

Table 10.7 Microbial metabolism at cold gas outlets

Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway(s)
S-Oxidation	S_{red}	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
H_2-Oxidation	H_2	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria, Firmicutes*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl pathway
Methanotrophy	CH_4	SO_4^{2-}	*Euryarchaeota*	Wood-Ljungdahl pathway
Sulfate reduction	H_2	SO_4^{2-}	*Deltaproteobacteria, Euryarchaeota, Firmicutes*	Wood-Ljungdahl pathway, (Reductive tricarboxylic acid cycle)

Table 10.8 Microbial metabolism in deeply buried marine sediments

Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway(s)
Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl pathway
Acetogenesis	H_2	CO_2	*Firmicutes*	Wood-Ljungdahl pathway
Sulfate reduction	H_2	SO_4^{2-}	*Deltaproteobacteria, Euryarchaeota, Firmicutes*	Wood-Ljungdahl pathway, (Reductive tricarboxylic acid cycle)

10.4.2.2.2.6 Mountain Sides

Rocks of the waste flanks of mountains are other significant, but as yet poorly explored, areas of chemoautotrophic biomass formation. Namely, iron, hydrogen, and/or sulfide oxidation have been identified as possible energy sources, fixing an estimated **~1 Tg** of **carbon per year** carried out on oceanic crust (Table 10.9).

Evidence suggests that autotrophic iron-oxidizing *Zetaproteobacteria* involved in biomass production in iron-rich hydrothermal systems and bedrock likely utilize the Calvin-Benson-Bassham cycle.

Table 10.9 Microbial metabolism on crusts of waste flanks of oceanic mountains

Metabolism	Electron donor	Electron acceptor	Groups	Carbon fixation pathway(s)
Fe^{2+} oxidation	Fe^{2+}	O_2, NO_3^-	*Zetaproteobacteria*	Calvin Benson Bassham Cycle
S-Oxidation	S_{red}	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
H_2-Oxidation	H_2	O_2, NO_3^-	*Gammaproteobacteria, Epsilonproteobacteria, Firmicutes*	Calvin-Benson-Bassham cycle, Reductive tricarboxylic acid cycle
Methanogenesis	H_2	CO_2	*Euryarchaeota*	Wood-Ljungdahl Way
Sulfate reduction	H_2	SO_4^{2-}	*Deltaproteobacteria, Euryarchaeota, Firmicutes*	Wood-Ljungdahl pathway, (Reductive tricarboxylic acid cycle)

10

Test Your Knowledge

- Which physico-chemical conditions influence the growth of microorganisms?
- In what form do microorganisms mainly occur in the soil?
- What is meant by the soil solution?
- Discuss the size comparison clay/silt/sand
- Describe the grain triangle.
- In which soil horizon is microbial activity mainly found?
- Explain the term syntrophy. Give examples.
- What ingredients do you find in EPS?
- Name problems caused by biofilms.
- Describe the behaviour of r- and K-strategists towards the substrate. Under what conditions does one type displace or outgrow the other?
- Describe the metabolism at the Black Smoker. What is the source of the Black Smoker? What does a Black Smoker have to do with Biofim? Describe the temperature curve.
- Explain gradients in nature using the example of a soil or in the ocean.
- What do maximum specific growth rate and affinity constant mean?
- Explain the terms oligotrophy, autochthonous, zymogenic, copiotrophic.
- Name forms of adaptation.
- What changes in organisms that can grow with solvents compared to "normal" organisms?
- Why do we find a minimum limit concentration during degradation that is not undercut?
- What do you understand by stratification, epilimnion, hypolimnion? Show the seasonal sequence in a lake.
- Where does Eutrophobia come from?
- Explain a color striped mudflat. Which metabolic processes depend on each other in such an ecosystem? Say something about gradients found there in the course of a day. Say something about the use of light?
- Water activity has a growth-limiting effect on an organism when the concentration of compatible solutes in its living environment increases. To counteract this situation, the organism

builds or accumulates intercellular compatible solutes that serve to maintain the cell in positive water balance. The evolution of some microorganisms has been to grow best at reduced water potential and some even require high salt concentrations to grow. What is the a_w of pure water? What is meant by a compatible solute and why is it needed? Name a compatible solute for the Halobacterium species?

- What do you mean by euphotic zone, oxygen minum zone, meso- and bathypelagic zone?
- How do you estimate the concentration of oxygen in the ocean in the different areas?
- How fast does mass transfer take place in the ocean?
- Does CO_2 formed in the ocean depths reach the ocean surface?

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Microbial Communities: Structural and Functional Analyses with Classical Approach

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_11

Microorganisms are involved in numerous processes in the environment in a desirable or undesirable manner. At the same time, they practically never occur in nature as pure cultures, but always together with other microorganisms and often also higher organisms. In this respect, the task of learning something about the state of microbial communities arises again and again. For example, one might want to know something about the microbial activity of a soil in order to be able to draw conclusions about the consequences of management measures. One may want to know whether the activated sludge of a sewage treatment plant is impaired in its activity by the introduction of toxic chemicals. The occurrence of certain physiological groups, for example nitrificants, may need to be monitored. Or one may be interested in whether the bacteria or the degradation genes necessary for a degradation process are present at a site and, if so, whether they have been induced. One might want to identify the carriers of a process in order to better control processes on the basis of their development or to be able to use the microorganisms in a targeted manner. Finally, one sometimes wants to ensure that certain bacteria are not present in a community or are not present in high numbers. The following chapter will explain some of the approaches and principles that can be used to address such or similar issues.

The following BOX lists different methodological approaches and strategies for the analysis of microbial communities.

Strategies for the Analysis of Microbial Communities

Characterization by detection of cell components

- Non-specific components
  - Protein determination
  - Biomass determination by CO_2-release
- Biomarker
  - Non-nucleic acid biomarker molecules
    - Phospholipids
    - Fatty acid pattern
    - Muramic acid
    - Ergosterol for fungi
  - Nucleic acid biomarker molecules

Characterization by evidence of activities

- Community Activities:
  - CO_2-release
  - O_2-consumption
  - Incorporation of radioactive compound e.g.^{3} H-labelled thymidine
  - Isotope fractionation
  - ATP content determination as a state description of cells
- Determination of enzyme activities
  - Hydrolases
  - Esterases
  - Lipases
  - Proteases
  - Dehydrogenases
- Presence of mRNA as an indication of the enzymes expressed and the metabolism used

11.1 Summary Methods

Summary methods attempt to say something about the biocoenosis as a whole and not about the occurrence or activity of individual taxonomic or physiological groups. A rough distinction can be made between methods that determine the number of cells or biomass and methods that directly tell us something about their activity.

11.1.1 Determination of Bacterial Counts and Biomasses

The **total bacterial count** in a sample (including cells that can no longer divide) can be determined relatively easily by microscopy

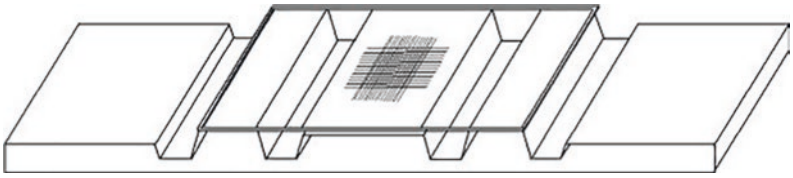


Fig. 11.1 Counting chamber for the determination of total microbial counts

using a **counting chamber.** Counting chambers are slides provided firstly with a grid defining the observed area and secondly with bars ensuring a defined distance of the coverslip and thus a defined volume over the surfaces of the grid (Fig. 11.1). Another possibility for counting bacteria is the **Coulter counter**, in which the passage of particles through a capillary is registered via changes in conductivity.

A common problem in microscopy, especially of prokaryotic microorganisms, is the low contrast to the surroundings. One measure to improve this is the use of **phase contrast objectives**, in which the phase shifts that occur when light passes through an object are exploited to increase contrast. Another measure, long used in microbiology, is the staining of cells with appropriate dyes. The use of **fluorescent dyes** is particularly effective (Fig. 11.2). Some of these dyes, such as fluorescein isothiocyanate, bind to SH groups of proteins; others, such as acridine orange and DAPI, bind to DNA. Propidium iodide (PI), like ethidium bromide, acts as a nucleic acid intercalator. Acridine orange changes its color from orange to green upon binding, and DAPI fluoresces blue. The available dyes differ in their ability to penetrate membranes. For example, while DAPI and SYTO® 9 also penetrate the cell through intact membranes, other dyes (for example propidium iodide and SYTOX® Green) require a damaged membrane to penetrate. With a suitable combination of interacting dyes of different membrane permeability and different fluorescence in the form of commercial ***viability kits,*** intact cells can be distinguished from dead or damaged cells. Some of the kits used for such staining are based on the activity of enzymes (e.g. esterases) in the cells in addition to membrane mobility. Depending on the procedure and the fluorescent dye used, in some cases one is dealing with an activity staining and not with a total bacterial count determination.

Flow cytometry is now of greater relevance. It describes a measuring method that is used in both medicine and biology. Flow cytometers (see schematic structure in Fig. 11.3) work with hydrodynamic focusing of suspended cells. Focused by the sheath current, the sample enters the microchannel of a high-precision cuvette made of glass or quartz. Each cell is thus guided one by one through the measuring range of one or more laser beams. The resulting scattered or fluorescent light is detected by photomultipliers. Using optical filters, possible fluorophores on or in the cells can be quantified by peaks of their emission spectra. Depending on the shape, structure and/or staining of the cells, different effects are produced from which the properties of each individual cell can be deduced. By analyzing a large number of cells within a very short time interval (>1000 cells/s), representative information about cell populations can be obtained quickly.

The amount of scattered light correlates with the size of the cell and with its complexity. Forward scattered light is a measure of the diffraction of light at a shallow angle and depends on the volume of the cell. Side scattered light is a measure of the refraction of light at right angles, which is affected by the granularity of the cell, the size and struc-

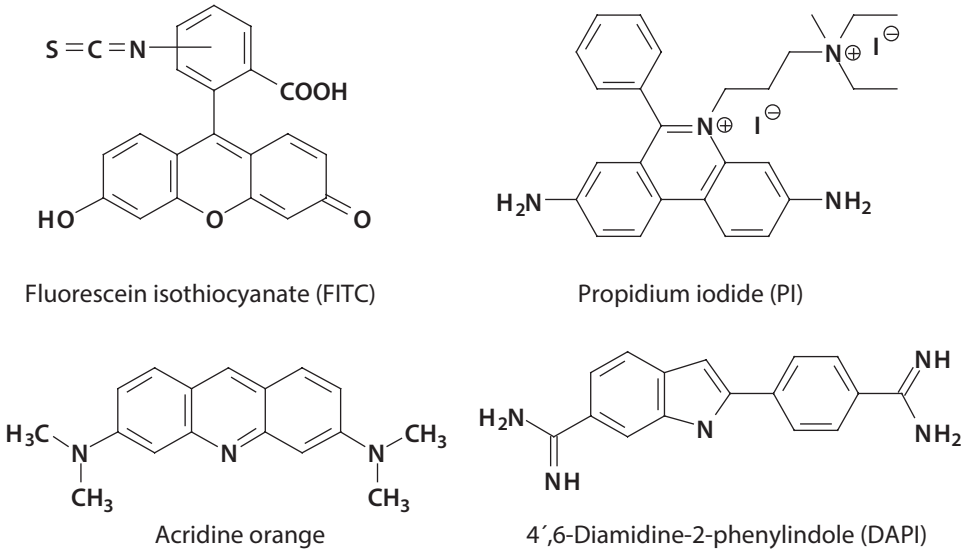


Fig. 11.2 Structural formulae of selected fluorescent dyes

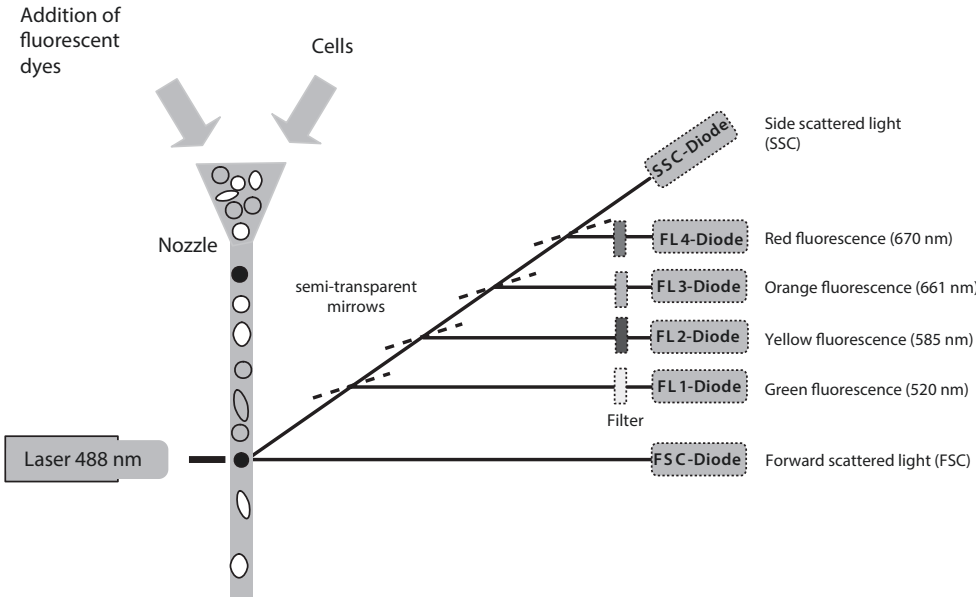


Fig. 11.3 Schematic structure of a flow cytometer

ture of the nucleus, and the amount of vesicles in a cell.

In the flow cytometer, fluorescent colors are measured simultaneously with the scattered light. These can be endogenous fluorophores such as chlorophyll. Since only a few cells emit fluorescent light per se, **fluorescent dyes** are added as mentioned above. If, for example, DAPI and propidium iodide are used, which bind to or intercalate with the DNA of a cell, the brightness can be used to determine how much DNA a cell contains.

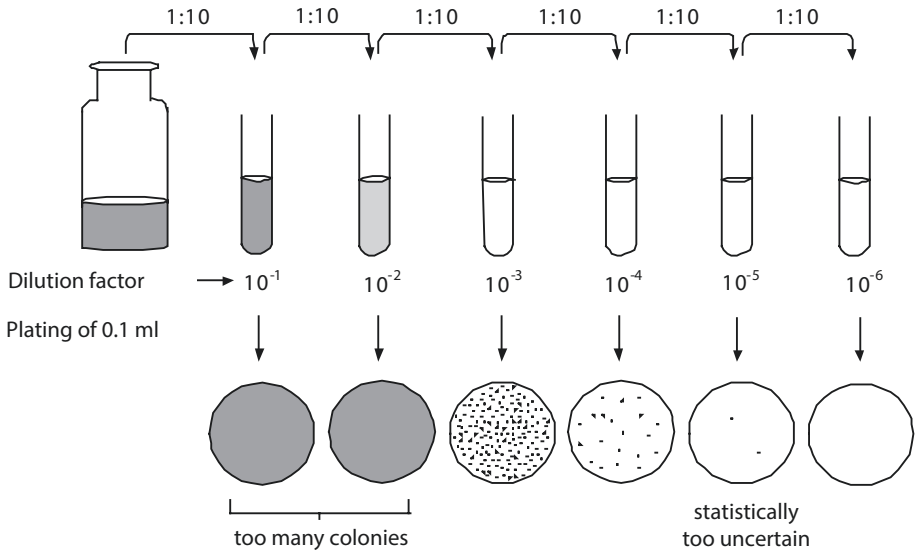


Fig. 11.4 Determination of the live germ count of a sample on solid culture media

The number of dyes that can be used and thus the information density can be increased by using different colored lasers and, above all, filters.

One application of flow cytometry is the quantitative examination of cells, but also a cell viability determination by distinguishing living from dead cells by staining with propidium iodide in combination with a DNA dye such as DAPI.

Since the method can also be used to determine the ratio of rather large to rather small cells, a possible change in a cell population as a result of a change in its environmental conditions can also be determined. Thus, flow cytometry was included in the Swiss Food Book ("Schweizerisches Lebensmittelbuch") as a recommended method for determining the total cell count in freshwater.

The determination of the **live germ count,** i.e. the number of divisible microorganisms, requires their cultivation on appropriate media. The easiest way to determine the live germ count is to create a dilution series of the respective sample and distribute an aliquot of each on agar plates (Fig. 11.4) or, in the plate casting method, to distribute it in the agar. By counting the colonies formed during an incubation, the number of **colony** forming units (CFU) per ml of the plated suspension is obtained, always taking into account that cells still attached to each other can only form one common colony. Provided that one wants to capture as many of the dividing cells as possible, it is important to use the least selective medium possible. This will normally be a **complete medium**, i.e. a medium containing a variety of compounds, usually derived from biological material and of limited definition, for example yeast extract, meat extract, peptone (polypeptide mixture from enzymatic cleavage of proteins) or casein hydrolysate. Since many bacteria tend to be adapted to the relatively low substrate concentrations found in nature (► Sect. 10.1.1), it is also important not to select too high a **concentration of** substrates in order to capture as many bacteria as possible, although compromises may have to be made to ensure sufficient size and hence visibility of colonies. Finally, incubation parameters such as temperature must also be taken into account

and, if possible, should be broadly similar to those of the sample's point of origin. Even if one tries to make the conditions for recording the live germ count as non-selective as possible, the number obtained will always be significantly smaller than the total germ count, especially in the case of environmental samples, because cells attached to each other only form one colony, because not all cells may be able to divide and, above all, because many of the microorganisms occurring in nature cannot be cultivated with the usual methods (see also ► Sect. 11.3).

A basic problem with microbial counts, especially in soil, is the possibility of **adsorption to surfaces,** which can lead to cells not detaching and therefore not being detected. At the same time, microbial counts do not always have to be determined starting from samples in the form of a suspension. Sometimes surfaces are offered directly in the habitat of the microorganisms so that they can adsorb or grow on them and then be counted.

Since many microorganisms do not grow well on agar plates or in agar, it is sometimes necessary to estimate the number of replicable organisms in a sample after cultivation or reaction in liquid media. As this is particularly important for the quantification of certain physiological groups, the approach (the **MPN method**) is explained in ► Sect. 11.2.

The determination of **biomass** in an environmental sample is highly dependent on the sample and its content of other solids. If the content of interfering solids is low, the organisms can be separated from the aqueous phase by **centrifugation or filtration** and then dried to obtain the dry biomass. This is obviously not possible for communities from the soil. Here, there are estimates that determine mass from **microscopic examination of** cell numbers and observed cell sizes using average densities.

Another method applied to soil is the so-called **chloroform fumigation incubation method.** Here, the cells of the soil sample are killed with chloroform and the cell contents are released. The mixture is then incubated with a non-treated soil sample and the CO_2 produced by **respiration of the biomass** is collected via alkali leach and determined (for methodology see below). The value obtained with a non-chloroform treated sample is subtracted from the value. Since not all biomass is released and oxidized, and for conversion to carbon mass, the mass of CO_2 obtained must still be multiplied by a correction factor, although it is doubtful whether the same factor can be used here for all soils.

In the **chloroform fumigation extraction method,** the procedure is similar in principle. However, here the batch of chloroform-treated cells is extracted with a solution of K_2SO_4. The organic carbon in the solution can then be oxidized with potassium dichromate ($K_2Cr_2O_7$) to CO_2 and determined from the change in absorbance that occurs upon reduction of the dichromate. Alternatively, the carbon content can be determined in an organic carbon analyser (DOC). The extraction method detects only about 70% of the organic compounds oxidized by the incubation method. The basic problem with biomass determinations is that it is hardly possible to differentiate between active and dead microorganisms.

Quantitative PCR, which is explained in ► Sect. 12.2, is becoming increasingly important for bacterial count determinations. As this method involves DNA isolation, the efficiency of which may be subject to fluctuations, it may be necessary to quantify using an internal standard.

11.1.2 Determination of Activities

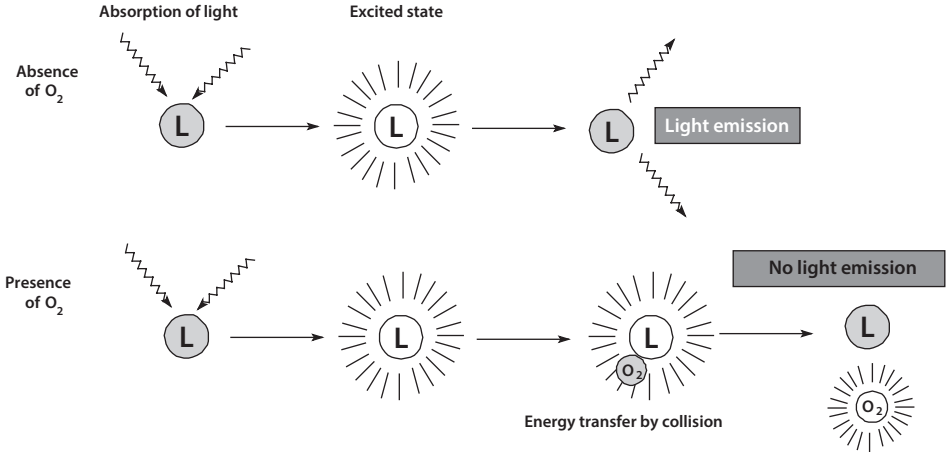
In many cases, it is not so much a matter of recording microbial counts or biomasses, but rather their activities. This is of interest, for example, when assessing the impact of pollutants or soil management practices on

microbial communities or the success of remediation measures. Of course, by tracking biomass or plate counts over time, one can also derive information about the activity of microbial communities. However, this is quite time-consuming. Depending on the investigated metabolic type, matrix, exact question and equipment, one can choose between several principally different approaches.

The **respiratory activity of** aerobic microorganisms is often determined. This can be basal respiration, i.e. O_2 consumption or CO_2 formation, which can be measured without the addition of an additional substrate. Or it can be substrate-induced respiration, which is observed after such an addition.

Respiratory activity is often determined by the rate of **oxygen consumption**. However, oxygen consumption is used not only to determine the activity of aerobic organisms, but also to describe the load of oxidisable compounds in waste water (► Sect. 14.1). The following methods, among others, are available for measuring O_2 concentrations:

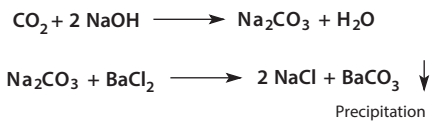
- The O_2 concentration can be monitored over time using an **O_2 electrode**, as often used in BOD measurements (► Sect. 14.1).
- Provided that CO_2 produced is captured by alkali hydroxide solutions, O_2 consumption in a closed system is accompanied by a reduction in pressure. Such **pressure changes** can be followed over time with a range of devices.
- In more complex devices, so-called **sapromats,** the O_2 consumption in the closed system is compensated by electrolytic simulation of O_2. The amount of consumed O_2 is also registered in this case via a pressure measurement and the simulated O_2 results from the current consumed for the electrolysis. An advantage of the sapromat is that a reduction of the O_2 concentration in the preparation and a resulting limitation of the reaction can be avoided.
- A measurement of the O_2 concentration is possible with **gas chromatographs.**
- The measurement of O_2 concentration with **optodes** is based on the quenching of the luminescence of a luminophore by O_2. In the absence of O_2, the light absorbed by the luminophore (for example polycyclic aromatic hydrocarbons or certain transition metal complexes) first leads to an excited state, which in turn emits light (◘ Fig. 11.5). In the presence of O_2, collision with the excited luminophore results in energy transfer from the luminophore to O_2, leading to quenching of the light emission.



◘ **Fig. 11.5** Principle of O_2 concentration measurement with optode. L = luminophore

The determination of **CO_2 formation** associated with respiration processes can also be used to assess respiration activities. Several possibilities are also available for this purpose:

- The formation of CO_2 can be monitored directly in the gas phase by **infrared** gas **analysis.**
- An analysis with **gas chromatography** is also possible directly in the gas phase.
- The classic method is to capture CO_2 formed with alkali hydroxide solutions and then precipitate it as sparingly soluble barium carbonate by adding $BaCl_2$. The concentration of unconsumed alkali hydroxide can be determined by titration with acids and used to calculate the alkali hydroxide consumed by CO_2 formation. Alternatively, after drying and weighing, the precipitated barium carbonate can be used as a measure of CO_2 formed (◘ Fig. 11.6).

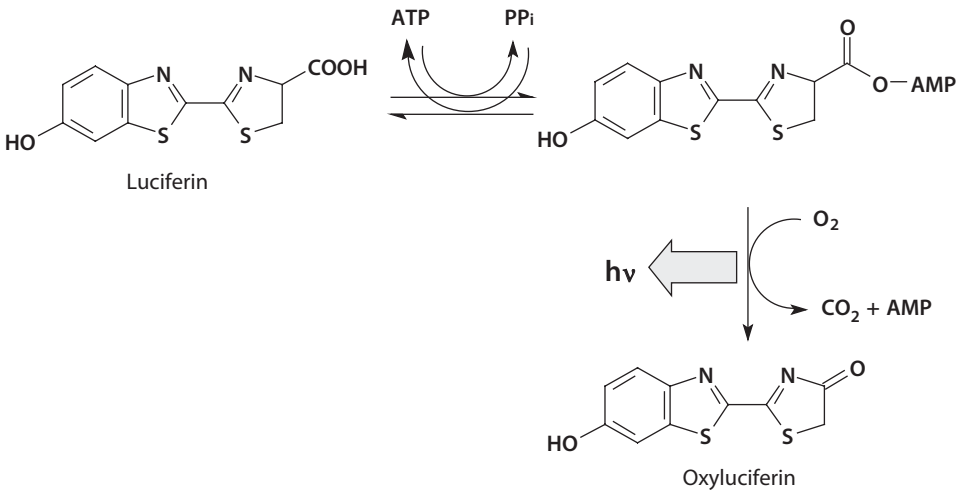


◘ **Fig. 11.6** Capture of formed CO_2 by alkaline solution and precipitation as barium carbonate

Since the condition of cells can be determined well on the basis of their **ATP content**, the ATP content in environmental samples is also frequently analysed. This is possible by HPLC measurements. It is also widely used to quantify ATP by bioluminescence. Firefly **luciferin** requires ATP for its activation, and adenylated luciferin reacts in the presence of oxygen under light emission to form oxyluciferin (◘ Fig. 11.7). The light emission is measured and in principle allows a sensitive ATP analysis, which is, however, sensitive to some accompanying substances from the environmental sample.

Since metabolic activity is inevitably associated with the **activity of enzymes**, the activities of **widespread and easily measured enzymes** are also frequently determined. In many cases, an attempt is made to follow the enzyme activity via consumption or formation of a dye, even if the substrate used in the measurement is a non-natural compound and is only converted by the enzyme by chance. The following examples of enzymes frequently found in environmental samples and the measurement principles are mentioned:

- Many of the respiratory chain **dehydrogenases** react not only with their natural electron acceptors but also with artificial



◘ **Fig. 11.7** Reaction of the luciferase of fireflies

ones. One such compound is triphenyltetrazolium chloride (TTC), which is reduced to the red dye trimethylformazan (TPF) (◘ Fig. 11.8). The activity of the dehydrogenases is dependent on the integrity of the cell.

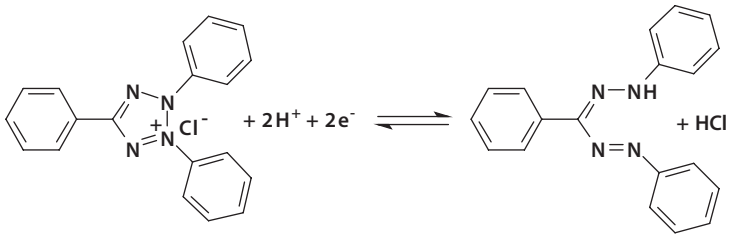
- Hydrolysis of fluorescein diacetate to green fluorescein by **esterases,** lipases, and proteases is also used as an indicator of microbial activity (◘ Fig. 11.9).

The measurement of such enzyme activities provides an accurate statement about the general microbial activity in the environmental sample (for example in the soil) only under certain **conditions**, namely when the respective enzyme is widely distributed, when the enzymes of the different organisms can be measured to a large extent with the possibly unnatural test substrate, when they react similarly fast with the test substrate and when they are also largely stable under the test conditions. These conditions are often only partially fulfilled.

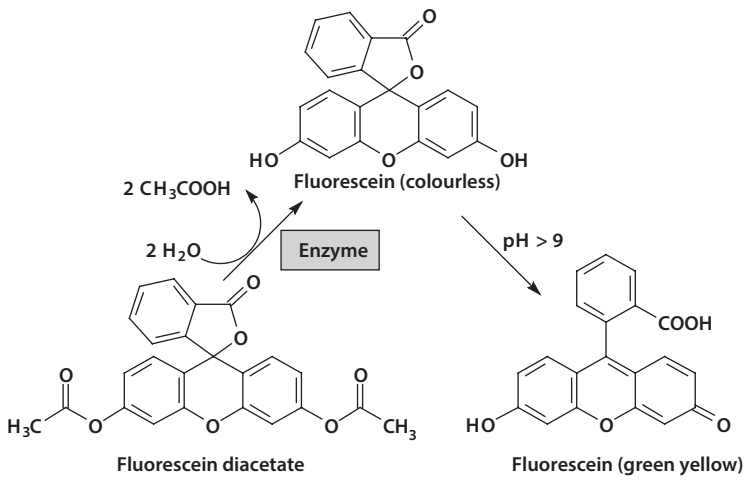
The heat tone associated with the metabolism of microorganisms can also be detected in communities by **calorimetry** and provides a very good measure of their activity.

Finally, the **incorporation of radioactive compounds,** such as ^{3}H-labeled thymidine into biomass is also used as a measure of metabolic activity.

In the microbial degradation of organic compounds, the formation of **heavy DNA** can be used as an indication of the utilization of the compound by feeding it with



◘ **Fig. 11.8** Measurement of dehydrogenase activities by reduction of triphenyltetrazolium chloride to triphenylformazan



◘ **Fig. 11.9** Measurement of the activities of esterases, lipases and proteases by hydrolysis of fluorescein diacetate to fluorescein

^{15}N-medium. Heavy and light DNA can be separated by centrifugation.

During the microbial degradation of organic compounds, molecules with the heavier ^{13}C isotope are in many cases converted somewhat more slowly than those without this isotope. In the course of degradation processes, this leads to an enrichment of the heavy isotope and to an increase in the $^{13}C/^{12}C$ isotope ratio in the unreacted residual fraction of a substance. The extent of such isotopic fractionation can be determined by mass spectrometric analysis and is given by the isotopic fractionation factor α. Knowing the **fractionation factor** α from comparative studies, using the Rayleigh equation for closed systems,

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\alpha-1} \quad \text{or} \quad \ln\left(R_t / R_0\right) = (\alpha - 1)\ln\left(C_t / C_0\right)$$

(R_t, R_0, $^{13}C/^{12}C$ isotope ratios at time points t and 0, respectively; C_t, C_0, concentrations at time points t and 0, respectively)

one can conclude from the observed isotope fractionation to the degradation that has already occurred, which is of great interest, for example, in the assessment of cases of damage involving organic pollutants that occurred a long time ago.

Another important quantity for describing isotope effects is the δ-value**.** To calculate the δ-value, the isotope ratio of a sample (R_{Pr}) is set in relation to that of a standard (R_{St}):

$$\delta = \frac{R_{Pr} - R_{St}}{R_{St}} \times 1000\left(^\circ/\infty\right)$$

Isotope fractionation caused by microbial activity is reflected in characteristic δ-values of the sample. For example, in the area of **inorganic compounds,** purely chemical sulfide oxidation leads to different $\delta^{18}O$ values in the resulting sulfate than microbially mediated sulfide oxidation (cf. ► Sect. 8.1.4), which may allow conclusions to be drawn about the important mechanisms at the respective site.

The NanoSIMS Laboratory

Research with the NanoSIMS combines for the first time the possibility to phylogenetically identify single cells from natural microbial communities and at the same time to gain knowledge about the respective metabolic function.

The NanoSIMS is a nanometer-scale secondary ion mass spectrometer with both extremely high lateral resolution and high mass resolution for analyzing the composition of solid surfaces and thin films.

The high mass resolution of the mass analyzer allows separation of the isotope (mass) of interest from interfering isotopes and/or molecular clusters with very similar masses (◘ Fig. 11.10).

Examples for the use of NanoSIMS are:

- Single cell analysis
- Degradation of plastic in the soil

▪ Individualism of Cells

NanoSIMS can be used to measure the food intake of individual cells.

Individual cells in bacterial groups that suffer from nutrient deficiency can react very differently. Although all cells in such a group are genetically exactly the same, they deal quite differently with the nutrients in their environment.

Bacteria of the species *Klebsiella oxytoca* prefer to take up nitrogen in the form of ammonium (NH_4^+), because this costs comparatively little energy. If there is not enough ammonium for all of them, some cells of the

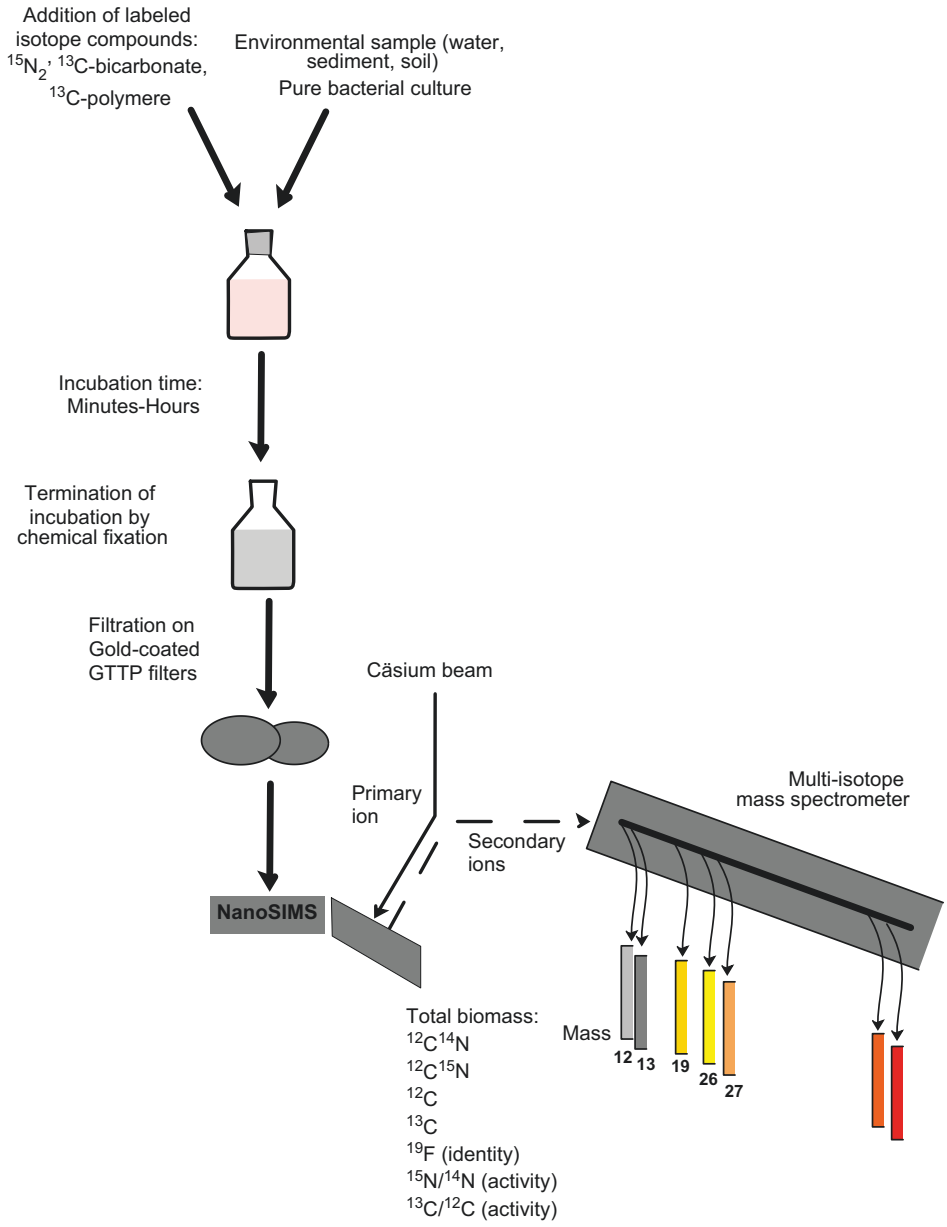


Fig. 11.10 NanoSIMS technique for the sensitive detection of activities (GTTP: hydrophilic, isopore, polycarbonate membrane filter)

group obtain their nitrogen by nitrogen fixation from elemental nitrogen (N_2), although this is considerably more costly.

The bacterial cultures of *K. oxytoca* were supplied with different concentrations of ammonium and an excess of $^{15}N_2$ in continuous cultures. The respective population was then examined with a NanoSIMS, thus visualizing the nitrogen fixation of individual cells in the bacterial culture (Fig. 11.11).

The level of NH_4^+ limitation affects the phenotypic heterogeneity of N_2-fixation. In

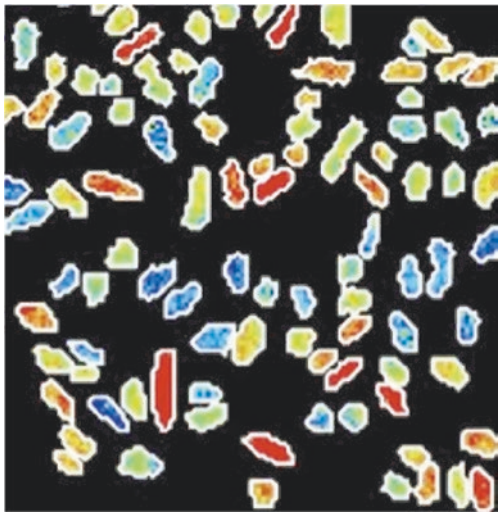


Fig. 11.11 The picture shows the enrichment of cells with heavy nitrogen (^{15}N) after they have been fed with heavy elemental nitrogen ($^{15}N_2$). The different coloration shows that the genetically identical cells in a population have different amounts of elemental nitrogen incorporated into the cell mass. The warmer the coloration, the more elemental nitrogen was incorporated
Inquiry: © Fray3enk Schreiber (BAM Berlin) and Sten Littmann (MPI Bremen)

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contrast, the rate of N_2-fixation of individual cells correlates positively with their growth rate during the shift to NH_4^+-depletion.

Although all individuals in the group are genetically identical and have been exposed to the same environmental conditions, the individual cells are different. Differences between individuals give the whole group new properties and thus allow it to cope with difficult environmental conditions. If the ammonium suddenly ran out altogether, these cells were well prepared for the deficiency. Even if individual cells suffer, the group as a whole can continue to grow.

It has been found that bacterial populations produce particularly large numbers of individualists when there are limited nutrients. This means that these bacterial populations do not only adapt to changing environmental conditions after the fact. The individualists can also be prepared for such changes in advance (Schreiber et al., 2016).

■ Microorganisms Can Decompose Plastic in the Soil

Since the 1960s, plastic films have been used in agriculture for so-called mulching (covering the soil). They have many benefits—for example, they allow better distribution and slower evaporation of moisture in the soil, ideal temperatures for plant roots and protection against weeds and insects. This improves conditions for plant growth, while reducing water consumption and the use of herbicides and fertilizers.

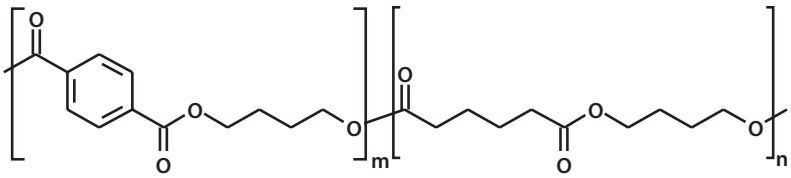
Usually, mulch films are made of non-degradable polyethylene (PE). If these PE films are not completely removed from the soil after harvesting, residues remain and accumulate in the soil over time. It is estimated that six million tonnes of plastic were used in agriculture worldwide in 2016, two million tonnes of which were in the form of mulch films alone.

A promising way to circumvent the accumulation of plastics in agricultural soils is to use films made of polymers that can be degraded by soil microorganisms. A promising polymer used in mulch films in this respect is PBAT (polybutylene adipate terephthalate).

To study biodegradation in soils in more detail, the researchers used special PBAT whose building blocks contained an increased amount of the stable carbon isotope ^{13}C instead of the conventional carbon ^{12}C. The researchers also found that the stable carbon isotope was enriched in the polymer. Since the ^{13}C isotope accounts for only about 1% of all carbon in the environment, it can be excellently used in enriched form in polymers to follow the flow of carbon atoms from the polymer during biodegradation in soils.

The degradation process in the soil takes place in two steps: First, the PBAT must be broken down (depolymerized) into its indi-

vidual building blocks by microbial enzymes. Then the small building blocks can be taken up and utilized by the soil microorganisms. To detect the incorporation of the ^{13}C-carbon into the biomass of soil microorganisms, the polymer samples from the soil experiments were analyzed using high spatial resolution secondary ion mass spectrometry (NanoSIMS). The measurements showed that both fungi and unicellular microorganisms (i.e. most likely bacteria) were involved in the degradation of the polymer. And most importantly, that all three components of the polymer were used by microorganisms (Zumstein et al., 2018).



11.2 Detection of Certain Microorganisms

In many cases, it is not so much a question of how many microorganisms occur in a community or how active they are, but rather of whether and in what numbers certain microorganisms occur. The bacteria to be detected may be specific taxa (e.g. certain species) or physiological groups independent of the taxon (e.g. sulfate reducers or nitrifiers). Classical methods for the detection of microorganisms are largely based on more or less **selective cultivation procedures.** In contrast to the least selective conditions mentioned in ► Sect. 11.1.1, in this case an attempt is made to allow predominantly or exclusively the microorganisms sought to develop by selecting the type and concentration of electron donor and electron acceptor, by incubation conditions (including temperature, pH), nitrogen source, inhibitors and others. Depending on the problem, isolated microorganisms may then have to be identified.

Quantification of microorganisms with certain physiological properties is often possible by a **live count determination on** a selective agar, by plating aliquots from a dilution series of the sample on an appropriate selective culture medium (compare ◘ Fig. 11.4). As noted above, some properties cannot be adequately tested on solid culture media, and estimation of abundances must be based on culturing or biochemical reaction in liquid culture. Since no enumeration of nascent colonies is possible here, one determines from which dilution level of the sample cells still growing or otherwise reacting in liquid medium are obtained. If only one liquid medium were inoculated from each dilution level, only the order of magnitude of cells contained in the original sample and capable of dividing or reacting in the liquid medium would be obtained, and this indication would have a considerable uncertainty because a different order of magnitude would be obtained depending on whether or not a cell was still accidentally captured during overinoculation from a high dilution level. To reduce this statistical uncertainty, the ***most probable number method*** (MPN method) overinoculates several times in parallel from the dilution levels of the sample into the liquid cultures. (◘ Fig. 11.12). The three highest dilution levels that still lead to growth or reaction are used to judge in how many of the liquid cultures prepared in parallel this occurs. Tables can then be used to determine probable numbers of dividing or

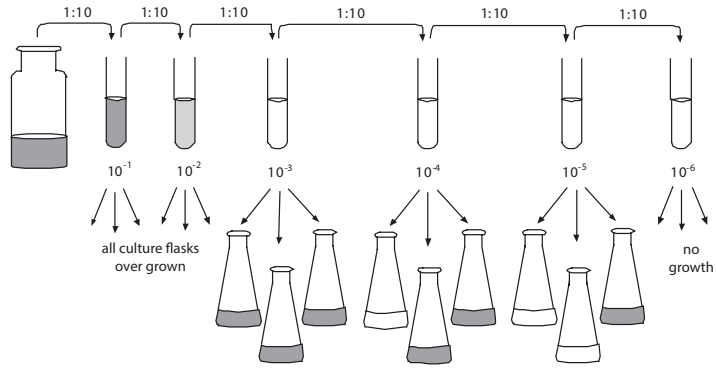


Fig. 11.12 Estimation of the abundance of bacteria with desired characteristics in liquid cultures using the MPN method

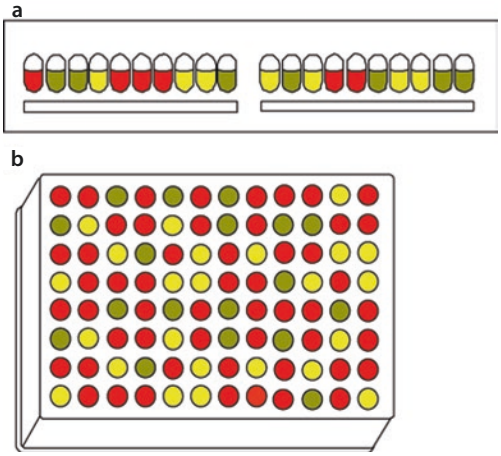
reactive microorganisms in the original sample. The accuracy of the indication depends on the number of parallel preparations analysed per dilution step.

A good example is the **detection of *Escherichia coli*** in drinking water. In addition to other microbiological parameters, the Drinking Water Ordinance specifies that no divisible cell of *E. coli,* which is used as an indicator bacterium for faecal contamination, may be present in 100 ml of water. This is tested according to DIN EN ISO 9808-1 by first filtering the sample through a membrane with a mean pore diameter of 0.45 µm and then incubating the membrane on an agar containing lactose and complete medium components as well as 2,3,5-triphenyltetrazolium chloride (TTC), sodium heptadecyl sulfate and bromothymol blue. Here, it is utilized that coliform bacteria such as *E. coli* can usually cleave lactose via β-galactosidase and degrade it with acid formation, resulting in the yellow coloration of the pH indicator. Sodium heptadecyl sulfate and TTC inhibit many Gram-positive bacteria. TTC is also reduced to a red dye in lactose-negative cells (◘ Fig. 11.8), whereas lactose-positive coliform bacteria appear yellow-orange due to weak TTC reduction. For more precise differentiation, it is then further checked that the bacteria of the yellow-orange colonies such as *E. coli* are negative in the oxidase test and positive in the indole test. The oxidase test checks for the presence of cytochrome oxidase by oxidizing tetramethyl-p-phenylenediamine to a blue dye. In the indole test, the tryptophan-cleaving enzyme tryptophanase is detected by the resulting indole reacting with *p*-dimethylaminobenzaldehyde under acidic conditions to form a red dye. Overall, *E. coli* detection is a multi-step and therefore relatively laborious procedure that utilizes several metabolic and resistance properties and takes two to three days in the standard procedure.

For example, if one is interested in the **ability** of the microorganisms of a soil or an activated sludge to **degrade organic pollutants**, one can attempt to demonstrate the presence of the pollutant-utilizing microorganisms by providing a mineral medium with such a compound as the sole source of carbon and energy. Growth in appropriate liquid cultures or colony formation on appropriate solid culture media indicate the presence of the pollutant-utilizing microorganism. When plating directly on solid culture media (i.e. without prior enrichment in liquid medium) or using the MPN method, the abundance of the microorganisms with the respective degradation property in the environmental sample can also be estimated.

A taxonomic analysis of microbial communities in environmental samples is a practically unsolvable problem with classical methods (even if only the cultivable bacteria are considered), because such a taxonomic analysis requires both an identification and a quantification of the microorganisms pres-

ent. However, an **identification of microorganisms,** i.e. their assignment to described species or other taxa, is usually not possible when using classical methods due to the small morphological differences with single cells. An exception is the identification of microorganisms using **immunological methods,** i.e. specific and labelled antibodies, which are usually only available for individual species and not for the majority of organisms in a community. Traditionally, therefore, microorganisms can usually only be identified when they are available as **pure cultures.** As demonstrated by the example of *E. coli*, adequate classification can then usually be achieved by **staining techniques** (e.g. Gram stain) and, above all, various tests for **metabolic properties** (utilization of carbon sources, relationship to oxygen, formation of metabolic products, detectable enzyme activities) (◘ Fig. 11.13). Today, **chemotaxonomic features** are also of great importance for taxonomy and thus for the identification of strains, e.g. the occurrence of characteristic fatty acids in the membrane lipids or the type of quinones in the respiratory chains. Such features can also only be studied using sufficient amounts of biomass from a pure culture.



◘ **Fig. 11.13** Bacterial identification by simultaneous testing of several metabolic properties in **a** "colored series" or **b** tests for substrate oxidation in microtitre plates in the presence of a redox dye

However, obtaining pure cultures for identification is at odds with the goal of quantifying species in the environmental sample, because isolation is necessarily selective to some extent. It cannot approximate all microorganisms present and is hardly feasible with hundreds or thousands of different bacteria in an environmental sample.

Although a taxonomic analysis of **communities** at the species level is usually not possible with non-genetic methods, an analysis at the level of higher taxonomic units can, however, be carried out partly on the basis of the aforementioned as well as other chemotaxonomic traits that are considered **biomarkers.** For example, **ergosterol,** which is abundant in fungi, can serve as a measure of fungal biomass in a community. **Muramic acid**, found in the cell walls of virtually all bacteria, is sometimes used as a marker of bacterial biomass, but it must be remembered that cell wall residues also occur in dead organic material, especially humic matter, and can greatly distort measurements. More accurate conclusions about the distribution of microbial taxa in a community can be drawn by analysing the **fatty acid pattern** of membrane lipids extractable from the community. However, the fact that a number of different fatty acids already occur in each individual taxon (and depending on the physiological state) tends to lead to very complex patterns with limited informative value in entire communities.

11.3 Microorganisms, from Nature to the Laboratory, the Isolation of Pure Cultures

The majority of information in microbiology was obtained with pure cultures. Isolated microorganisms are therefore of great importance for our knowledge.

11.3.1 Organisms That Cannot Be Cultivated?

As early as 1918, it was found that the available methods of cultivating bacteria on artificial media resulted in colony formation in only 1.5–10% of the bacterial cells present in the soil (Conn, 1918).

Even today, only a small proportion of naturally occurring prokaryotic microorganisms have been cultivated in the laboratory. It is still estimated that this proportion is less than 1% (Amann et al., 1995; Schloss & Handelsman, 2005).

The question arises: Is the majority of organisms not cultivable or can we not?

There are many reasons for the poor success in capturing microorganisms found in nature. In approaching the problem, we will first discuss what an organism needs in order to be cultured and at what point possible difficulties in isolation/enrichment from environmental media are to be expected.

The demands of various microorganisms on the composition of nutrient solutions and the milieu conditions are diverse. They depend on the nature of its energy metabolism and its biosynthetic services. Microbial growth is therefore influenced by

- the carbon sources,
- the electron donors and acceptors,
- the nitrogen sources,
- the sources of other major elements such as sulfur, phosphorus, magnesium and calcium,
- the need for trace elements,
- the need for vitamins and possibly growth factors,
- and some physico-chemical factors such as temperature, pH and the need for oxygen and salinity.

The natural site and the laboratory are two very different worlds. In the laboratory, it is often not possible to sufficiently simulate the abiotic and biotic conditions that exist at the bacteria's natural sites, so that the isolation of microorganisms has so far been unsuccessful.

- Enrichment cultures are often incubated as still cultures, with very high concentrations initially and low concentrations later.
- Aerobes are incubated on the shaker **in suspended form.** The partial pressure of O_2 may be very high. At natural sites, however, many bacteria form colonies or occur in flocs, mats or biofilms. Bacteria are also frequently present adsorbed on surfaces.
- At most natural sites, nutrients are only present in relatively low concentrations, usually much lower than those used in laboratory media. Very many microorganisms are particularly adapted to these low concentrations.
- In contrast to most laboratory media, there is rarely only one carbon source in nature.
- In nature, there are never pure but more or less complex mixed cultures. There are many different interactions between them, including symbiotic ones, which can also be obligate. In particular, some strict anaerobes are obligate to cooperate with partner organisms that consume, for example, H_2 and therefore cannot easily be grown in pure culture.

11.3.2 Isolation and Problems

Some species have such specific requirements that it requires very good microbiological knowledge, especially of metabolic physiology, to obtain them in pure culture. Extensive experience in microbiological working techniques, sure instinct and a lot of patience are also necessary. Basically, "You have to know what to look for." Thus, if one wants to enrich a very specific microorganism species from a mixed population, this requires precise knowledge of its nutrient requirements and optimal incubation conditions. Often, crucial information is not known.

Some microorganisms do not grow on plates that use agar as a solidifying agent. For acidophilic iron oxidizers, organic acids from agar and agarose are apparently toxic. Gellan (a polysaccharide from *Pseudomonas elodea*) or other solidifiers such as gelrite and silica gel can then often be used as an alternative agent.

It must also be taken into account that some microorganisms grow very slowly, which means that several weeks or even months may pass before visible growth is observed on solid media or in liquid culture.

However, although very important, reasons for the composition of a medium and the use of specific culture conditions are rarely addressed in the literature.

Methanomicrobium mobile, for example, could not be cultivated until the need for a new growth factor had been discovered. *Desulfomonile tiedjei* could only be captured as a pure culture when the biochemistry of dehalogenation resulting from the use of 3-chlorobenzoate as an electron acceptor was clarified. The cultivation of these specific sulfate-reducing bacteria from environmental samples was thus possible.

Another example is *Pelagibacter* ubique SAR11, which accounts for up to 50% of the total surface water microbial community in the Sargasso Sea. The oligotrophic organism relies on reduced sulfur compounds provided by other organisms in the habitat.

Many problems in the growth of microorganisms arise due to incorrect or uncontrolled incubation temperature or pH of the culture. Further, as addressed above, the lack of necessary nutrients, the less than ideal way of adding carbon or energy sources, and even the excess of some media components will result in poor or no growth or overgrowth by faster organisms in an enrichment culture.

Some compounds may even be toxic or insoluble at the concentrations commonly added to media. This is known from substrates such as formaldehyde, hydrocarbons and so-called xenobiotics.

For some toxic substrates, it is necessary to add them at low, non-toxic concentrations, track consumption, and resupply the culture with the toxic substrates when they are depleted.

11.3.3 Enrichment System

After the potential problems in isolating microorganisms have been pointed out, the enrichment technique developed by **Winogradsky** and **Beijerinck,** which is important for microbiology, will be presented. This technique allows the isolation of prokaryotes with specific metabolic capabilities by means of the selective properties of selected nutrient solutions. One establishes the appropriate environmental conditions in a nutrient solution by fixing a number of factors (energy, carbon and nitrogen source, electron acceptor, gaseous atmosphere, light, temperature, pH) and inoculating this nutrient solution with a mixture of organisms, such as those present in a soil, mud or water sample. In this way, an artificial ecological niche is created.

Due to the specifically tailored medium as well as the incubation conditions, individual organisms are preferentially induced to multiply over others, so that they eventually dominate in the liquid nutrient solution and can be easily isolated. Thus, in such an enrichment culture, the organism that can grow the fastest prevails. All other accompanying organisms are overgrown and escape isolation.

If the largest possible number of different strains is to be isolated under selective growth conditions, direct plating without prior enrichment in liquid culture is an option. If the inoculum is distributed on a solidified selective nutrient medium, the favoured metabolic types grow into colonies. With sufficiently wide colony spacing, they escape competition for nutrients; the slower-growing strains are not displaced by faster-growing ones and can thus be isolated separately.

The isolation of a pure culture can also be carried out in liquid nutrient media, but only if the desired organism far outweighs in number in the starting material. By **serial dilution** of a liquid enrichment culture transferred several times in the nutrient solution, it can finally be achieved that statistically only one cell is left in the last dilution stage, from which a pure culture then emerges.

To check the purity of a culture, one uses—in contrast to enrichment—the least selective, most complex nutrient medium possible to facilitate the growth of possible contaminants and thus make them easily visible.

Particularly selective enrichment conditions can be established for specialized microorganisms:

- A mineral medium lacking a nitrogen source selects for **nitrogen-fixing cyanobacteria** when exposed to light.
- Supplementing the nutrient solution with an organic energy and carbon source leads to the accumulation of the N_2-fixing aerobic soil bacterium ***Azotobacter*** **in** the dark under air.
- If this enrichment takes place without oxygen, the selection of **clostridia** then occurs.
- For the enrichment of fermenting organisms, the chemical character of the substrate is very important. For it to be degraded by fermentation, it must not be too oxidized or too reduced. Sugars are excellent fermentation substrates, but other classes of organic compounds with a similar oxidation state can also be fermented. The enrichment culture must be cultivated anaerobically only and in the absence of other good electron acceptors such as NO_3^- or Fe^{3+}, not only because some fermentative organisms are obligate anaerobes, but to avoid competition from aerobic forms or from denitrifiers or iron reducers.
- Nitrate should not be used as a source of nitrogen for the enrichment of fermentative organisms, as it allows the growth of denitrifying bacteria.

However, no more than the minimum needs of the metabolic type to be enriched may be met for enrichment to be successful:

- Oxygen must be excluded when isolating organisms that oxidize hydrogen with nitrate or sulfate as electron acceptors. Aerobic hydrogen oxidizers would overgrow the nitrate and sulfate oxygenators in the presence of oxygen.

For the enrichment of **chemoautotrophic** and **photoautotrophic** organisms, organic compounds must be avoided in the medium and CO_2 or bicarbonate must be added as the sole carbon source (◘ Fig. 11.14).

11.3.4 Analogue Enrichment: Sense or Nonsense?

Bacteria that can grow with xenobiotics such as chloraromatics are considered rare in nature. Therefore, one specifically increases the cell population by using a substrate analogue as the first enrichment substrate: Thus, if one wishes to isolate chlorobenzene utilizers, one can first begin by enriching a benzene culture, which is then slowly switched to chlorobenzene. After knowledge of the metabolism and the bottlenecks found, it can be said today that this procedure is rather bad, since such benzene utilizers accumulate the toxic chlorocatechols when chlorobenzene is administered, which runs counter to an enrichment. Thus, one is more likely to isolate organisms that can survive such stress. **Careful selection of chlorobenzene recyclers is the right way, which also allows a variety of organisms to be isolated** (reference to box "Substrate administration with lipophilic, liquid substrates").

11.3.5 Inoculum for Enrichment Culture

Suitable material for the enrichment of many microorganisms provide soil, water and sludge. Enrichment culture allows the enrich-

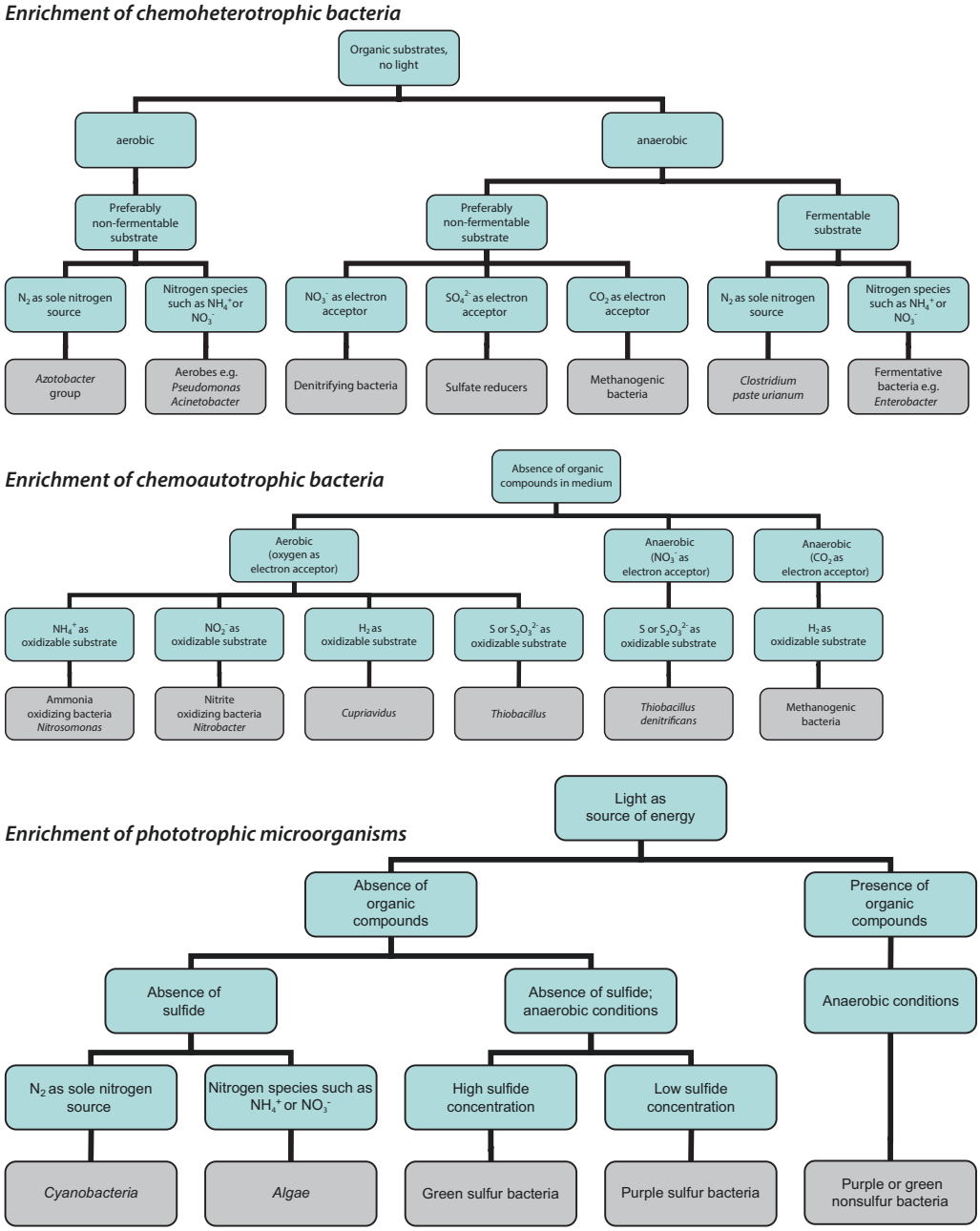


Fig. 11.14 Enrichments: Essential selective growth conditions are indicated in the flow diagrams for a selection of representative metabolic types. Fermentable substrates: generally glucose; for *Azotobacter:* alcohols, butyrate, benzoate; for denitrifiers: acetate, butyrate, ethanol; for sulfate reducers: lactate, malate Preferred non-fermentable substrates for aerobes: Lactate, benzoate, mannitol Preferred non-fermentable substrates for anaerobes: organic acids: acetate, butyrate; for methanogens: acetate, formate; Non-readily fermentable substrates for non-sulfur purple bacteria: Acetate, butyrate or malate

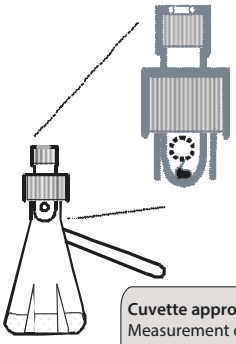
ment of microorganisms with a variety of combinations of nutrient requirements. The prerequisite is that the desired type occurs in nature at all and that the desired type of organism is present in the inoculum. This is not always self-evident despite the ubiquitous distribution of most microorganisms. Thus, microorganisms with certain performances will only be able to be isolated from certain habitats. One should therefore take the inoculum for an enrichment culture from a site that offers suitable growth conditions for the desired organism and where natural enrichment has already occurred:

- Carbon monoxide utilizing microorganisms from gas plant effluents,
- Hydrocarbon oxidizers from oil fields,
- Cellulose-utilizing fermenters from cowpea.

- "Standard laboratory conditions" are not suitable for insoluble and volatile substrates: Examples showing what to look for in the context of enrichment when adding substrates.
- There are some very good reasons why physico-chemical information of compounds is very necessary when enriching organisms. The compounds should be used **properly** in enrichment media. For example, it is often good practice to add water-insoluble solids (such as biphenyl) at concentrations far above their solubility in enrichment media. The compound will continuously dissolve as far as it is degraded in the liquid phase and thus rarely be toxic to bacteria.
- In contrast, compounds that are liquids at room temperature and insoluble in water (such as benzene, toluene) must be added carefully to the media. This is based on the fact that the formation of two liquid phases in enrichment media has been shown to be very toxic to many bacteria by the liquid extracting the lipids of the cell wall.
- This problem can be easily overcome by applying volatile, water-insoluble compounds as a vapour, as a liquid in a vessel above the growth medium in the enrichment vessel (◘ Fig. 11.15). This approach provides the bacteria with a continuous supply of the substrate without creating a two-phase system that would kill them.
- In this context, bacteria growing with aromatics such as benzene and toluene were considered to be rare in nature because toluene had been added directly to the enrichment media. But this finding was an artifact resulting from toxicity.
- The introduction of the vapor bulb technique led to the isolation of many bacteria growing with toluene/benzene and related aromatics (◘ Table 11.1).

Adding benzene or toluene directly to the medium as a liquid **will not result in growth**

When benzene is placed in a **block of paraffin**, very slow diffusion allows growth: the **technique of David T. Gibson**



Cuvette approach: Measurement of turbidity with out opening the flask

The vapor bulb technique: Microliter amounts of the liquin substrate (such as chlorobenzene) are added to the top fitting with a syringe via a septum. Evaporation and dissolution from the gas phase leads to very low concentrations in the medium (usually close to zero). Constant resolving allows growth.

The technique of adding the substrate via the vapor phase can also have negative effects on growth if the dose is too high. Even for organisms containing chloro-benzene like, every new dose in the upper approach causes a short growth disturbance.

Even the **hole size in the top approach** can affect growth: vessels suitable for chlorobenzene cultivation will not allow organisms containing tetralin to grow. On the other hand, a much smaller hole size in the approach allows growth with tetralin. Chlorobenzene and tetralin differ significantly in terms of log octanol-water coefficients. Tetralin is more membrane-dissolving, so its concentration in the medium must be very low.

Enrichment without direct contact: The medium with the inoculum without substrate is in the sealed Greiner tube (with polypropylene wall) in a Wheaton tube on a shaker. The liquid substrate vaporizes and diffuses through the polypropylene wall, providing the organisms with substrate whose concentration in the growth medium is always close to zero.
(Everything goes very slowly!!!!!)
Wheaton tube 500 mL, Greiner tube 50 mL, mineral medium 10–15 mL plus inoculum, liquid substrate 20 microliter.

Fig. 11.15 Growth success as a function of the type of addition of a substrate (example: benzene/toluene/chlorobenzene/tetralin)

Table 11.1 Physico-chemical data on three aromatic compounds investigated as growth substrates

	Benzene 	**Chlorobenzene** 	**Tetralin** 
Brief description	Colorless liquid with characteristic odour	Colorless liquid with a benzene-like odour	Colorless liquid with a naphthalene-like odour
State of aggregation	Liquid	Liquid	Liquid
Density (g/cm^3)	0.88	1.11	0.97
Melting point (°C)	5.5	−45.2	−35.8
Boiling point (°C)	80.1	132	208
Vapour pressure (hPa at 20 °C)	100	11.7	0.4
Solubility in water (mg/L or mM)	Bad: 1770 or 22.7	Very bad: 490 or 4.35	Very bad: 45 or 0.34
Log octanol-water coefficient	2.13	2.84	3.49
Henry's constant (atm m^3/mol)	$5.53 \cdot 10^{-3}$	$3.11 \cdot 10^{-3}$	$1.36 \cdot 10^{-3}$

Test Your Knowledge

- Name summary methods for characterizing microbial communities.
- What are fluorescent dyes used for?
- Compare a count by colony forming units with the use of a counting chamber.
- What is the MPN method?
- What can be used to determine the O_2 concentration?
- Describe the activity determination of an esterase.
- Why is fluorescein only green after the addition of NaOH? Draw the relevant structures.
- What does the firefly have to do with ATP quantification?
- In bacterial identification, the "colored series" is used. Please explain why "colored"?
- What is the basis for detecting microbial activity using isotope fractionation?

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Microbial Communities: Structural and Functional Analyses with Molecular Biological Approach

Contents

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The **classical approach** relies on enrichment techniques. The exploration of the abiotic and biotic factors of a habitat in conjunction with the flow of nutrients present there will allow conclusions to be drawn as to which metabolic types are to be expected in the respective habitat. With the general conditions maintained, it should then be possible to selectively enrich the microorganisms to be required in the habitat. Newer strategies, which will be discussed subsequently, dispense with the enrichment of the bacteria themselves.

It is now known that the vast majority of microorganisms occurring in nature are not readily cultivable. Figures for the **proportion of microorganisms that can be cultivated** vary for the different sites (◘ Table 12.1). Studies that depend on cultivation will generally only cover a small proportion of the microorganisms present and will therefore, at least if used alone, only provide statements with limited validity for describing the environment.

It is also now known that not only the number but also the **diversity of** microorganisms at natural sites is much greater than is reflected in cultivation-dependent methods. Thus, most of the normally used bacteria belong to a relatively limited number of phyla, while cultivated representatives of several putative phyla are not yet known.

Molecular genetic techniques are more informative even for the identification and classification of pure cultures than the traditional methods mentioned in ► Sect. 11.1.2 (determination of morphology, staining behaviour, metabolic properties and others). These are often sufficient for assigning individual strains to known species, and they also allowed a certain **classification of** the known microorganisms. However, it has also long been recognized that the classification of microorganisms based on traditional classification methods does not represent a **phylogenetic system**, i.e., a classification that reflects evolution, where the individual taxonomic groups thus correspond to units of descent in nature. In the meantime, identification and classification are largely based on sequence analyses of ribosomal RNA (rRNA) or the rDNA encoding it, as well as of genes of universally distributed enzymes, since this leads to phylogenetically more correct taxonomic groupings than traditional methods and since even very distant relationships can be analysed. The principles behind the most important molecular genetic methods for the identification and classification of isolated bacteria and for the characterization of microbial communities will therefore be briefly presented below.

◘ **Table 12.1** Proportions of culturable bacteria in different habitats

Habitat	Cultivability (%)
Seawater	0.001–0.1
Freshwater	0.25
Mesotrophic lake	0.1–1
Unpolluted water from estuaries	0.1–3
Activated sludge	1–15
Sediments	0.25
Floor	0.3

The numbers were compiled from Amann et al. (1995). Culturability was determined as culturable bacteria (colony forming units) related to the total cell number

12.1 Basic Molecular Genetic Methods for Classification and Identification of Pure Cultures

The basic assumption in the identification and classification of organisms on the basis of the sequences of macromolecules is that corresponding molecules of different organisms are homologous, i.e. that they have

emerged in evolution from an original molecule in the common ancestor of the organisms studied. Over time, sequence differences accumulated as mutations occurred. The degree of sequence similarity thus shows how closely related the respective organisms are to each other.

In the 1980s, the analysis of ribosomal RNA, and in particular of 16S rRNA and its coding genes, became the most important method for the classification and identification of microorganisms. In the meantime, there are hundreds of thousands of entries for 16S rRNA in the database of the Ribosomal Database Project. Sequence analyses have revolutionized the taxonomy of microorganisms and had a lasting impact on our ideas about the evolution of living organisms.

If you have a newly isolated bacterial strain and would like to identify it by means of 16S rRNA analysis, you must first isolate the corresponding nucleic acid in sufficient quantity. Whereas in the past the ribosomal RNA itself was usually isolated and analysed, today the gene, the 16S rDNA, is usually amplified using the *polymerase chain reaction* **(PCR)**. In this process, a specific piece of the DNA of the strain under investigation is repeatedly duplicated by copying using DNA building blocks (deoxynucleoside triphosphates) and a heat-stable DNA polymerase in approximately 20–30 cycles and is thus amplified exponentially. Which piece is copied depends on two so-called primers. These are oligonucleotides about 20–25 nucleotides long whose base sequence has been selected so that they each bind to a different strand at the front and back of the region to be duplicated, for example the gene of the 16S rRNA (◘ Fig. 12.1). Attempts are made to choose conditions (in terms of temperature, for example) stringent enough to prevent binding to other regions of the genome. Since each nucleic acid strand has a fixed direction and can only ever be extended at its 3′-end, the deoxynucleotides are added to the 3′-end of the primers by DNA polymerase. Cycling is accomplished by repeatedly heating, cooling, and incubating the reaction tubes at medium temperature. Heating to about 95 °C leads to denaturation of the DNA strands, i.e. separation of the two strands of the double helix. Cooling to (depending on the primer) approx. 50–60 °C leads to the reformation of hydrogen bonds between complementary strands, whereby the primers predominantly bind to their complementary regions on the matrix DNA. Heating to approx. 70 °C increases the activity of the DNA polymerases used for PCR and leads to elongation, i.e. the attachment of nucleotides to the primers.

While the nucleotides, the DNA polymerase and the buffer are often the same for different PCR reactions and are also marketed as ready-made mixtures, the primers are decisive for which piece of the matrix DNA is amplified. Thus, **primer design** is of great importance. In the case of 16S rDNA, for example, extremely conserved regions of these genes have been used to design so-called "universal" primers that bind to the same site on the rDNA or rRNA in almost all organisms. Other primers are specific to smaller or larger taxonomic groups. If one chooses a forward primer for the beginning of the 16S rRNA gene and a reverse primer for the posterior part of this gene, one can amplify almost the complete 16S rDNA (about 1500 base pairs). To control the reaction, an agarose electrophoresis can be used to check the size of the PCR product.

In many cases, a PCR product can be directly subjected to sequence analysis. However, **cloning** into a vector often takes place beforehand. For this purpose, the corresponding plasmid vector is cut with a restriction enzyme and the PCR product is linked to the vector DNA by a DNA ligase. Subsequently, the recombinant plasmid is usually transformed into an *E. coli strain,* during the division of which it is easily amplified and from which it can be easily isolated.

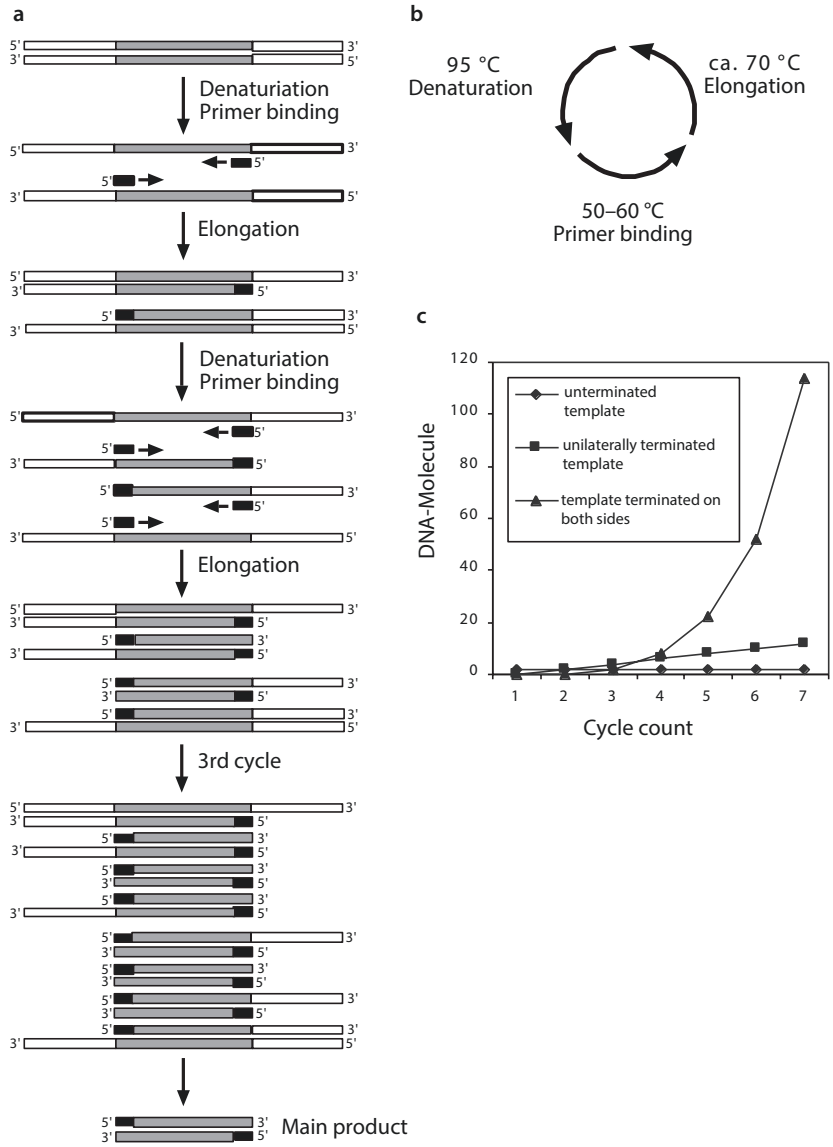


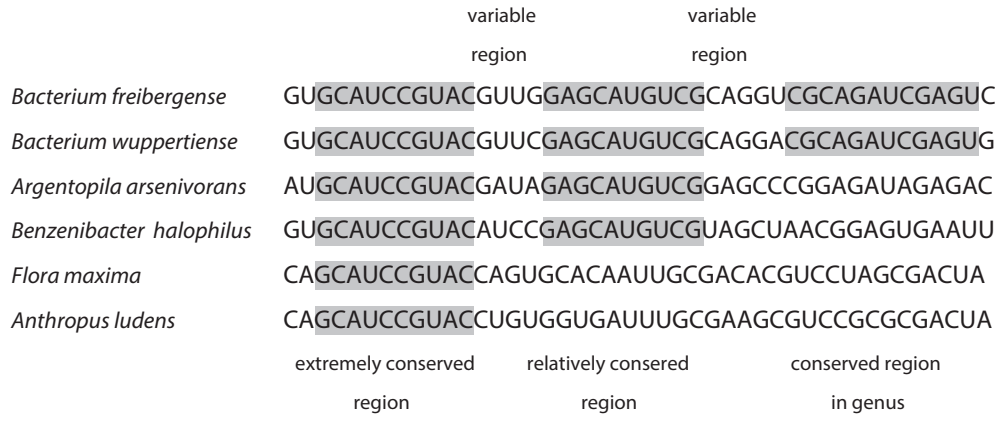
Fig. 12.1 Polymerase chain reaction (PCR). **a** Illustration of the amplification of a piece of DNA. Note that although the nucleotides copied directly from the template DNA do not have a precisely determined length, the vast majority of molecules in the PCR product ultimately do have a length that is precisely determined by the binding sites of the two primers. **b** Temperature cycle. **c** Growth curves for the two different PCR products

The availability of a 16S rRNA sequence alone says nothing about the taxonomic assignment of an assumed, newly isolated strain. Rather, this only results from comparison with the sequences of identified strains already available in the databases. It should be noted that databases are virtually flooded with sequences derived directly from the environment rather than from a characterized organism. In a first step, one can perform an ***on line database comparison with*** the new sequence and obtain a listing of the

most similar database entries together with a representation of the similarities found in the form of a pairwise sequence alignment in which the two sequences are placed next to each other in such a way that corresponding positions are on top of each other. In many cases, one then already knows quite well which genus the new isolate might be, for example, especially if one searches for sequences of so-called type strains of the species in the list obtained.

However, more meaningful analyses result from the classification of the sequence in a dendrogram, a family tree, of known sequences. In order to be able to create this, as well as to be able to compare several sequences with each other in a meaningful way, a **multiple sequence alignment** must first be created (by a computer). In this case, several sequences, selected for example by the database comparison mentioned above, are placed next to each other in such a way that identical or corresponding positions are superimposed (◘ Fig. 12.2). Where length differences in homologous molecules have occurred in the course of evolution due to insertions or deletions, the programs must insert gaps in one or the other sequence. Computationally, the alignment optimizes the number of matching positions in different sequences, taking into account, depending on the program, penalty points for introducing or lengthening gaps or for non-matching positions. From the alignments it becomes clear which regions are the same, i.e. strongly conserved, even in distantly related organisms, which regions are less conserved, for example within a phylum, and which regions are even more variable (◘ Fig. 12.2).

There are various principles and computational procedures for converting the sequence comparison in the alignment into a **dendrogram,** i.e. a phylogenetic tree. In many cases, the pairwise differences visible in the alignment are calculated in relation to the length of the compared region (◘ Fig. 12.3a), multiplied by a factor to account for multiple mutations, and then entered into a distance matrix (◘ Fig. 12.3b). In such a distance matrix, the pairwise similarities or differences between two sequences, for example the 16S rRNA sequences of two organisms, are each reduced to a number, the evolutionary distance. The distance values are then computationally converted into the branching pattern of a dendrogram, where the sum of the branch lengths between two sequences in the dendrogram corresponds to their evolutionary distance (◘ Fig. 12.3c, d).



◘ **Fig. 12.2** Multiple sequence alignment of RNA of hypothetical organisms from different domains of the organisms. Highly conserved, less conserved, and more variable regions are indicated

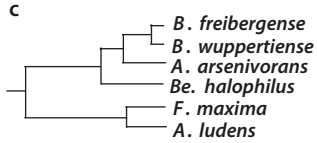
a

B.f.	0	3	9	12	23	21
B.w.	0.07	0	10	12	23	21
A.a.	0.20	0.23	0	16	23	24
Be.h.	0.27	0.27	0.36	0	23	22
F.m.	0.52	0.52	0.52	0.52	0	9
A.l.	0.48	0.48	0.55	0.50	0.20	0
	B.f.	*B.w.*	*A.a.*	*Be.h.*	*F.m.*	*A.l.*

b

B.w.	0.07				
A.a.	0.24	0.27			
Be.h.	0.34	0.34	0.50		
F.m.	0.90	0.90	0.90	0.90	
A.l.	0.76	0.76	0.97	0.82	0.24
	B.f.	*B.w.*	*A.a.*	*Be.h.*	*F.m.*

c



d

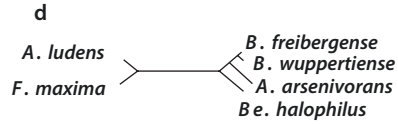


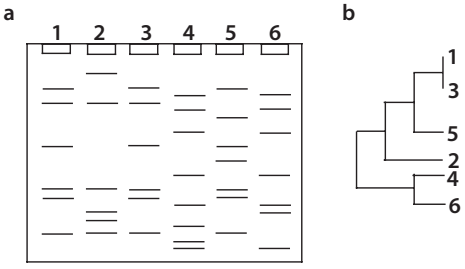
Fig. 12.3 **a** Matrix with observed differences between the sequences of the alignment in Fig. 12.2 (top right) and with the proportions of the differences in the total length of 44 positions (bottom left). **b** Matrix with evolutionary distances *D* calculated from the frequency f of the differences in a) taking into account multiple mutations at the same position, but without further weighting ($D = -\frac{3}{4} \ln(1 - \frac{4}{3} f)$). **c** Possible representation of the sequence similarities as a dendrogram with root assuming constant mutation rates. **d** Possible representation of the sequence similarities as a dendrogram without root

Just the more reliable computational methods lead to a dendrogram that shows only the branching patterns and distances between the compared sequences, without specifying where in the dendrogram to place the common root (Fig. 12.3d). (This would be equivalent, so to speak, to a "family tree" with "crown" only, where it is not clear where the stem attaches). Since the location of the root is nevertheless of interest, sequences are included in the analysis, so-called outgroups, which are known to be homologous to the sequences under consideration, but which have only relatively low similarity and lie outside the group of related sequences just considered.

The identification of the assumed newly isolated strain results essentially from the placement of its rRNA sequence in the dendrogram and, in particular, from the taxonomic affiliation of the strains that appear in the dendrogram as the closest relatives. The official taxonomic method for defining bacterial species, the pairwise hybridization of DNA from different strains, is much more laborious and is rarely used in routine strain identification studies.

If many, possibly also very closely related strains are to be compared with each other, the complete sequence analysis of all these strains with Sanger sequencing also becomes time-consuming and expensive. In such cases, fingerprinting techniques can be used to clarify in a first step which sequences are presumably very similar, i.e. of which only a few may need to be treated as examples, and which require more detailed analysis. A frequently used fingerprint method is **ARDRA** (*amplified ribosomal DNA restriction analysis*). The 16S-rDNA of the strains is also amplified in this case. However, the PCR products are then not all subjected to sequence analysis, but are cut with so-called restriction enzymes. Restriction enzymes recognise a specific sequence pattern and the vast majority cut the DNA double strand in the region of the recognition sequence. For ARDRA, restriction enzymes are relevant whose recognition sequence (usually 4 base pairs long) occurs statistically within a few hundred bases of each other on the DNA. In the case of two very similar sequences (from two closely related strains), the restriction enzyme sites on the 16S rRNA PCR product

will also generally be equally distributed and will therefore result in a similar banding pattern on electrophoresis (■ Fig. 12.4). If the sequences are very different, the interfaces will also be distributed differently and will accordingly give a different banding pattern. Similarity of fingerprint patterns can also be represented in terms of dendrograms. An analogous approach to ARDRA is used in the analysis of communities in the form of T-RFLP analysis.



■ **Fig. 12.4** Comparison of six bacterial strains by ARDRA. **a** Banding pattern on an electrophoresis gel after cutting amplified ribosomal DNA with a restriction enzyme. **b** Dendrogram illustrating similarities of the banding pattern of the six strains

Background: Ribosomal RNA and DNA

Ribosomes are the complexes responsible for protein synthesis in the cells of all living organisms. The so-called 80S ribosomes of eukaryotic organisms are slightly larger than the 70S ribosomes of prokaryotes (*Eubacteria* and *Archaea*). All ribosomes each have a large and a small subunit and consist of different proteins and ribosomal ribonucleic acids (rRNAs).

Bacteria have two rRNAs, the 5S rRNA and the 23S rRNA, in the large subunit of ribosomes and the 16S rRNA in the small subunit. The latter is normally involved in the recognition of the starting point of a gene on the mRNA and is used particularly frequently for taxonomic and ecological studies (■ Table 12.2).

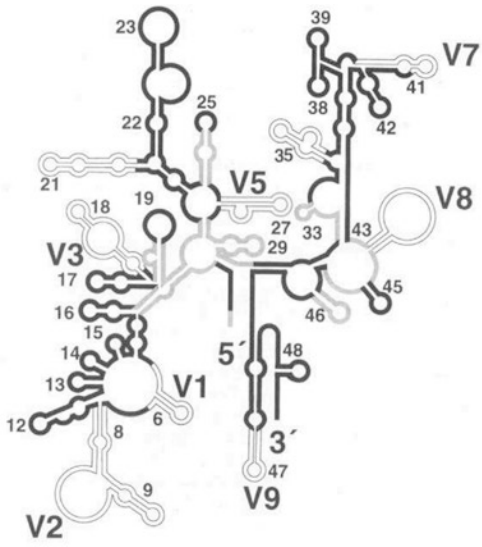
The importance of rRNAs for taxonomy is based in part on the fact that these molecules are universally distributed and perform the same function in all organisms. Importantly, rRNAs show a pattern of extremely conserved, less conserved and more variable regions. The former

■ **Table 12.2** Structure of ribosomes and their components compared in prokaryotes and eukaryotes

	Bacterium	Eukaryote (cytoplasm)
Ribosome size	70S	80S
Size of the large subunit	50S	60S
Number of proteins	36	≈ 49
rRNAs	5S (≈ 120 nucleotides)	5S (≈ 120 nucleotides)
	23S (≈ 3200 nucleotides)	28S (≈ 4700 nucleotides)
		5.8S (≈ 160 nucleotides)
Size of the small subunit	30S	40S
Number of proteins	21	≈ 33
rRNAs	16S (≈ 1540 nucleotide)	18S (≈ 1900 nucleotide)

After Nelson and Cox (2005)

provide the reference points when comparing sequences, which regions of molecules that are always slightly different in length correspond to each other. The variable regions, on the other hand, provide the differences for differentiation, for example between different species or genera. Not unimportant is also the aspect that the 16S- and especially the 23S-rRNA have a sufficient length so that many mutation events independent of each other can be analyzed. Finally, it should be mentioned that the genes of the rRNAs (the rDNA) are generally not subject to horizontal gene transfer into other microorganisms (◘ Fig. 12.5).



◘ **Fig. 12.5** Two-dimensional representation of 16S rRNA showing conserved sequence regions in black, less conserved regions in gray, and variable regions with double lines. (Modified figure by E. C. Böttger with permission of the author)

House-Keeping Genes

Alternative oligonucleotide probes with species-level resolution include several *house-keeping genes*. However, specific sequence databases contain considerably fewer entries for these alternative markers than the 16S rRNA databases (◘ Table 12.3).

Sanger Sequencing

The forms of **DNA sequence analysis** commonly used today extend DNA primers by attaching deoxynucleotides through a DNA polymerase. In this respect, a **sequencing reaction is** reminiscent of a PCR reaction. Classical sequencing according to Sanger assumes that DNA has been amplified in advance by PCR or by cloning and DNA isolation.

Since only one primer is used in sequencing, only one DNA strand is a template, in contrast to PCR, and only a linear, not an exponential, amplification occurs. Crucial for sequencing according to Sanger is that so-called didesoxynucleotides are added to the sequencing reactions. These lack an OH group at the 3′-position of the sugar building block (◘ Fig. 12.6). If such a didesoxynucleotide is incorporated into a growing oligonucleotide in the course of the sequencing reaction, chain termination occurs at this position because no 3′-OH group is available in the following cycle to which the next building block could be attached.

For each sequencing, **four reaction mixtures of** DNA to be sequenced, DNA polymerase and all four deoxynucleotides are incubated, whereby one of the four

Table 12.3 House-keeping genes

Gene	Protein
rpoB	DNA directed RNA polymerase beta chain
gyrA gyrB	Gyrase
recA	RecA protein family performing homologous recombination
tuf	Elongation factor Tu, EF TU
grossEL	Chaperone
atpD	F_1 F_0 ATPase, subunit D
ompA	Outer Membrane Protein
gapA	γ-Aminobutyrate permease
pgi	Phosphoglucose isomerase

House-keeping genes are genes that are generally always expressed and therefore involved in normal cell metabolism. They are essential for metabolic processes and substances

didesoxynucleotides is also added to each mixture. The dideoxynucleotides compete with the normal deoxynucleotides for incorporation into the growing chain, leading to chain termination with some probability. Thus, in each of the four reaction approaches, a mixture of oligonucleotides of different lengths is formed, with the oligonucleotides always terminating at a dideoxyadenine in one approach, dideoxycytosine in the second approach, dideoxyguanine in the third, and dideoxythymine in the fourth. In order to separate the mixtures of oligonucleotides of different lengths, **electrophoresis** is performed in which the oligonucleotides migrate at different rates through a polyacrylamide gel or capillary in an electric field. Short oligonucleotides travel faster than long ones. Because the newly synthesized oligonucleotides all start at the same site with the same primer, and because the separation conditions are chosen so that the incorporation of even one additional nucleotide slows the migration distance during electrophoresis, the base sequence of the DNA to be sequenced can be read from the comparison of the lengths of the oligonucleotides in the four approaches (◘ Fig. 12.7). In order to make the resulting oligonucleotides visible, they must be labelled. For this purpose, either primers provided with **fluorescent dye** or didesoxynucleotides carrying fluorescent dye are used today, so that the sequencing devices can then register the emission of the fluorescent dyes excited by a laser.

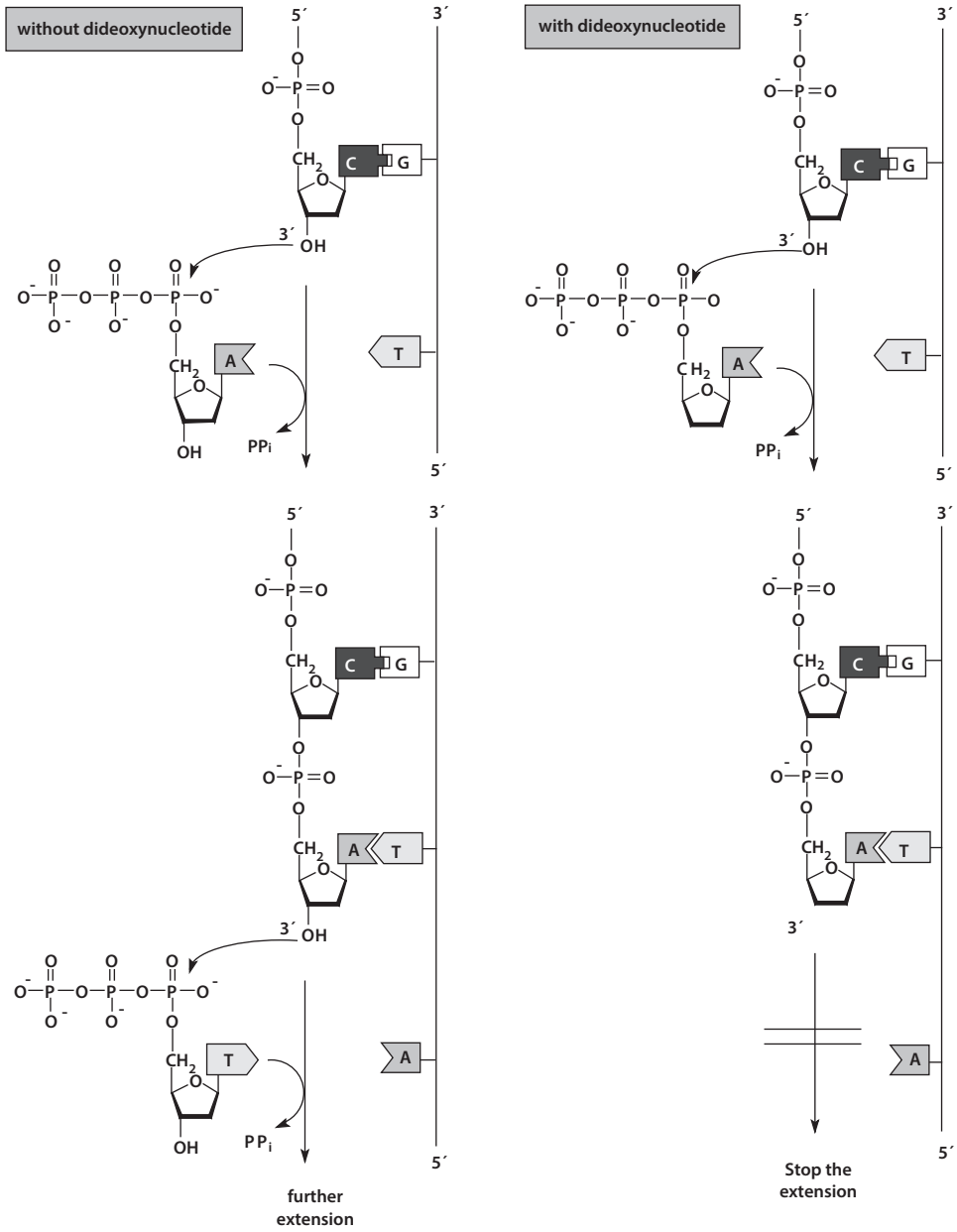


Fig. 12.6 Normal extension of a DNA strand by DNA polymerase and chain termination after insertion of a dideoxynucleotide

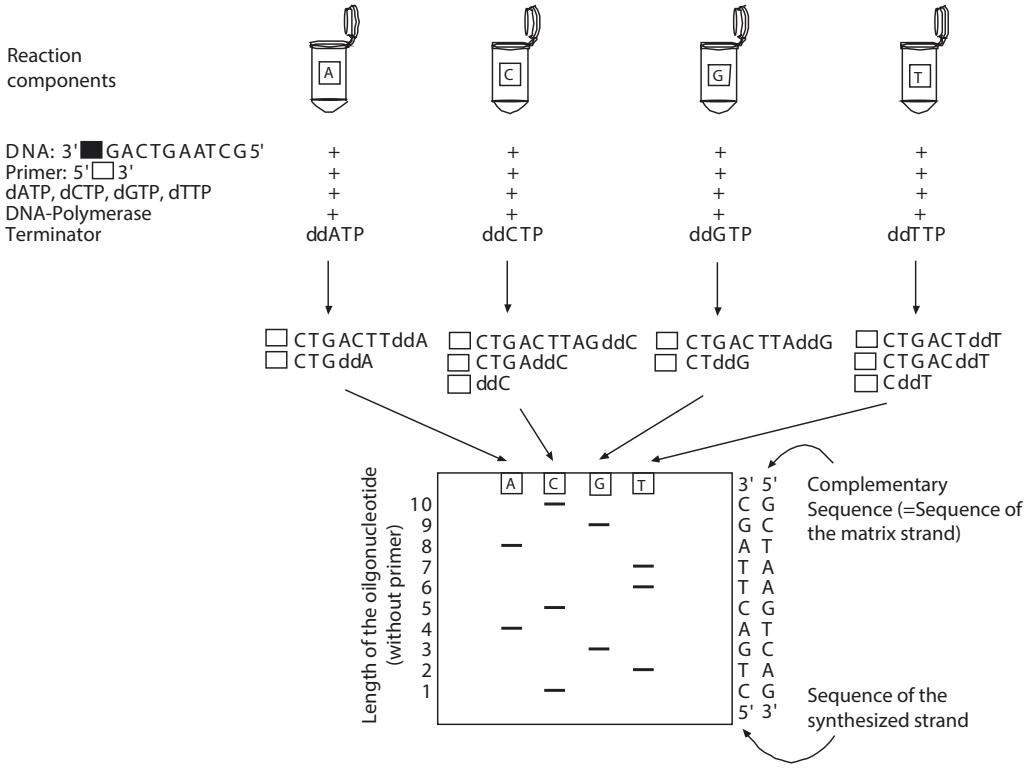


Fig. 12.7 Reactions for DNA sequencing according to Sanger in the presence of different dideoxynucleotides and gel electrophoresis to separate the resulting mixtures of oligonucleotides of different lengths

Pyrosequencing and Sequencing by Cyclic Reversible Termination

For decades, the actual determination of the DNA sequence was carried out almost exclusively by different variants of Sanger sequencing. For some years now, however, high-throughput sequencing techniques that are largely automated have increasingly come to the fore. They implement novel sequencing principles in which the respective PCR variant is closely coupled to the actual sequencing. Of the novel techniques, **pyrosequencing** and **sequencing by cyclic reversible termination** will be considered here. These and Sanger sequencing have in common that they (like PCR) start from an extension reaction catalyzed by a DNA polymerase. In all cases, a primer and the building blocks of DNA, the deoxynucleoside triphosphates (dNTPs), are also required.

Pyrosequencing was developed for high-throughput DNA sequencing in microtiter plates with very small wells. Since these can no longer be filled with PCR products by hand, pyrosequencing is very closely coupled to a special form of PCR known as emulsion PCR. In this process, DNA molecules that have been provided with two adapter sequences bind to beads that carry DNA complementary to one of the adapter sequences. This DNA acts as a primer through which the template DNA can be copied. Repeated amplification steps ensure that each bead is only occupied by one and the same PCR product.

In pyrosequencing, the actual sequence determination of the PCR products bound to the beads is not carried out after completion of the DNA extension reaction, but already during the reactions, i.e. after each individual extension step, by registering light signals. One after the other, the four deoxynucleoside triphosphates are offered to the DNA polymerase or the growing strain, respectively (strictly speaking, in the case of adenine, however, it is not dATP, but the sulfur derivative dATPαS, for reasons see below). Then, if the offered base fits, i.e. is complementary to the corresponding base in the template, the reaction occurs. This reaction releases pyrophosphate (PP_i) (◘ Fig. 12.8). In the presence of ATP sulfurylase, pyrophosphate reacts with adenosine phosphosulfate (APS) (see sulfate reduction, ► Sect. 8.1.1) to form sulfate and ATP. The ATP reacts with luciferin in the presence of luciferase and oxygen to form oxyluciferin, CO_2, AMP and PP_i , producing a light signal (see ◘ Fig. 11.7). The light signal is proportional to the ATP and thus to the PP_i released in the DNA elongation reaction, so that if several identical bases are present in succession, a larger light signal is also produced. To prevent the luciferase from already using the dATP offered to the DNA polymerase to produce a light signal, the aforementioned derivative dATPαS is used. After each reaction cycle, the apyrase must degrade the unincorporated nucleotides or unconverted ATP before the next reaction cycle can start with the next nucleotide.

Pyrosequencing achieves read lengths of a similar order of magnitude as Sanger sequencing (~ 700 bp per run). However, due to the high degree of automation, it is more cost-effective and thus also suitable for sequencing new genomes. Pyrosequencing is carried out in centres, be they sequencing centres of university research institutes or service companies, in order to utilise the capacity of the equipment.

In the case of **sequencing by means of cyclic reversible termination,** there is a decisive peculiarity already with regard to the PCR. This runs neither in solution nor as an emulsion of beads, but on the surface of a glass platelet, as a so-called bridge PCR. For this purpose, forward and reverse primers are applied in high densities to the glass surface. DNA molecules bound by complementary sequences bind to these primers. After bridging to the other primer, the latter can prime the extension reaction. PCR creates a patch of uniform DNA sequences around the first attachment point of the template fragment. For the actual sequencing reaction, in addition to DNA polymerase and dNTPs, dNTPs carrying a cleavable terminator (a 3′-O-azidomethyl group) at the 3′-position of the deoxyribose are used in this case. Thus, incorporation of these molecules leads to chain termination in a manner analogous to Sanger sequencing. However, the group can be cleaved off again before the next reaction cycle. In cyclic reversible termination sequencing, the four different nucleotides each carry a different fluorescent dye. After taking an image of the respective reaction cycle, the dye and terminator are cleaved off and a new reaction cycle is started.

Sequencing by cyclic reversible termination is once again significantly less expensive per sequenced base than pyrosequencing. However, the read lengths are significantly smaller (approx. 100 bp per run). Therefore, this form of sequencing is less suitable for the resequencing of genomes, but for transcriptome analyses of already sequenced genomes.

a Principle

Step 1: hybridization

single-stranded template DNA + primer $(dNMP)_n$

Step 2: DNA-Synthesis by DNA-polymerase

template DNA with attached primer $(dNMP)_n$ + a dNTP → extended primer $(dNMP)_{n+1}$ + PPi

Step 3: Detection of pyrophosphate

a) by ATP-sulfurylase: PP_i + adenosine-5´-phosphosulfate (APS) → **ATP** + Sulfate

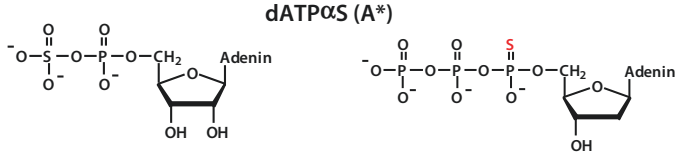
b) by luciferase: ATP + luciferin + O_2 → Oxyluciferin + CO_2 + AMP + PP_i + flash of light

Step 4: degradation by Apyrase

unincorporated dNTP and unconsumed ATP

The reaction cycle starts again at step 2 with a different nucleotide (4 cycles per base read).

APS **dATPαS (A*)**



b Result

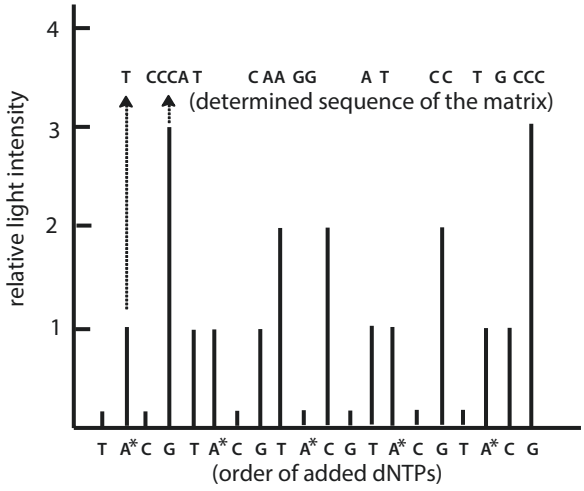
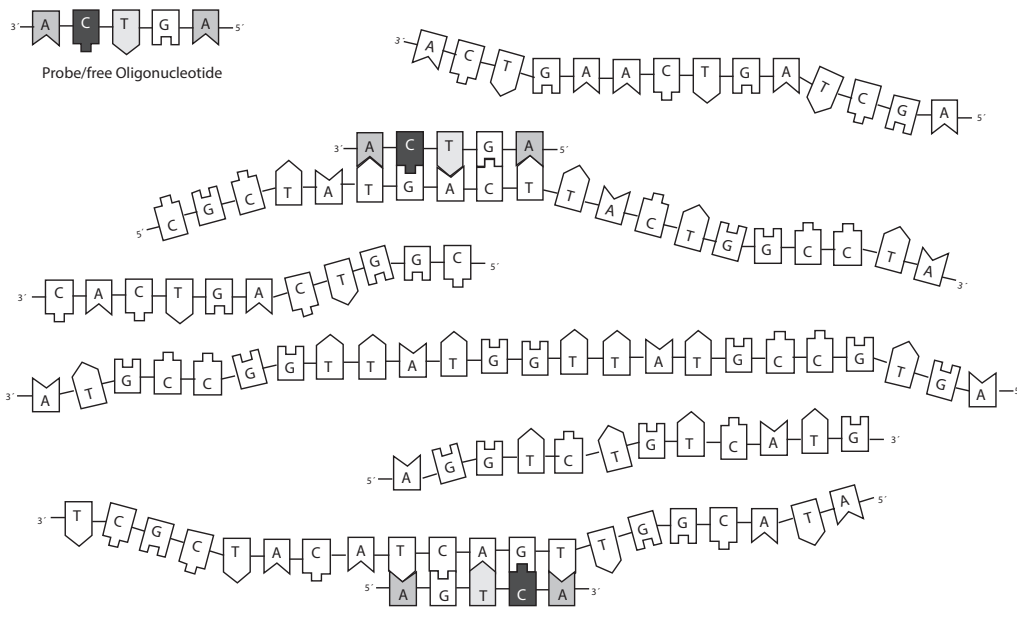


Fig. 12.8 Pyrosequencing. **a** Principle and **b** Result

12.2 Molecular Genetic Methods for Community Characterization

As already explained, there are numerous situations in which one would like to learn something about the composition of microbial communities, in which one would like to quantify the occurrence of certain species or in which one would like to analyse the frequency or the expression state of certain genes (for example of degradation genes). Certain conclusions about the composition of microbial communities can be obtained by isolating pure cultures and then identifying them. However, such an approach is limited to microorganisms that can be cultivated using standard methods, and these are very few. Therefore, efforts are being made to characterise microbial communities independently of strain cultivation using molecular genetic and increasingly also mass spectrometric methods.

An important basis of most molecular genetic methods for the characterization of communities, but also already for the performance of a conventional PCR, is the **specificity of the binding** between a synthetic oligonucleotide added to the sample and the DNA or RNA in the sample. This process of joining two strands of nucleic acid that (unlike the two strands of the DNA double helix) did not initially belong together is called **hybridization.** Just as the DNA double helix is held together by two hydrogen bonds each between adenine and thymine and three hydrogen bonds each between guanine and cytosine, and just as the identical duplication of DNA is ensured by the specificity of these bonds, shorter nucleic acids will also bind, depending on the conditions, only or primarily where as many of these hydrogen bonds as possible can be formed, i.e. where the oligonucleotide fits as optimally as possible (◘ Fig. 12.9). In principle, the same reasoning applies to the interaction between DNA and RNA, where the synthetic oligonucleotides are usually DNAs that bind to RNAs in the sample. Since uracil is present in RNAs instead of

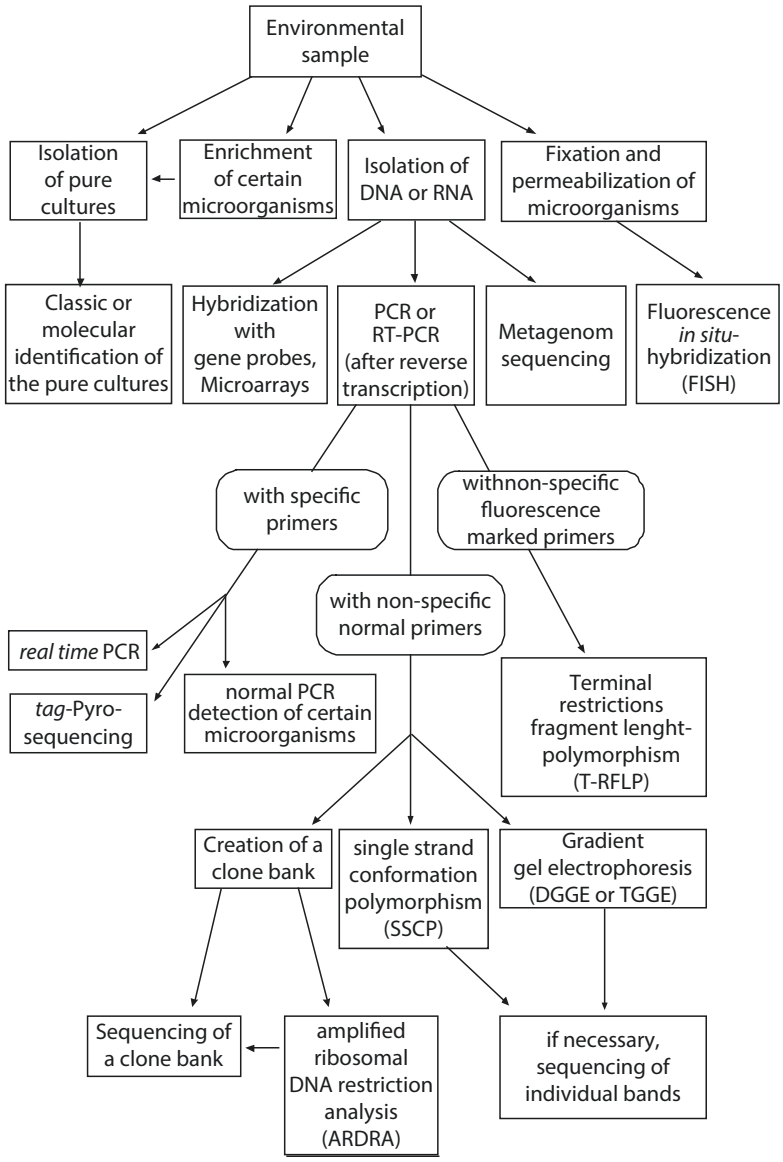


◘ **Fig. 12.9** Binding of an oligonucleotide to a mixture of single-stranded DNA molecules

thymine, there are also adenine-uracil interactions in DNA-RNA hybrids.

In recent years, numerous so-called molecular **ecological methods** have been developed for the investigation of microbial communities using **molecular** genetic techniques. Some important ones are summarized in ◘ Fig. 12.10, with mention of individual cultivation-dependent strategies. In principle, molecular methods can be differentiated between those in which nucleic acids (DNA or RNA) are first isolated from the community and those in which this is not done.

One method that does not involve the isolation of nucleic acids is **fluorescence in *situ*** hybridization **(FISH).** This procedure uses oligonucleotides that are labelled with



◘ **Fig. 12.10** Overview of some nucleic acid-based methods for characterizing microbial communities

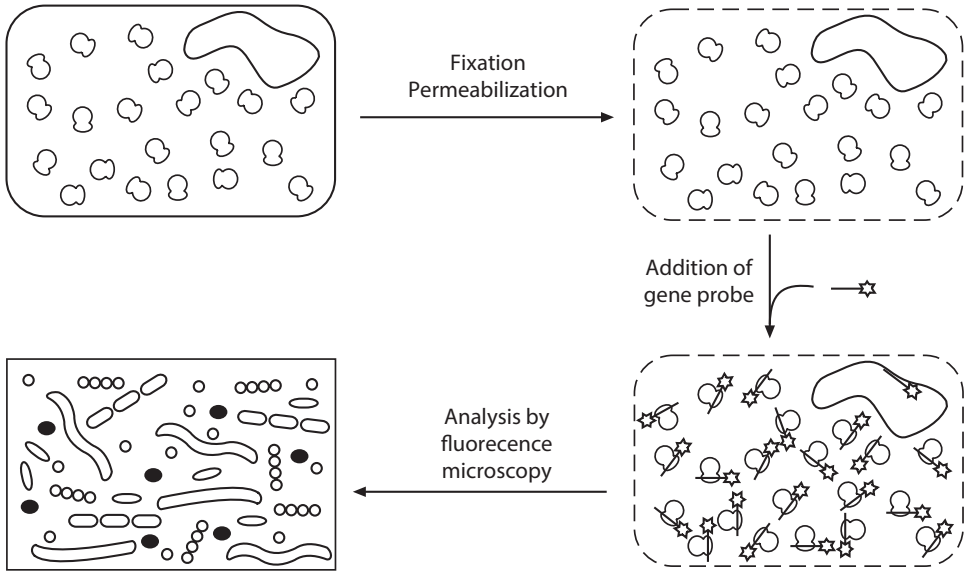


Fig. 12.11 Principle of fluorescence in *situ hybridization*

fluorescent dyes or with certain enzymes, so-called gene probes. To allow the gene probes to penetrate the cells, they must be fixed and permeabilized. If the gene probe binds sufficiently in the cell, the fluorescent dye is retained in the cell and the staining of the cell can be detected in the fluorescence microscope (Fig. 12.11). In the case of enzyme-labeled probes, the enzyme (for example, horseradish peroxidase) generates dye and many molecules of dye per bound probe, resulting in increased sensitivity. This is referred to as *catalyzed reporter deposition FISH* (CARD-FISH).

In microbial ecology, the FISH technique is mainly used in connection with gene probes for 16S rRNA or 23S rRNA. In the cell, the oligonucleotides bind not only to the genes of the ribosomal RNAs, but primarily to the RNAs themselves. Bacterial cells often contain several (1–15) genes for ribosomal RNA, but the number of ribosomes per cell is incomparably higher, often up to 100,000 or 200,000. The high number of ribosomes gives the method sufficient sensitivity for many questions. However, since the number of ribosomes correlates to a certain extent with the growth rate, slow-growing cells are sometimes poorly detected, a problem that is partly solved by CARD-FISH. In principle, FISH technology is suitable for quantitative questions, although the results can be falsified not only by the aforementioned problems with varying ribosome content, but also, for example, by differentially efficient permeabilisation of the cells or differentially efficient binding of the probes. Since only a very limited number of labels and thus probes can be applied to a sample at any one time, the FISH technique will never provide a largely complete overview of the microbial diversity of a community. In a sense, one only ever gets an answer to the question of how many organisms of a certain group occur in the community under investigation. For example, a relatively specific gene probe can be used to determine the abundance of certain species or genera in the community, and a probe specific to certain major groups, for example phyla, can be used to determine the occurrence of organisms of this major group. The number of cells that can be stained with DAPI (► Sect. 11.1.1) and the number of cells that

can be stained with a universal gene probe are used as reference values for quantitative data. A particular advantage of the FISH technique is that the cells can also be visualized in a largely intact confined environment. For example, it is possible to analyse the location of specific cells in a biofilm. However, for such analyses with a more complex, multilayered environment, a normal fluorescence microscope is not sufficient; instead, a confocal laser scanning microscope must be available.

In the majority of molecular ecological methods, DNA or RNA is first isolated from the biocoenosis, depending on the exact question. This can be done with direct extraction methods or after a first attempt to detach the microorganisms from the soil matrix, for example. Several relatively established methods are available for the **isolation of DNA** from an environmental sample, although isolation from soil and separation from humic substances in particular are still problematic, especially if the isolation must result in a purity that allows the DNA to be used even in subsequent enzymatic steps such as PCR. Despite the basic availability of suitable methods, the **isolation of RNA** is still much more problematic, firstly because RNAs can be destroyed by relatively stable RNases, i.e. RNA hydrolysing enzymes, and secondly because for many questions it must be ensured that the isolated RNA is free of any DNA. Despite these difficulties, RNA from environmental samples is increasingly being used. In taxonomic analyses, rRNA has the advantage over rDNA, which encodes it, that it is mainly the growing, i.e. active, cells of a community that are recorded, whereas DNA can still be intact in cells that are no longer able to divide and is also recorded in analyses targeting DNA if necessary. The mRNA from environmental samples makes it possible to analyse whether certain genes are induced under environmental conditions, information that cannot be obtained from DNA analyses alone.

Nucleic acids isolated from a community can be used directly for **hybridisation with a gene probe,** i.e. a labelled oligonucleotide. For example, hybridization between probe and mRNA allows the induction state of a gene in the community to be studied. The single-stranded DNA can be fixed on a membrane. With regard to complex samples, simple hybridization has the limitation that information is only ever obtained with regard to the gene probe used in each case. In order to circumvent this limitation, numerous gene probes are now combined in the form of so-called **microarrays.** For this purpose, numerous different oligonucleotides or PCR products are fixed on glass plates and then hybridised with the mixture of extracted nucleic acids. In all cases, direct hybridization requires that a sufficient amount of the nucleic acid can be extracted from the environmental sample for hybridization.

Many molecular ecological methods are based in one form or another on **PCR,** the principle of which has already been described in connection with the identification of pure cultures. If a PCR is to be applied to an RNA extract, the RNA must first be transcribed into DNA by a reverse transcriptase. This combination is therefore referred to as **rT-PCR. In** principle, PCR has the advantage over direct hybridization in that it uses very small amounts of nucleic acid. One disadvantage is that simple PCR is not a quantitative method in terms of its approach. Rather, exponential amplification often runs until some component of the reaction becomes limiting, so that even if the initial amounts of template DNA or RNA are different, similar amounts of PCR product and thus similar band intensities are obtained in subsequent electrophoresis. The performance of the PCR, and in particular the choice of primers, depends crucially on the question being pursued.

If the aim is to detect certain microorganisms or certain genes in an environmental

sample or to determine their abundance, relatively **specific primers** will be selected, precisely those that match the 16S rRNA or 16S rDNA of the taxonomic group or the gene sought. A **"normal" PCR** or "normal" rT-PCR will then, in the positive case, lead to a band in the electrophoresis gel, which proves the presence of the corresponding matrix DNA or RNA. As mentioned, this detection is not quantification. Since even a PCR performed with specific primers can lead to non-specific by-products, depending on the conditions, it is often necessary to perform a **check on the correctness of** the PCR product. For this purpose, one can, for example, determine the size of the product by comparison with size standards in electrophoresis. One can perform a "*nested* PCR" in which the PCR product of the first reaction is used as a template for a second reaction with primers that bind further in. Or one can determine the sequence of the PCR product as a control.

The fastest and simplest way to perform quantitative PCR is ***real time PCR.*** Here, the fluorescence of a dye is increased in different ways during the PCR (◘ Fig. 12.12). The decisive factor is that the fluorescence is already registered by the *real time-PCR instrument* during the reaction, so that the result can be assessed not only after the end of the reaction and electrophoresis, as is the case with normal PCR, but already during the course of the reaction. On the one hand, this enables a faster evaluation of results. On the other hand, higher numbers of matrix molecules in the exponential phase more quickly yield signals that exceed a threshold value. In this respect, quantitative differences are visible that often remain undetectable in a conventional PCR with many cycles For quantification, the number of cycles until the threshold value is reached is compared for sample and standards with known copy number. The correctness of the product can be analyzed by melting curves. Various approaches are used to increase fluorescence in the course of the PCR reaction (◘ Fig. 12.12).

- A dye such as SYBR Green I can be used, whose fluorescence increases when bound to double-stranded DNA (◘ Fig. 12.12a). As the concentration of double-stranded DNA increases during the course of the PCR, the fluorescence also increases. The effect on fluorescence is caused by any double-stranded DNA, which on the one hand is advantageous because one does not need (expensive) appropriately labelled oligonucleotides for each experiment. On the other hand, the non-specificity is a disadvantage, because even unwanted, non-specific PCR products give a positive signal.
- A specific signal only by the sought PCR products is achieved when both the fluorescent dye and a quencher are bound to an oligonucleotide (◘ Fig. 12.12b). As long as both are in close proximity to each other by binding to the same oligonucleotide, quenching of fluorescence by the quencher is successful. However, in the course of PCR, the oligonucleotides are destroyed by the 5′ → 3′ exonuclease activity of DNA polymerase, and thus the fluorescent dye and quencher are separated, leading to the increase in fluorescence.
- Various methods use oligonucleotides in which the fluorophore bound to them and the quencher, which is also bound, are brought into proximity to each other by forming a hairpin structure of the oligonucleotide (◘ Fig. 12.12c). Upon attachment to complementary DNA, the fluorophore and quencher are spatially separated, allowing fluorescence. As the PCR progresses, more and more complementary DNA is formed, as indicated by a stronger fluorescent signal.
- In another specific method, the 3′-end of one oligonucleotide is labeled with a green fluorescent dye and the 5′-end of a second oligonucleotide binding adjacent

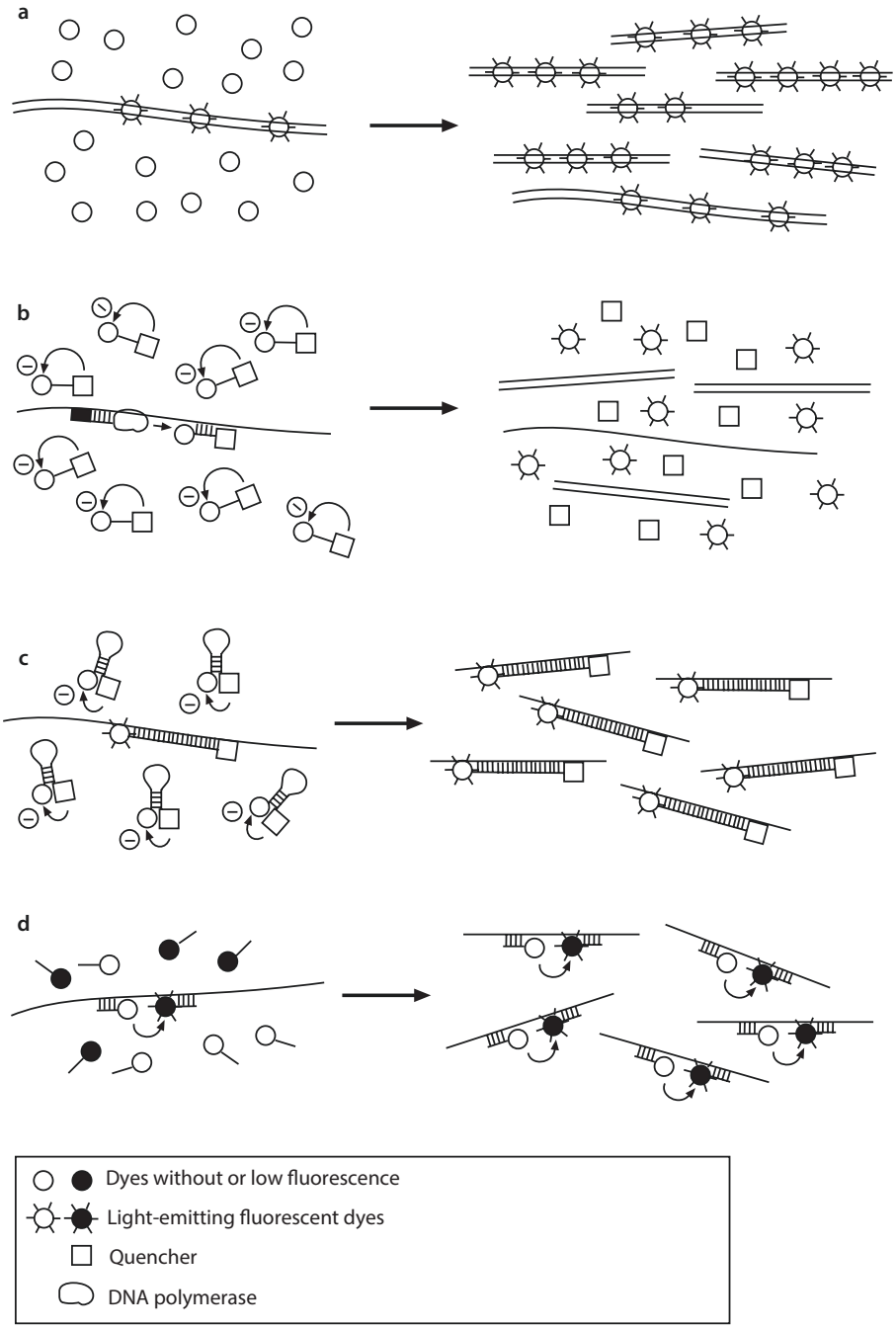


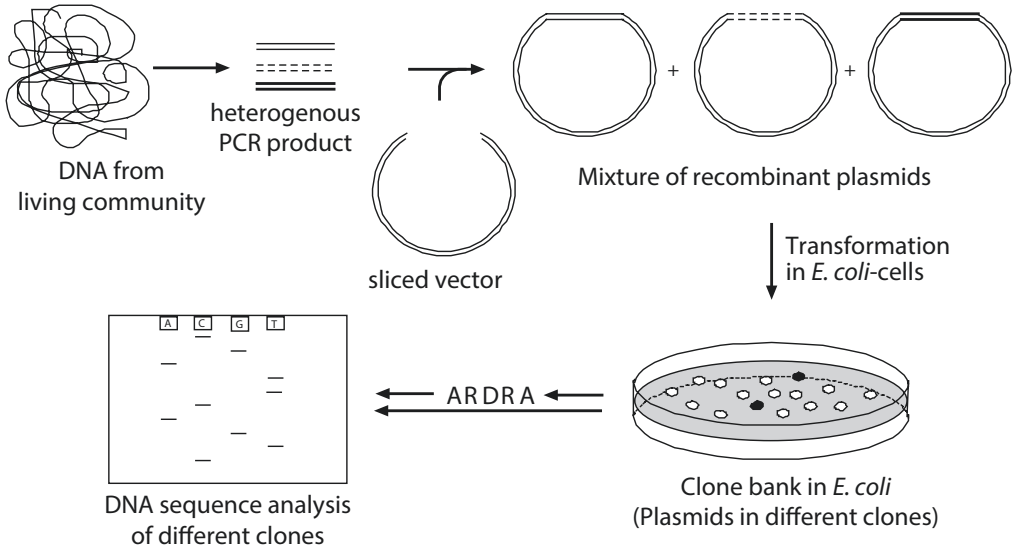
Fig. 12.12 Ways of quantifying genes by *real time PCR*. **a** Use of a dye that is more fluorescent when bound to double-stranded DNA. **b** Use of an oligonucleotide with fluorescent dye and quencher. Elimination of the effect of the quencher by 5′ → 3′-exonuclease activity of DNA polymerase. **c** Use of an oligonucleotide with fluorescent dye and quencher, wherein the quenching effect is removed by binding to complementary DNA. **d** Energy transfer from fluorescent dye at the 3′-end of one oligonucleotide to other fluorescent dye at the 5′-end of a second oligonucleotide

to the target DNA is labeled with a red fluorescent dye (◘ Fig. 12.12d). Binding to adjacent regions results in red fluorescence due to energy transfer from the first to the second dye. Adjacent binding is enhanced by formation of the PCR product. This system also avoids signal from non-specific PCR products.

In many cases, the aim of the investigation is not so much the qualitative or quantitative detection of specific strains or genes, but rather an inventory of what occurs at the site as a whole, i.e. for example a statement on the question of which microorganisms make up the biocoenosis. In such cases, PCR will be performed with relatively **non-specific primers** to account for the possible diversity at the site. "Relatively non-specific" can mean that one uses universal primers for taxonomic questions, but "relatively non-specific" can also mean that one is only interested in a particular group and the diversity within that group and therefore selects primers that cover an entire group. If one amplifies DNA (or reverse transcribed RNA) from a community with relatively non-specific primers, the **PCR product** will consist **of numerous different sequences.**

These different molecules, if they all originate from the same gene, e.g. the 16S rDNA of the respective microorganism, will have more or less the same length. Thus, a normal agarose electrophoresis will only show one (possibly somewhat broadened) band and will not reveal the diversity of different sequences behind it. However, if one is interested in the diversity at a site, be it the taxonomic diversity or the diversity of, for example, a degradation or resistance gene, then one must be able to analyse the heterogeneity of the superficially homogeneous PCR product.

One way of investigating the diversity of sequences occurring in a PCR product is to clone the PCR product into a vector and **create a clone bank**. In principle, each of the molecules produced during PCR is ligated into a different vector molecule and then transformed into a different bacterial cell. Different clones thus correspond to different molecules in the PCR product, and the molecules are separated from each other (◘ Fig. 12.13). Subsequent **sequencing of** numerous cloned sections can in principle



◘ **Fig. 12.13** Analysis of the diversity of a PCR product by creating a clone bank and sequence analysis

fully elucidate the diversity of sequences in the PCR product, and thus the putative diversity at the site. At the same time, the sequences found can be assigned to known sequences by database comparison, which leads to the identification of organisms present at the site in a taxonomic study of the 16S rDNA (provided that sufficiently similar sequences of identified organisms are recorded in the database).

Especially in a community with a strong dominance of individual species or genera, the case may arise that the same or extremely similar sequences are often obtained during sequencing. This would mean that unnecessarily high effort and costs are incurred in the characterization of the community. To limit this effect, clones obtained from the PCR product can first be subjected to **ARDRA analysis** by digesting the cloned sections with restriction enzymes. Only a few representatives of the different ARDRA groups are then sequenced, if necessary. Overall, depending on its size, the characterization of a clone bank may provide very comprehensive information on the occurrence of different microorganisms or genes at the site. At the same time, this strategy tends to be the most time-consuming, despite certain potential savings through ARDRA.

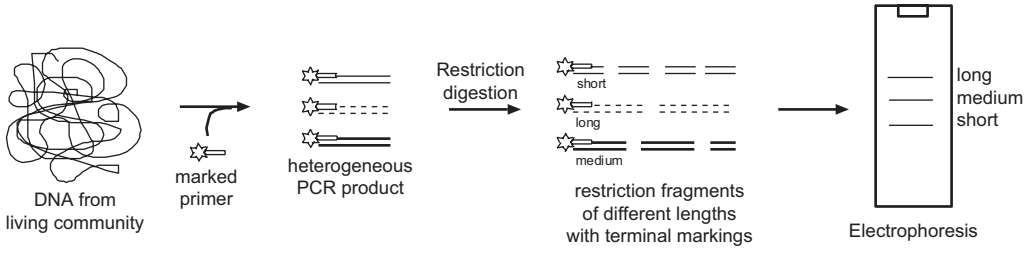
The new high-throughput sequencing techniques make it possible to obtain essentially the same information more easily than by clone bank analysis. The so-called ***tag pyrosequencing*** should be mentioned here in particular. In this strategy, the primers used to amplify the target gene (usually the 16S rDNA) are extended by additional sequences, the *tag*. These allow the sequence information obtained to be assigned to a sample. Then, a sufficient number of molecules of the PCR product are analyzed by pyrosequencing without prior cloning. Sequencing by means of cyclic reversible termination also allows a high degree of sequence information to be obtained, although the length of the individual sequence pieces is still much smaller than with pyrosequencing.

In addition to sequencing techniques, electrophoretic fingerprinting techniques allow conclusions to be drawn about the composition of microbial communities. It is important for this that special electrophoretic techniques can be used to separate DNA molecules of similar length from each other. One possibility is the analysis of ***single-strand conformational polymorphism* (SSCP)**. Single strands of DNA adopt different conformations depending on the sequence, which in turn influence the running speed in the electrophoresis gel. The sequence diversity of a PCR product is thus expressed in the number of bands obtained after denaturation on an SSCP gel. The intensity of the bands provides, to a limited extent (since PCR is not a truly quantitative method), information about the abundance of the sequences leading to the bands. To reduce the number of single strands to be electrophoretically separated per PCR product from two to one, and to circumvent problems due to unintentional clustering, a phosphorylated PCR primer can be used that results in phosphorylated single strands that can then be selectively degraded with an exonuclease. Assignment of bands to specific sequences, and thus for example in 16S rDNA analysis identification of species or genera, is possible by comparison with bands from PCR products of corresponding pure cultures or by purification of the DNA from the gel, re-amplification and subsequent sequencing.

A separation of DNA molecules of equal length and different sequence is also possible by gradient gel electrophoresis, in the course of which the DNA double strand is largely separated into single strands. **Denaturing gradient gel electrophoresis (DGGE)** and **temperature gradient gel electrophoresis (TGGE)** are commonly used. In DGGE, an increasing gradient of denaturing agents (formamide and urea) is polymerized into the polyacrylamide gel. In TGGE,

the gel is either subjected to a spatial temperature gradient (increasing temperature from top to bottom) or the gel is increasingly heated during the course of electrophoresis (temporal gradient). The increasing concentration of denaturant or the increasing temperature causes the hydrogen bonds holding the DNA double strand together to break. This occurs in a sequence-dependent manner, first in sections with a strong abundance of adenine and thymine (only 2 H-bridges) and later in sections where guanine and cytosine predominate (3 H-bridges). Sequence-dependent melting of DNA affects the rate of migration. Migration is particularly strongly reduced in Y-shaped structures, which are formed when the double strand is largely split but still hangs together at one end. In order to promote the formation of such structures, a GC clamp can be added to the PCR by selecting an appropriate primer. Also in DGGD and TGGE, the number of bands provides an indication of the sequence diversity of the PCR product and the intensity of the bands provides, to a limited extent, information on the abundance of the sequence type in the sample. Also with DGGE and TGGE an assignment of the bands to known sequences, i.e. with 16S rDNA analyses a statement about species or genera, is possible by comparison with bands of PCR products of pure cultures. In addition, it is possible to cut out bands from the gel, purify and clone the DNA from them and then carry out a sequence analysis of these bands.

For both SSCP and DGGD and TGGE, as mentioned, assignment of bands to known sequences is only possible by simultaneous examination of corresponding pure cultures or by subsequent excision, cloning and sequencing. This problem is avoided in another electrophoretic technique, **terminal restriction fragment length polymorphism (T-RFLP)** analysis. Here, a primer labeled with a fluorescent dye is used as early as the PCR stage. The PCR product, similar to ARDRA of pure cultures, is digested by a restriction enzyme that cuts relatively frequently, and the mixture of restriction fragments of the putative heterogeneous PCR product is electrophoretically separated in a polyacrylamide gel (◘ Fig. 12.14). When fluorescent light is measured after scanning with a laser, only the bands of the terminal restriction fragments are visible because only these carry fluorescent dye due to the labeled primer. The length of these terminal restriction fragments can be determined precisely and depends only on the sequence, the primer used and the restriction enzyme used. Therefore, one can basically calculate how long terminal restriction fragments to expect based on the primer sequence and the recognition sequence of the restriction enzyme for rRNA sequences in the databases. Conversely, it is also possible to draw conclusions in principle from the experimentally determined length of the terminal fragment directly (i.e. without applying PCR products from pure cultures and without subsequent sequencing) as to which organ-



◘ **Fig. 12.14** Characterization of a community by examination of the terminal restriction fragment length polymorphism (T-RFLP)

isms such a fragment could result from. In practice, however, it has proven useful to verify the fragment lengths to be obtained theoretically on the basis of the sequence by experimental analysis of corresponding clones. In contrast to ARDRA of pure cultures, T-RFLP only analyses the length of one fragment from each organism and not its whole banding pattern, because the superimposed banding patterns of all organisms in a community would be far too complex. Since T-RFLP uses less information than ARDRA, the taxonomic resolution of T-RFLP is lower, which means that, for example, a band of a certain length can result from the rRNA of several species, i.e. an unambiguous conclusion from fragment length to occurring species is not always possible.

A major challenge at present is not only to detect and, if necessary, quantify the occurrence of certain microorganisms in biocoenoses, but also to be able to **assign** the **activities that** are decisive for the respective process to **certain microorganisms.**

- One possible approach is to use radioactive substrates and, following fluorescence in *situ hybridization* (see above), to perform **microautoradiography** in order to spatially correlate the incorporation of radioactive isotopes (^{14}C) into the biomass with the presence of certain taxa. Recently, microautoradiography has also been successfully used in combination with microarrays for hybridization of rRNA in the form of so-called *isotope arrays.*
- In other strategies of activity detection, substrates labelled by **stable isotopes** (^{13}C) are used. Thus, the incorporation of the isotope into characteristic fatty acids can be detected. Also ^{13}C-labelled DNA or RNA can be obtained and analysed separately from unlabelled DNA or RNA by density gradient centrifugation in order to detect only those organisms that have converted the labelled compound (*stable isotope probing,* SIP).
- Metatranscriptome analyses also provide information on the function of certain taxa in a community. For example, by comparing the RNA sequences obtained by high-throughput sequencing using cyclic reversible termination with databases or the genomes occurring in a community, which taxa express certain genes.
- The same applies to metaproteome analyses. The coupling of liquid chromatography with mass spectrometric methods with double fragmentation (MS-MS) allows the determination of peptide sequences and thus the assignment of proteins isolated from the environment to specific organisms via database comparison.

DNA Microarrays

Microarrays can be used in microbiology for a variety of different applications, ranging from studies of gene regulation and bacterial response to environmental changes, genome organization and evolutionary questions to taxonomy and environmental studies.

The essence of microarray technology is the parallel hybridization of a mixture of labeled nucleic acids, called **target genes,** with a variety of individual nucleic acid species, called **probes,** which can be identified by their respective, fixed positions in a single experiment. The location of a specific probe on the array is called a **spot.** While the **probes** are immobilized on the solid support, the **target genes** are placed in solution on the array for hybridization after fluorescent labeling.

Gene-specific cDNA or oligonucleotide probes are "printed" on various carrier materials at defined positions of a grid as a probe (◘ Fig. 12.15).

The following main types of DNA microarrays are widely used:

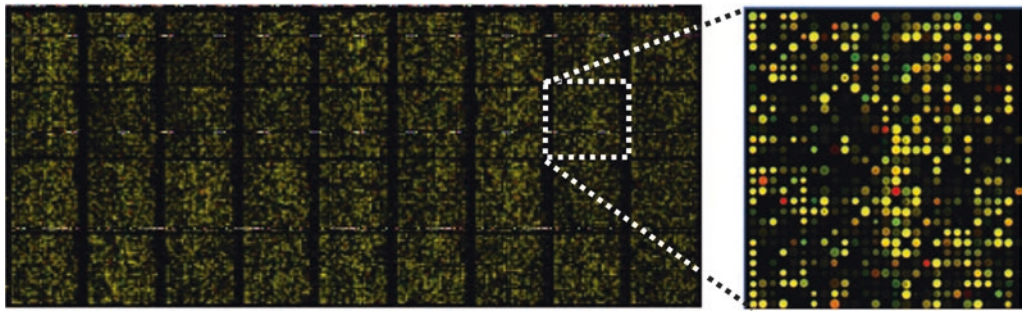


Fig. 12.15 Part of a microarray. (Left) Top view with 36 spot fields and (right) a spot field at the end of the assay after hybridization and laser scanning. Each color dot represents a specific oligonucleotide probe

- **Gene chips:** Microarrays in which probes are synthesized *in situ* nucleotide by nucleotide directly onto the surface of the chip.
- **Printed microarrays,** independently synthesized whole oligonucleotide probes are "printed" on special glass surfaces.

Gene chips are produced in a photolithographic process that is analogous to the methods developed for the production of microelectronic chips in combination with chemical reactions. The synthesis takes place at precisely defined positions on the glass surface. Tens of thousands of different oligonucleotides can be produced on a chip of 1–3 cm edge length, each in droplets of 100–200 µm diameter. Commercial high-density oligonucleotide arrays can have up to 400,000 features.

In repetitive cycles of masking, irradiation to remove blocking protecting groups, and chemical coupling of a nucleotide bearing a protecting group at the 3′-OH end, oligonucleotides of 25 nucleotides in length are synthesized on the chip surface.

Since the specificity of such a probe with 25 nucleotides is not high enough, each probe **("*match*")** is accompanied by a negative control in which a single base in the middle of the probe is altered, this is called a ***mismatch* probe.** Efficiency of **probe** and ***mismatch* probe** can be consequently used to detect and eliminate cross hybridization. **Probe** and ***mismatch* probe** are called **probe pairs.** Usually, 11–15 **probe pairs,** form a so-called **probe set,** which thus represents approximately a single gene.

The **printed microarrays** are synthesized independently and then sprayed onto the surface of the array by a microarray spotter like an inkjet printer. The main advantage of **printed microarrays** is their standardized size. Historically, the first microarrays were printed on microscopic slides, consequently the size 25 × 75 mm and a thickness of 1.0–1.2 mm was commonly in use. This allows free choice of the spotter, hybridization device, scanner and software from different suppliers or even the possibility of modifying the surface chemistry of the slides. Overall, **printed microarrays** have a much lower probe density compared to

that of gene chips: about 10,000–30,000 probes can be placed on a single **printed microarray.**

Three main categories of **microbial** diagnostic microarrays for microbial community analysis can be distinguished with respect to the nature of the probe and the target molecules used:

- Phylogenetic oligonucleotide microarrays (***phylochips***) use short oligonucleotides engineered against a phylogenetic marker gene.
- ***Functional gene arrays*** (FGAs) use gene fragments or oligonucleotides as probes that target genes encoding functions of interest.
- ***Community genome arrays*** (CGAs) use whole bacterial genomes as probes.

DNA microarrays are increasingly used in diagnostics and studies in differential gene expression. For many, the term chip analysis is therefore equivalent to conducting studies of transcription levels. DNA microarrays are thus used here to detect the RNA quantity of certain genes.

To study the **transcriptome of** an organism in specific physiological situations, RNA is isolated from cells cultured under appropriate conditions. Using reverse transcriptase, a mixture of randomly generated short oligonucleotides as primers and in the presence of a fluorescently labelled nucleoside triphosphate (green fluorescence for example growth on glucose (control), red for example growth on aromatic compounds), the mRNA is transcribed into cDNA.

The cDNAs are mixed and hybridized with the oligonucleotides immobilized on the chip. In this process, labeled cDNA pieces bind to their complementary counterpart on the array. After washing off the unbound cDNA pieces, the fluorescence signal of each position on the DNA microarray is recorded using two lasers of different wavelengths. The fluorescence value is a measure of the relative amount of transcript in the two mRNA populations. By superimposing the signals, it is determined which genes are expressed more strongly under one of the given physiological conditions (green or red), which are expressed under both conditions (orange-yellow) or not at all or below the detection limit (grey) (◘ Fig. 12.16).

The large amount of data is then statistically evaluated using bioinformatics methods and groups of genes can be formed that are coordinately regulated. This approach can therefore be used, for example, to detect global changes in the transcriptome of a cell.

The cDNA is then washed off the chip so that it can be reused.

Microarray analyses have the great advantage that the presence of many taxonomic groups or functional genes in a community or the expression of all genes of a genome or metagenome can be analysed in parallel. However, the production of corresponding arrays is correspondingly complex and expensive. Therefore, the production of an array is particularly worthwhile if the same question is to be investigated many times with different samples. Otherwise, it may make sense to switch to alternative high-throughput sequencing techniques such as *tag pyrosequencing* or expression analyses using high-throughput **sequencing based on cyclic reversible termination.**

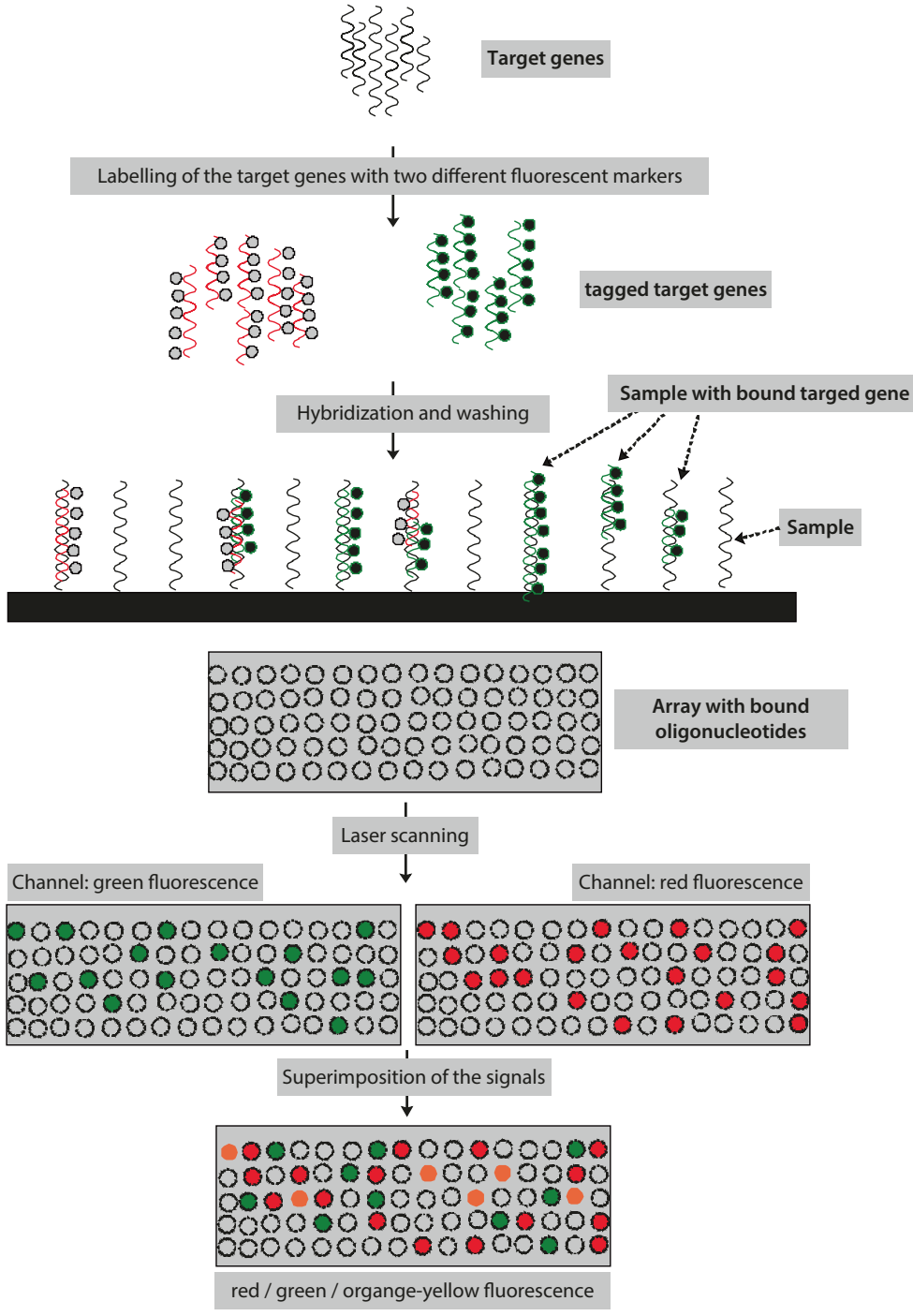


Fig. 12.16 Microarray experimental procedure showing the result of visualization of hybridization based on the fluorescence color of each probe. **yellow to orange-yellow:** target genes with both color markers have bound **gray:** none of the target genes have bound **red:** target gene with red fluorescence has bound **green:** target gene with green fluorescence has bound

12.3 Metagenomics

Metagenomics (*community genomics, environmental genomics* and *population genomics* are synonyms for the same way of working) is a comprehensive genetic approach to the molecular study of microbial communities. In this approach, DNA is isolated from environmental samples and sequenced randomly "shotgun sequencing". Previously, DNA was cloned into a suitable vector, the clones transformed into a host bacterium, and the resulting transformants screened. Clones can be screened for phylogenetic markers or "fixed points," such as 16S rRNA and *recA,* or for other conserved genes via hybridization, or for expression of specific traits, such as enzyme activity or antibiotic production. Each approach has its strengths and limitations. Together, these approaches have enriched our knowledge of the *uncultured world*, allowing insights into groups of prokaryotes that would otherwise have remained unknown.

Environmental genomics thus offers microbial ecology a new approach to explore the diversity of the microbial world in a way not previously possible. Metagenomics can thus reveal features of a microbial community that are missed by the single-gene approach. Thus, the metagenomic study of prokaryotes in the Sargasso Sea revealed an enormous diversity that had not previously been detected in community analyses based on 16S rRNA analyses. This is explained by the fact that not all 16S rRNA genes are captured by the universal or domain-specific primers used in PCR analysis. Genes that are not amplified naturally remain undetected. Environmental genomics circumvents this problem by shotgun sequencing of community DNA without first amplifying it by PCR. All genes are sequenced, whether they are amplifiable or not.

An extraordinary potential of metagenomics is to allow community-wide estimation of metabolic and biogeochemical function. Analysis of specific functions across all members of a community can generate an integrated model of how organisms realize the division of labor in maintaining the community's nutritional and energy budgets. The models can then be tested using genetic and chemical approaches.

Initially, nutrient-poor ecosystems were studied by extensive metagenomic sequencing. The microbial community of an acid mine drainage is an example of the analysis of such a community. This was later followed by nutrient-rich, complex habitats in which different sources of nutrients were present, such as plant material in the case of soils and lipid-rich bones in the case of deep-sea whale cemeteries. Determining the origin of DNA fragments and assigning them to functions is certainly much more difficult for such communities, which are phylogenetically or physiologically much more complex.

12.3.1 Community of an Acid Mine Drainage System

Near-complete sequencing of the metagenome of the Richmond Mine acid mine drainage community in Iron Mountain, California, is provided as an example of a community-wide assessment of metabolic and biogeochemical function. The microbial community forms a pink biofilm that floats on the surface of the mine water, which represents one of the most extreme environmental conditions on Earth. The water beneath the biofilm has a pH of 0.8 and contains high concentrations of Fe, Zn, Cu, and As. The solution around the biofilm water has a temperature of 42 °C and is microaerobic. The mine is rich in sulfides such as pyrite (FeS_2). There are no sources of carbon and nitrogen other than air.

The community is dominated by a few bacterial genera, such as *Leptospirillum* and *Sulfobacillus*, and less by *Acidimicrobium* and an *Archaea species*. *Ferroplasma acidarmanus*

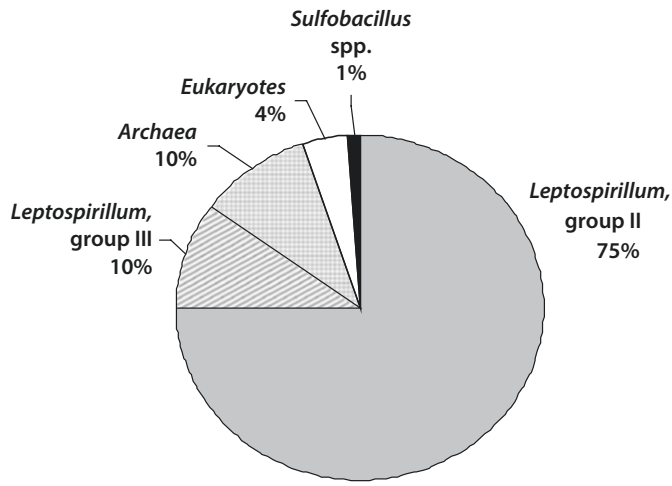
is present in relatively low abundance as are other members of the *Thermoplasmatales* group.

The simple structure of the community made it possible to clone all the DNA and obtain almost the entire sequence of the community with high coverage. The GC content of each clone formed a good indicator of its origin, as the GC content of the genomes of the dominant taxa in the mine differed substantially (◘ Fig. 12.17).

Almost the complete genomes of *Leptospirillum* group II and *Ferroplasma* type II were reconstructed. Essential sequence information of the other members of the community was reported. The metagenomic sequence allows important conclusions regarding metabolic activities. *Leptospirillum* group III contains genes similar to those that function in nitrogen fixation. This suggests that this group provides fixed nitrogen to the community. This was surprising as it had previously been suspected that the numerically dominant members of the community, such as *Leptospirillum* group II, would be responsible for nitrogen fixation. However, no such genes for nitrogen fixation were found in the *Leptospirillum* group II genome. Group III organisms therefore belong to a species which, despite being small in number, plays an indispensable role without which the community cannot function. *Ferroplasma* type I and II genomes also do not contain genes related to nitrogen fixation. However, they do contain many transporters that indicate that they take up amino acids and other nitrogen compounds from the environment (◘ Fig. 12.18).

Energy appears to be generated by *Ferroplasma* as well as *Leptospirillum* spp. by iron oxidation. The genomes of the two groups contain electron transport chains that differ considerably from each other. The *Leptospirillum* group II and III genomes encode possible cytochromes that typically have a high affinity for oxygen. The cytochromes may play a role in energy production as well as maintenance of low oxygen partial pressure, protecting the oxygen-sensitive nitrogenase complex of *Leptospirillum* group III.

All genomes in acid mine drainage are rich in genes associated with the removal of potential toxic elements from the cell. Proton efflux systems are important for energy production and also responsible for maintaining a neutral intracellular pH. Metal resistance factors pump metals out of the cells so that a non-toxic level exists within the cells.



◘ **Fig. 12.17** Relative microbial abundance determined by FISH. (After Tyson et al., 2004)

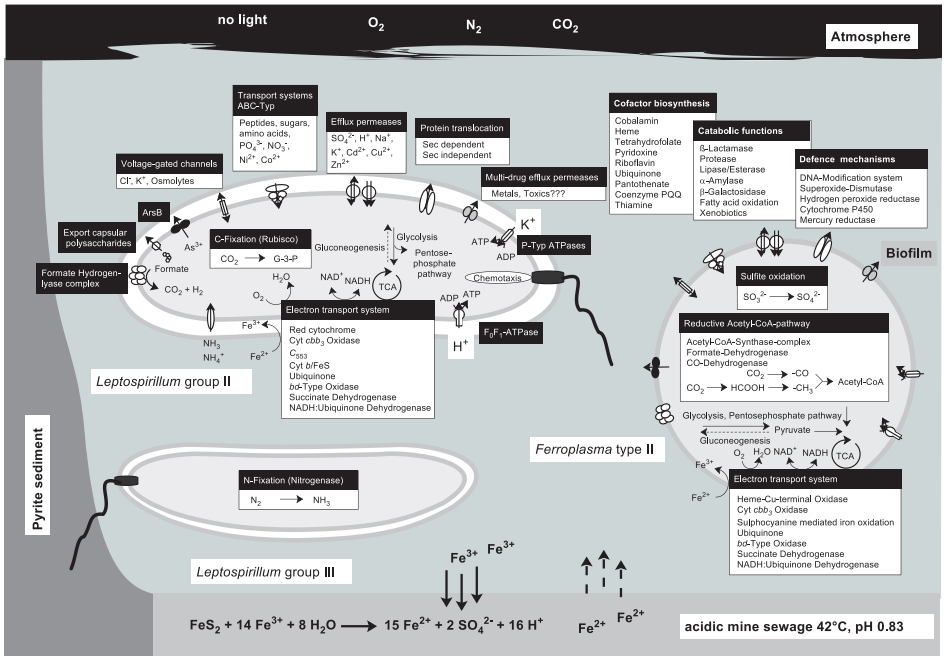


Fig. 12.18 Cartoons of the metabolites have been created after annotating the 2180 OFRs identified in the *Leptospirillum* Group II genome (63% with putative function) and the 1931 ORFs in the *Ferroplasma* Type II genome (58% with assigned function). *Leptospirillum* group III is added to document its indispensable role in the community, nitrogen fixation. Cells are shown in a biofilm located at the surface of acid mine water. The close coupling of iron oxidation, pyrite dissolution and acidification is shown. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase, THF, tetrahydro-folate. (Modified and supplemented after Tyson et al., 2004)

12.3.2 Community of the Sargasso Sea

The northwestern Sargasso Sea off the coast of the Bermuda Islands in the Atlantic Ocean is one of the best studied regions of the oceans (see Fig. 12.19). The Gulf Stream forms the western and northern boundaries of this region and provides a strong physical boundary separating the oligotrophic open ocean from the nutrient-rich waters of the U.S. continental shelf. The Sargasso Sea is thus a nutrient-poor area that marine biologists often compare to a desert.

In this region, the formation of subtropical upwelling water occurs each winter when the cold fronts above it break up the sea-

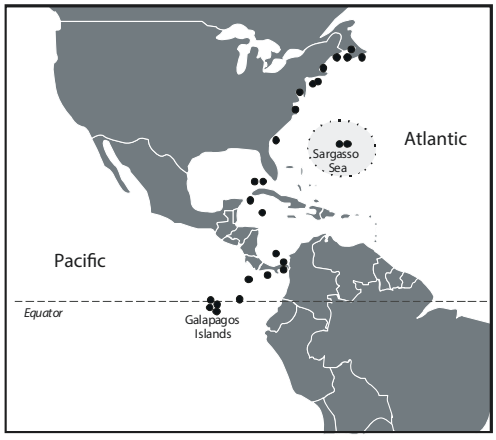


Fig. 12.19 Progress of the Sorcerer II Global Ocean Sampling Expedition (GOS). Sampling points are shown

sonal thermocline and cause convective mixing, resulting in mixed layers down to depths of 150–300 m. The formation of upwelling water in this region is a major cause of the seasonal thermocline. The influx of nutrient-rich deep water, which follows the collapse of the seasonal thermocline layers into the well-lit surface waters, results in the mass growth of single-cell phytoplankton, including the two cyanobacteria, *Synechococcus* and *Prochlorococcus,* which numerically dominate the photosynthetic biomass in the Sargasso Sea.

One of the most extensive microbial metagenome studies in the ocean was shotgun sequencing of the microorganisms of the Sargasso Sea (Venter et al., 2004). Genomic DNA was extracted from 170 to 200 L of surface water from filters with pore sizes ranging from 0.1 to 3.0 μm. Genomic libraries with insert sizes ranging from 2 to 6 kb were prepared, and the generated plasmid clones were sequenced from both ends.

The Sargasso Sea is a large-scale and complex ecosystem compared to the closed system of acid mine drainage. Thus, the inputs and outputs are much more difficult to quantify. The phylogeny of the community members has not yet been extensively and carefully studied.

Based on the sequence similarities and unique rRNA gene counts, the analyses suggest that the DNA fragments are from at least 1800 genomic species, and this includes 148 previously unknown bacterial phylogenetic types (Venter et al., 2004).

The study generated about 2 million pieces of sequence that contained over 1.6 billion base pairs of raw DNA sequences. Venter et al. claimed that 1.2 million new genes were discovered. The figure is astonishing, given that the 2004 SwissProt database contained entries for only about 140,000 proteins.

Stunning conclusions could be drawn due to the immense size of the data set. The authors assigned 794,061 genes to a conserved hypothetical protein group containing genes to which a function could not be assigned beyond doubt. The next widely distributed group was formed by 69,718 genes that likely encode proteins that function in energy production. Within the 1.2 million possible protein-coding genes, 782 new rhodopsin-like photoreceptors, the light-driven proton pumps of certain proteobacteria, were identified. This increases the number of sequenced proteorhodopsin genes 10-fold, reinforcing the importance of this type of phototrophy in the upper marine zone. Rhodopsin genes were shown to be linked to such genes that offer phylogenetic affiliation, such as genes encoding RNA polymerase subunits. Thus, the proteorhodopsins are distributed across taxa not previously known to have light-harvesting functions, including the Bacteroidetes phylum. Since cultures of these organisms have not yet been obtained, the connections between this form of metabolism and the specific phylum histories have not previously been suspected (◘ Table 12.4).

In an environment such as Sargasso Lake, which was actually thought to be species-poor, this metagenomic survey revealed tremendous diversity that had not previously been detected in community analyses based on 16S rRNA analyses.

Furthermore, in the Sargosso Lake study, genes for specific metabolic functions were found in some cases within the genomes of phylogenetic groups that were previously unknown. For example, the gene for ammonia monooxygenase – the key enzyme of ammonia-oxidizing bacteria – was discovered in the genome scaffolds of Archaea. Although ammonia-oxidizing archaea have never been described, environmental genomics has revealed that they almost certainly exist.

Another surprising observation is that many of the Sargasso Lake genomes showed similarity to those that function in phosphate uptake or utilization of polyphosphates and pyrophosphate, which are

Table 12.4 Gene assignment according to *The Institute for Genomic Research* (TIGR) categories

The Institute for Genomic Research (TIGR) Categories	Number of genes
Amino acid biosynthesis	37118
Biosynthesis of cofactors, prosthetic groups and carriers	25905
Cell envelope	27883
Cellular processes	17260
Central intermediary metabolism	13639
DNA metabolism	25346
Energy metabolism	69718
Fatty acid and phospholipid metabolism	18558
Functions to mobile and extrachromosomal elements	1061
Protein fate	28768
Protein synthesis	48012
Purines, pyrimidines, nucleosides and nucleotides	19912
Regulatory functions	8392
Signal transduction	4817
Transcription	12756
Transport and binding proteins	49185
Unknown function	38067
Miscellaneous	1864
Preserved, hypothetical	794.061
Total number of categories assigned	1242230
Total number of genes	1214207

Note that 28,023 genes were assigned to more than one category. 23 functional categories of expected genes include those known as COGs (clusters of orthologous groups), life processes such as: "information storage and processing", "cellular processes" and "metabolism"

necessary in the extremely phosphate-limited ecosystem. Understanding of nutrient cycling will grow through genome reconstruction and function-specific analyses.

Features of a microbial community missed by the single gene approach can be revealed by metagenomics. Environmental genomics can also detect new genes in known organisms and known genes in new organisms, among many other things. However, there are also possible **misinterpretations with metagenomics.** For example, a specific group of microorganisms was identified in the Sargosso Lake study. One organism, most likely a member of the genus *Burkholderia,* has 21-fold coverage and contains 38.5% of the sequence data in one of the four water samples. *Burkholderia* is typical of terrestrial environmental samples and the detection of the species in the ocean at such high frequency led the authors to suspect that terrestrial or coastal microorganisms play a significant role in marine microbial communities. However, this interpretation was challenged and the strong occurrence of *Burkholderia-like* sequences in one sample was attributed to possible contamination of the original water sample in the Venter et al. (2004) study. Such a revelation points to the need to exercise extreme caution when performing microbial metagenomic analyses. Nonetheless, the reconstruction of complete genomes indicates that shotgun sequencing of DNA from environmental microbial communities is a powerful approach in future studies of microbial ecology.

12.3.3 The Global Ocean Sampling Expedition

A metagenomic study was conducted as part of the Sorcerer II Global Ocean Sampling Expedition. Marine planktonic microorganisms were analyzed from samples in surface

water taken over several thousand km from the North Atlantic through the Panama Canal and ending in the South Pacific. A huge data richness of 7.7 million sequence pieces (6.3 billion bp) was generated.

Because PCR can introduce various systematic errors, the 16S rRNA genes were obtained directly from the original GOS. A total of 4125 whole or partial 16S rRNA genes were identified. Assembly of these sequences at 97% identity yielded a total of 811 distinct ribotypes. About half (48%) of the GOS ribotypes could be assigned to ribotypes that were available in public databases. This means that more than half of the ribotypes in the GOS dataset should be considered "new". Overall, the taxonomic distribution of GOS ribotypes collected by shotgun sequencing shows agreement with previously published PCR-based studies of marine habitats.

A small proportion of GOS ribotypes (16%) differ by more than 10% from the publicly available 16S rRNA gene sequences, meaning they are novel at the family level.

A survey of microbial ribotypes allowed us to identify the breadth of lineage and estimate their contribution to the GOS dataset. Of the 811 ribotypes, 60 contain more than 8-fold coverage of 16S rRNA genes. Together, these 60 ribotypes contribute to 73% of the total 16S rRNA gene sequence data. All but one of the 60 ribotypes have been previously detected, with only a few close relatives in complete or near-complete genome sequencing projects.

Several other common 16S rRNA gene sequences belong to known environmental ribotypes of which there are no cultured proxies (for example, SAR86, *Roseobacter* NAC-1-2, and branches of SAR11 other than that containing *Pelagibacter ubique*). Interestingly, *Archaea* are almost absent from the list of dominant organisms in these samples near the water surface. The distribution of ribotypes suggests distinct microbial communities. Only a handful of the ribotypes appear to be commonly distributed. These are dominated by relatives of SAR11 and SAR86.

Many of the ribotypes that dominate one or more samples appear to be present in one of the isolated marine surface habitats. For example, several SAR11, SAR86, and *Alphaproteobacteria,* as well as the *Acidimicrobidae group*, are widespread in surface waters, while a second niche is represented by tropical samples containing several different SAR86, *Synechococcus* and *Prochlorococcus,* and the *Rhodospirillaceae group.* Other ribotypes, related to *Roseobacter* RCA, SAR11 and *Gammaproteobacteria* are common in temperate samples. However, they have not been observed in the tropical or Sargasso Sea samples.

Not surprisingly, samples from non-marine environments, brackish water, and fractions from filtration with larger pore sizes had different characteristic ribotypes (◘ Table 12.5).

12.3.4 Sequence Data and Functionality: A Critical View

The daunting task of metagenomics is to access, analyse and exploit the enormous biodiversity of the microbial world. However, the limits of the technique must be made clear.

A major bottleneck in advances is the large number of misleading mappings in current databases. Many wrong or erroneous annotations are perpetuated and thus have a large impact. Without experimental validation, that is, plausibility checking, misinterpretations cannot be corrected. Databases are only as useful as the quality of the data they contain. Bioinformatics is only as good as the information that has been entered into the computers.

In general, there is a lack of functional analysis with sufficient quality of the rapidly

Table 12.5 Taxonomic assignment of GOS samples based on 16S rDNA data by shotgun sequencing

Phylum or Classa	Proportions (values are mean values of all samples)
Alphaproteobacteria	0.32
Non-classified *Proteobacteria*	0.155
Gammaproteobacteria	0.132
Bacteroidetes	0.13
Cyanobacteria	0.079
Firmicutes	0.075
Actinobacteria	0.046
Marine Group A	0.022
Betaproteobacteria	0.017
OP11	0.008
Non-classified *Bacteria*	0.008
Deltaproteobacteria	0.005
Planctomycetes	0.002
Epsilonproteobacteria	0.001

"candidate division" OP11 (Hugenholtz et al., 1998)

growing number of genomic sequences and genes.

Advances in microbial sciences are not only dependent on the availability of innovative sequencing platforms. They also need to rely on sequence-independent tools to gain insights into the functioning of microbial communities. The reasons for this are clear: any single-cell or environmental genome project adds a large number of "putative" genes whose functions are often unknown and, in the better case, inferred from sequence comparisons. Furthermore, even the best assignments can only provide hypotheses of protein functionality and substrate spectra. Experimental studies of classical disciplines such as physiology and biochemistry are necessary. Sequence data will hardly allow to estimate the specificity of enzymes.

Making sense of environmental sequence data is also often difficult due to the following fact: a significant amount of *open reading frames* cannot be characterized because similar sequences are not available in databases.

Test Your Knowledge

- Describe the basics of bacterial identification using DNA/RNA.
- From which basic processes in organisms was PCR derived? Are there also primers there?
- Why do you run the complex temperature cycle in PCR? What do thermophilic organisms have to do with PCR?
- What trick did Sanger contribute to DNA sequencing?
- What is meant by hybridization, what is meant by a gene probe?
- What is an "alignment"? What does it provide information about?
- You are confronted with the term "RT-PCR". Is it clear what is meant by this?
- Which measurement signal is used for pyrosequencing?
- Discuss the differences between pyrosequencing and Sanger's method.
- Name several ways in which a fluorescent signal can be generated in *real-time* PCR?
- What is meant by bridge amplification? In which technique does it play a role?
- Why does PCR not really allow quantitative analysis?
- Name several possibilities to obtain signals from the PCR product of the 16S rDNA from a microbial commu-

nity, which are characteristic for species or genera, for example!

- Name two ways of high throughput sequencing!
- Environmental genomics involves shotgun sequencing and analysis of the collective genomes of organisms present in a microbial community. What is a metagenome?

SAR 11

SAR11 is the numerically dominant microorganism in the ocean surface, renamed as *Pelagibacter ubique* SAR11 bacteria. It accounts for 35% of the total prokaryotes in ocean surface waters. To date, little is known about its involvement in marine biogeochemical cycles. Early studies reported that SAR11 bacteria are very small and they have few ribosomes. This suggests that SAR11 bacteria have low metabolic activities and they play only a minor role in the flux of dissolved organic matter despite their prevalence.

Microbial Communities, an Overview

Some metagenomic sequencing of both nutrient-poor ecosystems and nutrient-rich complex habitats is summarized in ◘ Table 12.6.

Looking at which organisms dominate the different ecosystems, we see clear differences in the occupancy of *archaea, eukarya, prophages, bacteria,* and *virus* (◘ Fig. 12.20).

◘ **Table 12.6** Examples of analyses of microbial communities by metagenomic sequencing

Ecosystems	Literature
Low nutrient	
Sargasso Lake	Venter et al. (2004)
Global Ocean Sampling	Rusch et al. (2007)
Plankton of the Chesapeake Bay	Bench et al. (2007).
Biofilm of the acid mine water	Tyson et al. (2004).
Microbial ecosystem of a deep mine	Edwards et al. (2006)
Urban indoor air	Tringe et al. (2008).
Nutrient-rich	
Human gastrointestinal tract	Gill et al. (2006), Qin et al. (2010), Walter and Ley (2011), Zhang et al. (2006)
Floors	Fierer et al. (2007), Riesenfeld et al. (2004), Rondon et al. (2000)
Worm, without mouth, intestine and nephridia	Woyke et al. (2006)
Termite Thick Intestine	Warnecke et al. (2007)
Deep sea whale carcass	Smith and Baco (2003), Tringe et al. (2005)

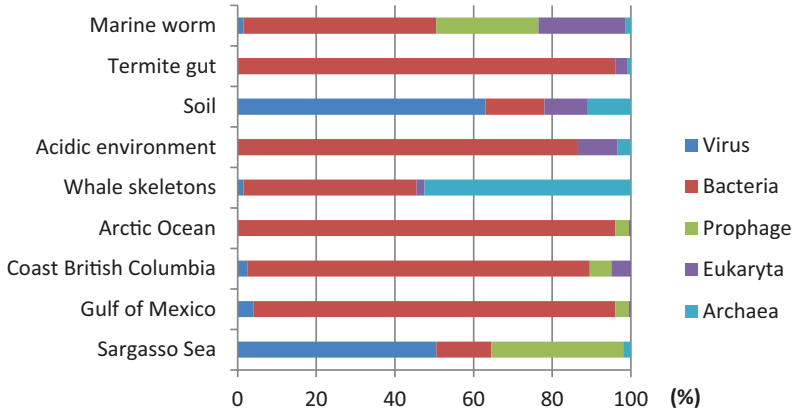


Fig. 12.20 Phylogenetic composition of a variety of communities in the environment. The results are averages of a total of 700 Mbp. As can be seen, the composition is very different between geographic and niche-specific ecosystems. (With data from Vieites et al., 2009)

Community Human Intestine

All plants and animals, including humans, are microbially colonized and contain ten times more bacteria than own cells and about 100–150 times more bacterial genes than own genes. New sequencing technologies have made it possible to gain both quantitative and qualitative insight into the bacteria that colonize us. The human gut is estimated to harbor 10–100 $\times 10^{12}$ bacteria representing hundreds, if not thousands, of different species (Turnbaugh et al., 2007).

Table 12.7 shows which organisms are present and where in the human intestine. Furthermore, their metabolism is addressed.

Human characteristics have been acquired through the acquisition of microbial symbionts such as the ability to break down a greater amount of plant polysaccharides. Microbial fermentation provides about 10% of the daily energy of the Western diet.

16S rRNA sequence-based methods reveal that two bacterial divisions, *Bacteroidetes* and *Firmicutes,* account for over 90% of the known phylogenetic categories and dominate the microbial population of the distal gut. *Bacteriodetes* are Gram-negative obligate anaerobic bacteria; *Firmicutes* are Gram-positive bacteria with the predominant genera being *Streptococcus, Lactobacillus, Bacillus* and *Clostridium.*

Despite the fact that a significant proportion of intestinal bacteria have not yet been cultured, over 1000 different microbial species found in the human gastrointestinal tract have currently been identified. The 1,057 species in the gut include Eukarya (92), Archaea (8) and Bacteria (957) and are based on phylogenetic studies of the sequences of the RNA genes of the small subunit of ribosomes. The detailed physiological and genetic information collected over 150 years from pure cultures isolated from the gut was mapped to the metagenome data (Rajilić-Stojanović & de Vos, 2014).

In a study, 1135 stool samples from Dutch participants were examined. A total of 126 factors were identified that influence the composition of the microbiome. These include 60 dietary factors, 12 diseases, 19 medications and four factors

Table 12.7 Genome sizes, habitats, and metabolism of human gut bacteria

Organism	Habitat	Metabolic activity	Genome size (Mb)
Bacteroides thetaiotaomicron	Colon	Saccharolysis	6.3
Bacteroides vulgatus	Colon	Saccharolysis	5.2
Parabacteroides distasonis	Colon	Saccharolysis	4.8
Escherichia coli	Small intestine, large intestine	Fermentation of simple sugars and amino acids	4.5
Roseburia intestinalis	Colon	Saccharolysis, butyrate former	4.2
Eubacterium rectale	Colon	Saccharolysis, butyrate former	3.4
Faecalibacterium prausnitzii	Colon	Saccharolysis, butyrate former	3.1
Bifidobacterium adolescentis	Colon	Dietary carbohydrates	2.1
Lactobacillus reuteri	Small intestine	Host-specific fermentation of simple sugars and 1,2-propanediol	2.0
Methanobrevibacter smithii	Colon	Methane generator	1.9
Helicobacter pylori	Stomach	Consumption of simple sugars and amino acids	<1.67

After Qin et al. (2010)

related to smoking. Our microbiome serves as a fingerprint of sorts, reflecting all sorts of signals of health. It has been found that women tend to have more microbial diversity than men and older people more than younger people (Zhernakova et al. 2016).

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Damage to Inorganic Materials Due to Microbial Activities, Biocorrosion

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_13

13.1 Iron Corrosion

The technically most important metal, iron (Fe^0) corrodes without a protective coating or without being alloyed with other, more expensive metals in an aqueous environment as a result of chemical and also biological processes.

The oxygen in the air progressively oxidises the solid metallic iron in a wet environment to form friable, hydrous oxides, whereby—chemically speaking—the metal becomes trivalent positive. During corrosion in air, electrons from the metallic iron react with oxygen and, with the participation of water, lead to hydroxyl ions (OH^-). These form rust, a complicated hydrous oxide of trivalent iron, with the released divalent iron (Fe^{2+}) with the further participation of oxygen. Corrosion with atmospheric oxygen is, according to current knowledge, a predominantly chemical (abiotic) phenomenon (◘ Fig. 13.1, top left).

Numerous ways in which microorganisms can affect the corrosion of iron have been proposed. In oxic environments, microbial corrosion typically occurs through colony formation and microbial O_2 consumption on iron surfaces, so that material loss can be triggered at these sites ("pitting"). Additional dissolution of protective rust deposits by aerobic iron-oxidizing microorganisms can also influence the rates of corrosion.

Even in the absence of oxygen, iron is not arbitrarily resistant, but is attacked by water alone. Flaky forms of Fe^{2+} and hydrogen are formed in the process. This corrosion under exclusion of air is—compared to rusting in air—very slow. It has long been assumed that the consumption of the hydrogen that forms slowly on iron in water, often referred to as "cathodic hydrogen", by sulfate-reducing bacteria causes the dissolution of the metal in the water (◘ Fig. 13.1, top right).

In oxygen-free environments such as cooling water circuits or buried pipelines, iron sometimes also corrodes very quickly as a result of microbial activity. Metal equipment used in oil production, as well as storage tanks, is also destroyed. The technical term microbially influenced corrosion (**MIC**) has become established for this. The economic costs of the resulting material destruction are enormous.

Under anoxic conditions or in systems with only temporary penetration of O_2, microbial corrosion is altogether more pronounced. Here, corrosion is caused by products of the fermentation of constituents, for example, of domestic wastewater by their organic acids and also hydrogen sulfide or other corrosive sulphur species.

Most studies have focused on the corrosive effects of sulfate-reducing bacteria (**SRB**) (see ► Sect. 8.1), but also other physiological groups such as thiosulfate-reducing, nitrate-reducing and acetogenic bacteria. Methanogenic archaea have also been associated with iron corrosion. Nevertheless, it is the physiological group of SRB that plays a key role in the anaerobic corrosion of iron and is widely distributed in many natural as well as engineered aqueous ecosystems. SRB obtain their energy for growth by the reduction of sulfate to hydrogen sulfide with electrons obtained from the decomposition of organic matter or from molecular hydrogen—normal fermentation products in soil, sediments and other anoxic habitats.

The hypothesized key role of SRB in biocorrosion is based on the following three observations:

1. Iron in anoxic environments such as marine sediments containing sulfate, i.e. the electron acceptor of SRB, is particularly susceptible to microbial corrosion. Particularly severe levels of corrosion are found in sulfate-containing waters and sediments.

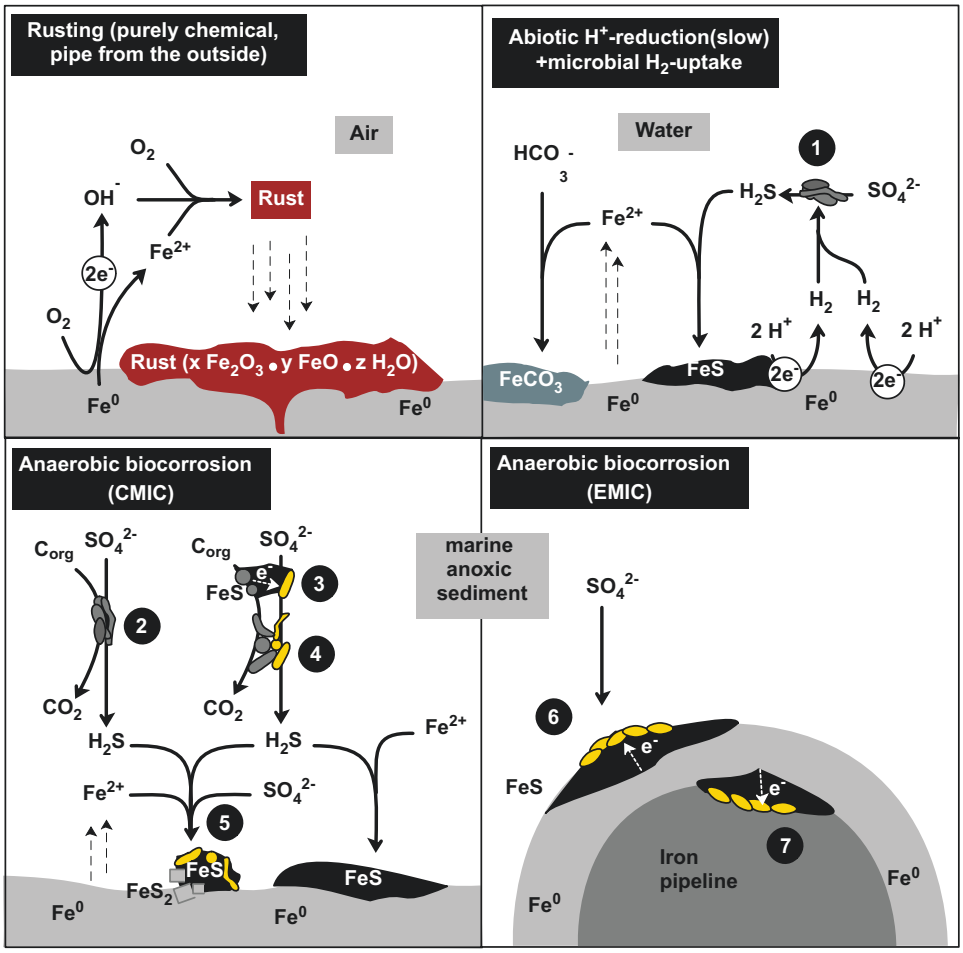


Fig. 13.1 Schematic representation of the different types of iron corrosion at neutral pH. Abiotic and biotic reactions are shown. All of the anaerobic processes shown can occur simultaneously on metal surfaces. However, they differ in rates and relative contributions to corrosion. The biotic reactions shown in anoxic habitats tend to proceed much faster than the abiotic corrosion reactions. **1.** slow, kinetically hindered reduction of H^+ ions to H_2 at iron surfaces. Consumption of H_2 by SRB does not accelerate the rate of H_2 formation. **2.** conventional organotrophic SRB. **3.** SRB interaction via electrically conducting iron sulfide with electron-donating organotrophic anaerobic microorganisms. **4.** SRB interaction in direct contact with electron-donating organotrophic anaerobic microorganisms. **5.** Speculative possibility of pyritization of FeS ($FeS + H_2S \rightarrow FeS_2 + 2H^+ + 2e^-$) as a direct electron source for sulfate reduction. **6.** Specially adapted lithotrophic SRB extract electrons from metallic iron as a source of electrons via electrically conductive iron sulfide (**EMIC**) on the outer surface of a pipeline. **7.** Specially adapted lithotrophic SRB extract electrons from metallic iron as a source of electrons via electrically conductive iron sulfide (EMIC) inside a pipeline. Note that CMIC is quantitatively dependent on the availability of biodegradable organic matter. Top left: x, y, z = positive ratios

2. SRB and their characteristic corrosion product FeS have been found ubiquitously on anaerobically corroded iron, i.e. they are always associated with severe corrosion damage.
3. At rates as high as 0.9 mm Fe^0 per year, SRB cultures grown in the laboratory corrode iron at rates that explain severe cases of corrosion. Field data indicate the prominent role of SRB in anaerobic iron corrosion, while laboratory studies provide plausible mechanistic explanations.

The observed material destruction by the sulfate-reducing bacteria is usually attributed to three complementary mechanisms of action:

1. Sulfide formed by SRB (H_2S, HS^-) has high reactivity towards iron.
2. The resulting FeS is thought to have a surface catalytic effect, accelerating the reduction of protons (from water) to molecular hydrogen.
3. It is assumed that the corrosion is accelerated by the microbial oxidation of the cathodic hydrogen on the iron or on the iron sulfides.

Recently, another mechanism was discovered on specialized lithotrophic isolates from enrichment cultures with metallic iron as the only electron donor. This is direct electron uptake from the metal by SRB. These lithotrophic strains corrode iron massively, forming electrically conductive mineral crusts on the metal. Their action is explained by the establishment of a galvanic element, iron being the anode, while the cells catalyze the cathodic reaction, directly affecting the rate of iron dissolution.

The electron flow proceeds accordingly from the metal ($4Fe^0 \rightarrow 4Fe^{2+} + 8e^-$) through the predominantly mineral corrosion crust to the mineral-associated SRB ($8e^- + SO_4^{2-} + 10H^+ \rightarrow H_2S + 4H_2O$).

It is therefore assumed today that the sulfate reducers cause anaerobic corrosion of the iron by directly utilizing metallic iron as an electron donor, thus destroying metal equipment in oil production, but also pipelines and storage tanks. The Fe^{2+} ions released in this process react with the H_2S formed to form insoluble FeS, the remainder precipitates with dissolved bicarbonate as poorly soluble $FeCO_3$.

Reaction diagram of microbial iron corrosion:

13

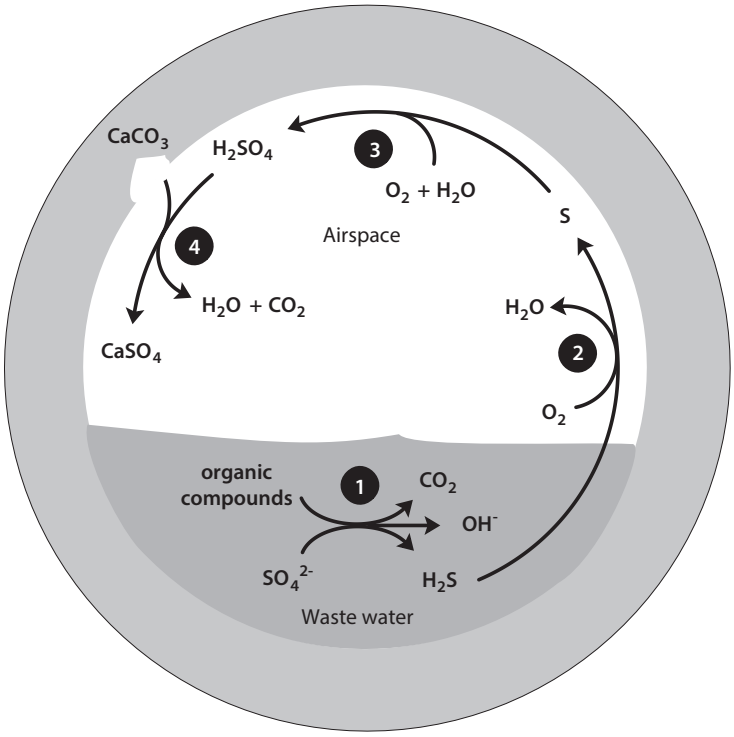
$$4Fe + SO_4^{2-} + 3HCO_3^- + 5H^+ \rightarrow FeS + 3FeCO_3 + 4H_2O\left(\Delta G^{0'} = -347\,kJ/mol\right)$$

Under appropriately adapted cultivation conditions, technically highly relevant rates of bioelectrical corrosion could be demonstrated. The corrosion of iron by direct microbial electron uptake is referred to as electrical microbially influenced corrosion (EMIC). This is mechanistically fundamentally different from the material destruction resulting from the chemical reaction of biogenic hydrogen sulfide with iron (chemical microbially influenced corrosion, **CMIC**).

A study was conducted to investigate the relative influence of the two mechanisms, indirect effects and direct interactions, on the corrosion of iron in a natural sulfate-containing ecosystem. Careful sampling and chemical analysis of the corrosion products of steel coupons buried in the tidal flats allowed a quantitative statement to be made on this issue. Surprisingly, the microbial corrosion under these conditions was exclusively bioelectrical in nature, i.e. EMIC represented the sole cause of the observed corrosion damage.

13.2 Concrete Corrosion

Acidithiobacillus thiooxidans is significantly involved in the corrosion of concrete pipes commonly used for underground sewerage. In this corrosion, two reactions of the sulfur cycle interact, dissimilatory sulfate reduction and sulfur oxidation. In addition, sulfide formation from anaerobic protein degradation also plays a role. Especially the sulfate reducers form H_2S in the partially filled wastewater pipes, which is further oxidized to sulfuric acid in the air phase directly or after chemical and biological oxidation to elemental sulfur by *Thiobacillus* species. The sulfuric acid leads to dissolution of the $CaCO_3$. This results in fractures in the upper part of the pipes (◘ Fig. 13.2). Concrete with a plastic lining is used for wastewater pipes unless the formation of H_2S can be reduced by constructive means (rapid wastewater flow and aeration).



◘ **Fig. 13.2** Bacterial corrosion of concrete sewer pipes. Reactions leading to dissolution of concrete wall in exposed part of sewage pipes: (1) Bacterial fermentation of organic matter and sulfate reduction. (2) Chemical or biological oxidation of H_2S. (3) Sulfur oxidation by thiobacilli. (4) Dissolution of $CaCO_3$

13.3 Building Corrosion/Damage to Stone

Environmental problems related to nitrification (see Sect. ► 7.3) result from the increased input of nitrogen compounds into the biosphere. Natural nitrification causes the breakdown of minerals, for example the release of potassium, calcium and phosphate, through the formation of nitric acid. The process leads to corrosion of structures made of sandstone and cement. Some nitrifiers, for example *Nitrosovibrio* as predominant species of nitrite oxidizers on buildings, colonize porous sandstones to the depth of several centimeters. This endolithic lifestyle is made possible by ammonium emissions, mainly from factory farming of pigs and poultry. Acid formation leads to the destruction of the sandstone structure, as has been demonstrated at Cologne Cathedral and other historic buildings (see ◘ Fig. 13.3).

Nitrificants find particularly good development conditions on limestone, lime mortar or concrete, if the building materials are exposed to vapours from stables or sewage plants and are also moist. Once nitrification has begun, ammonia should be increasingly "trapped" as the pH is lowered. Such trapping of the substrate would be tantamount to self-stimulation of the nitrification process.

The extent to which nitrifiers are involved in the corrosion of buildings when there are no conspicuous sources of ammonia in the immediate vicinity is being investigated.

◘ **Fig. 13.3** Influence of the weathering process on the angel statue at the Peters Portal of Cologne Cathedral. (From Warscheid & Braams, 2000). Cologne, Cathedral, west building, south portal, angel figure, state 1880 © Hohe Domkirche Köln, Dombauhütte. Cologne, Cathedral, west building, south portal, detail view: angel figure, state 1992 © Hohe Domkirche Köln, Dombauhütte

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Biological Waste Water Treatment

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_14

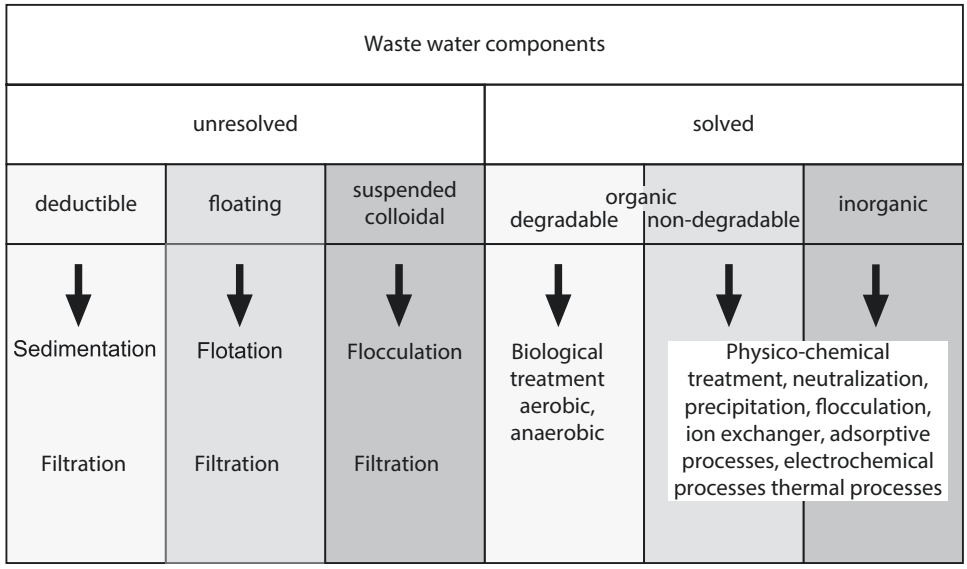
14.1 Formation and Composition of Waste Water

The Water Resources Act regulates the direct or indirect use of water bodies, which results in the requirements for the discharge of wastewater into a water body. **Wastewater** is "water that has been altered by use and any water that enters the sewer system" (DIN 4045)—i.e. also rainwater. A distinction is made between indirect and direct dischargers: Indirect **dischargers** are wastewater producers whose wastewater enters a public (municipal) treatment plant together with other (e.g. domestic) wastewater. **Direct** dischargers discharge their wastewater—like the municipal wastewater treatment plant—directly into a body of water after appropriate treatment. Roughly speaking, a distinction can be made between **municipal wastewater,** the various **types of industrial wastewater** and **mixed wastewater.**

Among the **constituents** in wastewater, a distinction is first made between **undissolved** and **dissolved substances.** The undissolved substances (which can be filtered as a whole) can be further subdivided into **settleable** and **non-settleable substances,** depending on the particle size. The non-settleable substances include "floating", very finely distributed particles as well as suspended or colloidal particles which can be separated analytically by ultrafiltration. The proportions of dissolved substances are divided into **organic, degradable** and **organic, non-degradable (or difficult to degrade)** and **inorganic substances** (◘ Fig. 14.1).

If one disregards extremely unbalanced industrial wastewater, the qualitative and quantitative composition of wastewater cannot be adequately characterised by individual parameters (e.g. fat, sugar, chlorides). Attempts have therefore been made to determine the sum of the organic pollutants contained in the wastewater indirectly, for example by oxidising them under defined conditions and recording the quantity of oxidising agent required for this. Typical **sum parameters** for wastewater include:

- The **biochemical oxygen demand (BOD)** serves as a measure of the microbially degradable organic substances. The BOD_5 is the amount of oxygen in mg/L that has been consumed for biodegrada-



◘ **Fig. 14.1** Composition of waste water and its possible treatment

tion in the dark at 20 °C after 5 days. The methodology simulates the degradation process that occurs when pollutants are discharged into waters that contain sufficient microorganisms. In this standard method, not all substances are recorded, but only the easily degradable components. These are sugars, proteins and amino acids, organic acids and lipids. The complete oxidation of the unit weight of 1 g of these substances requires different amounts of oxygen (theoretical oxygen demand, ThSB). The respiration of 1 mol of glucose consumes 6 mol of oxygen, which means in units of weight that 1.07 gO_2 is required for the decomposition of 1 g of glucose. For more oxidized substrates, the theoretical values for complete oxidation are less than one; for reduced substrates, they are greater than one (g O_2/g substance): Acetic acid 0.94; proteins 1.46; butyric acid 1.82; triglycerides 2.85; phenol 2.39; methane 4. Since municipal wastewater contains mainly carbohydrates and proteins, a conversion factor from BOD_5 to degradable organic matter of about 1.3 can be derived from this. However, oxygen is not only consumed in the oxidation of organic matter, but also in the oxidation of the ammonium resulting from protein degradation to nitrate (nitrification). The BOD_5 values vary for waters and wastewaters of different origins (◘ Table 14.1).

◘ Table 14.1 BOD5-values of various wastewaters

Waste water/water	BOD_5 values (mg O_2/L)
Municipal waste water	300–430
Biologically treated waste water	15–40
Pure river water	1–3
Dairies and breweries	500–2000
Industrial animal production (manure)	10,000–25,000

Data Fritsche (1998). Environmental Microbiology. Gustav Fischer Verlag, Jena

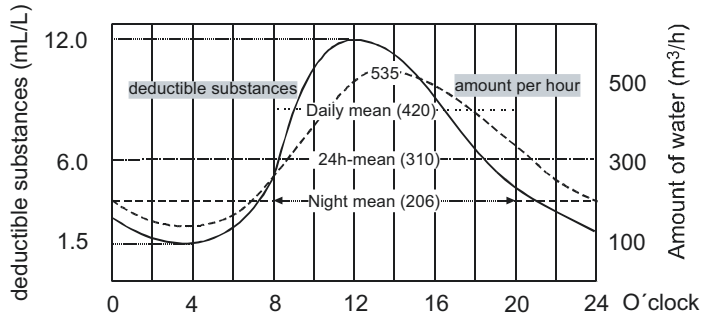
- Not all organic substances in wastewater are microbially degradable. In order to determine as far as possible all organic substances present in wastewater, the **chemical oxygen demand (COD)** is determined. When determining **COD** using chemical oxidation by potassium dichromate, either the amount of oxidant consumed or the equivalent amount of oxygen calculated from it (g O_2/L) is given. The COD value is higher than the BOD_5 value, as it includes not only the readily degradable compounds but also the poorly degradable natural substances and xenobiotics. For municipal wastewater, the COD:BOD ratio is around 1.5–2, for industrial wastewater it is often above two.
- The **DOC (dissolved organic** carbon**)** or dissolved organic carbon forms the **total organic** carbon **(TOC)** together with the undissolved **particulate organic carbon (POC)** and the **volatile organic carbon (VOC).** To determine the DOC, the dissolved organic compounds are completely oxidized after filtration and the resulting carbon dioxide is determined using infrared spectroscopy in more recent methods. The result is the concentration of the element carbon, which is present in organically bound form. For natural waters, DOC values are in the range of a few milligrams per litre. Typical values are 0.7 for groundwater, 2–10 for rivers and 10–60 mg/L for swamps/moors. Experience has shown that the element carbon accounts for about 50% by weight of organic compounds. Thus, with a DOC of 15 mg/L, there are about 30 mg/L of organic compounds present in the sample.
- The sum parameter **AOX** records the **"adsorbable organic halogen compounds"** in water (in chemistry, X stands for the halogens fluorine, chlorine, bro-

mine and iodine). In the analytical determination of AOX, the compounds in question are adsorbed on activated carbon. The carbon is then burned and the resulting halogen acids are measured by precipitation with silver ions and the resulting power consumption. The AOX thus covers the majority of all organochlorine, bromine and iodine substances contained in a sample. When evaluating the measurand AOX, it must be noted that certain polar chlorine compounds adsorb poorly on activated carbon and are thus only partially detected.

Municipal wastewater is defined as the wastewater generated by households, municipal facilities (schools, hospitals) and small businesses (slaughterhouses, laundries, restaurants) within a municipality, including the precipitation that enters the sewer system. Over the course of the day, both the **volume of wastewater** per inhabitant and the **composition** and **concentration of** pollutants show characteristic fluctuations (◘ Fig. 14.2). The quantity and composition of the wastewater also change depending on the day of the week (working day—holiday), the time of year (seasonal operation, holiday period) and the operating conditions of trade and industry. For example, uncontrollable and unpredictable events (e.g. "operational breakdowns" in businesses, thunderstorm rainfall after a long dry period) can cause the substrate supply to increase several times over in the short term (load surges) or the volume and thus the concentration and retention time in the plant can change. Therefore, there is neither qualitatively nor quantitatively a uniform substrate supply for the organisms contained in the wastewater treatment plant. Domestic wastewater contains mainly organic substances originating from urine, faeces, rinsing, cleaning and washing water. Wastewater contains a wide range of different substances: from organic substances that are easily degradable by microorganisms (carbohydrates, proteins, fats), urea from excrement, salts and sand to substances that have a disturbing effect in wastewater treatment plants, such as surfactants (from detergents).

The **population equivalent** (PE) is a calculated value for wastewater treatment. It is a measure of the load of commercial and industrial wastewater with organically degradable substances—measured as BOD_5, which indicates the number of inhabitants to which this load corresponds. It is therefore used to compare industrial, commercial and domestic wastewater and is used, among other things, as a measure for calculating the size of sewage treatment plants (wastewater treatment). A population equivalent corresponds to the amount of organic pollutants discharged daily into the wastewater by one inhabitant. The value of this pollutant quantity in Germany is on average 60–65 g BOD_5 per inhabitant and day. According to an average wastewater volume of 150–200 L (per inhabitant and day), this results in a BOD_5 in untreated wastewater of 300–430 mg/L.

Industrial wastewater is the wastewater produced in industrial and commercial



◘ **Fig. 14.2** Variations in the volume of wastewater and the settleable substances it contains in a city of 50,000 inhabitants over the course of a day. (After Imhoff & Imhoff, 1993)

Table 14.2 Wastewater load from various industries

Operation	Wastewater load (population equivalents)	During the processing or production of
Brewery	150–350	1000 L beer
Dairy (without cheese dairy)	100–200	1000 L milk
Starch factory	500–900	1 t maize or wheat
Wool Laundry	2000–4500	1 t wool
Paper mill (depending on paper quality)	200–2000	1 t paper
Slaughter-house	3000	100 pigs

Modified according to Präve et al. (1994)

operations in connection with production processes. The quantity and composition **are** therefore determined by the **type of raw products** and **processing methods.** In principle, wastewater from all enterprises that process **vegetable** or **animal** substances is amenable to biological treatment, e.g. food industries, distilleries, paper mills, tanneries, wool laundries (see Table 14.2). However, organically contaminated wastewater from the **chemical** industry can also be treated by biological processes.

14.2 Waste Water Treatment in Mechanical-Biological Treatment Plants with Aerobic Stage

A **wastewater treatment plant** is a plant for the treatment of wastewater (elimination of the constituents) with the aim of reducing the harmful effect. As an important ecological link in the material cycle, a wastewater treatment plant thus has the task of returning water of comparable quality to the receiving watercourse (a natural flowing watercourse, stream, river) and humus-like sludge to the soil. The wastewater must therefore be treated to such an extent that the water bodies can be used for fishing and recreation after discharge and aquatic ecosystems that are as close to their natural state as possible can develop. This also means that preventing the eutrophication of water bodies, including the seas, is a central task of wastewater treatment.

The various purification processes of a common wastewater treatment plant can often be summarized in three **stages** (Fig. 14.3):

- mechanical cleaning,
- aerobic biological purification and
- post-treatment to reduce the inorganic mineralisation products ammonium, nitrate and phosphate produced during degradation. Today, this step is often integrated into the second stage.

These three stages are often followed by **treatment of** the **sludge** produced in the three stages by aerobic composting or anaerobic digestion. The sludge can thus be used for biogas production, and if the pollutant content is low, it can also be used for soil improvement. Otherwise, it must be disposed of after dewatering by sewage sludge landfilling or incineration.

In the first stage, **mechanical cleaning** is carried out by **rakes** for the coarse cleaning of pieces of wood, leaves and fabric residues as well as by a **grit trap,** in which reduced flow velocity ensures that sand or other granular mineral substances washed in with the water are removed from the water by settling in long grit traps or circular basins. Fats and oils are separated in **oil separators** due to their lighter specific gravity. **Preliminary clarification** begins in a sedimentation tank, where settleable (sedimentable) pollutants settle to the bottom during

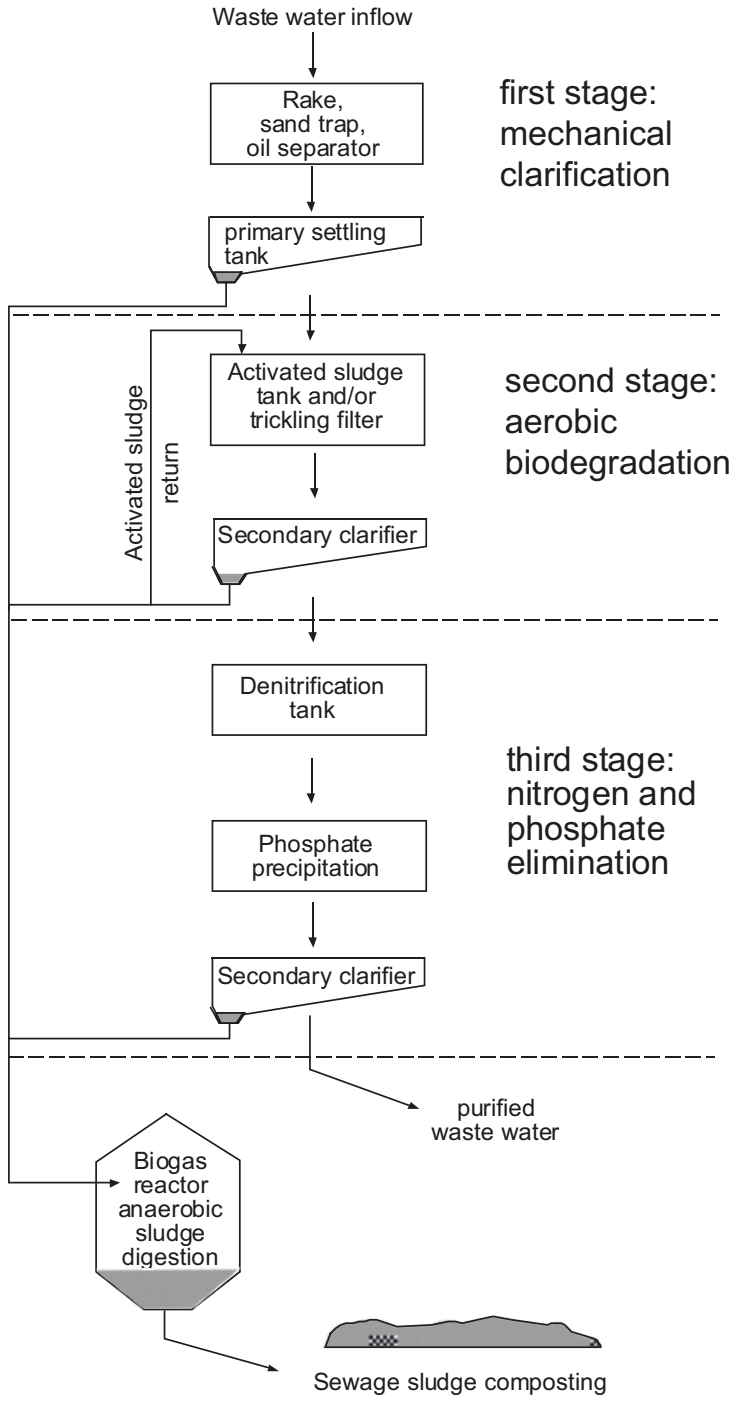
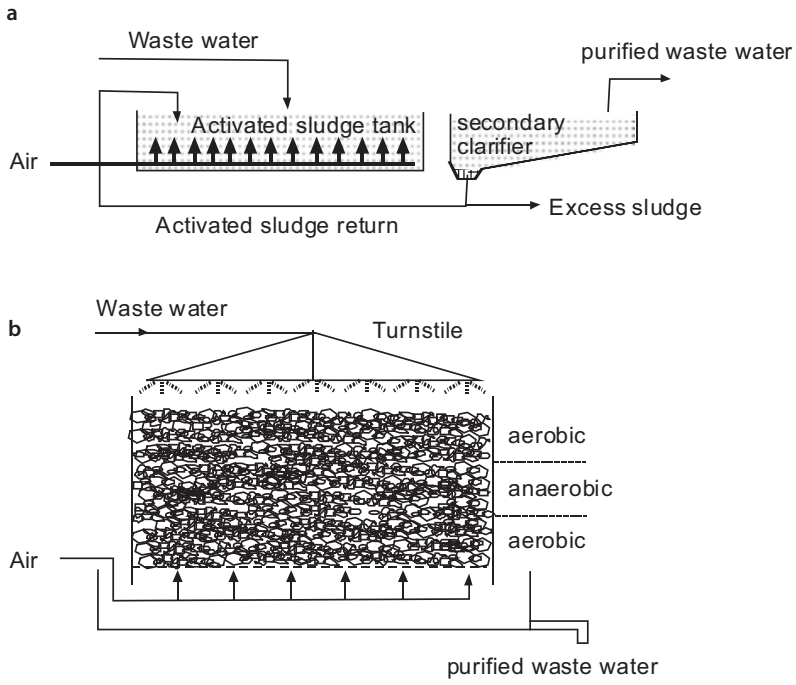


Fig. 14.3 Design of a three-stage municipal wastewater treatment plant with subsequent sludge digestion and biogas generation

a residence time of several hours at a significantly slowed flow. Here already 30–35% of organic matter is retained. The resulting sludge is fed to a central after-treatment by anaerobic digestion or composting. Two thirds of the organic pollutant load remains as dissolved or finely distributed (suspended or colloidally distributed) substances, which are mainly accessible to aerobic degradation by the bacteria already present in the water. This supernatant is fed to the second stage.

In the second stage, the **aerobic biological degradation processes** usually take place. In the case of more highly polluted wastewater, the degradation of organic substances is limited by oxygen. The oxygen demand required for substrate degradation by respiration is considerable. Respiration of 1 g of glucose requires about 1 g of oxygen as discussed above. Since microorganisms require dissolved oxygen, but the solubility of oxygen in water is very low (8.9 mg O_2/L at 20 °C at normal pressure), oxygen deficiency occurs very quickly if oxygen is not continuously replenished by aeration. The core of every aerobic wastewater treatment plant is therefore an effective aeration system, and the **type of air supply** determines the technology of aerobic wastewater treatment, whereby the activated sludge, trickling filter and immersed disk processes should be mentioned here (◘ Fig. 14.4).

The three processes mentioned differ fundamentally in the microorganisms and their growth behaviour or localisation. In the activated sludge process, the degrading microorganisms are in **suspension** in flocs. They therefore essentially correspond to the planktonic organisms in the lake or river (although the latter do not sediment in the secondary clarifier like the activated sludge organisms). In the trickling filter or submersible disk, on the other hand, the degrading microorganisms form a **biofilm** on a solid surface. This corresponds to the growth behaviour of benthic microorganisms



◘ **Fig. 14.4** Types of aerobic wastewater treatment reactors. **a** Aeration tank, **b** Trickling filter

(periphyton) on stones or plants in the water body.

The most widely used process is the activated **sludge process**. According to the principle of **continuous culture**, the microorganisms in the activated sludge tank are constantly supplied with new substrate, and due to the supply of nutrients as well as abundant supply of oxygen, the bacteria multiply, biomass is formed. However, except for a few highly concentrated industrial wastewater species, the substrate concentration in the wastewater is so low and the flow rate so high that in a flow-through clarifier the leaching rate is always higher than the growth rate of the organisms. The processes must therefore include means for **biomass recirculation** or retention. In a downstream non-aerated settling tank **(secondary clarifier),** the activated sludge sediments and is thus separated from the treated wastewater. A long storage time of the activated sludge in this anaerobic phase should be avoided. Part of this biomass is returned to the aeration basin in order to concentrate the degrading organisms and thus increase the degradation performance. The excess sludge is fed into the central sludge treatment plant. Due to the necessity of biomass recirculation, the aeration and secondary clarification tanks form a unit whose function is only guaranteed if the settling properties of the activated sludge flocs allow their separation and thickening in the secondary clarification tank. Biomass enrichment in the aeration tank can also be achieved by installing additional growth surfaces, for example fixed beds made of plastic elements or foam cubes, thus adopting the principles of biofilm reactors (compare trickling filters).

For purification, close contact between wastewater, activated sludge flocs and oxygen is required by sufficient **circulation** in the tank. The **aeration** takes place either by compressed air or by surface aerators. The necessary circulation can already be achieved by the aeration or can be realized separately. Instead of air, technically pure oxygen can also be used for gassing the wastewater. Especially in the case of odour-intensive waste water (animal carcass processing, fish processing), **oxygen gassing** offers advantages due to the low waste gas flow.

Depending on the type of feed and design of the aeration tank (2–4 m depth), a distinction is made between the **totally mixed tank with** uniform loading of the activated sludge and the "classic" **tank with longitudinal flow,** in which a decreasing loading gradient forms from the inlet to the outlet. However, a more uniform load can also be achieved by distributed wastewater feed (step loading). In **cascade basins**, on the other hand, an alternation between high loading in the first stage and low loading in the following stages is deliberately forced. Further variants are the 2-stage activated sludge plants with separate sludge circuits, whereby combinations between trickling filter and activated sludge processes are also possible.

In municipal wastewater treatment plants, the **concentration of** the influent substrate is around a value of 400 mg BOD_5/L, the effluent around 30 mg BOD_5/L. Due to the degradation processes, the dissolved oxygen decreases from 8.9 to 2 mg O_2/L. This content still ensures a steady aerobic degradation process. The activated sludge content is in the order of 3 g BOD_5/L. The generation times of the microorganisms of the activated sludge flocs are 1–10 days. For the control and dimensioning of plants, the **sludge age,** the average residence time of the microorganisms in the activated sludge tank, is a decisive parameter, on which the residual contamination is essentially dependent.

In the activated sludge tank, the microbial conversion of organic pollutants to inorganic end products or their incorporation into the activated sludge takes place. Most of the microorganisms do not simply float around as single cells in the aqueous medium, but are embedded in **activated sludge flocs** of 50–300 µm. These are bioce-

noses of varying composition surrounded by a common slime matrix. About 70% of the flocs are organic components, the remaining part are inorganic intercalations, for example of clays, of SiO_2, of calcium carbonates and of iron oxides (Fe_2O_3). Already with traditional, culture-dependent methods, several hundred species of microorganisms had been detected in the activated sludge and, in this respect, quite a considerable **diversity.** In the meantime, several studies have also been carried out using molecular ecological methods. A complete description of the diversity found in activated sludge is still far away, but at least there are some insights. *Proteobacteria* were found to be particularly abundant in activated sludge, and among these, *Betaproteobacteria* are especially dominant. Within this group, *Zoogloea ramigera* (a species in which the single cells are held together by mucus) and members of the genera *Alcaligenes, Azoarcus* and *Brachymonas* have been described as quantitatively important. Other important groups seem to be the *Alphaproteobacteria* especially with *Sphingomonas* species, *Gammaproteobacteria, Epsilonproteobacteria* with *Arcobacter* sp. as well as the *Bacteroidetes* (CFB group), the *Chloroflexi,* the *Nitrospirae* and the *Planctomycetes*. Numerous **protozoa** are also present in the activated sludge. Since different protozoan species prefer different loads and oxygen concentrations, and since they can be easily differentiated by microscopic examination, they are also used to assess sludge condition. For the function of an activated sludge plant it is important that the sludge sediments well in the secondary clarifier. If this process is disturbed by increased development of filamentous bacteria, **bulking sludge** is formed. The CO_2 formed during respiration drives the larger flocs to the surface of the aeration basins, thereby reducing the degradation efficiency and making sedimentation more difficult. Among the filamentous bacteria in wastewater, the physiologically versatile *Thiotrix* sp. (belonging to *Gammaroteobacteria*) and the long known excretory "wastewater fungus" *Sphaerotilus natans* (belonging to *Betaproteobacteria*) play a role, but also other *Proteobacteria* and representatives of various other phyla such as *Actinobacteria, Bacteroidetes* or *Planctomycetes.* The modern possibilities of bacterial identification allow a better understanding of the reasons for the formation of bulking sludge and should help to avoid such problems more easily in the future.

Instead of or in addition to the activated sludge process, **trickling** filters are also used, which can be derived from soil filters (trickling filters) (◘ Fig. 14.4b). These are **fixed-bed reactors** filled with carrier materials such as slags, lava tuffs or plastics. The first mentioned **filling materials,** with a grain size of 40–80 mm, have both a large specific surface area (90–100 m^2/m^3) and a large void fraction (40–60%), allowing good cleaning performance with good aeration. More recently, molded plastic internals have also been developed, allowing even larger surface areas (up to 250 m^2/m^3) and void fractions (up to 95%). The trickling filter itself consists of a cylinder about 2.80–4.20 m high, the diameter of which is determined by the wastewater volume and BOD_5 load and the selected space load. Above the bottom of the body, at a distance of approx. 0.5 m, a grate made of prefabricated concrete elements or similar is attached, on which the trickling filter filling material is supported. The side walls are provided with openings to allow air circulation through the trickling filter.

In order to prevent clogging of the trickling filter by the sludge substances contained in the raw wastewater, an upstream mechanical treatment stage (rake, grit trap, sedimentation tank) is particularly important here. The wastewater is distributed as evenly as possible over the surface with the aid of **rotary sprinklers** and trickles over the trickling filter in 20–60 min. The microorganisms settle as a slimy lawn **(biofilm)** on the carrier materials and are supplied with substrates

by the inflowing wastewater. On the bottom of the hollow bottom, the wastewater that has trickled through the body collects with the flushed out parts of the trickling filter lawn and flows off to the secondary clarifier.

The trickling filters are **aerated** naturally, (a) by the uniform trickling of the wastewater through the rotary sprinkler and (b) by the temperature differences between the inside and outside air. The air in the trickling filter takes on the temperature of the wastewater (heat from the metabolic processes), i.e. it is colder in summer and warmer in winter than the outside air. Accordingly, the air flows downwards or upwards through the body (chimney effect). A prerequisite for good ventilation is sufficient cavity volume throughout the body. In special cases, the trickling filter can be covered and artificially ventilated, which may be necessary, for example, to protect nearby residential areas against odour nuisance and trickling filter flies.

In the trickling filters, communities of **bacteria, fungi, protozoa, small crustaceans, worms** and **insect larvae** develop according to the different milieu conditions. **Blue and green algae** can also grow on the exposed surface. According to the changing nutrient and oxygen supply from the surface to the trickling filter bottom, a vertical **stratification of** the biocenosis can be observed. Furthermore, especially with larger biofilm thicknesses (up to approx. 1 cm), there is also a stratification from the biofilm surface to the substrate: while the organisms at the surface are well supplied with nutrients and oxygen, diffusion processes and swirling movements of the organisms are not sufficient to ensure a good supply in deeper layers as well. Therefore, nutrient deficiencies and anaerobic conditions can occur here, resulting in cell death and detachment of parts of the biofilm. In the upper aerobic zone, a biocenosis forms that is adapted to high substrate concentrations and grows particularly quickly. Here, the detachment of cells occurs again and again. In the lower, also aerobic zone, slower oxidative processes such as the nitrification of NH_4^+ to NO_3^- take place. In addition, anaerobic conditions can occur in deeper layers, which lead to the fermentation of wastewater constituents and the formation of reduced degradation products.

In addition to the described self-regulation of the biofilm thickness through the death of deeper layers, two other factors contribute significantly to the fact that the trickling filters do not become clogged:

- The biofilm of bacteria and fungi is **grazed** by protozoa, nematodes and insect larvae. This process takes place mainly in the upper zones, where there is a particularly strong biofilm increase due to good substrate and oxygen supply.
- The wastewater volume and the **flushing effect** it produces are also essential. In the case of a high BOD_5-space load, it must be kept so high by recirculating treated wastewater that excess biomass is flushed out immediately (flushing trickling filter).

Depending on the BOD_5-space load and the amount of water, there are differences in the biocoenosis as well as the cleaning effect and sludge production. In **lightly loaded** trickling filters, almost all microorganisms adhere to the surfaces of the fillers. The formed trickling filter lawn is only flushed out slowly. In these trickling filters, in addition to a very extensive BOD_5 elimination, nitrification of the ammonium usually also takes place. The organic sludge particles are already largely degraded in the trickling filter. The flushed sludge is thus reduced in quantity, it is relatively low in water and has a low putrefactive capacity. In the case of **highly loaded** trickling filters, on the other hand, organisms are flushed out so that slow-growing bacterial species such as nitrifiers can no longer settle stably, at least in the upper zone. In this case, the trickling filter is only used for wastewater treatment and no longer has the function of sludge treatment, so that

secondary sedimentation is required. The rinsed sludge is still putrescent and rich in water.

Compared to activated sludge processes, trickling filters have the advantage that they have a highly differentiated biocoenosis, which leads to extensive mineralisation without a large amount of activated sludge. Disadvantages are the **reduced possibilities for process control,** for example in the case of intermittent accumulation of toxic substances, as the dilution effect is missing. With the increasing possibilities of measurement and control technology and the necessary elimination of nitrogen and phosphorus, the trend is therefore towards activated sludge processes.

In contrast to the classic trickling filter, in which the biomass is fixed and the wastewater is moved, there are biofilm processes in which the biomass is moved through the wastewater. A special form are the **disc immersion filters.** These are round discs several meters in diameter, spaced about 10 cm apart on a shaft. The discs represent the growth surface for microorganisms. Half of the slowly rotating disc packs are immersed in the wastewater. Through slow rotations of the roller, the biomass on the discs is alternately immersed in wastewater and air, resulting in an alternating supply of substrate and oxygen to the organisms.

14.3 Biological Phosphate Elimination

Phosphorus is required by organisms as a vital nutrient in building material and energy metabolism and is therefore absorbed from the environment as phosphate. Phosphates must be removed from wastewater to **prevent eutrophication of** water bodies. Phosphates lead to the temporary mass development of algae and cyanobacteria (algal blooms), 1 g P is sufficient for about 100 g biomass dry matter. For cyanobacteria, phosphorus is the element that limits growth, especially since many species are not dependent on fixed nitrogen due to their ability to fix atmospheric nitrogen. Phosphorus is present in wastewater mainly as phosphate, which comes from the breakdown of nucleic acids of food substances and from detergents. Approximately 3 g of phosphorus per inhabitant and day is produced, two thirds of which is due to **foodstuffs and** one third to **detergents.** The proportion of phosphates from detergents has fallen sharply in recent years as the phosphates added to surfactants to soften water have been replaced by more environmentally friendly agents such as zeolites (sodium aluminium silicates) and sodium citrate (low-phosphate and phosphate-free detergents). However, as the phosphate content from the human diet remains constant, phosphate elimination is still necessary. For aerobic growth, a balanced ratio of C:N:P is theoretically about 100:14:3. In pretreated domestic and municipal wastewater, the ratio is about 60:12:3, so the P content of the wastewater greatly exceeds the amount needed for normal growth of the activated sludge or biofilm. Therefore, the wastewater treatment microorganisms assimilate only about 30% of the phosphate present in the wastewater. The effluent of the activated sludge tank still contains 10–20 mg/L total phosphorus. The EU directive (91/271/EEC) stipulates that the P content (total phosphorus) should be below 1 mg/L for more than 100,000 population equivalents.

In the context of **chemical phosphate elimination**, phosphate ions can be removed from the water by precipitation with the aid of Fe^{3+} or Al^{3+} ions. Precipitation can take place at three different points in a wastewater treatment plant. If the precipitant is already added in the area of the aerated grit chamber or directly in the inlet to the primary clarifier, the phosphate fraction introduced (without the phosphate released during further degradation) is precipitated together with organic substances. In the case of simultaneous precipitation, the precipi-

tant is added in the area of the aeration tank. The floc separation takes place in the secondary sedimentation. The settling properties of the activated sludge are improved in this most widely used and operationally reliable process. An average concentration in the effluent of about 1 mg/L total P is achieved. The third process, post-precipitation, requires a separate precipitation and settling tank. It is the most effective, biologically undisturbed, but also the most expensive process variant. The precipitant is dosed at points of high turbulence. In the zones of lower turbulence, floc growth is then favoured. The combination of precipitation and flocculation filtration achieves effluent values between 0.2 and 0.3 mg/L. Chemical phosphate elimination by precipitation with iron and aluminium salts (chlorides, sulfates) is expensive due to the cost of the precipitants, leads to the salinisation of the treated water and to metal contamination from the sludge produced.

Due to the above-mentioned disadvantages of the established chemical processes, **biological phosphate elimination** processes are also becoming increasingly important. The basis of the biological elimination of larger amounts of phosphate by normal assimilation (**EBPR**, **enhanced biological phosphorus removal**) is the ability of some bacteria to store phosphate as **polyphosphate.**

The **polyphosphate-accumulating organisms** (PAOs) are obligate aerobes and are enriched by an alternation of anaerobic and aerobic conditions in wastewater. The following processes take place in them:

- In the **aerated stage,** anaerobically stored lipid reserve substances and easily degradable exogenous substrates, for example the fermentation end products from the anaerobic stage and easily degradable organic substances from the influent, are used as a source of energy and carbon for growth (◘ Fig. 14.5). By utilizing the endogenous storage substances, the cells can quickly adapt to the aerobic environment and immediately begin respiratory metabolism and growth. The energy gain from the respiration of the endogenous and exogenous substrates serves simultaneously for the uptake of phosphate, its **storage as polyphosphate** and the synthesis of polyglucose (glycogen-like).
- In the **anaerobic stage of** the wastewater treatment plant (oxygen- and nitrate-free zone), various metabolic processes take place which are important for the subsequent increased phosphorus removal from the wastewater in the aerated zone (◘ Fig. 14.5). The fermentation metabolism of facultative anaerobic bacteria produces predominantly short-chain organic acids (for example, acetate and propionate). These are taken up by the polyphosphate-storing bacteria, converted and stored as lipid reserve substances (for example, poly-β-hydroxybutyric acid, PHB). The energy for their synthesis comes from the polyphosphates previously stored, under aerobic conditions, so phosphate is partially released again from the cells. The polyphosphates probably also serve the cells as a source of energy for survival in the anaerobic environment.

Thus, the organisms have three types of storage substances, the relatively reduced poly-β-hydroxybutyric acid, which is deposited in the cell in the form of large inclusions, the small granules of polyphosphate, originally called volutin, and the relatively oxidized polyglucose (glycogen-like). By storing organic reserve substances under anaerobic conditions, the obligate aerobic polyphosphate-storing bacteria appear to have growth advantages over the other obligate aerobes, which aerobically depend exclusively on exogenous substrates for their syntheses. It is likely that these metabolic characteristics are responsible for the accu-

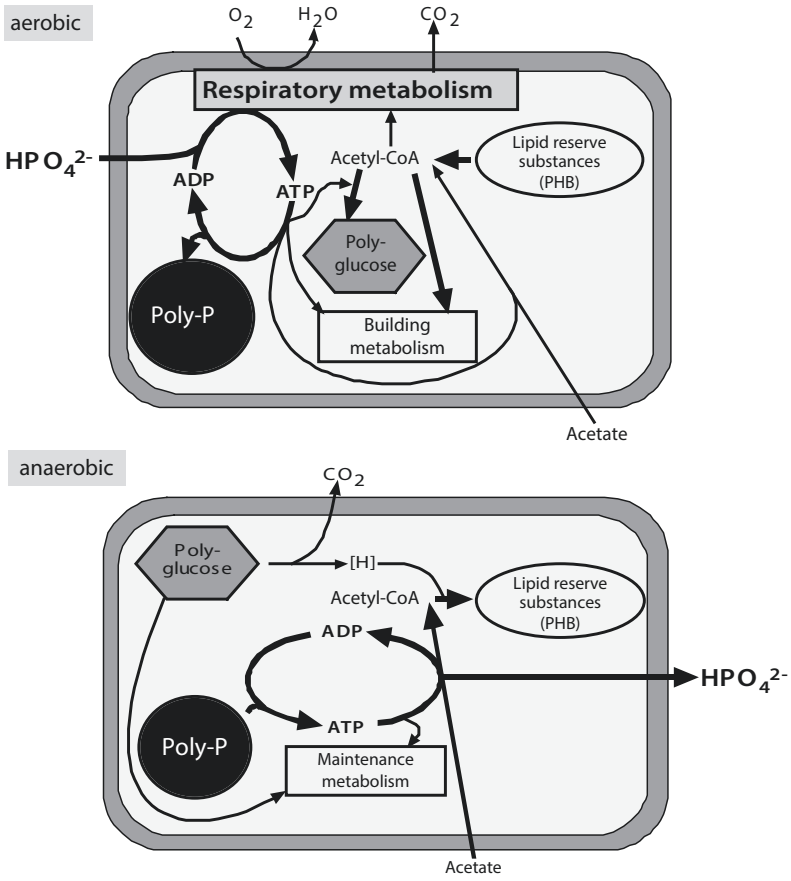


Fig. 14.5 Schematic representation of the metabolism of obligate aerobic, polyphosphate-storing bacteria under aerobic and anaerobic conditions. *Poly-P* polyphosphate; *PHB* poly-β-hydroxybutyric acid; *[H]* reduction equivalents. (Modified after Lemmer et al., 1996)

mulation of polyphosphate-storing bacteria in the activated sludge under appropriate anaerobic-aerobic processes. Figure 14.6 shows schematically the material changes during the change from anaerobic to aerobic conditions.

Acinetobacter calcoaceticus has long been mentioned as a typical representative of polyphosphate-accumulating organisms. However, on the basis of molecular biology studies, other bacteria such as *Accumulibacter phosphatis*, which belongs to the *Betaproteobacteria,* have been found to play a more significant role in the process of extensive biological phosphorus removal.

Other so-called **glycogen-non-polyphosphate-accumulating organisms** (GAO) were discovered in addition to the PAO in the sewage treatment plants. In terms of carbon, GAOs have a metabolism comparable to that of PAOs and compete with PAOs for the organic compounds formed under anaerobic conditions. However, GAOs do not release phosphorus during the anaerobic-aerobic cycle, nor do they accumulate it as PAOs do. All the energy for GAO during the anaerobic phase is thought to be provided by glycolysis of the stored glycogen. Thus, one of the problems for the operator of a wastewater treat-

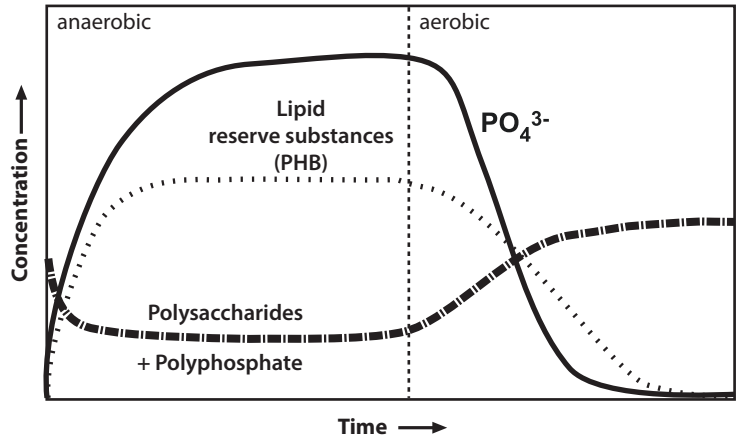


Fig. 14.6 Schematic representation of the material changes in phosphorus and carbon during the biological phosphate elimination process. (Modified from Blackall et al., 2002)

ment plant is how to exclude the GAOs and promote the PAOs, since the identical conditions (anaerobic-aerobic cycling of biomass) select both types of organisms, with the GAOs running counter to the process of phosphorus elimination by competing for the organic compounds.

The effectiveness of the EBPR nevertheless becomes clear when the following comparison is made: While the P content of a typical activated sludge is 1.5–2% P per dry mass, the EBPR achieves 4–5% and in laboratory systems even up to 15%.

Two process principles are used for biological phosphate elimination, main stream and side stream processes. In the **main stream process,** the phosphorus stored as polyphosphate is removed with the cell biomass. A simple variation is the A/O process shown in ◘ Fig. 14.7a. It is carried out in a cascade of tanks downstream of the aeration tank. Nitrogen must have been eliminated beforehand, as nitrate interferes with the processes. In the upstream anaerobic stage, organic acids are formed by fermenting organisms, from which polyphosphate-accumulating organisms form PHB. At the same time, a degradation of polyphosphate takes place, which leads to phosphate redissolution. In the subsequent aerobic stage, polyphosphate is synthesized again. This phosphorus-rich activated sludge is largely removed, a small part is recycled.

In the **bypass process,** the biomass, which contains a lot of polyphosphate, is fed into a bypass stream. This contains an anaerobic pool known as a stripper, in which phosphate is dissolved back. The low-phosphate biomass is then returned to the main stream, while the concentrated phosphate is precipitated from solution with $Ca(OH)_2$. The resulting calcium phosphate can be used as a fertilizer. ◘ Figure 14.7b shows in the lower part the flow diagram for the **phostrip process,** the most common variant of the side stream process. It is the combination of a biological enrichment process with a chemical precipitation process.

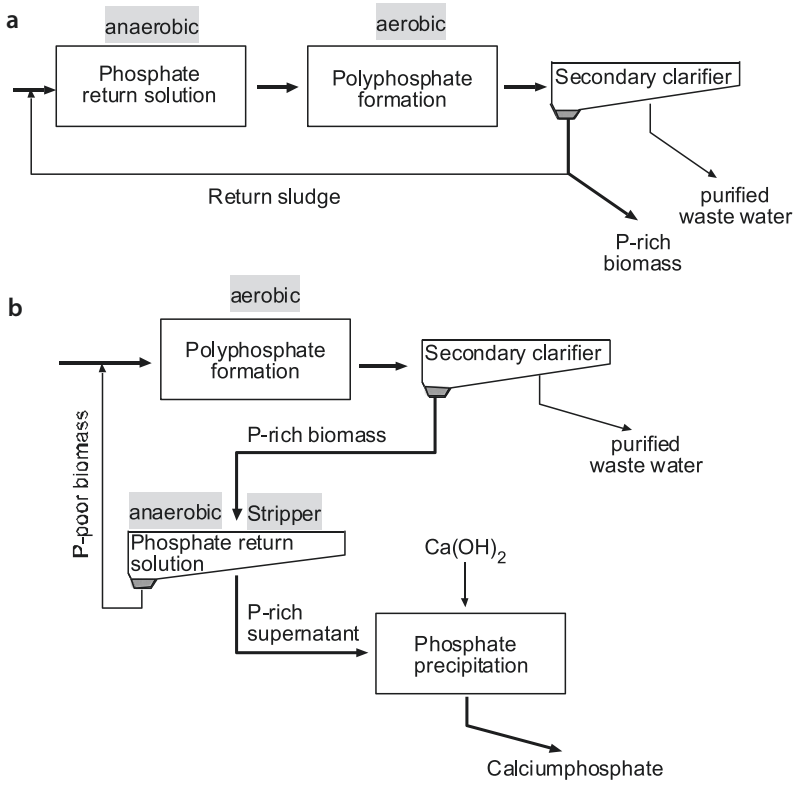


Fig. 14.7 Process variants of biological phosphate elimination. **a** A/O process, **b** Phostrip process

14.4 Nitrogen Elimination During Waste Water Treatment

Domestic and municipal wastewater contains up to 30 mg ammonium/L, the levels of which increase further as a result of the decomposition of organic nitrogenous substances during wastewater treatment. Since NH_4^+ is both a fish poison and leads to high oxygen depletion in water bodies through oxidation to NO_3^- (nitrification), it must be oxidized in a wastewater treatment plant and the NO_3^- formed must also be removed as N_2 if possible.

The inorganic compounds contribute to the eutrophication of water bodies. Municipal wastewater has lower C:N ratios of 100:20, but since the C:N ratio encountered in cells is 100:10 to 100:15, only a portion of the nitrogen enters the formation of the activated sludge biomass and is thus removed from the wastewater.

The elimination of nitrogen compounds can be achieved in the activated sludge process through the coupled use of nitrification and denitrification processes (cf. ► Chap. 7). Nitrogen is usually present in wastewater in reduced form (NH_4^+) alongside organic compounds. Therefore, ammonium must first be oxidized to nitrate by **nitrifiers.** Therefore, a mixed biocoenosis between organo-heterotrophic bacteria and the litho-autotrophic nitrifiers forms in the aeration tank. As **ammonium oxidizers** among the nitrifiers, the *Nitrosomonas europaea/ Nitrosomonas eutropha* group, the *Nitrosomonas marina* relatives and the *Nitrosococcus mobilis* group seem to be of

particular importance in wastewater treatment plants. Among the **nitrite oxidizers,** non-cultivated members of the genus *Nitrospira* seem to dominate in many plants due to higher affinity for NO_2^- and O_2 compared to the *Nitrobacter* species previously considered important. Process-wise, a key bottleneck of biological nitrogen elimination is the **low growth rates of nitrifiers.** Therefore, the **sludge age** (retention time of the biomass in the system) must be selected high enough to allow the relatively slow-growing nitrifiers to be present in the mixed biocoenosis.

A second central problem of biological nitrogen elimination lies in **denitrification**, namely in the **provision of the H donors** in the form of biodegradable substrates. Dentrification of NO_3^- to N_2 requires nitrification of the reduced N compounds. Since this occurs simultaneously with the aerobic degradation of the organic compounds in the aeration tank, no H donors are subsequently available for rapid and complete denitrification of the nitrate formed. However, denitrification, a form of anaerobic respiration, requires organic matter. This fact has led to the following process options (◘ Fig. 14.8):

- In the previously dominant **downstream denitrification,** organic substances, for example methanol, are added to the water from a nitrification stage in a non-aerated basin as an H-donor. This expensive and complex arrangement has been replaced by modern processes of upstream and simultaneous denitrification.
- In **upstream denitrification**, the nitrate formed in the aeration tank is transported by recirculation of water from the nitrification together with the return sludge from the secondary clarifier into an upstream anoxic denitrification tank or into the initial area of the aeration tank where the highest concentration of organic matter is present. By not aerating, the anaerobic conditions required for denitrification are obtained. In these cases, we speak of anoxic conditions, since no oxygen is present, but nitrate or nitrite are present as electron acceptors. Nitrate can only be reduced according to the return/recirculation conditions. Denitrification is therefore limited here by the hydraulic load and the unintended introduction of oxygen into the denitrification zone.
- In **simultaneous denitrification**, the wastewater flow passes through unaerated and aerated zones one after the other, denitrification and nitrification take place in one basin. Alternation between aerobic and anoxic phases in the aeration tank is achieved by interrupting aeration or alternation of aerobic and anoxic zones in a recirculation tank by staggered aeration.

A fundamental **deficit of conventional processes** for the removal of nitrogen from wastewater is that the **ammonium** is first oxidized to nitrate with considerable oxygen consumption, and this must then be reduced again (◘ Fig. 14.9a). If, as in the case of downstream denitrification, extra organic compounds are added to the water as electron donors, the high oxidation level of the nitrate also increases the consumption of these. Both increase the costs of water purification. With regard to the consumption of electron acceptor and electron donor, it would actually be more favourable if the ammonium were only oxidised to nitrite and then reduced to molecular nitrogen (◘ Fig. 14.9b). It would be even more advantageous, especially in ammonium-rich effluents, if the ammonium itself could act as an electron donor. The part oxidized directly to N_2 would then not have to be oxidized further at all, and in addition the electron donors could be largely saved (◘ Fig. 14.9c, d). Recent studies on the microbiology and process engineering of

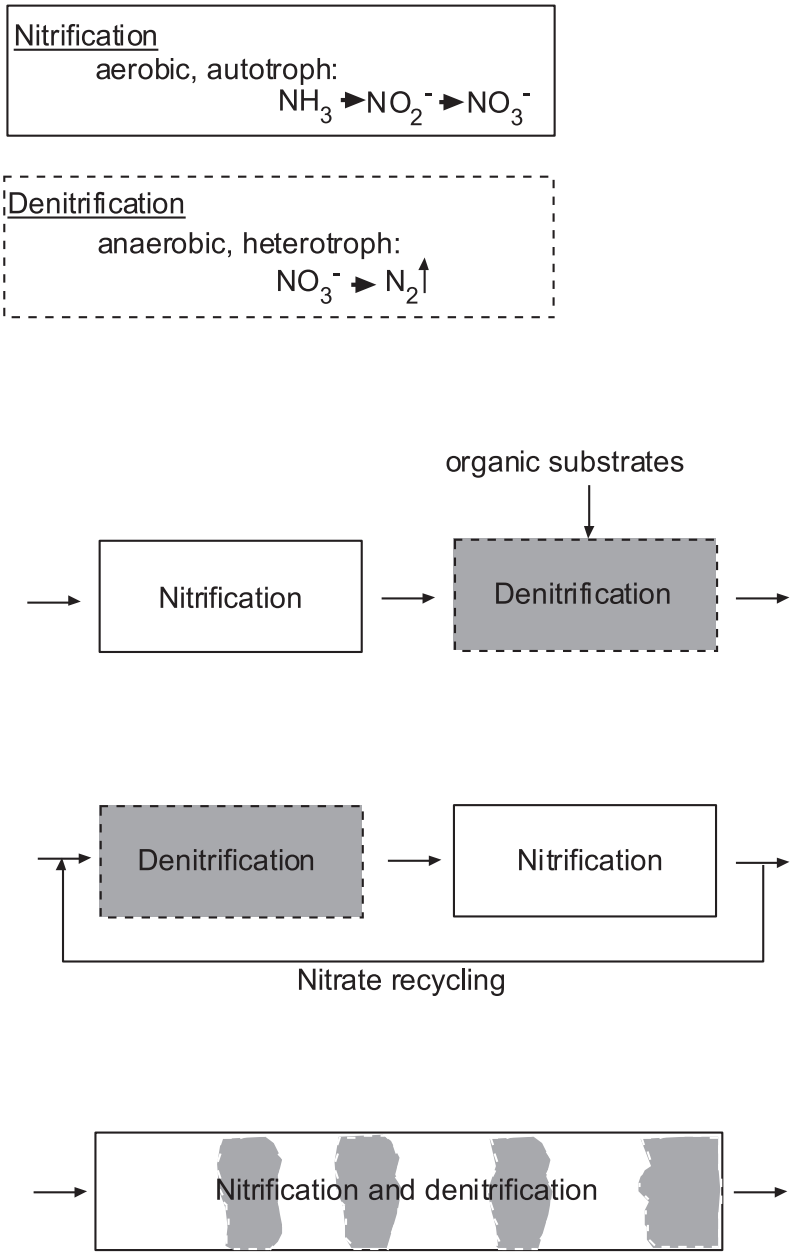
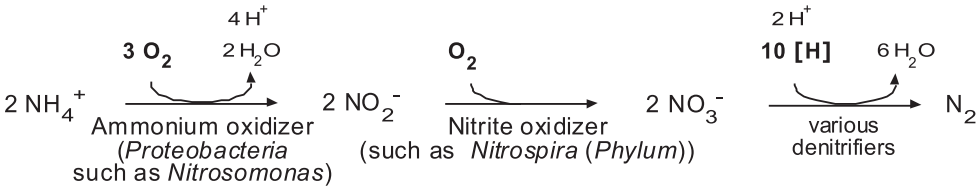


Fig. 14.8 Different process combinations of nitrification and denitrification processes

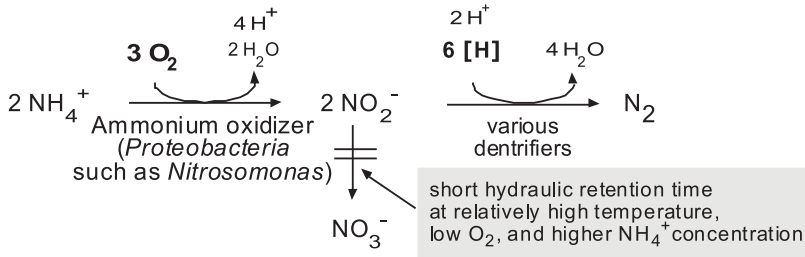
nitrogen transformations show that such **processes,** which are **more advantageous** with regard to the redox ratios, are possible in principle.

The consumption of oxygen and electron donor can already be limited if it is possible to carry out only **partial nitrification** and to couple this with **conventional denitrification** (albeit with nitrite as the first electron acceptor) (Fig. 14.9b). Partial nitrification can be implemented by running nitrification at relatively high temperatures (>26 °C) and

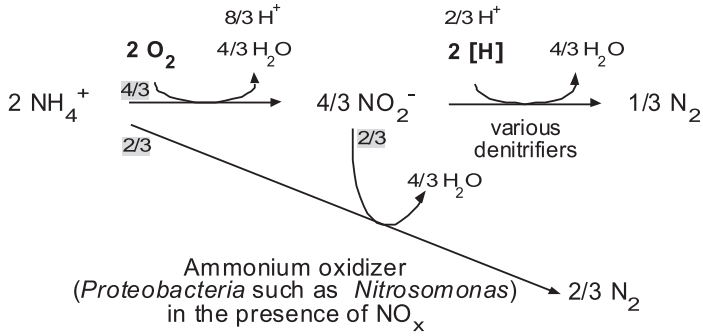
a **Conventional nitrification and dentrification**



b **Partial nitrification and conventional denitrification**



c **NO_x-process with ammonium oxidizing denitrification**



d **Partial nitrification and Anammox-process**

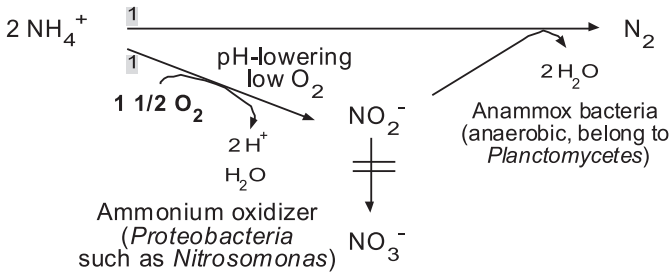


Fig. 14.9 Schematic process, microorganisms involved and consumption of O_2 as well as reduction equivalents in conventional **a** and various alternative **b–d** processes for removing ammonium from wastewater. For better comparability, all figures refer to the oxidation of two ammonium ions to one nitrogen molecule. The values for the consumption of O_2 and reduction equivalents ([H]) are highlighted in each case

relatively short residence times without sludge recirculation. Ammonium oxidizers grow faster than nitrite oxidizers at higher temperatures and are not washed out, while nitrite oxidizers are washed out due to the lack of sludge recirculation. Additionally, at limiting O_2 concentrations and excess ammonium, nitrite oxidizers are inhibited and cannot grow, which could also allow processes without elevated temperature.

It has been found that conventional proteobacterial ammonia oxidizers can denitrify not only under anaerobic conditions but even under aerobic conditions, provided that the presumably regulatory effective (and at the same time toxic for nitrite oxidizers) nitrogen oxides NO or NO_2 are present. This effect can be exploited to save even greater amounts of O_2 and electron donor in nitrogen removal compared with conventional methods (◘ Fig. 14.9c). In the so-called **NO_X process,** proteobacterial ammonium oxidizers related to *Nitrosomonas* cause, on the one hand, a **partial nitrification of** ammonium to nitrite under the influence of NO_X. On the other hand, part of the nitrite is then used as an electron acceptor for an **ammonium-oxidizing denitrification.** Another part of the nitrite still has to be reduced to N_2 by conventional **denitrification**, although the consumption of electron donor is significantly reduced.

The maximum theoretical reduction in the consumption of O_2 and electron donors is achieved by coupling partial **nitrification** and the **anammox process** (◘ Fig. 14.9d). Ammonium oxidation to nitrite by conventional proteobacterial ammonium oxidizers is limited to 50% by limiting oxygen and allowing a pH decrease that occurs as a result of acid formation. Under anaerobic conditions and in the absence of any other electron donor, **anaerobic ammonium oxidizing** bacteria then prevail. These anammox bacteria belong to the planctomycetes and obtain energy by **ammonium-oxidizing denitrification** (Sect. ► 7.4). As the anammox bacteria grow very slowly, corresponding plants currently still have an extremely long start-up time. In addition, sufficient sludge recirculation is particularly important.

14.5 Anaerobic Sludge Treatment, Direct Anaerobic Wastewater Treatment and Biogas Production

During the various steps of wastewater treatment, **sludge** rich in organic substances accumulates in the clarifiers. In the first mechanical stage it is particulate pollutants (primary sludge), in the following stages the bacterial flocs with adsorbed pollutants. Per inhabitant value (60–65 g BOD_5 per inhabitant and day) about 10 g sludge dry matter are produced in a sewage treatment plant, the share of organic substances is about 70%. The sludge is very rich in water (about 90%), because the bacterial slimes (extracellular polysaccharides) bind water. **The aim of sludge treatment** is to recycle and dispose of the sludge. In the process, no odour nuisance should occur (deodorisation) and the end products should be hygienically harmless (hygienisation). In addition to incineration and landfilling, the **processes used** include composting (► Sect. 17.2) and anaerobic digestion in conjunction with biogas production.

Anaerobic **sludge digestion** combined with biogas production is the most attractive process, as it combines waste disposal with energy production. However, this process requires relatively complex equipment, the digestion towers or digesters. In modern wastewater treatment plants, they stand out as large pear-shaped or cylindrical structures, usually in multiple versions. The large dimensions are due to the fact that the digestion process is slow. Since the retention time of the sludge is several weeks, great efforts are made to speed up the process.

The microbiological basis of sludge digestion is the **methanogenic food chain** discussed in ► Sect. 4.5.1 (► Fig. 4.23). Four metabolic types interact in this process: Primary fermenting organisms convert the initial substrates to organic acids and alcohols as well as H_2 and CO_2. Acetogenic bacteria obtain energy by forming acetate from the formed H_2 and CO_2. Methanogenic archaea form methane from the acetate or directly consume H_2 and CO_2. Finally, an important link in this food chain is the fourth type, secondary fermenters, which further oxidize the initially formed organic acids and alcohols to acetate or CO_2, releasing H_2. These reactions are often endergonic under normal conditions. Therefore, such organisms can only grow if the hydrogen formed is constantly removed by acetogenic or methanogenic microorganisms. The associations here are very close, including the occurrence of symbiotic relationships of interspecies hydrogen transfer. To ensure these **syntrophic relationships,** close contact between the microorganisms involved is necessary, which must not be disturbed by strong turbulence in the reactors. Another prerequisite for effective sludge digestion is the constant consumption of the acids formed in the initial reactions. Both fermentations and acetogenesis produce organic acids. If they accumulate, methanogenesis, which proceeds optimally in the neutral pH range, is inhibited. As the pH decreases, the organic acids are increasingly present in the undissociated and more inhibitory form. The maintenance of an optimal pH value in the reactors is achieved with the help of buffer tanks.

The organisms in the methanogenic food chain gain only little energy from the respective material conversions, much less than, for example, aerobic organisms. Therefore, little biomass is formed, or a great deal of organic material is converted to the respective metabolic product for biomass formation. In anaerobic sludge digestion, about 10% of the compounds used are assimilated and 80–90% are converted to biogas. This **carbon balance** of sludge digestion is advantageous in two ways. First, due to the low new biomass formation, only **small amounts of digested sludge are** ultimately produced. Although the digested sludge can in principle be used as a fertilizer, sewage sludge utilization, depending also on the pollutant load (see below), poses problems that are difficult to solve, especially in urban areas. Secondly, by far the **greater part of** the organic material is converted into **biogas**, which is a versatile source of energy. Biogas is a mixture of 60–70% methane, 30–40% CO_2 and small amounts of H_2S and NH_3.

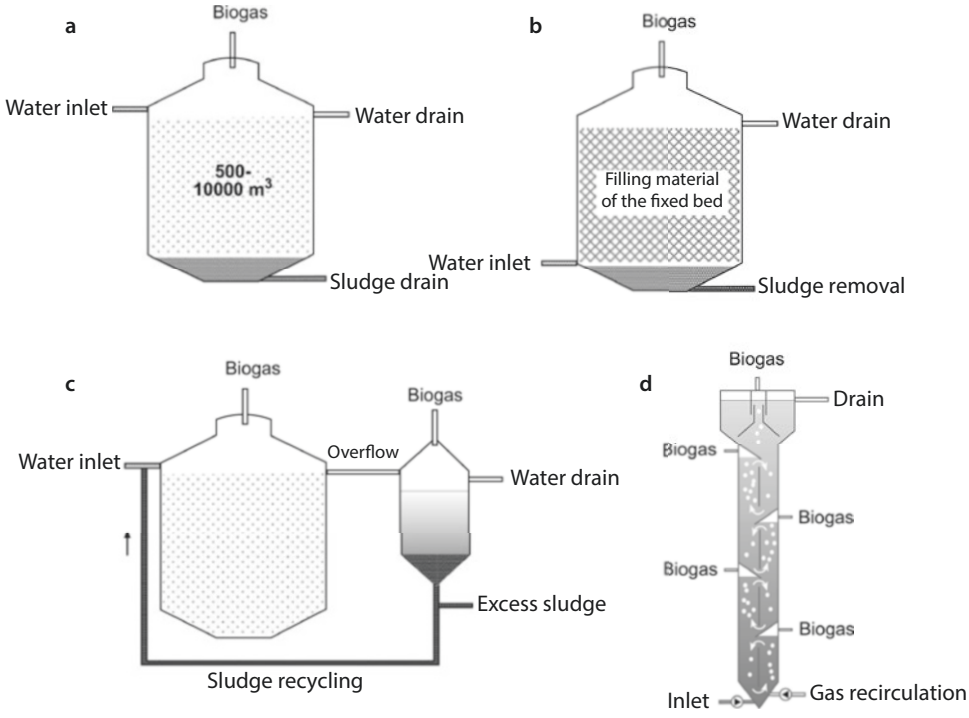
Biogas (Gülzower Expert Talks, 2003)

- Biogas is enjoying enormous popularity as an energy source, especially in the agricultural sector. In 2003, more than 2000 biogas plants with an installed electrical capacity of over 250 megawatts were in operation in Germany. In agricultural biogas plants, only one third of the fuel energy is converted into electricity. The remaining 2/3 is generated as heat, which is rarely used to any significant extent.
- Nevertheless, the potential for energy generation from biogas is far from exhausted today. New options of biogas for the future are (a) use in fuel cells or micro gas turbines, (b) feeding into public gas grids or (c) use as fuel.
- An important problem to be solved is gas purification.

From an energy point of view, **biogas formation** is a very effective process. More than 80% of the energy contained in the organic matter is converted into methane. Up to 1 m^3 of biogas is produced from 1 kg of organic matter, which is equivalent to the energy content of 0.5 L of crude oil. The calorific value of biogas is 20,000–25,000 kJ/m^3, which is about half the calorific value of

natural gas. The necessary gas purification (drying and removal of H_2S) reduces the achievable energy. Considering that large amounts of wastewater and thus sewage sludge are produced from agriculture and the food industry, this results in significant alternative energy sources from wastewater and waste. For example, each dairy cow produces about 45 L of liquid manure with 5 kg of dry matter per day, from which about 3 m^3 of biogas is formed. In the biogas reactors commonly used today, about 1 m^3 of biogas is formed per 1 m^3 of reactor volume and day, and the retention time is between 10 and 30 days.

The design of **biogas reactors** (◘ Fig. 14.10) must always take into account the low growth rates of the microorganisms involved in methanogenesis, in addition to the quantities produced and the possible technical effort required. In order to achieve an intensification of sludge digestion and biogas formation, the biomass once formed is used in high concentration for as long as possible. For this purpose, as already discussed in ► Sect. 14.2 for aerobic plants, an **enrichment and recycling of the bacterial biomass** is necessary. At the same time, the cells with the various metabolic activities are kept together in **functional associations.** Since aggregation of the cells into sedimenting flocs is difficult to achieve, inert particles such as sand can be added for colonization. This ensures that the biomass is sedimented rather than washed out. The forming sludge beds are flown through from below and thus kept in suspension **(fluidized bed reactor)**. **Fixed bed reactors** are completely loaded with carrier materials such as plastic fillers, which have the lowest possible weight and a



◘ **Fig. 14.10** Process variants for biogas production. **a** Digester with homogeneous sludge distribution by circulation, **b** Anaerobic fixed-bed reactor in which the bacteria grow on filling material, **c** Contact process with separation of the biomass in a settling tank and recirculation of the biomass, **d** Biogas tower reactor with freely suspended biomass

high settlement area. In the case of the **biogas tower reactor** (pilot plants: 20 m height, 1 m diameter, volume: 15 m^3), the biomass is present in granular or pellet form. The biogas is drawn off laterally in a controlled manner through internals in order to avoid critical gas loading in the upper area. The internals as well as a sedimenter installed in the reactor head ensure biomass retention.

Sludge digestion is predominantly carried out at 30–37 °C (mesophilic range). In order to maintain these microbially required optimal temperatures, heat (exchange) exchangers are installed upstream of the digesters or inside. Thermophilic processes (50–55 °C) are also being tested.

The **digested sewage sludge** produced in addition to the biogas can be easily dewatered. Provided it does not contain high concentrations of pollutants, it can be used as a nitrogen-rich organic fertiliser. Among other things, the contents of toxic heavy metals as well as dioxins and furans are problematic. For example, sewage sludge from urban areas often has cadmium and mercury contents of more than 10 mg/kg. According to the current German sewage sludge ordinance, the limit values for cadmium are 1.5 and for mercury 1 mg/kg dry matter. For other heavy metals the limits are higher, for lead 100, copper 60, nickel 50, chromium 100 and zinc 200 mg/kg sewage sludge dry matter. The use of more highly contaminated sewage sludge in agriculture is prohibited. A considerable proportion of the sewage sludge produced must therefore be landfilled or incinerated.

Aerobic wastewater treatment is a very complex process, as aeration requires a lot of energy and incurs relatively high costs. Anaerobic treatment, on the other hand, produces energy in the form of biogas. **Direct anaerobic wastewater treatment** is attractive if the process can be carried out stably and at a high throughput rate. An essential prerequisite for this is **highly contaminated wastewater,** such as that produced in the food industry and agriculture. In principle, the fresh sewage sludge discussed so far also represents such a concentrated wastewater. Wastewater with a content of 1–100 g/L organic matter can be treated directly anaerobically. Wastewater from sugar and starch factories, dairies and breweries as well as slaughterhouses have concentrations of 1–10 g/L, while sewage sludge and liquid manure have concentrations of 10–100 g/L of organic matter.

Wastewater constituents such as carbohydrates, proteins and fats are readily utilized by the microorganisms of the methanogenic food chain, whereas lignin is not. Higher concentrations of heavy metals, chlorinated organic solvents (e.g. chloroform), cyanides and hydrogen sulfide have an inhibiting effect in wastewater. Hydrogen sulfide, which is formed from sulfate by sulfate reduction, has an inhibitory effect mainly in its undissociated form (at acidic pH values). On the other hand, however, it can also contribute to the detoxification of heavy metals by sulfide formation.

The processes shown in ◘ Fig. 14.10, in which the bacterial biomass is retained in the reactor, achieve high cell densities (around 50 g/L) which, in continuous operation, lead to a degree of purification of the wastewater of 80–90% with residence times of 10–24 h. The degree of purification clearly depends on the composition of the wastewater, so that post-treatment may be necessary.

14.6 Treatment of Industrial Waste Water

Industrial wastewater is often more polluted than municipal wastewater. The content of organic substances is 2–3 g/L, some of which is difficult to degrade. This has led to the development of much more effective activated sludge processes in the form of biohigh reactors or tower biology (◘ Fig. 14.11). These are enclosed biore-

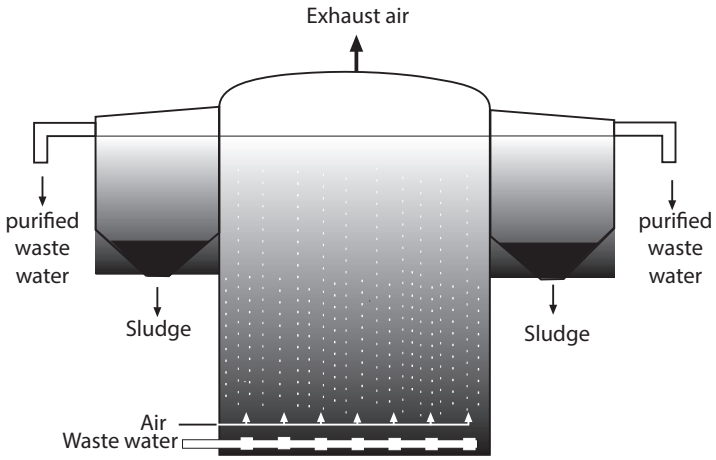


Fig. 14.11 Biohigh reactor or tower biology

actors 20–30 m high and more than 10,000 m^3 in volume. The high water columns increase the O_2 saturation value, at 8 m water depth it is 17, at 20 m water depth 28 mg O_2/L. This increases the oxygen yield, and the introduced air is used much more effectively for degradation processes than in shallow, open basins. Special constructions at the bottom of the tower reactor mix the introduced wastewater flow with the air flow and accelerate the mass transfer. The upflowing and circulating wastewater streams carry the activated flocs upwards, where they sediment in the settling basin arranged in a ring around the tower reactors and are also partly returned to the aeration chamber. Modern plants of large chemical companies achieve the purification of 100,000 m^3 wastewater/day with 160 t BOD_5/day by this intensive technology with several reactors. The closed plants prevent gaseous waste substances from being released into the atmosphere.

While it was previously considered that concentrated wastewater from the chemical industry had to be "diluted" with municipal wastewater before biological treatment, experience has shown that it is often more economical to carry out separate treatment.

Today, the chemical industry avoids (a) generating large quantities of diluted wastewater and (b) combining streams of the most diverse wastewaters from the production plants in a common plant, since mixtures are generally more difficult to treat. Instead, concentrated wastewater from each production stream is treated individually in decentralized treatment plants. Since such wastewaters are sometimes very toxic, i.e. not "biologically compatible", physicochemical methods are often used.

14.7 Near-Natural Wastewater Treatment Processes

In addition to high-tech tower biology plants, simple technologies also have a future. However, such near-natural wastewater treatment processes are still rare in Germany. They are particularly suitable as decentralised solutions for outdoor areas with smaller connection sizes (up to 1000 PE) due to the partly high space requirement (5–10 m^2/PE for a constructed wetland), the low hydraulic load, biomass concentration and oxidation rate as well as the low operating and energy expenditure.

In constructed wetlands, the wastewater is fed into a sandy-gravelly soil body covered with selected marsh plants (helophytes such as reed: *Phragmites australis;* wattle: *Schoeneplectus lacustris* and also cattails:

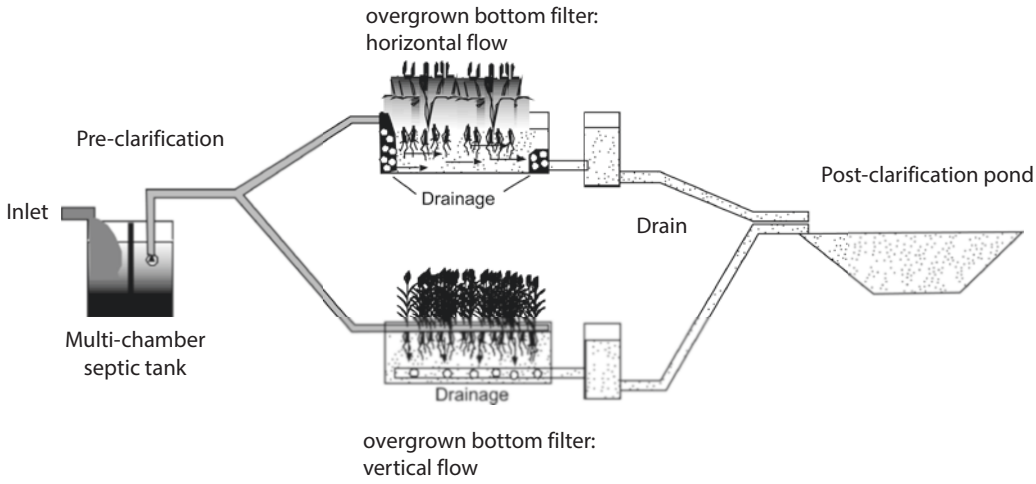


Fig. 14.12 Schematic of constructed wetlands. Horizontal flow at the top, vertical flow at the bottom. (Modified according to UBA, 2003)

Typha latifolia) for treatment. The soil body is flown through or over horizontally or vertically. The purification processes are mainly based on the activity of the settled microorganisms. The marsh plants are only indirectly involved in the purification process. Their role is to create favourable conditions for the degrading bacteria. This includes, for example, the transport of oxygen through the roots into the soil and the maintenance of the hydraulic permeability of the soil through root growth. There are aerobic but also anaerobic zones in the root zone, so that a complex biocenosis with diverse degradation services develops (Fig. 14.12).

An annual harvest of the above-ground plant parts can remove about 5% of the N and P load from the system. Due to the relatively slow development of the above- and below-ground plant parts, a constructed wetland requires a break-in period of at least one growing season. However, full development takes considerably longer.

Test Your Knowledge

- What do the sum parameters BOD, COD, DOC, TOC, AOX mean?
- What is a population value?
- What is a receiving water?
- Describe an ordinary sewage treatment plant with its substeps.
- Compare the treatment of wastewater in the aeration tank and by trickling filters. Highlight the advantages and disadvantages of each.
- Why is sludge recirculation often used?
- What is bulking sludge?
- What conditions are required for the elimination of phosphate from wastewater by "polyphosphate-accumulating organisms"?
- What metabolism can easily remove the nitrogen load from a wastewater? You have operated the aerobic stage of wastewater treatment and then want to perform N removal: What problems do you have with this sequence?
- What is the anammox process?
- Compare the biomass formation in the aerobic and anaerobic part of a wastewater treatment plant. How much of the resulting compounds is assimilated during sludge digestion and how much is converted to biogas? What is the composition of biogas?

- What sense does the use of tall reactors make in the treatment of industrial wastewater?
- How does a constructed wetland work? Where can it be used?

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Biological Exhaust Air Treatment

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_15

» "The **Technical Instructions on Air Quality** serve to **protect** the general public and the neighbourhood against harmful effects on the environment caused by air pollution and to **take precautions** against harmful effects on the environment caused by air pollution in order to achieve a high level of protection for the environment as a whole."

The **protection** against harmful effects on the environment caused by odour immissions is not regulated in the TA-Luft; on the other hand, **precautions** against harmful effects on the environment caused by odour emissions are regulated in this administrative regulation.

The cleaning of waste gas/exhaust air streams containing gaseous and aerosol air pollutants must therefore be carried out.

15.1 Problems with Exhaust Air Flows

Odour-intensive waste air flows represent a significant problem. Odorous substances occur in the exhaust air from **composting plants, sewage treatment plants, animal husbandry** and **food production**. Amines, amides, indole, skatole (3-methylindole), ammonia, hydrogen sulfide, dimethyl sulfide, mercaptans, butyric acid, n-butanol, isobutanol, 2-butanone, acetic acid butylester and butylene glycol are to be mentioned. These substances are characterised by the fact that their odour threshold, i.e. the smallest amount of substance that can still be perceived by most people, is very low. With regard to drinking water contamination, odour thresholds are given for different substances as their concentration per litre of water. ◘ Tables 15.1 and 15.2 make it clear that for some substances, quantities of µg/m^3 in the air or ng/L in drinking water already cause annoyance, while others are only perceived in the high range of mg/m^3 and µg/L respectively.

Odor Unit Is the Unit of the Odorant Quantity

One odour unit corresponds to the quantity of an odorous substance or odorous substance mixture which—distributed at 20 °C and 1013 Pa in 1 m^3 of neutral air—just about triggers an odour perception according to the definition of the odour threshold.

The European Standard (EN 13725: 2003) defines a method for the objective determination of the odorous substance concentration of a gaseous sample by application of dynamic olfactometry with persons as testers and for the determination of the emission flows of odorous substances from point sources, area sources with defined volume flow and area sources without defined volume flow.

DIN EN 13725: 2003-07 Air quality; determination of odour concentration by dynamic olfactometry; German version EN 13725:2003. Beuth Verlag, Berlin.

Inorganic substances originate from agriculture and the combustion processes of the energy industry.

Volatile organic compounds enter the air from a wide range of emission sources from industry, commerce, municipalities and agriculture.

In the **industrial sector,** organic **solvents** are the main air pollutants. The following list provides a selection:

1. Hydrocarbons: Methane, ethene, pentane, hexane, cyclohexane, heptane.
2. Aldehydes and ketones: Formaldehyde, acetone.
3. Aromatics: Benzene, toluene, xylene, phenol, styrene, naphthalene.
4. Chloroaliphatics: Dichloromethane, dichloroethane, trichloroethane.
5. Chloroaromatics: Chlorobenzene, 2-chlorotoluene.

Table 15.1 Odour threshold values of substance groups and individual components in air

Substance groups:Substance	mg/m³	Substance groups:Substance	mg/m³
Aromatic hydrocarbons		*– carboxylic acid esters*	
Benzene	16.2	Ethyl acetate	22.0
Toluene	7.6	Acetic acid butyl ester	0.03
1,3,5-Trimethylbenzene	2.0	Acetic acid pentyl ester	0.4
Isopropylbenzene	0.04	Methacrylic acid methyl ester	0.2
		Acrylic acid ethyl ester	0.002
Oxygen-containing compounds		*– phenols*	
– alcohols			
Methanol	5.3	Phenol	0.2
Ethanol	19.1	Cresole	0.004
Isopropyl	7.5	**Sulfur-containing compounds**	
Isobutanol	2.2		
n-Butanol	0.4	Methyl mercaptan	0.04
n-amyl alcohol	0.7	Ethyl mercaptan	0.003
Isoamyl alcohol	0.2	Carbon disulfide	0.6
2-Ethylhexanol	0.4	Hydrogen sulfide	0.003
		Nitrogenous compounds	
– ether		Ammonia	1.9
Diisopropyl ether	0.04	Dimethylamine	0.09
– aldehydes		Trimethylamine	0.0005
Formaldehyde	0.1	Diethylamine	0.06
Acetaldehyde	0.4	Triethylamine	0.4
– ketones		Dibutylamine	1.4
Acetone	48.0	Pyridine	0.07
Methyl ethyl ketone	6.0	2,4-Toluylene diisocyanate	14.4
– carboxylic acids		Nitrobenzene	0.03
Formic acid	1.9	**Halogenated hydrocarbons**	
Acetic acid	2.5		
Propionic acid	0.2	Dichloromethane	706.0
Butyric Acid	0.004	Carbon tetrachloride	640.0
		Trichloroethene	109.0
		Tetrachloroethene	34.0
		Chlorobenzene	0.9

The quantitative release of such compounds via exhaust air is often underestimated. Calculations show that in Europe about 1,360,000 t of benzene, 1,650,000 t of toluene and 50,000 t of styrene are emitted per year (from Fritsche, 1998).

In connection with odour nuisances, there are often fears that **bioaerosols** may cause **adverse health effects.**

Bioaerosols are airborne particles of biological origin (DIN EN 13098). Bacteria, fungi, viruses and pollen are relevant to

Table 15.2 Odour threshold concentration (GSK) in water

Substance groups:Substance	GSK (µg/L)	Substance groups:Substance	GSK (µg/L)
Aromatic hydrocarbons		**Nitrogenous compounds**	
Benzene	2000	Trimethylamine	0.4–1
Naphthalene	500	Pyridine	100
Oxygen-containing compounds		Indole	300
– ***alcohols***		3-Methylindole	3
Geosmin (bicyclic alcohol)	0.006	Nitrophenol	10,000
Menthol (monocyclic Monoterpene alcohol)	6	**Halogenated hydrocarbons**	
– ***ketones***		Tetrachloroethene	300
Acetone	5000	Chlorophenols	10
– ***carboxylic acids***		Trichloroanisoles	0.0002–0.003
Butyric Acid	50	Chlorobenzene	100
– ***phenols***		Dichlorobenzene	10–30
Phenol	1000	Trichlorobenzene	5–50
Sulfur-containing compounds		Tetrachlorobenzenes	20–400
Dimethyl sulfide (thioether)	0.03–1	Pentachlorobenzene	60
Thiophenol	1	Hexachlorobenzene	3000
Hydrogen sulfide	10–20		

environmental medicine. They can either form particles or adhere to particles. Furthermore, it is possible that only fragments of bacteria, fungi, viruses or pollen or their metabolic products adhere to particles.

Possible sources of bioaerosols are waste treatment and disposal plants (such as waste sorting plants, transfer stations, waste wood treatment plants, composting plants, fermentation plants, landfills), plants for the production of agricultural products, food, luxury food and animal feed (e.g. livestock farms, slaughterhouses, rendering plants), sewage treatment plants, biological exhaust air purification (e.g. biofilters), biogas plants and cooling towers.

According to the current legal situation—§ 5 para. 1 no. 2 Federal Immission Control Act (BImSchG)—and from a precautionary point of view, it is at best desirable at present to avoid any increase in ambient air concentrations compared to background values.

For the **waste sector,** the following **plant-related lead parameters** apply: *Aspergillus* spp., *Aspergillus fumigatus* (for composting plants), *Penicillium* spp.

For **animal husbandry/food production**, the following **facility-related lead parameters** apply: *Staphylococcus aureus,* Staphylococci, Enterococci, *Enterobacteriaceae*, Endotoxins in animal houses (Table 15.3).

Infectious agents of relevance include *Legionella pneumophila* from cooling towers.

Table 15.3 Origin and guiding parameters of bioaerosols in waste management and agriculture (VDI 4251 Part 1, VDI; 4250 Part 1-E 2009, new table as VDI 4250 Part 3)

Area	System type	Plant-related and protected good-related measurement parameters relevant to environmental medicine Guiding parameters
Recovery and disposal of waste and other materials (disposal installations)	Recyclable waste sorting plants Commercial waste sorting plants	*Penicillium* spp., *Aspergillus* spp.
	Recycling of separately collected/sorted recyclable materials (metals, plastics)	*Penicillium* spp., *Aspergillus* spp.
	Waste wood processing plants	*Penicillium* spp., *Aspergillus* spp.
	Composting plants Soil and humus plants	*Penicillium* spp., *Aspergillus* spp., thermotolerant fungi
Food, beverages and animal feed, agricultural products	Animal husbandry	Staphylococci, *Staphylococcus aureus*, *Enterobacteriaceae*, Intestinal Enterococci
	Slurry storage and processing, manure drying	Enterobacteriaceae, Intestinal enterococci
	Slaughterhouses	Staphylococci, *Staphylococcus aureus*, *Enterobacteriaceae*, Intestinal Enterococci
	Rendering plants	Staphylococci, *Staphylococcus aureus*, *Enterobacteriaceae*, Intestinal Enterococci

The VDI Guideline Series Provides the Basis for the Measurement and Assessment of Bioaerosol Immissions

- **VDI 4250** Part 1–3 (Bioaerosols and biological agents) describes the **effect of microbial air pollutants on humans**.
- **VDI 4251** Part 1–3 (Detection of airborne microorganisms and viruses in outdoor air) specifies the conditions that must be taken into account when **planning immission measurements of** microbial air pollutants.
- **VDI 4252** (Detection of airborne microorganisms and viruses in outdoor air) describes the various methods for **sampling** bioaerosols and specifies the requirements for carrying out immission measurements.
- **VDI 4253** (Bioaerosols and biological agents—Laboratory analysis) specifies the conditions for the cultivation and detection of microorganisms as well as for the **analysis of viruses** and builds on the sampling described in VDI 4252.
- **VDI 4254** (Bioaerosols and biological agents—Laboratory analysis) deals with the **analysis of gaseous air contaminants of microbial origin** such as Microbial Volatile Organic Compounds (MVOC), Endotoxins, Mycotoxins and Glucans.

- **VDI 4255** Part 1–3 (Bioaerosols and biological agents—Emission sources and abatement measures) describes the different **emission sources of microbial air pollutants** and describes methods for abatement of these emissions.
- **VDI 4256** (Bioaerosols and biological agents—Determination of process parameters) defines the **statistical parameters** necessary for the description and comparability of the processes.
- **VDI 4257** (Bioaerosols and biological agents—Measurement of emissions) describes the planning, implementation and various methods of **emission measurement of microbial air pollutants**.
- **VDI 4258** (Bioaerosols and biological agents—Measurement of emissions) will describe the production of test aerosols for the validation of measurement methods.

15.2 Microbial Exhaust Air Purification, General Principles

There are various efforts to prevent air pollution, to clean the exhaust air and even to recover some substances from the exhaust air. In principle, adsorptive, absorptive, thermal, catalytic and biological processes can be used for **exhaust air purification** of vaporous and gaseous pollutants.

- If the substance concentrations are above a few grams per m^3 of exhaust air, they can be effectively removed and possibly recovered by **physical** and **chemical processes.**
- Low concentrations in the range below 1 g/m^3 are difficult to remove by the methods mentioned. It is particularly difficult to clean large quantities of exhaust air from mixtures of substances in low concentrations. For these concerns, the biological processes described in more detail below are increasingly being used.

A decisive prerequisite for biological exhaust air purification is the **microbiological degradability** of the potential pollutants. The list of biologically eliminable exhaust air constituents is long. In principle, all of the organic compounds selected above are microbially metabolizable. For some, however, microbiological degradability has not yet been demonstrated on an industrial scale of exhaust air purification. Aliphatic and aromatic hydrocarbons, for example, are growth substrates for a number of microorganisms. Halogenated hydrocarbons can be partially transformed productively and otherwise often cometabolically, i.e. in the presence of a growth substrate. However, substances that are only degraded very slowly cannot be satisfactorily removed from exhaust air by means of biological purification.

The microorganisms are usually ubiquitous **microorganisms.** They have the ability to adapt to changes in nutrient supply within a wide range. As a rule, many different microorganism species are involved in the degradation processes. A change in substrate supply leads to changes in composition without affecting the performance of the system. Spontaneous changes in the exhaust gas composition are unfavourable for biological systems.

The natural selection of **degradation specialists** for certain substrates is sometimes slow in air pollution control plants, so that **inoculation with special cultures** may be appropriate.

Volatile substrates can only be degraded by microorganisms when they reach the cells in dissolved form. Therefore, a sec-

ond central requirement for biological exhaust air purification is that the substances to be degraded must have **sufficient water solubility.** The Henry's constant from Henry's Law (cf. ▶ Sect. 5.1.2) serves as a parameter.

◘ Table 15.4 gives the Henry constants for a large number of compounds whose biological elimination from exhaust air streams has been investigated. ◘ Table 15.5 makes it clear that the different processes—biofilter, bio trickle bed and bioscrubber processes—have different limits on the pos-

◘ Table 15.4 Suitability of biofilters (VDI 3477) and bio trickle bed reactors (VDI 3478, Sheet 2) for the elimination of substances/substance groups from exhaust air streams and the Henry constant

Substances/Henry constant (Pa m^3/mol)[a]	suitability of the process		Substances/Henry constant (Pa m^3/mol)[a]	suitability of the process	
	VDI 3477	VDI 3478 Sheet 2		VDI 3477	VDI 3478 Sheet 2
Aliphatic hydrocarbons			– ***ketones***		
– ***saturated aliphatic hydrocarbons***			Acetone/3.8	+	+
Methane/72000	0	–	Methyl ethyl ketone/3.5	+	+
Pentane/130000	–	–	Methyl isobutyl ketone/46	+	
Hexane/17000	–	0	– ***carboxylic acids***		
Longer chain hydrocarbon		?	Butyric acid/0.05	+	
– ***unsaturated aliphatic hydrocarbons***			– ***carboxylic acid esters***		
Acetylene/2500		–	Acetic acid ethyl ester/13	+	+
Longer-chain hydrocarbon		?	Acetic acid butyl ester/21	+	+
– ***cyclic aliphatic hydrocarbons***			Methacrylic acid methyl ester	+	+
Cyclohexane/18000		0	Glycolic acid ester	+	+
Aromatic hydrocarbons			– ***phenols***		
Benzene/510	0	0	Phenol/0.04	+	+
Toluene/680	0	+	Cresol/0.25	+	+
Xylene/580	0	+	**Sulfur-containing compounds**		
Ethylbenzene/790	0				
Styrene/270	0	+	Sulfides (thioethers)	0	?
Naphthalene/51	0	?	Sulfur heterocycles	0	?
Oxygenated compounds			Mercaptans/140	0	+
– ***alcohols***			Carbon disulfide/2300	–	+
Methanol/0.58	+	+	Hydrogen sulfide/1000	0	+
Ethanol/0.64	+	+			
Isopropyl alcohol/0.84	+				

(continued)

Table 15.4 (continued)

Substances/Henry constant (Pa m³/mol)[a]	suitability of the process		Substances/Henry constant (Pa m³/mol)[a]	suitability of the process	
	VDI 3477	VDI 3478 Sheet 2		VDI 3477	VDI 3478 Sheet 2
Butanol/0.78	+	+	**Nitrogenous compounds**		
Glycol/0.006		+			
Diglycol		+	Ammonia/5.7	0	+
Butyl glycol		+	Amide	+	?
			Amine	+	+
			Nitrogen heterocycles		?
			Isocyanates		?
– ***ether***			Nitro compounds	0	?
			Nitrile	+	?
Tetrahydrofuran/5.9		?	Isonitrile	0	?
Diethyl ether/110		?			
Dioxane/0.59		?	**Halogenated hydrocarbons**		
– ***aldehydes***			Dichloromethane/1000		+
			1,2-dichloroethane/630		+
Formaldehyde/0.03	+	+	1,1,1-Trichlorethane/1900		–
Acetaldehyde/6.0	+	+	Vinyl chloride (chloroethene)/2700		0
					0
			Trichloroethene/920		–
			Tetrachloroethene/1800		+
			Chlorophenols/0.84		

+ well suited; 0 conditionally suited; – not suited; ? no reliable findings on an industrial scale
[a]Sander (2015)

sibility of purification with respect to the Henry constant.

For the effectiveness of an exhaust air purification system it is essential that the **mass transfer from the gas to the liquid phase** takes place at a high rate. This applies to both the substances to be decomposed and the oxygen. Thus, the largest possible contact area between the gas phase and the liquid phase must be created in order to ensure rapid and intensive exchange. Even if the degradation takes place in biofilms on solid fillers, as in biofilters and trickling filters, there is a water film between the gas and solid phases in which the microorganisms grow. This mass transfer across phase interfaces depends on the exchange surface (in biofilters, the pore volume, particle size and structure of the filler material). The presence of water as a mediating agent is therefore a basic requirement. Furthermore, a sufficiently high water supply is elementary for the function of the degradation processes. Thus, the topic of water balance/substance transport in the filter material is of particular importance.

The preferred working range is in the so-called mesophilic temperature range (about 20–40 °C).

The biomass in the biofilm depends on the concentration of degradable pollutants, the oxygen supply and the nitrogen

Table 15.5 Comparison of the main technologies of biological exhaust air purification

Characteristics	Biofilter	Bio trickle Bed Reactor	Biowashers
Reactor design	Single reactor	Single reactor	Two reactors
Installation and operating costs	Low	Comparatively higher	Comparatively higher
Carrier	Organic or synthetic	Synthetic	No carrier
Space requirement	Large areas are necessary	Compact system	Small volume of the plant
Mobile phase	Gas	Liquid	Liquid
Surface area	High	Low	Low
Process control	Limited process control	Limited process control	Good process control
Gas flow rate	100–150 m^3/m^2 h	100–1000 m^3/m^2 h	3000–4000 m^3/m^2 h
Operation	Simple commissioning and operation	Relatively complicated commissioning	Relatively complicated commissioning
Operating stability	Channeling of the air flow normal	Channeling of the water flow normal	High operational stability
Pressure drop	Medium to high	Medium to high	Low
Concentration of the target connection	<1 g/m^3	<0.5 g/m^3	<5 g/m^3 (1 g/m^3, VDI3478, sheet 1: 2011-03, section 1)
Suitable for connections with Henry constant	<2500 Pa m^3/mol	<250 Pa m^3/mol	<25 Pa m^3/mol
Nutrients	Nutrients can only be added with a complex process	Addition and control of nutrients is possible	Addition and control of nutrients is possible
Biomass	Fixed biomass	Fixed biomass	Suspended biomass
Packing clogging	Constipation problems	Constipation problems	No clogging problems
Excess sludge	No such problems	Elimination is necessary	Elimination is necessary

and phosphorus sources necessary for growth. In biofilters, these substrates may come from the organic fillers such as compost or bark. In other cases, an addition of nutrient salts may be necessary. If leaching of cells occurs, recirculation of biomass is beneficial. If more substrate diffuses into the biofilm than is enzymatically converted, sufficient degradation will not occur.

A permanently optimal performance of the microorganisms is only guaranteed if the

milieu conditions with regard to factors such as mesophilic temperature range, sufficient water supply, neutral to slightly acid pH range and balanced nutrient supply are maintained within certain limits. Since microorganisms are influenced by changes in their living conditions, adjustment times are generally to be expected when a plant is commissioned or when its operating conditions change.

Table 15.4 gives an overview of the types of waste gas and/or substances that have been treated so far with biofilter plants and bio trickle bed reactors.

The various types of exhaust air purification are described in more detail below.

In cases that cannot be reliably controlled with **a** system of waste gas purification equipment alone from a process engineering, economic or ecological point of view, combinations of biological waste gas purification and other waste gas treatment processes are used.

Normally, comparative plants and/or the results of pilot tests are used for design. As a general rule, the suitability of air pollution control systems must be tested by long-term prior pilot tests (at least 3 months).

Systems of Biological Exhaust Air Purification

The guidelines VDI 3477 and VDI 3478 Part 1 and Part 2 describe the state of the art in biological waste gas purification using the tried and tested **biofilter, bioscrubber** and **bio trickle bed reactor (trickling filter)** systems. The differences are in part minor and the transitions are fluid.

Where biological exhaust air purification processes can be used, they usually represent a simple, cost-effective and operationally reliable alternative to "conventional" chemical or physical processes.

Chemical processes include acidic or alkaline scrubbers, for example using sulfuric acid or caustic soda as make-up chemicals. Alternative **physical processes** include adsorption on activated carbon, UV, ionization or ozone oxidation systems.

In the **biofilter,** exhaust air substances are sorbed on the biofilter material, diffuse into the aqueous phase and are converted or degraded there. The filter regenerates itself through biological activity.

In the **bioscrubber,** the exhaust air substances are dissolved directly in the water phase. The regeneration of the scrubbing medium takes place through the activity of microorganisms, which are usually located in an apparatus separate from the scrubber.

If absorption and regeneration take place in the same apparatus (=reactor), one generally speaks of the **trickling filter plant** or the **bio trickle bed reactor.**

A film is formed in the **bio trickle bed reactor,** while in the **scrubber** the microorganisms are suspended in the scrubbing liquid.

15.3 Exhaust Air Purification Systems: Biofilter

Biofilters are systems with a solid matrix on which the microorganisms grow in the biofilm. They are the simplest concept of a biological exhaust air purification, because pollutant absorption by the aqueous phase and its microbial regeneration occur both locally and temporally coupled without movement of large amounts of water. They can remain in use for several years.

The exhaust air to be cleaned is passed from the bottom to the top at 0.02–0.1 m/s past the single- or multi-stage filter bed (single-layer and multi-layer biofilters). In this way, the water-soluble pollutants are transported into the aqueous phase prevailing at the surface of the filter material and thus reach the microorganisms.

Exhaust Air Purification Systems in Animal Husbandry

The exhaust air purification systems in animal husbandry are generally technically simpler in design than systems for industrial applications. The concentrations of odorous substances and other trace gases in the exhaust air from livestock facilities are comparatively low, unlike in many industrial processes. The high air rates required to remove heat, carbon dioxide and moisture and to comply with the Animal Welfare Livestock Ordinance result in the low concentration levels. In conventional fattening pig farming, air rates can vary between 7 and 100 m^3/(animal h) depending on animal weight, time of day and season, and in broiler farming between 0.3 and 4.5 m^3/(kglive weight h). In summer, air rates of up to 150,000 m^3/h can occur at the end of fattening in fattening pig houses with 1500 animals, and up to 300,000 m^3/h in a broiler house with 40,000 birds and a weight of about 1.7 kg.

Exhaust air purification systems in animal husbandry must be designed for rapidly changing operating conditions and have a high degree of elasticity. Due to the ventilation control according to the difference between outdoor and indoor temperature, both the contact time and the pollutant gas or particle concentrations can change quickly. Despite comparatively low pollutant gas concentrations, exhaust air purification systems must therefore achieve satisfactory efficiencies even with large emission mass flows.

The general requirements for the cleaning performance are specified in the test framework "Exhaust air cleaning systems for livestock facilities" in such a way that 70% of the total dust and ammonia load must be separated. Furthermore, the odour concentration in the clean gas must not exceed 300 GE/m^3 in any sample, as otherwise raw gas odour is to be expected.

Exhaust air purification systems for pig farming which have been tested according to the Cloppenburg guidelines or the DLG test framework reliably meet the specified purification requirements when operated correctly.

In poultry farming, on the other hand, there is still a considerable need for research with regard to odour reduction.

- VDI 3477:2014-08 Biological waste gas purification; biofilters. Berlin: Beuth Verlag.
- DLG test report 5944: Two-stage exhaust air purification plant. Groß-Umstadt, 2010.
- Hahne, J. 2013: Biofilters in animal husbandry. Gefahrstoffe—Reinhaltung der Luft 73:187–191.
- DLG Test Centre for Technology and Equipment (n.d): Test framework for exhaust air purification systems for livestock facilities, Groß-Umstadt, in the current version in each case.

Biofilters are open or closed fixed-bed reactors filled with bulk layers of partially mixed organic materials (leaves, soil, brushwood, wood and bark chippings, root wood, composts from organic waste, tree bark or composts of other origin, fibrous peat and heather, as well as their mixtures with each other or with other structuring materials). Plastics such as polystyrene or inorganic

materials with a large internal surface area and a corresponding microorganism population such as expanded clay or volcanic slag are added to the organic base materials to improve the structure and as a supporting material. In individual cases, activated carbon is added.

Organic materials have prevailed in biofilters because they already have a high population density and a large variety of microorganisms by nature.

The organic materials are mixed with the inert materials to improve the structure and service life behaviour. The positive effects of both materials complement each other in this case. Special foam glass and plastic products are also produced as filter material.

The filter materials must meet the following conditions:

- **Large effective surface area** for mass transfer and colonization by microorganisms. The large surface area and low water content of biofilters makes them useful for the treatment of less hydrophilic pollutants.
- **High porosity and homogeneity** to ensure a good and uniform flow (about 50% air space). The cost of fan operation is kept within limits due to the high porosity and the associated limited pressure drop.
- **High water holding capacity to** counteract dehydration. Since the heat generated by the biological reaction and the low moisture content of the input air determine the rate of water loss, humidification is necessary.
- **Heavy degradability,** so that the longest possible service life of the filters is achieved.

A water and biofilm forms on the surfaces of the packing, on which the biological degradation of the raw gas contents to be separated takes place. At the same time, the organic substances of the packed layers serve as nutrients for the microorganisms. As a result, the structure is destroyed over time and the filter material itself is degraded. There is a tendency for increased nitrous oxide formation due to ammonia input in decayed filter material during stable air purification. From a certain level of degradation, this must therefore be renewed.

The microbial degradation processes of organic carbon compounds run undisturbed in the neutral to slightly acidic pH value range. The effect of degradation products or by-products is usually buffered in the filter material. The deviation to extreme pH-values can be tolerated in special applications, for example hydrogen sulfide elimination (pH-value 1–2).

For maximum performance, the nutrient supply must also be balanced. In addition to carbon, the microorganism needs nitrogen, phosphate, sulfur and trace elements.

According to the arrangement of the filter material, a distinction is made between panel filters and high filters (stack filters, honeycomb filters, tower filters) in open and closed construction, which can be assembled in modular form from container-shaped units in some cases.

Large-area open biofilters are operated primarily for odour-intensive exhaust air constituents, such as those produced during waste composting. The filter area is in the order of 500–1000 m^2. The structural properties of the structure determine the layer height of the packed filter, which is typically 1–2 m.

Waste gas volumes of 1000–70,000 m^3/h are cleaned with an efficiency of 80–95%. Closed systems in the form of containers

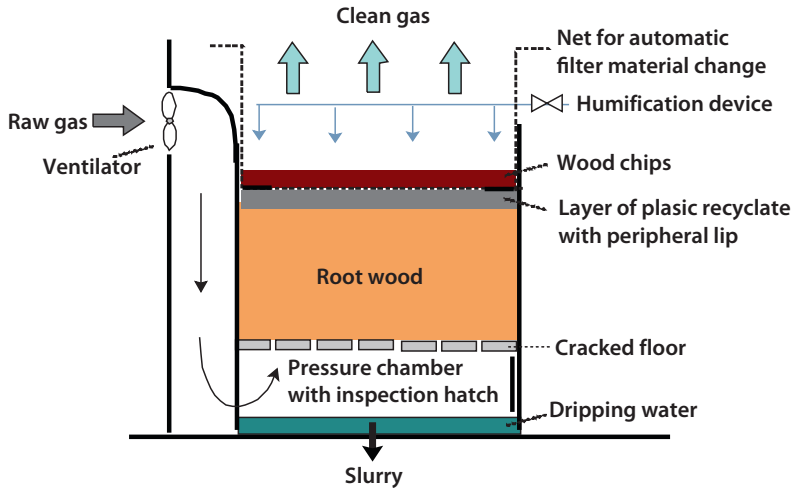


Fig. 15.1 Schematic of a biofilter approved by the German Agricultural Society (DLG) for odor and total dust separation in litterless pig farms

are increasingly being used for organic solvents. They allow better control of the processes (humidity, temperature) and analysis of the purification efficiencies. The organism density is 10^9 bacteria per g of filling material.

In **multi-layer biofilters** (Fig. 15.1), the bottom layer consists of wooden boards stacked crosswise with open gaps and the top layer of wood chips. The middle layer consists of plastic granulate. In this system, dust separation takes place mainly in the lowest layer of the biofilter. Humidification of the stable exhaust air takes place via the dripping water in the pressure chamber and via the moist boards of the lowest layer. Water is periodically sprayed onto the top layer with nozzles. This moistens the filter material and at the same time ensures a regulated washing out of degradation products from the filter.

Biofilters and bioscrubbers are successfully used in the plants listed in Table 15.6. Despite the numerous practical applications of biofilters, their use should continue to be tested in the future in cases where there is no transferable experience to date. Furthermore, a comparison with other exhaust air purification processes is recommended.

With regard to **the reduction of bioaerosols**, biofilters (surface filters) have been tested which have been conceived and designed with regard to the odour reduction of various composting plants. Measurements of composting-specific parameters (fungi, 20 °C; thermotolerant fungi, 45 °C, *Aspergillus fumigatus,* 45 °C; bacteria, 37 °C; thermophilic actinomycetes, 50 °C) showed lower concentrations in the clean gas of the biofilters than in the untreated raw gas (Schilling, 2003).

Table 15.6 Possible applications of biofilters (F) and bioscrubbers (W) for waste gas purification (according to VDI 3477 and 3478, Sheet 1), arranged according to industries

Asset types	Smell	Aliphatic hydrocarbons	Aromatic hydrocarbons	Oxygen-containing compounds	Sulfur-containing compounds	Nitrogenous compounds	Halogenated hydrocarbons	Essential oils	Hydrogen sulfide	Ammonia
Food and stimulant production										
Aroma extraction	F							F		
Fried Fish making	F			F	F	F			F	
Cereal production	W			W						
Fryers	F W	F W		F W						
Flavour production	F									
Yeast fermentation	F W			F W	F W					
Coffee Roasters	F W			F W	F W	F W		F W		
Cocoa roasting	F W			F W	F W	F W		F W		W
Herb processing	W			W						
Oil Mills	F				F					
Slaughterhouses	F			F	F	F			F	F

Tobacco processing	**F W**		**F W**	**F W**		**F W**				**F W**
Animal husbandry	**F**			**F**	**F**	**F**			**F**	**F**
Seasoning	**F W**				**F W**					
Sugar factory	**F**	**F**								
Recycling of residual materials/waste										
Beer yeast drying	**F**			**F**		**F**				
Fat Melting	**F**			**F**	**F**	**F**			**F**	**F**
Fishmeal factories	**F W**			**F W**	**F W**	**F W**		**F W**	**F W**	**F W**
Gelatine production	**F**			**F**	**F**					
Bone processing	**F**			**F**	**F**	**F**			**F**	**F**
Composting	**F W**	**F W**		**F W**	**F W**	**F W**	**F W**		**F W**	**F**
Fecal drying	**F**	**F**		**F**	**F**	**F**			**F**	**F**
Rendering	**F**			**F**	**F**	**F**	**F**		**F**	**F**
Disposal										
Waste storage and treatment	**F W**	**F W**	**W**	**W**	**F W**	**F W**	**F W**		**F W**	**F W**

(continued)

Table 15.6 (continued)

Asset types	Smell	Aliphatic hydrocarbons	Aromatic hydrocarbons	Oxygen-containing compounds	Sulfur-containing compounds	Nitrogenous compounds	Halogenated hydrocarbons	Essential oils	Hydrogen sulfide	Ammonia
Landfill gas disposal	F			F	F				F	
Sewage treatment plants, industrial	F W	F W	F W	F W	F W	F W	F W	F W	F W	F W
Sewage treatment plants, municipal	F W	F W	F W	F W	F W	F W	F	W	F W	F W
Sewage sludge drying	F	F	F	F	F	F	F		F	F
Chemical products										
Essencing	F	F		F						
Production and processing of paints and varnishes	F W	F W	F W	F W						F
Photographic film production	W	F W		F W						
Impregnating agent production and processing	F W			F W		F W				

Adhesive production	**F W**			**F W**						
Plastics processing	**F W**	**F W**	**F W**	**F W**		**F W**				
Glue production	**F**			**F**						
Oils and greases, technical	**F**	**F**	**F**		**F**		**F**			
Polymer concrete production				**F**	**F**					
Polyester production					**F**	**F**				
Tank farm	**F**	**F**	**F**							
Other										
Coating plants	**W**	**W**	**W**	**W**						
Bitumen processing	**F**	**F**	**F**	**F**						
Printers	**F W**	**F W**	**F W**	**F W**						
Foundries	**F W**	**F W**	**F W**	**F W**						**F W**
Rubber processing	**W**		**W**	**W**	**W**					

(continued)

Table 15.6 (continued)

Asset types	Smell	Aliphatic hydrocarbons	Aromatic hydrocarbons	Oxygen-containing compounds	Sulfur-containing compounds	Nitrogenous compounds	Halogenated hydrocarbons	Essential oils	Hydrogen sulfide	Ammonia
Semiconductor processing	W	W								
Wood-based material production e.g. chipboard, hardboard production (press exhaust air MDF)	F W	F W	F W	F W						
Solvent cleaning systems	W	W	W	W						
Pharmacy, fermentation of drugs	W	W		W						
Friction lining production	W	W	W	W		W				
Sandpaper production	W		W	W						

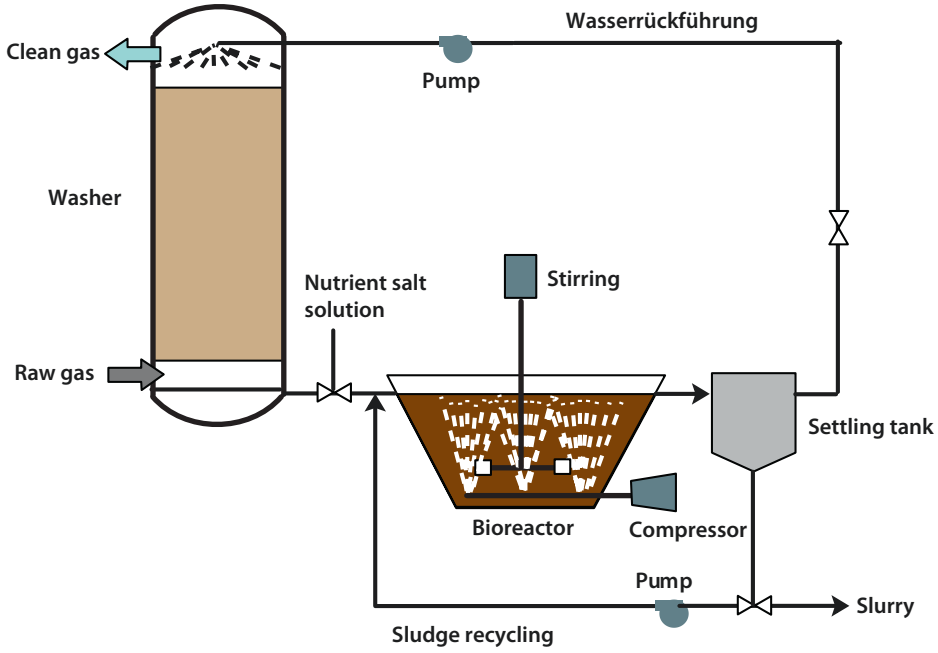
15.4 Exhaust Air Purification Systems: Bioscrubbers

As far as the actual washing process is concerned, bioscrubbers correspond in structure and mode of operation to the general principles of physical absorption (◘ Fig. 15.2). The special feature of the process is that the pollutants dissolved in the absorbent are broken down by the metabolic activity of microorganisms. In contrast to biofilters, the microorganisms in the bioscrubber are in suspension. In bioscrubbers, the washing out and absorption of the volatile air constituents takes place in the upper part of cylindrical systems. The raw gas introduced at the bottom (typical gas velocities are 1–3 m/s in packed scrubbers) is met by sprayed solution flowing from the top, which absorbs the contents in the absorption zone (sieve trays or packed columns). The gas-liquid mass transfer zone is designed to promote mass transfer from the air to the liquid phase, reducing the size of the system and resulting in a small pressure drop. The small specific gas-liquid mass transfer area limits the use of bioscrubbers to contaminants with low Henry constants (<25 Pa m^3/mol). The constituents are mainly degraded in the separate part of the plant, which corresponds to an aeration tank of wastewater treatment (compare ▶ Sect. 14.2). The concentration of nutrients and the pH-value are constantly controlled to ensure microbial growth and a high activity. During degradation, regeneration of the wash solution takes place and it is repeatedly pumped over. Microorganisms are present in high density in the circulating wash solution.

The excess biomass and also by-products are continuously removed from the system.

In practice, bioscrubbers are usually designed in two stages to ensure the effectiveness and safety of the cleaning effects. It is also possible to connect them to existing wastewater treatment plants.

Compared to biofilters, these systems have a higher degradation capacity per reac-



◘ Fig. 15.2 Schematic of a bioscrubber system

Table 15.7 Advantages/disadvantages of bioscrubbers in stable air purification

Advantages	Disadvantages
– Low space requirement – Low pressure difference – Good degradation of odours – High air flow rate per m^2 filter area – Faults can usually be rectified quicklywith a controlled sludge removal rate and a controlled pH range in the circulating water, there are further advantages: – long-term stability – Also suitable for higher substance concentrations – Very good degradation of ammonia – Low nitrous oxide formation	– Nitrous oxide formation with insufficient blowdown rate – Degradation reduction due to fluctuating pH in the circulating water – Risk of clogging – Limited degradation of odor at low odorant concentrations – For high ammonia reduction rates of well over 50%, high blowdown rates are required, which can lead to bottlenecks in slurry storage capacity

tor volume due to the higher density of organisms (the biomass concentration is around 5–8 g/L). They are more susceptible to failure in the case of varying amounts of exhaust air and do not have the buffer capacity inherent in biofilters. Operation requires additional aeration (Table 15.7).

Most Frequent Errors in Biological Exhaust Air Purification Systems According to a Study on the Purification of Stable Exhaust Air

The reasons for subsequent failures in biological exhaust air purification systems in agriculture are often due to a lack of knowledge on the part of the operators and system suppliers.

Most common mistakes with biofilters

- For reasons of economy (water consumption/energy), a humidification system is simply switched off or not installed at all.
- Due to incorrectly dimensioned ventilation or unsuitable air routing, an uneven inflow of air to the filter occurs, resulting in filter breakthroughs.
- Filter material is very old or already rotten.

Most common mistakes with organic scrubbers

- Bioscrubbers are operated with too low blowdown rates.

Most frequent faults with biofilters and bioscrubbers

- For cost reasons, plants are undersized.
- The fan capacity is too small to overcome the flow resistance of the exhaust air purification system at the summer ventilation rate required for the barn.
- No attention is paid to the pH value in the filter material, in the sump of a biofilter or in the wash water of a biowasher. A corresponding disturbance is often not noticed.

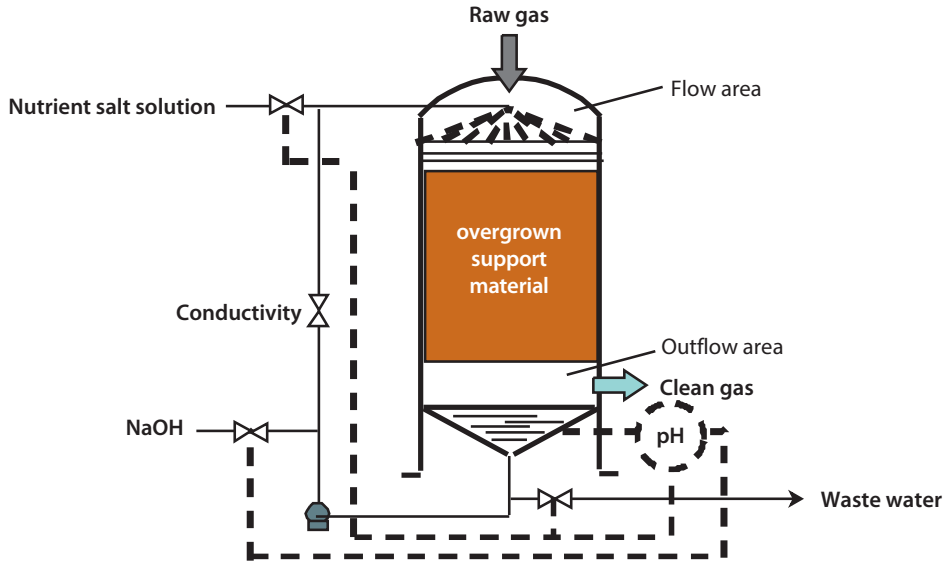
15.5 Exhaust Air Purification Systems: Trickling Filter Scrubbers

The trickling filter or bio trickle bed process is an independent process of biological exhaust air purification, which, however, overlaps in several respects with the two other biological exhaust air purification technologies, biofilter and bioscrubber. The trickling filter variant was adopted from biological wastewater treatment, where it

has been one of the standard methods under the same name for almost 100 years. The trickling filter is similar to a bioscrubber with packing. In addition to its dissolving function, the water has the function of a rinsing medium to keep the bacterial film, which forms as a biofilm on the packed surfaces, thin and thus active. The organisms must therefore adhere well to the material. To provide the moisture essential for the microorganisms, the packing is regularly sprayed with a liquid film to which nitrogen or phosphorus components are added as required. A pH control is integrated into the sprinkler water circuit to maintain a constant pH value. The sprinkling is designed in such a way that no leaching effect can be attributed to the moisture film.

As it trickles down over the biological lawn, the pollutant-absorbing water is regenerated at the same time, i.e. absorption and regeneration are coupled in terms of time and place, as in the case of the biofilter. As with the bioscrubber, the water can only optimally fulfil its transport function if it trickles as evenly as possible through all areas of the packing covered with biological turf. Inert materials, such as lava slag, are used as carriers. The raw gas is introduced into the sprayed and recirculated scrubbing solution. The contact time between gas and liquid must be between 5 and 20 s for readily soluble and readily degradable compounds, while it is much longer for poorly soluble and poorly degradable components, depending on the input concentration. Washed-out biomass can be added back to the recirculated wash water. The exhaust air flows through the open system while the washing solution circulates (◘ Fig. 15.3).

A bio trckle bed reactor containing only fixed microorganisms or microorganism agglomerates is certainly a borderline case. A technical bio trickle bed reactor also contains agglomerated microorganisms suspended in the water to a certain degree. The proportion depends, among other things, on the design of the bio trickle bed reactor and the materials used—especially the surface structure. The suspended microorgan-



◘ Fig. 15.3 Schematic of a trickling filter scrubber system. The designs in use are: Raw gas: 100–1000 m^3/m^2 h and <1.5 g/m^3; Height: 0.5–3 m, pressure difference 50–250 Pa/m; Wastewater: 0.1–1 m^3/h; Conductivity: <5 mS/cm; Irrigation liquid: 0.1–0.5 m^3/m^2 h

isms also degrade pollutants dissolved in the liquid and can thus contribute to increasing the reaction rate and improving the separation efficiency of the bio trickle bed reactor.

The water phase must be renewed when its conductivity has reached a certain value (usually approx. 1000 mS/m). The maximum conductivity is technology-specific (microorganism strains) and can be far above 1000 mS/m in individual cases.

Regeneration is usually carried out continuously by blowing down the circuit water and filling it up with fresh water. Depending on the amount of pollutants (dust, grease, salt), it may be necessary to increase the blowdown rate. This is usually determined during trial operation. The usual values for the blowdown rate are 200–1000 L for a circulation quantity of 70 m^3/h in 24-h operation.

Table 15.8 lists the bio trickle bed plants successfully operated on an industrial scale for various industries.

Table 15.8 Examples of applications for large-scale bio trickle bed systems, sorted by industries

Industry	Raw gas content to be separated
Food and stimulant production	
Tobacco industry	Smell
Food production	Smell
Sugar industry	Odor, hydrogen sulfide
Slaughterhouses	Smell
Disposal	
Biowaste composting plants	Smell
Biowaste fermentation plants	Smell
Waste paper recycling	Odor, hydrogen sulfide
Municipal wastewater treatment plants	Hydrogen sulfide
Brewery wastewater treatment plants	Odour, hydrogen sulfide, mercaptans
Industrial wastewater treatment plants	Odour, toluene, xylene, isobutanol, acetone, hydrogen sulfide
Recycling	Acetone
Landfill gas cleaning	Hydrogen sulfide
Soil remediation	Gasolines, high boilers
Print and color	
Ink production	Toluene, ethyl acetate, ethanol
Lacquer processing	Acetone, methyl isobutyl ketone, ethanol, ethyl acetate, butyl acetate
Flexo printing, gravure printing	Ethanol, ethyl acetate, Industrial methylated spirit (propanol, methanol, ethanol), hexane, Gasoline, heptane, isopropanol, methyl ethyl ketone
Screen printing	Aromatics, ketones, acetates
Coating industry	Formaldehyde, butyl acetate, propanol, ethanol, acetone
Other	
Shoe production	Toluene, hexane, methyl ethyl ketone, acetone
Pulp industry	Carbon disulfide, hydrogen sulfide
Pharmaceutical industry	Smell
Manufacture of adhesives, detergents, etc.	Dimethyl chloromethane, toluene, xylene, ethyl acetate

Table 15.8 (continued)

Industry	Raw gas content to be separated
Production of plant protection products and fertilizers	Toluene, xylene, dichloroethane, isopropanol, methanol, methyl isobutyl ketone, pyrrolidone
Semiconductor industry	Propylene glycol monoethyl ether acetate, n-methyl pyrrolidone, n-butyl acetate, acetone, xylene, isobutyl acetate, methyl ethyl ketone, gasoline, methoxyethanol, mesitylene, ethyl acetate

15.6 Exhaust Air Purification Systems: Membrane Reactors

A technology still under development for the purification of relatively poorly water-soluble air pollutants, for example dichloroethane, benzene or xylene, is **membrane reactors.** Membranes can be constructed of very different materials and have different chemical (solubility and selectivity) and physical properties (mechanical strength, pore size, thickness and porosity). In semi-permeable hydrophobic membranes, such as latex or silicone, the mass transfer rate depends on the solubility and diffusivity of the contaminant (Fig. 15.4).

In these processes, the exhaust air to be purified is passed through hollow fibres made of polydimethylsiloxane, Teflon, polypropylene, polytetrafluoroethylene (PTFE) and various composites, with nutrient solution containing microorganisms flowing around the outside. They have pore sizes of 0.1–1.0 µm, small diameters (200–400 µm ID), and are usually packed in bundles in reactors containing between 30 and 100 cm^2/cm^3. The membrane reactors contain the very large number (for example, 1000) of hollow fibers to achieve a large exchange area.

The lipophilic substances are dissolved in the hollow fibre membrane and degraded by the microorganisms on the outside of the membrane.

An important characteristic of membrane bioreactors is the fact that the exhaust air and the biomass are physically separated from each other, which makes biological exhaust air purification possible even in certain applications, such as indoor air.

Nutrient solution containing the microorganisms is pumped around to supply the cells with oxygen and to remove the degradation products (CO_2) (Table 15.9).

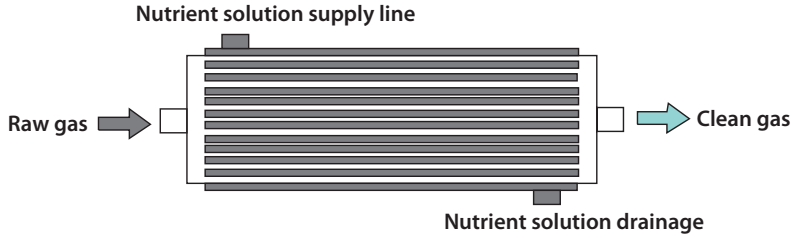


Fig. 15.4 Schematic of a membrane reactor

Table 15.9 Comparison of the limits of membrane biofilters and conventional biofilters (page 196, M.W. Fitch)

Characteristics	Conventional biofilter	Membrane biofilter
Clogging and pressure drop	Main problem. Dominant topic of biofiltration	No clogging of the gas phase, in line with a moderate pressure drop
Channeling, overflowing or foaming	Important topics depending on the operation	Not available
Mass Transfer	Direct Gas Biofilm	Important obstacles of the membrane and liquid phases
Pollutants with low water solubility	Poor removal due to mass transfer	Good disposal
Nutrient Control	Potentially difficult	Simple due to the separation of the liquid phase
Humidity control	Requires humidification of the gas phase	Minor or negligible issue; condensation is sometimes observed on the gas side of the membrane
Reaction to impact load	Poor response depending on nutrient status	Poor response
Maximum pollutant load	50–300 g/m^3 h, 50–2000 ppm_v	Membrane flow limit can allow the treatment of an enormous loads
Acid by-products	Lowered pH, can occasionally lead to destruction of the filling material	The pH control of the liquid phase is simple
High temperatures	Limited up to 80 °C	With an external cooling loop for gas up to 150 °C possible
Operation	humidification is required, replacement of the filling material is common at intervals of 3–5 years	Periodic addition of nutrient solution
Cost of capital	Low	High, the most important planning problem
Application on an industrial scale	Many	Not yet until 2005

15.7 Selection Criteria for Procedure Selection

The following **criteria** can be used to decide between the three established process variants described (see Table 15.5): Due to their low flow velocity, biofilters require a larger construction volume, which can be realised as surface or high filters. In terms of acquisition and operating costs, biofilters have advantages over bioscrubbers and trickling filters, since they require less equipment and less monitoring, no chemicals are needed and large quantities of

water do not have to be pumped. Biofilters are thus an inexpensive and effective method of odour control in agriculture. Due to the possibility of regulating the pH-value as well as dosing nutrients and thus influencing the microbial decomposition in a targeted manner, bio-scrubbers and trickling filters can be used more flexibly. The bridging of standstill periods is easier with the biofilter, as the filter material serving as a nutrient medium only has to be aerated and kept moist, whereas with bioscrubbers and trickling filters nutrients also have to be dosed in order to maintain the biomass. While no reaction products have to be disposed of regularly in the case of biofilters, small amounts of excess sludge with approx. 1% organic dry matter accumulate in the case of bioscrubbers and trickling filters.

Test Your Knowledge

- What are the basic problems with exhaust air?
- Name any sources of bioaerosols?
- Describe biofilters and bioscrubbers. Name the advantages and disadvantages of each
- What are bio trickle bed reactors?
- For which concentration range in g/m^3 exhaust air is biological exhaust air purification with the different biological systems useful?
- What properties besides biodegradability are required for biological exhaust air purification?
- Discuss bioaerosols and legionella in the exhaust plumes of cooling towers (Ulm, Warstein, etc.).

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Biological Soil Remediation

Contents

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16.1 Contaminated Site Issues

It was not until the seventies of the last century that the dangers posed by soil contamination known as contaminated sites were recognised. **Contaminated sites** are deposits and old sites containing substances that pose or are expected to pose hazards to the environment, especially to human health. **Old deposits** are abandoned or disused dumping grounds for waste and production residues, for example "wild" rubbish tips. Abandoned **sites** are disused areas and facilities where hazardous substances were handled in the past. The problem of contaminated sites is illustrated by the number of more than 360,000 suspected contaminated sites in the Federal Republic of Germany. Examples of contaminated sites and the groups of substances present there are listed in ◘ Table 16.1.

◘ **Table 16.1** Widespread contaminated sites and the main pollutants occurring there

Legacy	Pollutant groups
Mineral oil processing and storage, petrol stations	Aliphatic and aromatic hydrocarbons
Gas works, coking plants	Phenols, PAH
Freight stations, airfields	Aliphatic and aromatic hydrocarbons
Chemical and pesticide production	Chlorinated hydrocarbons, PCBs
Ammunition factories	Nitro aromatics like TNT
Dry cleaners	Tri- and tetrachloroethene
Metalworking	Heavy metals, cyanides
Unclassified landfills, waste disposal sites	Heavy metals, sulfates, nitrates

The hazards posed by soil pollution were neglected for a long time because the self-purifying powers of soils were overestimated and the transfer pathways from soil to groundwater and the food chain were insufficiently understood. But even after these hazards had been recognised, there was irresponsible dumping and backfilling with hazardous substances in order to avoid disposal costs. Only now, with the regulations on soil protection and the Closed Substance Cycle Economy and Waste Management Act, are the foundations being laid to ensure that no "new burdens" are added to the legacy of industrial development and disorderly waste disposal. However, the value of soil for the function of ecosystems as a filter and buffer system for material cycles is still not recognised to its full extent, as the continuing sealing and building development show.

Today and in the future, the aim is to identify the dangers posed by contaminated sites and, where necessary, to remediate the soils. The term **soil remediation** covers all measures that lead to the soil being cleaned to such an extent that there is no danger to protected assets such as groundwater, surface water, soil and air. Even the best and most costly measures will not be able to restore the soil to its original uncontaminated state. This once again underlines the necessity of preventive environmental protection: prevention is always more cost-effective than remediation.

Which **remediation targets** are to be achieved? The substance- and concentration-related criteria for assessing contamination in environmental media are controversial. Use-related criteria are increasingly being discussed. An area that is to be used as a children's playground or for sports and leisure purposes after remediation must be cleaner than future car parks or industrial and commercial areas. However, it is difficult to determine types of use for long periods of time. Therefore, reference and test values have been established in the Netherlands for a larger number of organic

and inorganic pollutants, which form an important basis for limit values. These values have been summarized in the **Dutch List** (amended 1994), which includes values for both soil and groundwater. In ◘ Table 16.2 some values from the list are given to illustrate the approach. The table distinguishes between the categories of reference and intervention values. Reference values are individual or average values determined on land outside the area of impact of contaminated sites and correspond approximately to natural values (background concentration). Intervention values signal the existence of serious soil contamination if exceeded. The legal consequence of this is the obligation to carry out a use-dependent current risk assessment and the resulting determination of the remediation urgency (0–4 or 4–21 years). The intervention values are based on human and ecotoxicological criteria as well as the exposure factors for a standard soil underlying the standard scenario "living with garden". The intervention values are considered to be exceeded if a minimum volume of >25 m^3 soil or >100 m^3 pore water saturated soil/aquifer is affected.

◘ **Table 16.2** Reference and intervention values for soil and groundwater contamination for the protection of humans and ecosystems (Altlasten Spektrum, 3/95, 165–166)

Substance	Soil/sediment (mg/kg dry matter)		Groundwater (µg/L)	
	Reference values	Intervention values	Reference values	Intervention values
Metals				
Arsenic	29	55	10	60
Cadmium	0.8	12	0.4	6
Chrome	100	380	1	30
Cobalt	20	240	20	100
Copper	36	190	15	75
Mercury	0.3	10	0.05	0.3
Lead	85	530	15	75
Nickel	35	210	15	75
Aromatic compounds				
Benzene	0.05 (d)	1	0.2	30
Ethylbenzene	0.05 (d)	50	0.2	150
Toluene	0.5 (d)	130	0.2	1000
Xylenes	0.5 (d)	25	0.2	70
Phenol	0.05 (d)	40	0.2	2000
Polycyclic Aromatic Hydrocarbons (PAH)				
Naphthalene	–	–	0.1	70
Anthracene	–	–	0.02	5

(continued)

Table 16.2 (continued)

Substance	Soil/sediment (mg/kg dry matter)		Groundwater (µg/L)	
	Reference values	Intervention values	Reference values	Intervention values
Phenanthrene	–	–	0.02	5
Fluoranthene	–	–	0.005	1
Chrysen	–	–	0.002	0.05
Benzo(a)pyrene	–	–	0.001	0.05
PAH (sum of 10)	1	40	–	–
Chlorinated hydrocarbons				
Tetrachloroethene	0.01	4	0.01 (d)	10
Trichloroethene	0.001	60	0.01 (d)	500
Vinyl chloride	–	0.1	0.01 (d)	0.7
Carbon tetrachloride	0.001	1	0.01 (d)	10
Trichloromethane	0.001	10	0.01 (d)	400
Dichloromethane	–	20	0.01	1000
Chlorobenzenes (total)	–	30	–	–
Monochlorobenzene	–	–	0.01 (d)	180
Dichlorobenzenes (total)	0.01	–	0.01 (d)	50
Trichlorobenzenes (total)	0.01	–	0.01 (d)	10
Chlorophenols (total)	–	10	–	–
Monochlorophenols (total)	0.0025		0.25	100
Dichlorophenols (total)	0.003		0.08	30
PCB (sum of 17)	0.02	1	0.01 (d)	0.01
Pesticides				
Aldrin, Dieldrin, Endrin (total)		4		0.1
α-HCH	0.0025			
β-HCH	0.001			
γ-HCH	0.05 µg/kg		0.02 ng/L	
Carbaryl		5	0.01 (d)	0.1
Atrazine	0.05 µg/kg	6	0.0075	150
Other compounds				
Pyridine	0.1	1	0.5	3
Styrene	0.1	100	0.5	300

Table 16.2 (continued)

Substance	Soil/sediment (mg/kg dry matter)		Groundwater (µg/L)	
	Reference values	Intervention values	Reference values	Intervention values
Phthalates (total)	0.1	60	0.5	5
Mineral oil (total)	50	5000	50	600

Standard soil: 10% organic matter, 25% clay
(d) limit of quantification; if no values are given, the chemicals are not detectable by current methods

16.2 Methods of Biological Soil Remediation

At first glance, biological soil remediation seems to be something paradoxical: Microorganisms are supposed to remove pollutants whose biological degradation has either not occurred for years or decades or has proceeded very slowly. The question is therefore why the degradation of compounds that are in principle degradable fails to occur at some sites or proceeds at a very slow rate. According to the available experience, the **main reasons for the lack of degradation or slow degradation** are, depending on the site and the compound:

- Since oxygen is needed both as a reagent in the oxygenase reactions (see Sect. ► 6.1) and as an electron acceptor in the fast aerobic processes, a **lack of oxygen** is often the main problem. This can have a particular effect in the **absence of alternative electron acceptors.**
- Another major problem is the **lack of bioavailability** of many contaminants. This can result from poor water solubility of the compound. Particularly in soil, the sorption of pollutants to soil components also plays a decisive role. Both hydrophobic bonds to organic material and the sorption of polar substances to humic acids or clays are important. In the case of fine-grained material with relatively large surfaces, sorption processes play a particularly important role. The age of a contaminant can also contribute to the reduction of bioavailability via the diffusion of pollutants into the smallest pores over time or sequestration in the humic matrix.
- At the site, **nutrients** such as nitrogen or phosphorus can be limiting.
- Biological activity is often high at soil **pH values** between 6 and 8. pH values that deviate strongly from this can have an inhibiting effect on activity and thus on the degradation of pollutants.
- For reductive or cometabolic processes (Sect. ► 6.2), an **electron donor** or an **auxiliary substrate** may also be missing.
- The **toxicity of** the pollutant in question or of concomitant substances such as heavy metals or biocides may inhibit microbial activity.
- Normally, soil remediation relies on the activity of bacteria and fungi already present in the soil. Especially in the case of compounds that are foreign to nature (xenobiotics) or that occur very rarely or normally in low concentrations, an **insufficient number of degrading microorganisms** can be a decisive problem. The time factor and possible genetic adaptation processes (Sects. ► 6.2 and 10.1.2) also play an important role here.

Biological **remediation processes** generally aim to compensate for all or the most impor-

tant of these deficiencies in each case, thus enabling or accelerating degradation or even immobilisation. **Complete biodegradation** occurs when the organic pollutants are converted to mineral end products (carbon dioxide, chloride) on the one hand, and to biomass on the other. **Incomplete biodegradation** can also be used to clean contaminated soils, either by converting the pollutants into harmless metabolites (intermediate products) or by fixing them in the soil through incorporation reactions (**immobilisation/humification**).

Biological soil remediation techniques are divided into ***ex situ* techniques** (with soil excavation) and ***in situ* techniques** (without soil excavation), with *ex situ remediation* being applied as *on-site* (at the remediation site) or *off-site* (away from the remediation site), depending on the location of the treatment facility.

While *ex situ* and *in situ* methods are aimed at controlling and accelerating degradation or immobilisation processes, another strategy relies solely on "**natural attenuation**" (NA), i.e. on the fact that naturally occurring degradation and retention processes can slow down the spread of contaminants in the unsaturated and saturated soil zone. The reduction in the contaminant load of a contaminated system under natural conditions, identified by the term "Natural Attenuation", may be associated with a reduction in the mass, toxicity, mobility, volume or concentration of the contaminants in the soil or groundwater. In conjunction with long-term monitoring of identified contaminants, Natural Attenuation processes can be used as remediation strategies, according to the U.S. Environmental Protection Agency (EPA). The term "**Monitored Natural Attenuation**" (MNA) has been proposed for this approach.

While the term "natural attenuation" refers exclusively to the uninfluenced use of natural retention and purification processes in the subsurface, the application of methods to stimulate self-purification processes is referred to as "**Enhanced Natural Attenuation**". This strategy aims at harnessing natural pollutant degradation by enhancing, optimizing or accelerating self-cleaning processes by modifying the physical or chemical conditions at the site of pollutant degradation through simple technical measures. As this cannot be clearly separated from *in situ* processes, the Enhanced Natural Attenuation strategy is not listed separately here.

Frequently Used Abbreviations in Soil Remediation

BTEX	Benzene, toluene, ethylbenzene, xylenes (mineral oil hydrocarbons)
cDCE	*cis*-1,2-Dichloroethene
DNAPL	Dense non aqueous phase liquids
HCH	Hexachlorocyclohexane (lindane)
MKW	Mineral oil hydrocarbons
MNA	Monitored Natural Attenuation
MTBE	Methyl *tert*-butyl ether
NA	Natural Attenuation
ORC	Oxygen Release Compounds
PAH	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated biphenyls
PER, PCE	Tetrachloroethene
RDX, HMX	Royal Demolition Explosive
TCE	Trichloroethene
TNT	Trinitrotoluene
TPH	Total Petroleum Hydrocarbons
VC	Vinyl chloride (=chloroethene)
VOXs	Volatile halogenated organic compounds (C_1/C_2-alkanes/ alkenes with boiling points <150 °C)

16.2.1 *Ex Situ* Procedure

All *ex situ* methods allow a relatively **intensive treatment of** the contaminated soil compared to the *in situ* methods.

- Microbial activities can be promoted by aeration, nutrient supply and, if necessary, pH adjustment.
- Homogenization leads to an increase in the bioavailability of the contaminants and can also reduce toxicity problems through more uniform distribution.
- If necessary, a lack of degrading bacteria can be compensated for by adding special cultures.

In addition to such procedural aspects, *ex situ* procedures have the advantage over *in situ* procedures that the contaminated site is available for new use very quickly. At the same time, they presuppose that an *ex situ* solution is still practicable and financially viable in terms of its scope (contaminated area, depth of contamination).

In *ex situ* processes, mechanical **soil pre-treatment is** carried out at the beginning of the processing. In this way, impurities such as plastics, coarse building rubble and metals can be removed or, if necessary, coarse particles can be crushed to the required grain size by means of a crusher. This achieves the desired homogenization. For further processing, mainly windrow technology and reactor processes are used.

16.2.1.1 Rental Technique

The term "windrow technology" refers to processes that lead to the biological degradation of pollutants in the form of constructed soil windrows. The windrows, usually referred to as soil or regeneration windrows, are heaps of different shapes and sizes to which the soils are piled. Depending on the shape of the soil windrow, a distinction is made between table (rectangular) windrows, trapezoidal windrows and pyramidal windrows. The height of the ground heaps is up to 3 m, in exceptional cases high heaps with more than 3 m height are created. The windrow technique is carried out on sealed ground in halls or tents. Flat windrow methods are also used under the terms "bio-beds" or "bed method".

The limitations of microbial degradation mentioned above are already partially reduced during mechanical **soil pre-treatment**. This leads to **homogenization** and loosening of the material with positive effects in terms of bioavailability of pollutants and oxygen supply. Soil pre-treatment is usually combined with the incorporation of nutrients and structural and aggregate materials. The most important **nutrients** include organic and inorganic nitrogen and phosphorus compounds. On the one hand, these should be present in sufficient amounts, and on the other hand, they should be adjusted to a favourable ratio of the nutrient contents to each other. The so-called C:N:P ratio should be between 100:15:2 and 100:10:1 (weight units). **Structural and additive** substances are substances which are added to the soil at the beginning of the treatment and which lead to a loose soil structure and good aeration. Bark products (bark mulch, bark humus, bark), straw, composts are used. The addition of these substances also increases the organic content of the soil and thus promotes its biological activity. **Inoculation** with special cultures or organisms previously isolated from the site can also be carried out as part of the pre-treatment.

Windrow techniques are generally aerobic processes and aeration is already improved by the aforementioned loosening and the structural and aggregate materials. Depending on the type of additional **oxygen supply**, a distinction is made between passive and active aeration. **Passive aeration** of soil windrows is achieved by a layered windrow structure consisting of an alternating sequence of soil and aeration layers (◘ Fig. 16.1c). Coarse structural materials

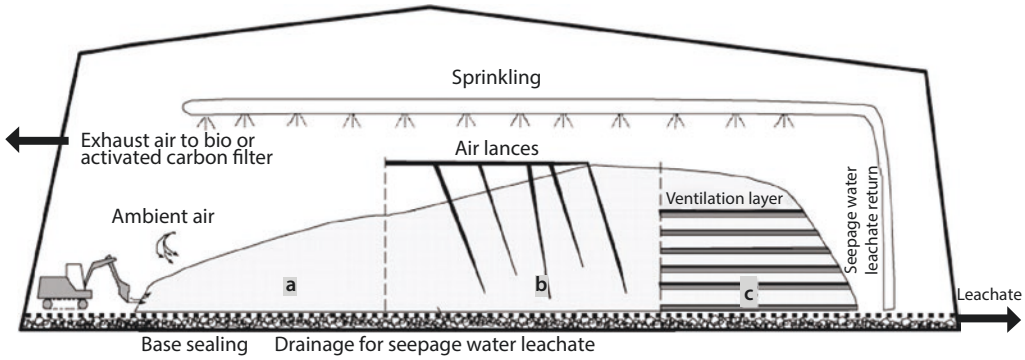


Fig. 16.1 Schematic structure of a floor stack with **a** dynamic ventilation, **b** passive ventilation and **c** forced ventilation respectively

such as wood chips are used in the aeration layers and in this way a supply of atmospheric oxygen to the soil layers is achieved by passive diffusion. The supply of atmospheric oxygen is also improved when the windrow body is heated by the biological activity (chimney effect).

The **active ventilation of** floor heaps includes forced ventilation and dynamic ventilation. In the case of **forced aeration**, the soil windrow is supplied with atmospheric oxygen with the aid of technical installations or measures. Sieve trays, drain pipes or air lances are used (Fig. 16.1b). Depending on the arrangement of the blower devices, a distinction is made between pressure and suction aeration. In the presence of highly volatile components (e.g. BTEX aromatics, short-chain aliphatics, VOX), suction aeration offers the advantage that emissions are avoided if an exhaust air filter is installed downstream of the suction system. **Dynamic aeration** is achieved by regularly turning or repositioning the floor heaps. In this process, the soil is picked up with the aid of turning and repositioning machines, piled up to form a new windrow and the ambient air is thus introduced into the soil windrow.

An essential prerequisite for biological activities in the soil is an optimal **water content**. Depending on the type of soil windrow irrigation, a distinction is made between dry windrows (dry rotting process) and wet windrows (windrows with process water circulation). In the case of dry windrows, a water content of up to about 30% by weight is set at the beginning of treatment, which corresponds to the natural soil moisture, so that no free water is produced during operation. To prevent the windrow from drying out during the course of treatment, the water content is regularly checked and adjusted if necessary. In the case of wet windrows, irrigation is carried out during operation using sprinkler systems. This produces free process water which, depending on the load, has to be cleaned and can then be reused for irrigation (process water circuit). Due to the necessary installations, such as drainage pipes or drainage layers, wet windrows are static windrows.

The windrow technology is aimed at the treatment of aerobically degradable pollutants, regardless of which type of aeration is selected. An exception are modified windrow processes, which allow anaerobic or two-stage **(anaerobic—aerobic) process control** due to the quantity and composition of the aggregates. Due to the high proportion of easily utilisable, organic aggregates, these lead to a biological oxygen depletion within the soil windrow, so that anaerobic conditions quickly develop in the windrow body. Anaerobic-aerobic windrow processes have

been tested on a technical scale in the biological treatment of soils contaminated with explosives.

The **bioavailability** of pollutants, which is **limited** by low water solubility and sorption to organic matter and/or to the clay content in the soil, can ultimately only be improved to a limited extent in soil windrows. Due to their high sorption capacity with respect to the pollutants and at the same time poor aeration, loamy and clayey soils in particular are only suitable for the windrow technique with restrictions. In general, the particle size distribution of the soil is an important parameter for biological soil treatment. A high proportion of organic matter in the soil binds lipophilic pollutants and thus limits their bioavailability. If the bioavailability of the pollutants is low, the use of substances with solubilising properties (e.g. surfactants, emulsifiers) can be useful.

Windrow methods are predominantly used for soil contamination with mineral oil hydrocarbons and BTEX aromatics. This is due to the widespread contamination by mineral oil products (petrol, diesel, heating oils), but is also based on the good biodegradability of these substance groups. In isolated cases, windrow processes are also used for polycyclic aromatic hydrocarbons (PAHs), but mainly when only or predominantly low-condensation PAHs (2- to 4-ring compounds) are present. Other classes of substances such as phenolic compounds, nitrated and chlorinated hydrocarbons are only treated with the windrow process in rare cases.

In practice, windrow techniques are not only used as the sole remediation technique, but also in **combination** with non-biological techniques. The most common are combinations of biological windrow technology and thermal soil treatment. In this case, highly contaminated soil areas are subjected to thermal treatment (for example, if mineral oils are present in the liquid phase), while less contaminated soil batches are treated using the windrow technique. A combination with soil washing processes can also be useful (see also ► Sect. 16.2.1.3).

16.2.1.2 Landfarming

Landfarming is the treatment of contaminated soils using agricultural equipment such as ploughs and tillers to loosen and aerate the soil and add nutrients and aggregates. This method can be used to treat large areas of contaminated soil *in situ* and after excavation in separate storage areas. With this simple method there is a risk of leaching of mobilised contaminants and volatilisation. In Germany, therefore, the contaminated soil is applied to a base liner such as foil to prevent leaching. The excavated soil is spread over a wide area in a kind of shallow trough, with a layer height of about 0.4 m. The soil is then spread over the surface. At this layer height, treatment with traditional agricultural techniques is possible. The beds are usually not sealed at the top, so that they are exposed to the weather and emissions of outgassing pollutants can occur. In addition, the amount of land required is very large, about 3000 m^2 is required for 1000 m^3 of soil. Landfarming is therefore rarely used in Germany.

16.2.1.3 Reactor Process

Technologies that lead to biodegradation of pollutants in closed systems are the reactor processes. Biodegradation of pollutants in bioreactors is achieved analogously to windrow technology by removing factors that limit degradation. In bioreactors, this goal can be achieved **faster and more effectively than in soil windrows** because a much higher degree of mixing of soil and additives can be achieved and because a closed system is easier to monitor and control. The higher degree of mixing results in better bioavailability of contaminants, and rapid equalization of contaminant levels can help reduce toxic effects from locally high concentrations. Oxygen supply is ensured to a greater extent. If nutrient additions are required, local nutrient deficiencies can be compen-

sated for by the high degree of homogenization in bioreactors. Organic co-substrates, buffer substances, redox-active substances, complexing agents, detergents and solubilizers can be used as further additives in bioreactors. This also makes pollutants with low water solubility accessible for treatment, and multi-stage, for example anaerobic-aerobic processes such as for the degradation of higher chlorinated hydrocarbons (Sect. ► 6.2) or explosives (Sect. ► 6.3) can be more easily implemented. In the case of inoculation of bioreactors with microorganisms (e.g. with activated or digested sludge or with pre-cultures of microorganisms), bioreactors as closed systems offer the advantage that added cultures can be better controlled and the growth conditions required for the establishment of added microorganisms can be better adjusted. In the case of pollutants with a high volatility, the closed design ensures that the substances remain in the gas phase of the reactor or are specifically extracted and purified via exhaust air filters.

Overall, bioreactors lead to a significant expansion of the spectrum of treatable pollutants and soils compared to conventional aerobic windrow technology. The spectrum of successfully treated pollutants ranges from BTEX to low-condensation PAHs and highly chlorinated hydrocarbons (chloroethenes, chlorophenols) and compounds typical of explosives. At the same time, reactor processes have the disadvantage that they are associated with significantly higher costs than windrow processes.

Bioreactors for the purification of contaminated soils can be constructed in very different ways. Depending on the water content of the soil to be treated, a distinction is made between solid and suspension reactors. Despite the diversity, there are common basic elements:

- Reactor vessel for receiving the soil (or the soil suspension).
- Mixing aggregates for homogenization of the soil and distribution of additives.
- Gassing devices for the supply with oxygen, if necessary also with inert gases.
- Exhaust air filter for cleaning contaminated process exhaust air.
- Dosing devices for the addition of additives (nutrients, electron acceptors and donors, pH-active substances).
- Temperature control devices for generating and controlling the desired temperature.

In **solid reactors**, earth-moist soils are treated so that no free water occurs in the form of process water. The moisture content is usually between 50 and 70% of the maximum water holding capacity of the soils to be treated. The setting of the water content has a decisive influence on the biological activity. At low moisture contents (below 50% of the maximum water holding capacity), there is not enough water available for the pollutant-degrading microorganisms, while at high moisture contents the oxygen supply is limited. In solid reactors, mixing units ensure regular or even continuous contact of the soil with oxygen. In fine-grained/cohesive soils, the oxygen supply to the microorganisms is hindered by the formation of undesirable soil pellets.

Solid fuel reactors are **classified** according to the respective design principle. On the one hand, **rotary drum** or **rotary tube reactors** are used (◘ Fig. 16.2a). The reactors consist of a rotating drum with permanently installed mixing equipment and can have a capacity of up to 100 m^3. Rotary drum plants can be operated in batches (batch operation) or continuously. In contrast to rotary drum plants, tank and tube reactors have a fixed (static) reactor housing and mobile mixing equipment. **Tub reactors** (◘ Fig. 16.2b) consist of individual segments (modular design) and can thus be adapted to the required treatment capacity. The system is closed by covering the segments. A mixing unit with vertically arranged agitator shafts runs through the entire tank reactor and leads to a high degree

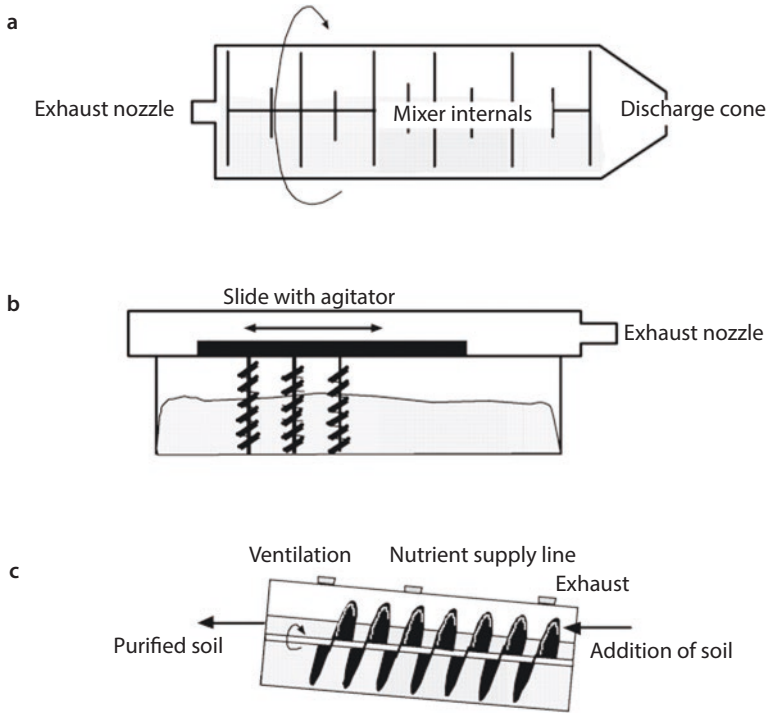


Fig. 16.2 Schematic representation of solid reactors. **a** Rotary tube reactor with fixed mixing internals, **b** Tub reactor, **c** Tubular reactor with transport screw

of homogenization of the soil. **Tubular reactors** (Fig. 16.2c), on the other hand, have a moving screw that moves the bottom in the reactor and thus homogenizes it. **Flat-bed reactors** are another type of reactor used. They consist of one or more flat containers which can be stacked. For homogenization of the soil, the individual containers have several parallel horizontal stirring shafts. Aeration takes place via aeration hoses at the container bottom. Optionally, the soil to be treated can also be irrigated via a water circuit (sprinkler system, perforated container floor, collection tray).

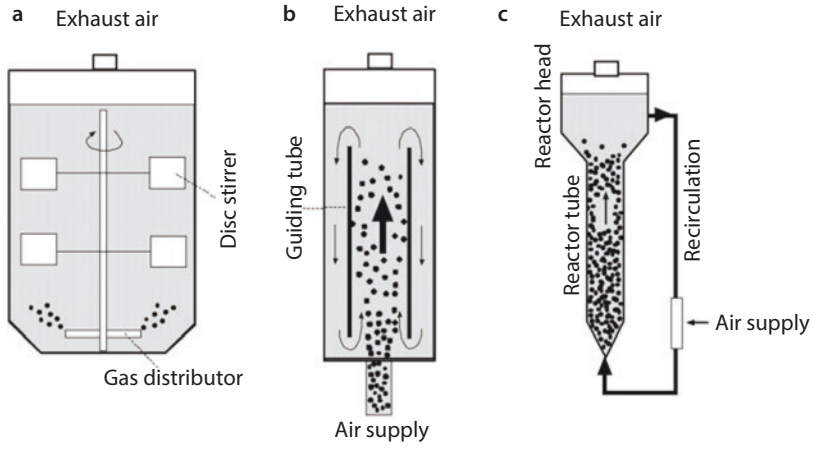
In contrast to solid reactors, ***suspension or slurry reactors*** use soil slurries by adding water or aqueous media. Suspension reactors are usually operated with solids contents of between 30 and 50 wt.%. In terms of process technology, soil suspensions offer two major advantages compared to solid processes. Firstly, they lead to extremely homogeneous mixtures, the treatment of which is correspondingly **easy to monitor and control**, for example with regard to combined anaerobic-aerobic processes. Secondly, suspension processes can be used to biologically clean **fine-grained, cohesive and poorly permeable soils** or soil fractions that cannot be treated as solids or can only be treated inadequately. Thirdly, suspension reactors ensure permanent contact between contaminated soil particles and the surrounding aqueous phase, thus **promoting mass transfer**. Fourthly, solid pollutant matrices (inclusions, crystals) can also **dissolve** in suspension reactors. A disadvantage is an increased effort in the production of bottom suspensions and in the dewatering of the suspension after treatment. The construction principles of suspension reactors essentially originate from wastewater technology and the associated sludge treatment processes. In suspension reactors, oxygen is introduced via supply air lines with

gas distributors, injection nozzles or gassing membranes, whereby both compressed air and technical oxygen can be used. In principle, the input of oxygen is also possible via dissolved oxygen carriers such as hydrogen peroxide or **oxygen release compounds** (ORC, effective component magnesium peroxide, which splits into oxygen and magnesium hydroxide).

Suspension reactors are classified according to the respective process principle, although combinations can also exist in one reactor type. **Stirred reactors** (◘ Fig. 16.3a) consist of a static housing and a stirring system which, on the one hand, keeps the bottom suspension in suspension and, on the other hand, ensures a uniform distribution of the air supply or the dosed additives in the suspension. In addition to stirred reactors, loop reactors and fluidized bed reactors are also used for the treatment of soil suspensions. In order to keep the soil particles in suspension and to achieve a high degree of mixing with the added additives, the suspension is circulated. In **loop reactors** (◘ Fig. 16.3b), the flow pattern is that of a loop, due to the installation of a concentric guide tube inside the reactor. In **fluidised bed reactors** (◘ Fig. 16.3c), on the other hand, circulation is achieved by withdrawing the suspension from the upper part of the reactor (reactor head) and feeding it back in via a pump at the bottom. If there are aeration devices at the bottom of loop or fluidised-bed reactors, settling of bottom particles by rising air (gas) bubbles can be avoided (airlift principle).

An important influence on the **selection** of the reactor process is the **texture of the soils** to be treated:

- Solid matter reactors are mainly suitable for coarse-grained and less cohesive soils; only with these is sufficient permeability of the soil (oxygen supply) guaranteed.
- Suspension reactors, on the other hand, are primarily aimed at treating fine-grained soils or soil fractions. Coarse fractions are separated and can be treated separately. Fine-grained soil particles can be kept in suspension by various systems (agitator, airlift, liquid circulation), which leads to optimum contact with the surrounding medium. This in turn is the prerequisite for desorption of the pollutants, which are available to microorganisms after transfer to the aqueous phase.



◘ **Fig. 16.3** Schematic diagram of suspension reactors for soil treatment. **a** Stirred tank reactor, **b** Airlift loop reactor, **c** Fluidized bed reactor

The development of bioreactors in soil remediation is increasingly moving towards suspension (slurry) processes. This is due to the fact that suspension processes extend the application range of biological soil remediation in the direction of fine-grained soils. In contrast, solid reactors, which are primarily suitable for the purification of well permeable, coarse-grained soils, are in competition with other remediation processes such as soil washing.

On a technical or industrial scale, suspension reactors are currently used in combination with soil washing processes. In this process, the contaminated soil is first broken down in a washing plant and then separated into different particle size classes. The contaminated fine and ultra-fine grain fractions are treated biologically in suspension reactors. The treatment of ultra-fine fractions (grain sizes below 5 μm) using bioreactors is of great interest. In general, however, the use of bioreactors for the treatment of contaminated fine and ultrafine fractions from soil washing is in strong competition with the usual disposal of these residual materials by landfilling.

16.2.2 *In Situ* Soil Remediation

Biological *in situ* soil remediation refers to processes in which the contaminated soil (unsaturated soil zone) or the contaminated aquifer (saturated soil zone) remains in its natural position. Excavation of the contaminated soil does not take place. Ideally, the subsoil should be regarded as an oversized "reactor" in which the biological remediation process takes place. Such methods are particularly suitable when the extent of the damage is so great, due to the area or depth of contamination affected, that *ex situ* remediation becomes too complex and expensive. In the case of more limited damage, *in situ* procedures are used where the accessibility of contaminated soil areas is restricted. This is the case if there are buildings, plants, sewer systems, pipelines or other infrastructural facilities on the contaminated site that need to be protected.

In situ processes are based on physical, chemical as well as biological processes. Physical and chemical processes lead to a removal, transformation or immobilisation (precipitation, sorption) of the pollutants in the subsurface. Biological processes can not only eliminate degradable contaminants, but also initiate or support physicochemical processes. Conversely, biological degradation reactions occur as "secondary effects" when physical or chemical processes are used. In this respect, biological *in situ* processes are rather combinations of processes. The factors limiting degradation, such as a lack of nutrients or oxygen, must be eliminated for this purpose.

In principle, *in situ* procedures require extensive **information about the contaminated site.** For the treatment of the unsaturated zone, knowledge of soil **properties** such as soil structure (aggregation, presence of macropores), soil texture (grain size distribution), porosity/storage density, water content and organic matter content is important. These factors influence the air permeability on the one hand and the sorption behaviour of the soil on the other. Aeration processes for the unsaturated soil zone require sufficient air permeability throughout the soil body to be decontaminated. Understanding the **hydrogeologic system** is important for treating the saturated soil zone. Thus, knowledge of the horizontal/vertical layer sequence, the hydraulic permeability of the water-bearing layers, the geo- and hydrochemical milieu conditions in the groundwater and potential sorbents (clay, organic matter) is necessary.

The possibility of using biological *in situ* processes also depends on the **properties of the pollutants**. Pollutants with severely limited water solubility and bioavailability, such as tar contaminants, are hardly an option. Physical and chemical properties such as vapour pressure, saturation concentration,

mobility, distribution equilibrium between gas and water phase, possibility of formation of explosive gas mixtures must also be taken into account when planning biological *in situ* processes.

Heavy metals play a special role in *in situ* processes, as they are not subject to degradation. Various phytoremediation processes can play a role in remediation here. In some cases, however, the mobility of the metals can be influenced by additives and process control, which in principle can be used for remediation by controlled leaching or by targeted immobilization.

The distribution of contaminants in the subsurface is of great importance, in particular the question of whether areas near the surface, deeper-lying but not water-saturated or water-saturated zones are affected. In addition, extremely inhomogeneous contamination distributions in the subsurface lead to inconsistent degradation, which can manifest itself in the fact that contaminant foci are only slowly degraded from the edges.

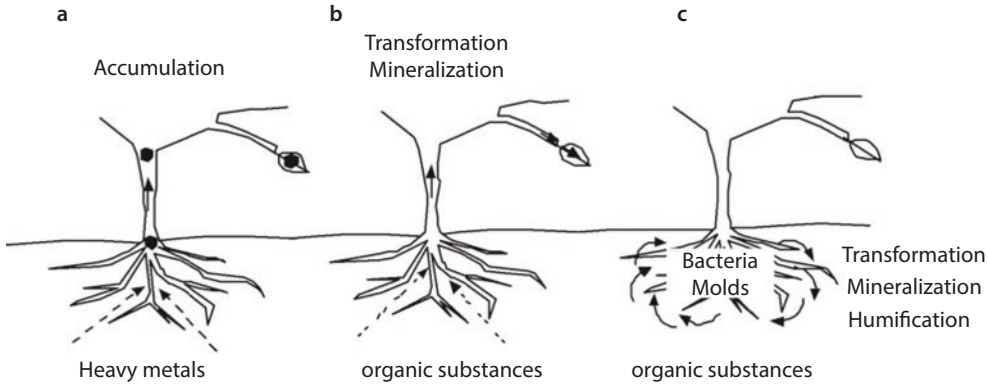
The different soil zones can be considered as remediation areas for *in situ* procedures, whereby a classification of the *in situ* procedures is made according to the principle of the respective technique used:

- The near-surface unsaturated soil zone can be treated with phytoremediation techniques and soil vapour extraction.
- Deeper areas of the unsaturated soil zone are reached by infiltration and aeration methods.
- In the saturated soil zone, infiltration and aeration processes are also widely used.

16.2.2.1 Phytoremediation

In phytoremediation, higher plants are used to clean contaminated soils (◘ Fig. 16.4). By their very nature, the processes are aimed at decontaminating soil contaminants close to the surface. Phytoremediation processes can be applied to contamination with both organic and inorganic pollutants (heavy metals). Various processes play a role in the cleaning of contaminated soil surfaces with plants.

Plants are most commonly used for **phytoextraction** of pollutants. In the case of contamination with heavy metals, phytoextraction leads to the removal of the pollutants from the soil by the heavy metals being taken up and accumulated by the plants. In particular, plants with high accumulation capacities, so-called hyperaccumulators, are used here. In this process, the plants take up the soluble and thus mobile portion of heavy metals in the soil. After accumulation of the heavy metals in the plants, they are harvested and the contaminated plant biomass



◘ **Fig. 16.4** Schematic representation of phytoremediation processes. **a** Phytoextraction, **b** Phytodegradation and **c** Rhizosphere degradation

must then be disposed of (for example by thermal processes). The plants should be harvested including their roots, because heavy metals accumulate after uptake, especially in the root tissue. Even with good enrichment factors, extremely long remediation times are to be expected in some cases.

When organic pollutants are taken up by plants, **phytodegradation** or **phytotransformation** can occur, i.e. a degradation of the compounds in the plant tissue.

In principle, the treatment of highly volatile pollutants in the soil can also lead to **phytovolatilization**, i.e. the pollutants enter the plant and are possibly emitted into the atmosphere via transpiration processes after transformation (◘ Table 16.3).

The degradation of organic pollutants in the rhizosphere, the root zone of the plant, is another possibility of phytoremediation processes (**rhizosphere degradation**). The degradation processes are caused either by the enzymes present in the root exudates (for example peroxidases), by the additional carbon sources or by associated rhizosphere organisms (soil bacteria, soil fungi, mycorrhizal fungi). Root zones are characterized by much higher microbial activities than the root-free soil. The function of the plant here is to provide the root structure and supply the soil organisms with oxygen and nutrients through the root exudates.

Planting also contributes to reducing the spread of pollutants (**phytostabilisation**). By reducing the formation of leachate through transpiration of the plants, the uncontrolled leaching of pollutants is counteracted. The vegetation also reduces soil erosion.

◘ **Table 16.3** Phytoremediation variants with the plants used according to (► www.gwrtac.org/pdf/phyto_e_2002.pdf)

Application	Contaminations	Typical plants
Phytoextraction	Metals: Pb, Cd, Zn, Ni, Cu	Mustard *(Brassica juncea)* Sunflower (*Helianthus* spp.) *Thlaspi carulescens*[a]
Phytotransformation	Chlorinated aliphatics: TCE MTBE Munitions waste: TNT, RDX, HMX Herbicides	Poplar, Willow grasses (rye, fescue, bermudagrass, millet, sedge) Legumes (clover, lucerne, bean)
Phytovolatilization	Metals: Se, As, Hg VOX	Mustard *(Brassica juncea)* Groundwater dependent trees
Rhizosphere degradation	Degradable organics: BTEX, TPH, PAHs Pesticides	Grasses with fibrous roots (bermudagrass, wheat, fescue, rye) Phenol releasers (mulberry, apple, oranges) Groundwater dependent trees
Phytostabilization	Metals: Pb, Cd, Zn, As, Cu, Cr, Se, U Hydrophobic organics	Grasses with fibrous roots for erosion control

RDX (cyclo-1,3,5-trimethylene-2,4,6-trinitramine), HMX (1,3,5,7-tetranitro-1,3,5,7-tetraazocyclooctane), TPH (total petroleum hydrocarbons)

[a] Small, little biomass

16.2.2.2 Infiltration Method ("*Pump and Treat*" Technology)

In infiltration processes, additives are infiltrated into the deeper soil zones via trickling, lances and wells, which are suitable for stimulating biological degradation or removing limiting conditions for pollutant degradation in the subsoil. The following additives can be used for this purpose:

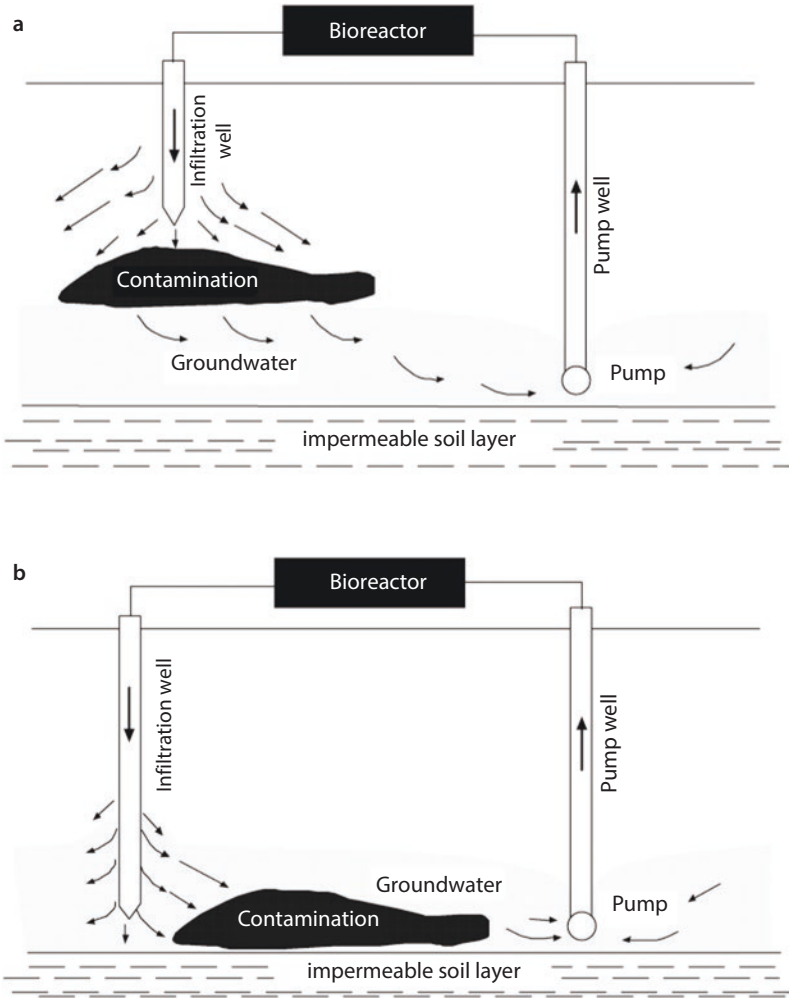
- The most important **nutrients** in the soil include nitrogen and phosphorus compounds, which are added to the contaminated soil area under appropriate deficiency conditions to stimulate microbial growth.
- **Co-substrates** are added to initiate cometabolic xenobiotic degradation. For example, the pollutant trichloroethene (TRI) is known to be degraded under aerobic conditions only in the presence of specific carbon sources.
- Dissolved oxygen (O_2) or its precursors hydrogen peroxide (H_2O_2) and ozone (O_3) are infiltrated as **electron acceptors**. Furthermore, so-called oxygen release compounds or oxygen releasing materials such as magnesium peroxide have been used recently, which lead to a release of oxygen through decay. Molecular oxygen is also a substrate for oxygenases, important reactions in the aerobic degradation of many hydrocarbons. In addition to compounds that lead to the release of oxygen, other electron acceptors such as nitrates (potassium and ammonium nitrate [KNO_3, NH_4NO_3]) are also used.
- A modification of the milieu conditions can also be achieved by adding **electron donors.** These are easily utilizable substrates that cause oxygen depletion or release hydrogen (in the form of so-called reduction equivalents). Pollutants such as higher chlorinated hydrocarbons, which can only be degraded under anaerobic conditions or a sequence of anaerobic and aerobic conditions, can be converted in this way.
- **Buffer substances** (acids, alkalines) can be used for pH regulation.
- **Detergents** serve to increase the bioavailability.
- In order to achieve or accelerate the degradation of pollutants in the subsoil, **microorganisms** can also be added.

When adding additives for *in situ* processes, it must generally be taken into account that, in addition to the effects described, **undesirable side effects** may occur. For example, the introduction of oxygen compounds can lead to oxidation and precipitation of iron, manganese and other metal compounds in the subsurface, which can lead to technical problems (clogging of remediation wells). When nitrate is used as an additive for *in situ* processes, there is a risk of groundwater contamination. Similarly, nutrients, electron donors and detergents should generally be carefully dosed to avoid additional groundwater contamination. Excessive bacterial growth due to nutrient additions can lead to clogging of the pore spaces ("bioclogging"). When microorganisms are added, uniform dispersal is often not achievable due to their tendency to sorption to surfaces and biofilm formation within the contaminated body. In addition, added microorganisms are in competition with the autochthonous microflora.

In infiltration processes, the required water is previously extracted from the aquifer. Depending on the location of the contamination, it can be little to heavily contaminated, can and normally must be subjected to **water purification** on site before infiltration. So degradation processes take place in the soil or aquifer itself on the one hand, but also in water purification plants at the site on the other. Which effect predominates may vary depending on the type and location of the contamination relative to the water table. As long as infiltration

processes are used as the sole remediation method, relatively long remediation times often result.

For *in situ* remediation by **infiltration of the water-saturated soil zone**, additives are added to water taken from the aquifer, purified if necessary, and infiltrated into the unsaturated soil zone (◘ Fig. 16.5a). Depending on the extent of the contamination, infiltration takes place either via individual filter pipes or wells connected via a pipe system. Depth-adjustable insertion pipes allow the contamination body to be supplied with additives section by section, which means that differently contaminated depth areas can be targeted more precisely. A homogeneous distribution of the additives is decisive for the success of infiltration processes, because otherwise only a partial treatment of the contaminated body is achieved. In general, it should be noted that additives introduced into the water-saturated soil zone exclusively by infiltration have only a relatively small range of action (limited



◘ **Fig. 16.5** Schematic representation of infiltration processes for the **a** unsaturated and **b** saturated soil zone

mass transport, sorption processes), so that a high density of infiltration wells may be required. For this reason, infiltration of the unsaturated soil zone as the sole remediation method is not very common in practice. The fact that infiltration and water extraction take place in different soil layers does not create a closed loop. Care must therefore be taken to limit the quantity of infiltration medium in order to avoid infiltration-induced spreading of contamination through seepage processes.

For *in situ* remediation by **infiltration of the water-saturated soil zone**, the infiltration medium is recirculated (◘ Fig. 16.5b). For this purpose, contaminated water is extracted, treated *on site* (e.g. with a biological treatment stage) and re-infiltrated into the subsurface in such a way that it can flow through the contaminated area again. This is usually where a significant amount of degradation takes place in the water treatment plants. However, during infiltration, additives can be added again to induce biodegradation in the contaminated body as well. Depending on the arrangement of the infiltration and extraction units, different flushing systems can be generated in the subsurface (horizontal flushing, vertical flushing), whereby not only the saturated but also the unsaturated soil zone can be flushed and cleaned. In order to avoid an outflow of contaminated water during flushing procedures, for safety reasons the withdrawal quantity in the remediation area is generally significantly greater than the infiltration quantity flowing through the contaminated area. The treated excess water (blowdown) can be returned to the aquifer or discharged to a river outside the remediation area, subject to compliance with discharge regulations. As a further protective measure, protective infiltration can be carried out to permanently separate the contaminated zone from the uncontaminated aquifer. For this purpose, groundwater from other groundwater zones or aquifers is used. Infiltration procedures for the saturated soil zone are used for contamination with Mineral oil hydrocarbons and BTEX, and occasionally also for PAHs (insofar as these are water-soluble) and VOX.

16.2.2.3 Aeration Process

Techniques are used that result in direct or indirect aeration of the soil and **reduction of existing oxygen limitation**. The techniques used to aerate the subsurface are derived from processes aimed at **stripping** volatile contaminants from soil air and contaminated groundwater. Aeration measures can be carried out both continuously and discontinuously. In this way, an induction of aerobic contaminant degradation occurs.

In the case of the **unsaturated soil zone**, in the simplest case compressed air is injected into the soil body ("**bioventing**"). For this purpose, aeration lances or levels are installed which project into the contamination area (◘ Fig. 16.6a). Depending on the soil structure and the arrangement of the levels, the oxygen introduced with the air is distributed and leads to a stimulation of aerobic pollutant degradation as well as to a stripping of the pollutant. Indirectly, aeration of the soil is also achieved in **soil vapour extraction**, which has been known for a long time. A soil air flow is induced by the suction, which leads to a supply of ambient air to the contaminated area. With the combination of both methods, on the one hand a release of stripped, volatile pollutants can be avoided and on the other hand a well controllable oxygen supply can be achieved.

For the **saturated soil zone**, methods are used in which compressed air is injected into the saturated area. For this purpose, wells are installed and filtered in a suitable manner so that the compressed air reaches the contamination body as completely as possible via cone-shaped aeration zones (◘ Fig. 16.6b). This also has two main effects here: Firstly, **contaminant desorption** and **stripping** is achieved by the air flow in

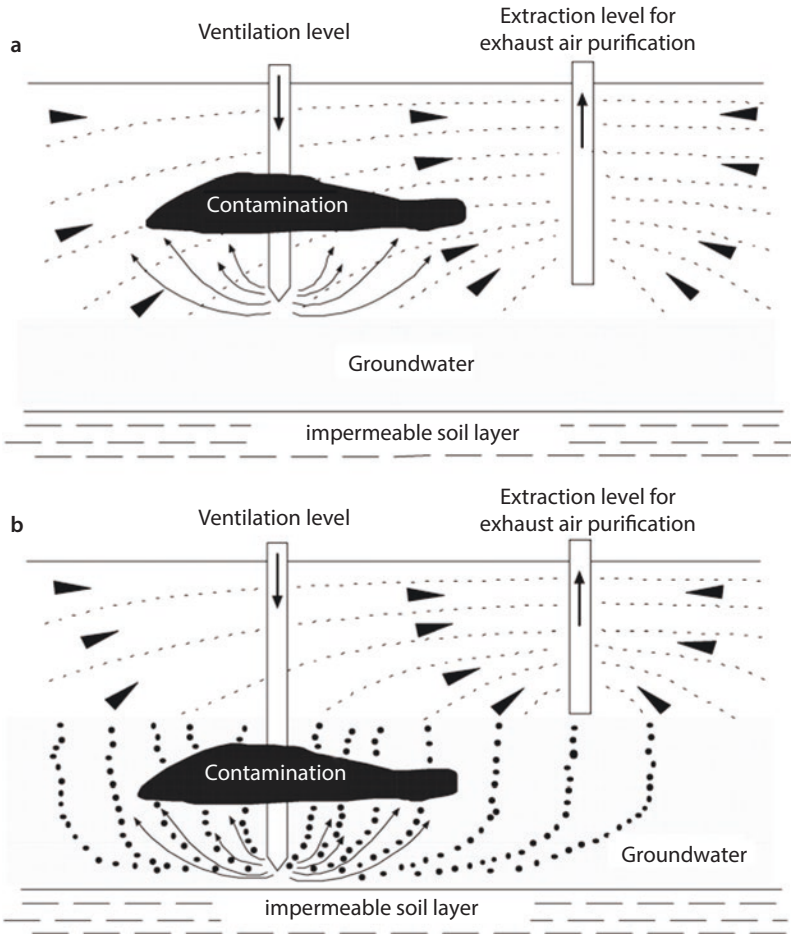


Fig. 16.6 Schematic representation of aeration processes for the **a** unsaturated and **b** saturated soil zone

the aquifer, and secondly, biological degradation of the contaminants is induced via the oxygen supply ("**biosparging**"). These processes, known as air sparging, are usually used in combination with soil vapour extraction of the unsaturated soil zone. In this way, contaminants spanning the saturated and unsaturated soil zone can be treated, and controlled airflow in the unsaturated soil zone can prevent contaminants from spreading to surrounding areas. The success of aeration measures depends largely on the subsurface properties, in particular the density and gas permeability (expressed as a permeability coefficient) of the soil. In principle, the entry of compressed air into contaminated groundwater zones can lead to the drift of pollutants into uncontaminated zones of the aquifer. If oxygen is introduced into the aquifer, it should also be noted that precipitation in the form of iron and manganese oxides may occur. Important questions are also how quickly the air bubbles dissolve and to what extent they can clog an aquifer.

Direct aeration processes are currently **used** for contamination with mineral oil hydrocarbons (well below the saturation limit), BTEX and, in individual cases, low-condensation PAHs. For mineral oil hydrocarbons, even if not fully chlorinated, such

as trichloroethene, these processes are less suitable, as aerobic degradation is slower than for mineral oil hydrocarbons or BTEX and therefore stripping rather than quantitative biodegradation is to be expected. A combination of these processes with measures for soil vapour extraction of the unsaturated soil zone is widespread.

In remediation practice, **combinations of aeration and infiltration processes** are also used. For example, aeration processes (bioventing, air sparging) can be combined with the application of nutrients, whereby the installed compressed air lances can be used for both aeration and nutrient infiltration, so that no additional installations are necessary. In addition, the spreading of the additives in the subsoil is promoted by the aeration process.

Test Your Knowledge

- What is the balance of nutrient contents (C:N:P) to ensure aerobic growth of microorganisms?
- What is the list of reference and intervention values for the purification of soil and groundwater?
- Give reasons (properties of chemicals, types of contaminants, soil types, nutrients) for failure or slow microbial degradation of a contaminant.
- What does "Natural Attenuation" and its modifications involve?
- Distinguish *in situ, ex situ, on site, off site* procedures.
- Bioaugmentation, biostimulation: What do these terms mean?
- Say something about the bioavailability of PAHs in soil.
- What are the advantages and disadvantages of using a slurry reactor for floor cleaning?
- What is the purpose of O_2 in cleaning PAH contaminated soil? Can NO_3^- perform the function? What does biosparging mean?
- What is soil vapour extraction? For which contaminations is it suitable?

16

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Biological Waste Treatment

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_17

17.1 Waste Issues

Domestic, commercial and hazardous waste represent a major environmental problem. The high volume of waste and the limited landfill space have led in recent years to legal principles of **recycling and waste management.** Waste prevention has found its way into the laws as the primary goal. Recycling success varies and is still low in some cases. There is high recycling potential in domestic waste, a large proportion of which consists of compostable organic waste and recyclable packaging materials (◘ Table 17.1).

In addition to reducing the amount of waste to be disposed of through recycling or prevention measures, the equally important objective of waste management must be to reduce pollutants in waste in order to keep emissions from treatment facilities (landfill, waste incineration, pyrolysis, composting) low.

Society's production and goods flows are reflected in the **volume and composition of municipal waste.** In Germany, around 570 kg of household waste is generated per inhabitant and year. The amount of other municipal waste, such as municipal waste from greenery and tree cuttings, is around 50 kg. Added to this is the sewage sludge produced during wastewater treatment. If these waste quantities are added up, conservative estimates result in a waste volume of about 630 kg per inhabitant and year, almost half of which is organic material.

Organic wastes of a special nature are the **hazardous** wastes (for example, oily wastes, varnishes and paints, halogen-free and halogen-containing solvents). They must be disposed of as hazardous waste by special processes of recycling, detoxification, incineration or landfilling. In general, environmentally sound disposal of unavoidable waste is the goal of recycling and waste management.

Energy and material waste recovery includes waste incineration and composting. Thermal recovery (which does not mean waste incineration) accounts for only a small proportion in the 1% range of waste recovery. On behalf of the Federal Ministry of Consumer Protection, Food and Agriculture, an investigation was carried out to determine whether there is greater potential in the use of biogenic residual and waste materials for heat and electricity production. ◘ Table 17.1 shows the data determined in Germany in 2016 on the generation and recycling of municipal waste.

◘ Figure 17.1 shows the municipal waste generation in the EU-28 countries in 2016. According to Eurostat, the Danes were the citizens producing the most municipal waste in 2016 with 777 kg per person, while the Romanians produced the least waste with 261 kg. Denmark is followed by Norway (with 754 kg per person), Switzerland (720 kg per person) and Iceland (656 kg per person). Germany also ranks at the top end of the scale and above the European average with 626 kg of waste per capita per year, while at the other end of the list come three Eastern European countries: Poland produces only 307 kg per capita, followed by the Czech Republic (339 kg of waste per person) and Slovakia (348 kg of waste per person). On average, Europeans produce 480 kg of waste, an amount matched by Greeks (497 kg per person), Italians (495 kg per person) and Britons (495 kg per person).

Table 17.1 Municipal waste generated and recycled in Germany in 2016

Waste generation	Total volume (1000 t)	kg/inhabitant	Recycling rate in % total
Total municipal waste	52,133	634	94
Typical household municipal waste	46,605	567	95
Household waste, commercial waste similar to household waste collected together via the public waste collection service	14,631	178	85
Bulky waste	2541	31	94
Compostable waste from the organic waste bin	4446	54	100
Biodegradable garden and park waste (including cemetery waste)	5925	72	100
Other separately collected fractions	19,062	232	100
Glass	2629	32	100
Paper, cardboard, cartons	7814	95	100
Mixed packaging/valuable materials	5844	71	100
Waste electrical equipment	617	8	99
Other (composites, metals, textiles, etc.)	2159	26	99
Total other municipal waste	5528		92
Household-type commercial waste, delivered or collected separately from household waste	3320		99
Street sweepings/non-degradable garden and park waste	942		59
Biodegradable kitchen and canteen waste	986		100
Market waste	70		91
Fluorescent tubes and other waste containing mercury	11		96
Other separately collected fractions	217		86

Federal Statistical Office (2018)

Overall, 30% of waste was recycled, 27% incinerated, 25% landfilled and 17% composted in the EU in 2016. The share of municipal waste recycled or composted in the EU has steadily increased over the period from 17% in 1995 to 46% in 2016.

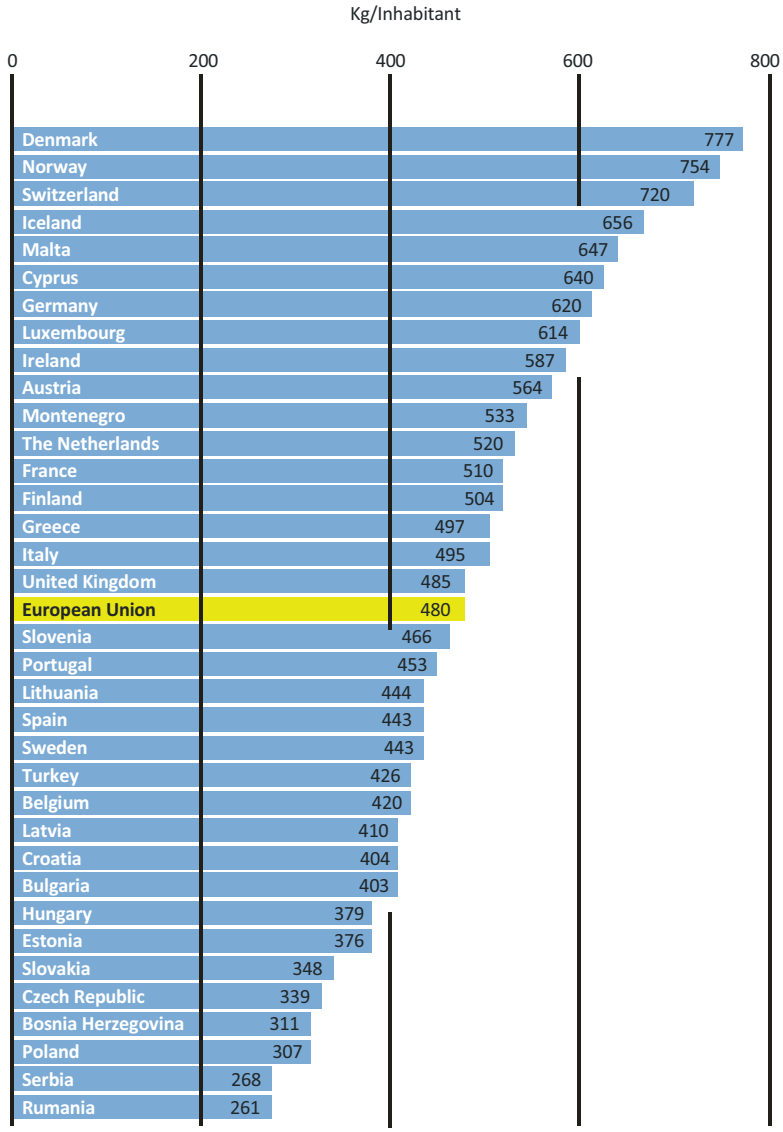

Fig. 17.1 Waste in kg per inhabitant in Europe 2016. The Eurostat data refers to the information from European municipalities and the amount of waste they collect. It mainly includes waste originating from private households, but also from shops, offices and public institutions. Data on municipal waste vary within EU Member States. They can explain some discrepancies in the data

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17.2 Biological Waste Treatment Processes

In waste management, **composting** is referred to as a "cold process". It is a sensible waste recycling method with a low environmental impact. However, this requires that the pollutants in the waste, especially the heavy metals, are significantly reduced in the initial product. The composting of municipal waste stands or falls with this. Good pre-sorting of household waste is a prerequisite for problem-free recycling, as compost must not

contain any glass, metal, plastic or household chemicals.

Composting (rotting) is an ancient method of converting organic residues. Compostable is part of the household waste, sewage sludge and a large part of all organic matter such as leaves, wood, garden and agricultural waste. The finished compost is then a humus-like, plant-compatible soil conditioner.

Composting will be carried out on a large scale to recycle municipal waste. Composting plants are used to control the rotting process in a targeted manner.

Organic waste can be assigned to an aerobic or anaerobic process by its structure:

- Waste that is rich in structure and thus usually well aerated is particularly suitable for composting (e.g. plant residues, bark).
- Liquid waste and waste with a high water content and/or weak structure (for example, waste from factory farming) are more suitable for anaerobic treatment (biogas production).

17.2.1 The Composting Process

Composting or rotting takes place under aerobic conditions. The components of the biomass enter into two microbial processes, mineralisation and humification.

Since the temperature development in the windrow runs parallel to the intensity of the decomposition, conversion and build-up processes and the hygienisation, the temperature development is particularly suitable for characterising the composting process. Based on the temperature curve, the composting process can be divided into four phases:

- **Initial phase** (adaptation and development of the microflora)
- **Thermophilic phase** (period of most intensive conversion with maximum heat accumulation)

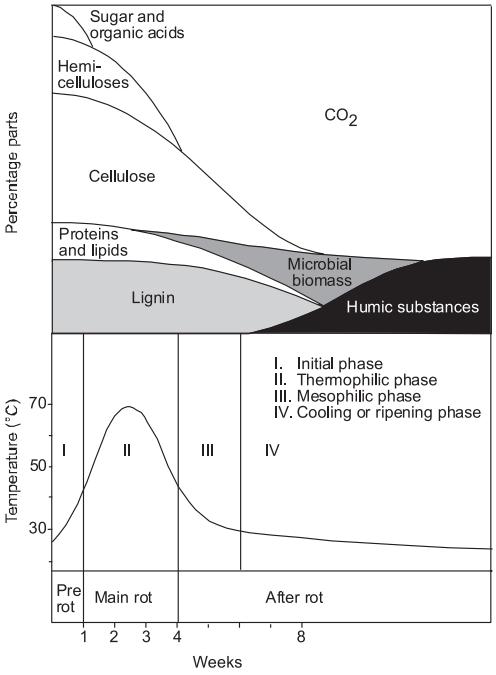


Fig. 17.2 Sequence of the composting process. The components of the biomass are microbially mineralized and assimilated at different rates depending on their degradability. The lignin component, which is difficult to degrade, is depolymerized to aromatics and transformed into humic substances together with other degradation products. The duration of the individual phases is strongly dependent on the type of composting process used

- **Mesophilic phase** (period of decreasing flow processes with low heat accumulation)
- **Cooling** or **ripening phase** (period of low turnover activities, start of humification processes).

The **composting process** is strongly schematized in Fig. 17.2. The easily and moderately degradable components of the biomass are assimilated and oxidized to CO_2 and water, while the lignin, which is difficult to degrade, is transformed into humic substances after partial degradation. This humification process also involves degradation products of proteins, carbohydrates and lipids as well as secondary metabolites of the microorganisms.

A **succession of** microorganisms is involved in the rotting process. The stages of composting are generally named as follows:

- **Pre-rotting:** The bacteria present in the organic waste multiply strongly (about 10^{10} germs/g dry weight). The easily degradable components are assimilated and mineralised, the organic matter decreases by about half. The high metabolic activities lead to heating, the mesophilic phase (20–40 °C) changes into the thermophilic phase (40–75 °C). If the air supply is insufficient, fermentation occurs in this phase, during which unpleasant-smelling fermentation products are also released. The acids formed during fermentation also inhibit the further composting process.
- **Main rot:** The temperature increase to 40–75 °C leads to the preferential development of thermophilic aerobic Bacillus species. At 75 °C, *Bacillus stearothermophilus* predominates. Bacilli are characterized by the ability to form extracellular enzymes that depolymerize proteins, hemicelluloses and cellulose. In this thermophilic phase, the total bacterial count decreases to about 10^8–10^9 microorganims/g dry weight. Many gram-negative bacteria are killed. Since this group also includes potentially pathogenic species, the process is associated with the **sanitization of** the composting material. Seeds are also inactivated to a large extent at this high temperature.
- **Post-rotting:** After consumption of the rapidly utilizable substrates, metabolic activity and thus exothermic self-heating decline. In succession, mesophilic microorganisms increase, especially actinomycetes and fungi. The actinomycetes are mainly represented by *Streptomyces* species, whose spore masses are macroscopically visible as a mealy coating on the substrate. The fungi are mainly *Aspergillus, Geotrichum, Mucor* and *Penicillium* species, but Basidiomycetes also occur. These groups of microorganisms degrade lignocellulose through extracellular enzymes. During the degradation, aromatic compounds are formed, which are converted into radicals (radical phase). They are the primary starting materials for **humification** (described in detail in ► Sect. 4.4.4.6).

The high-molecular stable humic substances formed during humification give the compost and humus of the soil its brown to black colour. Humic substances are amorphous, organic colloids (<2 µm) with a large specific surface area and the ability to accumulate water molecules and ions reversibly. Humic substances are particularly important for water retention, structure binding and nutrient salt adsorption of the soil due to their good water holding and adsorption capacity. Compared to clay minerals, they have a higher water holding and adsorption capacity and also a higher cation exchange capacity. Because of their dark colour, they also have a positive influence on the heat balance of the soil. They often form very stable compounds with inorganic clay minerals (= clay-humus complexes) and thus give the humus a high structural stability. They are important for soil fertility.

A finished, stable compost should have the following microbial population densities in CFU/g dry weight:

10^8–10^{10} heterotrophic aerobes; the ratio between aerobes and anaerobes should be 10:1; 10^3–10^4 yeasts and fungi, 10^3–10^6 pseudomonads and 10^3–10^6 free-living nitrogen fixers (Compost Microbial Guidelines).

17.2.2 Composting Processes

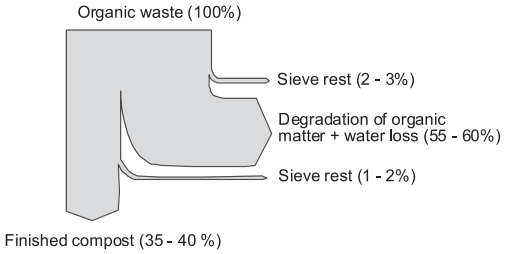
The aim of all processes is to ensure sufficient **aeration** and to **accelerate** the aerobic decomposition process. The times shown in ◘ Fig. 17.3 refer to the classic compost heap. In many cases it is necessary to intensify the processes in order to save space and time (intensive rotting). In order to avoid

odour nuisance, closed systems are used (tents, reactors). The exhaust air is cleaned by air filters.

The composting processes are to be outlined under the aspect of increasing intensification, which is at the same time associated with increasing investment expenditure. The actual composting process is preceded by **mechanical processing steps** in which the feedstock is sorted, screened and shredded. The mechanical shredding increases the attack surfaces for microorganisms. Further comminution is carried out by soil animals. The water content should be about 50%; material that is too moist (for example sewage sludge) is mixed with drier material, for example wood cuttings, in some plants. ◘ Figure 17.3 shows an example of the mass flows to be expected in the composting of biowaste.

The simplest method is **open heaps** in the form of triangular or panel heaps, which should have a bottom seal at the bottom. Aeration and mixing is effected by turnover devices, more rarely by so-called forced flow by means of aeration pipes. Mixing and aeration are particularly important in the preliminary and main rotting stages to prevent the formation of anaerobic zones and desiccation. The influence of climatic factors is reduced by roofing and enclosure.

Intensive procedures are carried out in reactors. A distinction is made between static and dynamic procedures. Static procedures are carried out in **closed containers,** boxes or tunnel reactors which allow ventilation through perforated floors. A dynamic process is the **rotting drum.** These are thermally insulated rotary tubes with a diameter of about 3 m and a length of 9–50 m. They are usually used only for homogenization. They are usually only used to homogenise the rotting material in the pre-rotting stage. In a quasi-continuous operation, the continuous mixing achieves an optimal process control and shortens the pre-rotting period and part of the main rotting period to 1–2 days. The discharged material is further composted statically, for example in windrows, in the subsequent post-rotting stage. Fungi, which do not tolerate constant movement, play an essential role in the post-rotting process.



◘ **Fig. 17.3** Mass flows in the composting of biowaste

The product of waste composting, **compost,** must be carefully controlled for contaminants, especially heavy metals, before it can be used for soil improvement.

17.2.3 Anaerobic Waste Treatment by Digestion

In the **anaerobic wet processes of waste fermentation,** the coarsely comminuted biowaste is suspended in water, resulting in a two-phase system of organic waste and water (about 30% waste dry matter). An alkaline pre-treatment for better pulping is being tested. This suspension is fed into special biogas reactors, which are operated discontinuously or semi-continuously; the residence time is 15–20 days. The microbial processes follow the methanogenic food chain described in ► Sect. 4.5. Both mesophilic (32–38 °C) and thermophilic (50–55 °C) processes are under development. Products of the process are biogas and the non-fermentable biomass fraction. The process requires subsequent dewatering and aerobic post-rotting. Basically, the fermentation process replaces the pre-rotting and part of the main rotting. The subsequent post-rotting only takes 4–8 weeks, as the fermentations break down the biomass to a greater extent.

The need for dewatering is eliminated in the **Anaerobic Dry Fermentation** process. The process works without the addition of water. As a result, the amount of wastewater can be significantly reduced. Fermentation is carried out in upright or horizontal tubular reactors, which require gas-tight feed and discharge nozzles for the solid biomass. The material remains in the anaerobic reactor for 15–25 days and is then aerobically post-fermented for 4–6 weeks.

Advantages of the digestion process are the acceleration of waste treatment, the prevention of odour nuisance and the production of biogas.

Test Your Knowledge

- How do you dispose of hazardous waste? What does it include?
- Describe the partial steps in composting with the temperature curve. Say something about the microorganisms involved.
- What are humic substances (see also ► Chap. 4)?
- Would you like to live near a composting plant? What problems can occur there?
- How much garbage do you produce in about a year?

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Biotechnology and Environmental Protection

Contents

W. Reineke, M. Schlömann, *Environmental Microbiology*, https://doi.org/10.1007/978-3-662-66547-3_18

The areas of application of **traditional environmental biotechnology** ("grey biotechnology") are expanding:

- In Germany, about 10,000 municipal wastewater treatment plants are operated with biological treatment stages.
- In Germany, 7.6 million tonnes of biowaste per year are used in biological recycling plants.
- Overall, the contribution of biomass use to electricity production increased from 222 GWh in 1990 to 5140 GWh in 2003.

The fields of application of **modern biotechnology** are:

- "Red biotechnology" (healthcare biotechnology): Bioproducts (proteins, antibodies, enzymes) are said to already have a market share of ~20% of pharmaceutical products and new developments are said to be 50% bioproducts.
- "Green biotechnology": the use of genetically modified plants, which began about 20 years ago, reached 45% for corn, 86% for soybeans and 76% for cotton in the US in 2004. Current developments aim to use plants (and animals) for the low-cost production of pharmaceutical products and industrial raw materials.
- "White Biotechnology": Use of biotechnology as a building block for sustainable future-compatible chemistry ("Green Chemistry") and its application in the food industry. Products include bulk and fine chemicals, foodstuffs as well as food additives and feed additives, agricultural and pharmaceutical precursors, auxiliary materials for processing industries such as technical enzymes and biofuels. It has been projected that by 2010, approximately 20% of the sales of the entire chemical industry should be attributable to the use of white biotechnology.
- "Grey biotechnology": the field of environmental protection listed above has now been joined by environmental diagnostics.

All biotechnology fields are directly or indirectly related to environmentally relevant areas.

18.1 Biological Pest Control

The concerns of environmentally sound pest control are increasingly leading to the search for microorganisms that can be used as antagonists against so-called harmful insects or whose metabolic products are suitable as new active substances.

18.1.1 Bioinsecticides

Bacteria, fungi, viruses, proteins or low-molecular secondary metabolites are used as bioinsecticides. ◘ Table 18.1 summarizes some examples and the insects controlled.

18.1.1.1 *Bacillus thuringiensis* and *B. sphaericus*

Bacillus thuringiensis is the best studied insect pathogenic bacterium. It was isolated as early as 1901 in Japan as the bacterial causative agent of Sotto's disease (flaccidity) of silkworms. It has been known as the causative agent of flour moth caterpillar disease since 1911.

Bacillus thuringiensis produces toxins in the form of crystals during endospore formation that affect a wide range of Lepidoptera (butterflies), but not other animals or humans. Other subspecies have been found to be pathogenic to Diptera (two-winged moths: mosquitoes, flies) and some Coleoptera (beetles). Today, there are also known to be active against Hymenoptera (hymenopterans such as bees, wasps and ants), Homoptera (cicadas, aphids), Orthoptera (grasshoppers and crickets), Mallophaga (animal lice such as hair and feather lice), Nematodes (roundworms and nematodes), mites and Protozoa (◘ Table 18.1).

Bacillus thuringiensis forms several virulence factors. The most important and selective

Table 18.1 Microorganisms, viruses and metabolites as bioinsecticides

Agent	Insect pests
Bacteria	
Bacillus thuringiensis (B. t.)	
B. t. subsp. *thuringiensis*	Lepidopteran caterpillars, for example goldafter, cabbage white butterfly
B. t. subsp. *thuringiensis, β*-exotoxin producing strains	fly maggots, for example *Musca domestica*
B. t. subsp. *kurstaki*	Gypsy Moth
B. t. subsp. *tenebrionis*	Beetle larvae, for example potato beetle
B. t. subsp. *israelensis*	Mosquito larvae, for example *Anopheles, Aedes* species, meadow snails
Paenibacillus popilliae	Japanese beetle grubs *(Popillia japonica)*
Mushrooms	
Beauveria brogniartii	Maybug
Beauveria bassiana	Potato beetle, bark beetle
Metarrhizium anisopliae	Weevils, for example thick-mouthed weevils, bark beetles
Verticillium lecanii	Aphids, whitefly
Viruses	
Granulose virus (CPGV)[a]	Codling moth *(Cydia pomonella)*
Nuclear polyhedrosis virus (LdNPV)	Gypsy moth *(Lymantria dispar)*
Nuclear polyhedrosis virus (LmNPV)	Nun *(Lymantria monacha)*
Secondary Metabolites	
Abamectin	Leaf-mining diptera or plant sap suckers, mites
Spinosad	Lepidoptera, Diptera, Blattodea, Coleoptera

[a] The insect virus name is derived from the Latin names of the insects and the abbreviation for the virus type

factor is the crystalline *δ*-endotoxin (Fig. 18.1), which consists of proteins of different composition. A strain usually synthesizes one to five different toxins, which appear in the form of a single crystal or a multitude in a cell. Approximately 170 naturally occurring Bt toxins with varying ranges of activity are known today. The *Bacillus thuringiensis* toxins enter the larvae, maggots or caterpillars of the pests with food and develop their pathogenic effect in the intestinal tract. The steps in the pest are the following:

1. Dissolution of the crystal in the midgut of the caterpillar (pH > 9.5),
2. proteolytic cleavage of the N- and C-terminal part of the protoxin by the midgut proteases,
3. Binding of the activated toxin to the receptor in the epithelial cell membrane in the midgut,

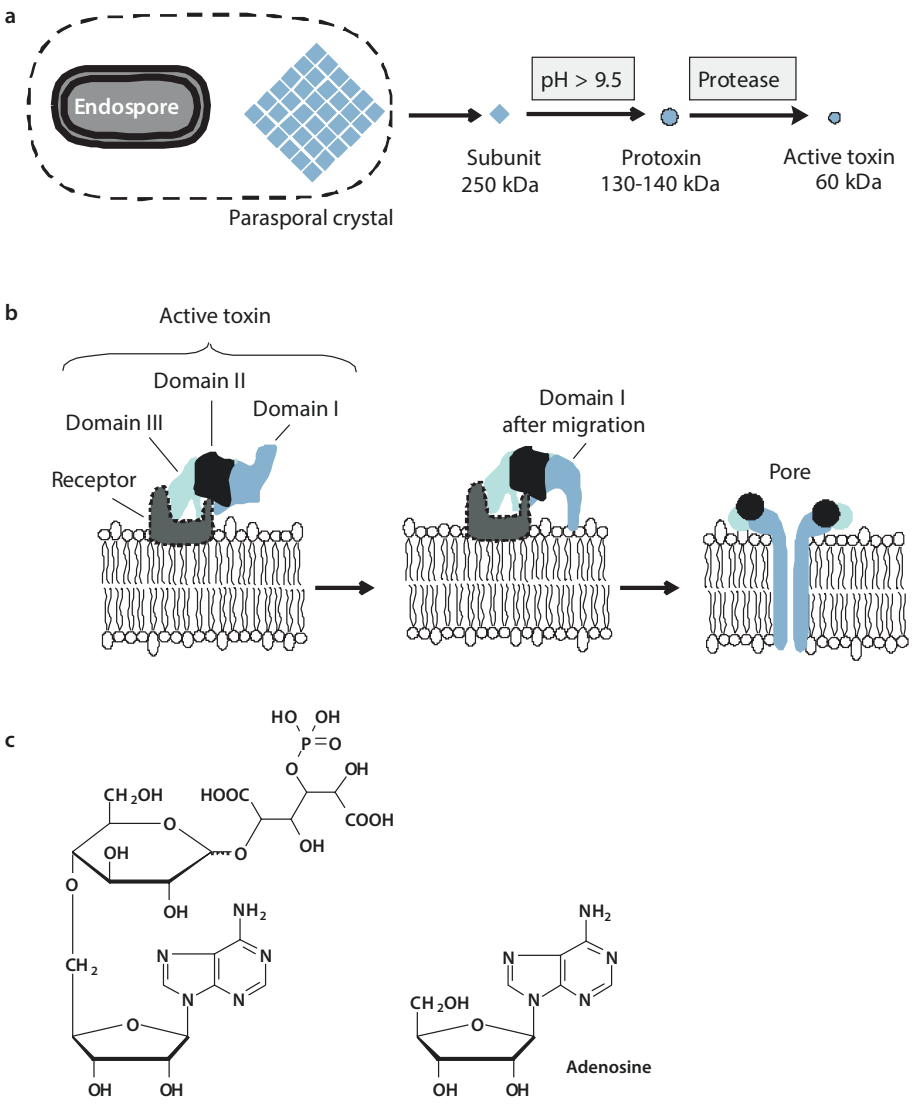


Fig. 18.1 **a** Schematic representation of the paracrystalline δ-endotoxin in the cell of *Bacillus thuringiensis* and activation of the protoxin intermediates by specific proteases in the intestine of the caterpillars to form the toxic peptide, **b** Attachment to the receptor, rearrangement of the toxin and formation of a pore, **c** Structure of β-exotoxin compared to the natural nucleoside

4. integration of the toxin into the membrane so that ion channels or pores are formed and the cell membrane of the intestinal epithelial cells is irreversibly damaged, and
5. due to the destruction of the intestinal barrier, the bacterial spores enter the body, they germinate and cause septicemia.

The primary damaging effect therefore comes from the δ-endotoxin. *Bacillus thuringiensis* is only weakly infectious and therefore does not spread epidemically. *Bacillus thuringiensis* preparations are therefore used as biopesticides in a similar way to a chemical insecticide and thus differ from biological control agents,

which spread autocatalytically in the insect pest population until the number of individuals has become too low for transmission.

A few strains of Pathovars *thuringiensis* produce other exotoxins in addition to the δ-endotoxin, such as the low molecular weight *β*-exotoxin. The structure of this unusual nucleotide is shown in ◘ Fig. 18.1c. It is an antimetabolite of DNA-dependent RNA polymerase. Because its action is non-specific, it also affects vertebrates. Strains producing *β*-exotoxin in addition to *δ*-endotoxin have been tested as agents against fly maggots. The preparations are added to cattle feed, pass through the intestine without acute damage and thus reach the excrement in which the fly maggots live.

Bt preparations have been approved as plant protection products in Germany since 1964 and are available on the market under various names. They are mainly used in maize, potato, vegetable and fruit cultivation. They are of greater importance in organic farming. Commercially available Bt preparations consist of dried bacterial spores and the crystalline toxin. The bacteria cultivated in the fermenter on simple media are separated from the nutrient medium and formulated with adhesion and light protection agents. For successful use, it must be taken into account that only the feeding caterpillars or larvae take up the preparations.

Comparing the speed of action of Bt preparations with that of other insecticides, it is clear that longer durations are required before the bioinsecticide takes effect.

Genetic engineering has been used to transfer the ability to produce *δ*-endotoxins to rhizosphere bacteria, such as *Pseudomonas fluorescens,* with the aim of controlling earthworms.

The Bt toxin genes *(cry)* isolated from *Bacillus thuringiensis* were transferred to plants. These now produce the active ingredient themselves, which is toxic to feeding pests. In order to produce genetically engineered insect resistance, different variants of Bt genes were used, for example *cry1Ab, cry1Ac* and *cry9c* in maize. These differ both in length and in the promoters used. Depending on the Bt gene variant, the transgenic maize varieties differ both in the amount of Bt toxin and in its distribution in the plant. The first commercially grown Bt maize plants contained high levels of Bt toxin in all parts of the plant (pollen, stalks, cobs), higher than necessary to achieve the desired effect. Newer Bt corn varieties not only produce lower amounts of Bt toxin, but also produce it only where it is needed, which is in the stalk. This is achieved by providing the *cry* genes with tissue-specific promoters that only "kick in" in certain parts of the plant.

In the case of maize, the Bt concept appears to be particularly attractive, as it makes it possible for the first time to control the corn borer caterpillars ***in*** the plant. However, Bt genes have not only been transferred into maize, but also other variants into cotton or the potato.

In 2004, 81 million ha of genetically modified organisms (GMO) crops were harvested worldwide. Of these, 19% were insect-resistant Bt plants and a further 9% were a combination of herbicide and insect resistance. At 72%, herbicide resistance is the dominant trait.

The cultivation of Bt maize in the USA was ten million ha in 2004 and accounted for about 45% of maize cultivation. Other countries where Bt corn is grown are Argentina, Canada, Spain and South Africa.

Bt cotton was grown on about 2.5 million ha in the USA in 2004, which accounted for about 76% of the cultivated area. Bt cotton is also grown in Argentina, Australia, China, India, Indonesia, Mexico and South Africa.

Work is being carried out on about 30 plant species to create resistance to various insect pests by transferring Bt genes.

One advantage of the Bt toxin is its specificity. It attacks the respective pests and spares other animals, especially the beneficial insects. Expectations do not always seem to be fulfilled in this way. Insect pests develop

resistance to the insecticides used over time. With the exception of a few isolated cases, this has not yet happened with the classic Bt preparations. However, it is feared that large-scale cultivation of Bt plants will accelerate the development of resistance: "Since the active ingredient is present in the genetically modified plants throughout the entire growing season, pests can develop resistance more easily than if farmers only spray the insecticide now and then." In the US, the cultivation of Bt crops has been accompanied by mandatory resistance management. According to this, areas with conventional varieties without Bt toxin must be planted proportionately as refugia to avoid "wild plants" becoming "BT plants".

Mosquito pathogenic bacteria have been known since the 1960s when the first isolates of ***Bacillus sphaericus*** with larvicidal properties were discovered. Around 1980, isolates were found from adult black flies in Nigeria, which together with *Bacillus thuringiensis* subsp. *israelensis* became prominent in the biological control of biting and black flies.

The toxic effect of *B. sphaericus*, as with *B. thuringiensis,* is based on the formation of parasporal protein crystals, which in *B. sphaericus* are located in a "spore-crystal complex" enveloped by a membrane. In contrast to *B. t.* subsp. *israelensis*, *B. sphaericus* has a binary toxin consisting of two proteins with molecular weights of 51.4 and 41.9 kDa. Both are necessary for mosquito toxicity. The mechanism of action of the binary toxins, similar to that of *B. t.* subsp. *israelensis*, is based on binding to receptors. Additional toxins (Mtx toxins) with a molecular weight of up to 100 kDa can be produced in vegetative *B. sphaericus* cells. These Mtx toxins are also toxic to mosquito larvae, but are not homologous to either the binary toxins or those of *B. t.* subsp. *israelensis.*

In recent years, *B. sphaericus* has gained importance, mainly because of its particular spectrum of activity and its ability to be reused or persist under certain conditions. Reuse can result in a long-term effect, increasing the time interval for re-treatments.

Target organisms are representatives of different mosquito genera, such as *Aedes, Anopheles* and *Culex*. However, there are large differences in sensitivity among species within a genus. Some mosquito species, predominantly *Culex* species, but also some *Anopheles* species are particularly sensitive to *B. sphaericus,* while other species, such as *Aedes aegypti,* are almost insensitive. In contrast to *B. thuringiensis* subsp. *israelensis, B. sphaericus* does not kill black fly larvae.

The protein toxins of *B. sphaericus* are harmless to warm-blooded animals and other non-target organisms.

18.1.1.2 Bioinsecticides from Actinomycetes

Active ingredients from actinomycetes are other bioinsecticides. **Abamectin** is a mixture of active ingredients that was first registered as an acaricide/insecticide for crop protection purposes in 1986 and is now used in over 80 countries on a wide range of crops. It is derived from a microorganism isolated in Japan that showed good activity against nematodes. A total of eight similar macrocyclic lactones, the avermectins (see ◘ Fig. 18.2), were isolated from the mycelium of *Streptomyces avermitilis* and identified as active compounds. The avermectins show anthelmintic (against worms) properties but have neither bactericidal nor fungicidal activity. The major component B_1 is extremely active against a range of mites and insects. This was therefore selected for use in crop protection.

Abamectin mainly shows a feeding effect. The contact activity is rather limited due to the rapid oxidative degradation on leaf surfaces. The half-life of abamectin is less than 4 h. The physiological mode of action in the insect is through stimulation of the release of the neurotransmitter γ-aminobutyric acid

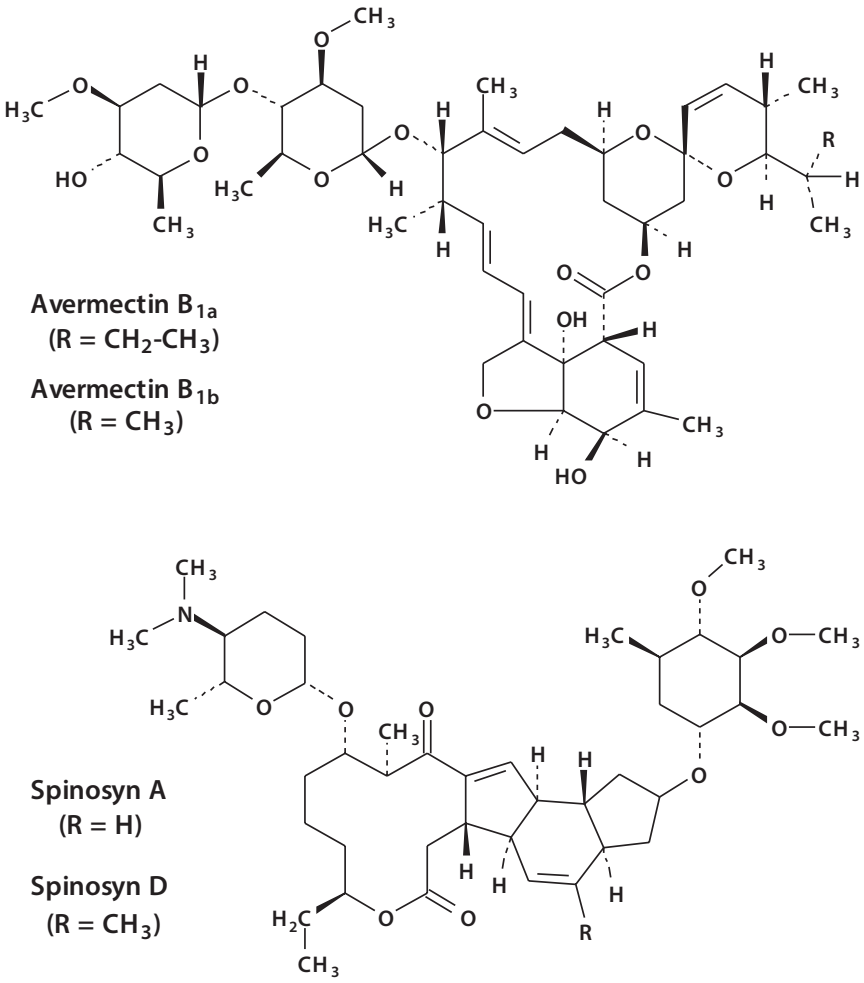


Fig. 18.2 Active substances against insects from actinomycetes

in inhibitory cells of the nerve/muscle plate, leading to paralysis via sustained suppression of muscle contraction. It occurs within hours after ingestion of a toxic dose, is irreversible and leads to death.

Abamectin is effective against all mobile stages of insect pests that feed on plant tissue or suck plant sap, but it has no ovicidal activity. The main target organisms are mites and leaf-mining dipterans. Abamectin is rapidly absorbed into the leaf after treatment, where it is protected from degradation by UV radiation and from being washed off by rain. Since abamectin is very strongly bound to soil particles, it is immobile in soil and shows no leaching. It shows no bioaccumulation and is rapidly degraded by microorganisms in the soil. It is insoluble in water. Degradation in plants occurs mainly at the leaf surface by photolysis.

Spinosad is another insecticidal active ingredient. It is produced by the actinomycete *Saccharopolyspora spinosa*, is characterized by a broad host spectrum and a rapid action comparable to that of synthetic insecticides (Table 18.2). *S. spinosa* was discovered in 1982 on a Caribbean island in the

Table 18.2 Speed of action of insecticides

Insecticide category	Effect after
Carbamates/ Organophosphates	Hours
Bt preparations	Days
Spinosad	Minutes to hours
Synthetic pyrethroids	Minutes to hours

soil of a former rum distillery. Since 1994, the development of spinosad as an insecticidal preparation has been pursued worldwide, and in 1997 the first approval was granted in the USA.

S. spinosa produces a number of metabolites, the spinosynes, with only the two biologically active forms spinosyn A and D being present in spinosad. Spinosyn A and D are macrocyclic lactones, with very little difference between the two isomers (see Fig. 18.2). Spinosyn A and D have a quantitative ratio of about 85–15% in spinosad.

The active ingredient is absorbed through feeding activity and contact. The efficacy due to feeding activity is 5–10 times higher than that due to contact. The effect of spinosad is based on influencing the neuronal activity of the insects. The active ingredient affects the nicotinic acetylcholine receptor localized in the postsynaptic cell and causes an ion influx, resulting in hyperactivity of neurons and muscle activity, ending with the complete, irreversible paralysis of the insect. In addition to the effect of spinosad on the nicotinic acetylcholine receptor, it is also thought to affect the γ-aminobutyric acid receptor. The binding sites of spinosad are different from those of other insecticidal agents.

Spinosad is used on a wide range of crops: potato, vegetables, lettuce, corn, canola, cotton, woody plants, ornamentals and turf. Butterflies, bipeds, cockroaches as well as beetles that can be controlled with spinosad are: Cotton owl (*Helicoverpa armigera* as well as *Spodoptera littoralis*), sugar beet owl (*Spodoptera exigua*), crossed grape berry moth (*Lobesia botrana*), fruit pod moth (*Adoxophyes orana*), corn borer (*Ostrinia nubilalis*), cabbage whitefly (*Pieris* spp.), coal owl (*Mamestra brassicae*), house fly (*Musca domestica*), leaf miner (*Liriomyza* spp.), German cockroach (*Blatella germanica*) and Colorado potato beetle (*Leptinotarsa decemlineata*).

Spinosad has a low acute toxicity to mammals and birds and a favourable environmental and beneficial insect profile. It is judged to be non-carcinogenic, non-teratogenic, non-mutagenic and non-neurotoxic.

Degradation of spinosad in the environment occurs mainly by photolysis and microbial metabolism. The potential to migrate in soil is very low to low depending on the soil type.

18.1.1.3 Mushroom Preparations

Important fungal pathogens of insects are listed in Table 18.1. These fungi infect the insects, penetrate the insect cuticle by means of chitinases, multiply in the insect body and form low-molecular toxins. Thus, *Beauveria* forms the cyclodepsipeptide beauvericin, *Entomophthora* two azoxybenzene toxins. The mycelium proliferates through the dead insect and penetrates to the outside, where sporulation occurs. The spores can spread to other individuals and germinate there (Fig. 18.3).

Preparations of some fungi already exist, others are being tested. *Beauveria bassiana* preparations consist of spores formed on the mycelium in submerged and surface culture on substrates such as molasses or bran. The ecological conditions under which effective infection occurs are crucial for successful use. In the field, such conditions are difficult to predict. Therefore, some preparations are being developed for use in greenhouses.

Beauveria bassiana may be produced as an insecticide (against bark beetles) according to § 6a para. 4 sentence 1 no. 3 letter b of the Plant Protection Act for agricultural, forestry or horticultural purposes for use on the own farm. Also *Beauveria brongniartii*

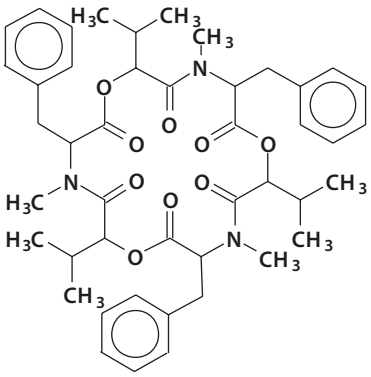


Fig. 18.3 Beauvericin is a mycotoxin produced by many *Fusarium* species and has been isolated from *Beauveria bassiana.* It is a bioactive cyclodepsipeptide similar to that of valinomycin and contains three *D*-α-hydroxy-isovaleryl and *N*-methyl-*L*-phenylalanyl groups in alternating sequence

(= B. tenella) against cockchafer and *Metarhizium anisopliae* against weevil and bark beetle.

18.1.1.4 Virus Preparations

In agriculture and forestry, **baculoviruses** have long been used for biological control of insect pests, but have so far played only a minor role because of their slow action compared to chemical agents.

Baculoviruses are the largest and most diverse insect-specific virus group. The viruses have a double-stranded circular DNA genome of 90–190 kb. The family Baculoviridae has previously been grouped into 2 genera, the nucleopolyhedro- and the granuloviruses. This classification is based predominantly on the morphology of the inclusion bodies of these viruses. If a virion is surrounded by a capsule, they are called **granulose virus (GV)**, if numerous virions are enclosed by a capsule, they are called **nucleopolyhedron virus (NPV)**.

Baculoviruses are pathogenic for larvae, especially of butterflies, bipeds, hymenoptera and beetles, which means that they can only be used in very specific developmental stages of the insects. If this time is missed, the viruses are ineffective. Baculoviruses infect intestinal epithelial and fat body cells and cause the death of the larvae or caterpillars. The specificity of the viruses used is high, so that toxic and environmentally harmful side effects are not to be expected.

The first large-scale use for pest control was with the nuclear polyhedrosis virus against the pine sawfly (*Neodiprion sertifer*). In the meantime, effective preparations against the codling moth, the gypsy moth and the coal owl (*Mamestra brassicae*) are in use or being tested. Preparations are made with the help of caterpillar cultures. Efforts are being made to produce them using insect cell cultures.

Genetic engineering methods are to be used to modify the viruses so that they are effective over a longer period of time. Work is also currently being carried out on optimisation, for example on killing the target organisms more quickly or on expanding the host spectrum by incorporating genetic information for the production of poisons against scorpions, spiders or mites. As a strategy to improve the biosafety of transgenic baculoviruses, the gene encoding the coat protein has been removed. As a result, baculoviruses can no longer form their envelope, which protects them from environmental influences—but their infectivity remains intact. The release trials of transgenic baculoviruses conducted to date in the USA, Canada and Great Britain were aimed at investigating their effectiveness in the field and their effects on non-target organisms.

18.1.2 Biofungicides and Herbicides

The use of mycoparasitism is being investigated for the **biological control of phytopathogenic fungi. Mycoparasites** are fungi that parasitize on other fungi by invading the hyphae of the host fungus and utilizing the cytoplasm as substrate.

The use of the antagonistic fungus *Peniophora gigantea* against root rot of pine

caused by *Heterobasidion annosum* (*Fomes annosus*) has gained practical importance as a fungicide. *Heterobasidion annosum* colonizes the snags of freshly felled trees. The pathogen spreads to adjacent healthy trees via the root system. If the fresh cut surface is inoculated with spores of the antagonist *P. gigantea*, the pathogen can no longer colonize the surface (possession principle). The spores can be brought onto the cut surface of the trees with the lubricating oil of the chainsaws.

An underdeveloped area in Europe is the use of phytopathogenic fungi for **weed control**, the so-called **mycoherbicides**. In the USA, the spores of the fungus *Colletotrichum gloeosporioides* are used in rice and soybean cultivation against the weed *Aeschynomene virginica.* Trials are ongoing in Europe with the leaf spot pathogen *Curvularia lunata* against chicken millet (*Echinochloa crus-galli*) and the rust fungus *Puccinia striiformis* against field thistle (*Cirsium arvense*). *Chondostereum purpureum* is approved by the Federal Office of Consumer Protection and Food Safety as a herbicide against American weeping cherry.

18.2 Design of New Chemicals

Environmental microbiology is currently strongly characterized by aftercare measures, so-called "end of the pipe" activities. A paradigm shift towards precautionary and integrated environmental protection is called for. *Green chemistry,* or sustainability in chemistry, aims to achieve this. It is the term for efforts to change chemical manufacturing processes or to develop new ones in order to ensure a safe and clean environment in the twenty-first century.

What do products (chemicals) have to look like in order to comply with this? Are there rules for this? Or does every new chemical have to be tested for degradability? What does this mean, what is the catalogue of criteria that leads to a classification?

Green Chemistry

In the context of sustainability of chemicals, the term **green chemistry is** often used. Green chemistry is the term used to describe the type of chemistry that seeks to curb pollution by preventing it at the point of origin, to save energy and to produce in the most environmentally friendly way possible. At the same time, hazards of the production and the product are to be avoided. Only materials that are environmentally friendly or biodegradable at the end of their use and do not accumulate in the environment or form mountains of waste should be produced.

The so-called 12 basic principles of *Green Chemistry* were developed by Anastas and Warner (1998):

1. **Avoid pollution:** It is better to avoid waste than to treat or remove it after it has been produced.
2. **Atomic economy:** synthesis methods should be designed and used in such a way that no/few atoms or molecules of the starting reagents are left over or undesirable substances are produced.
3. **Less hazardous chemical syntheses:** Wherever possible, synthetic methods should be designed and used to produce substances that pose little or no risk to humans and the environment.
4. **Design for safer chemicals:** The aim is to create products with high functionality while reducing toxicity.
5. **Safer solvents and auxiliary substances:** The use of auxiliary substances (such as solvents, release agents) should be eliminated whenever possible and should be harmless when applied.
6. **Design for energy efficiency:** The need for energy should be identified and minimized in terms of environmental impact and cost. If possible,

reactions should be carried out at room temperature and under normal pressure.

7. **Use of renewable raw materials:** Wherever technically and economically feasible, renewable rather than limited raw materials should be used.
8. **Reduction of derivatives:** Whenever possible, unnecessary intermediates in chemical processes (blocking group, protecting group/deprotection, temporary modification) should be avoided.
9. **Catalysis:** Catalysts (as selective as possible) should be used in syntheses instead of stoichiometric reagents.
10. **Design for degradability:** Chemicals and products should be designed in such a way that they can be naturally degraded after use and no degradation products are produced that harm the environment.
11. **Real-time analysis to prevent pollution:** Analytical methods need to be further developed so that control and management of all operations can be achieved through real-time monitoring to prevent pollution and contamination and thus waste.
12. **Safe chemistry:** Substances and the form of substances used in chemical processes should be selected to minimize the risk of accidents such as release, explosion and fire.

18.2.1 Structure-Activity Relationship/Predictability of Degradation

Attempts have long been made to determine whether structural properties of substances provide clues to expected degradation behavior and can be used to classify degradable and persistent substances. Of the numerous **structure-activity relationships (SARs)** or **quantitative structure-activity relationships (QSARs)** that have been developed in recent years to estimate biodegradation, the BIOWIN program developed by the *Environmental Protection Agency* (EPA) as part of EPI-Suite will be addressed here. This is a freely available software package that can be used to estimate parameters such as **aerobic biodegradability**, octanol/water partition coefficient, melting points, hydrolysis rates and much more.

It includes three types of mathematical models for estimating aerobic biodegradability:

1. Linear and non-linear biodegradation model (Biowin1 and Biowin2) modeled using 295 chemicals.
2. Ultimate and Primary Biodegradation Model (Biowin3 and Biowin4) (an assessment using expert interviews).
3. Linear and Non-Linear MITI Biodegradation Model (Biowin5 and Biowin6) (Established using 884 experimentally studied compounds).

For this purpose, a molecule is broken down into different parts, "fragments", to each of which a positive or negative influence on degradability is attributed (or a numerical value is calculated). Furthermore, the size of the molecule is taken into account. With increasing size, biodegradability is considered to be more difficult (see ◘ Table 18.3).

It is a common observation that substances are "easily degraded" that have hydrolyzable groups such as carboxylic acid esters (C(O)OR), amides ($C(O)NR_2$) and anhydrides (C(O)O(O)CR) or phosphoric acid esters. This is not surprising since these are found in proteins, polysaccharides and lipids. Consequently, all organisms have hydrolytic enzymes to cleave such functional groups.

Furthermore, the presence of hydroxyl (-OH), formyl (-CHO) and carboxyl (-COOH) groups usually gives an indication that the degradation of a compound is "easy", because oxygen-bearing structures

Table 18.3 Structural fragments considered in the model calculation for the degradability of a chemical and assessed as positive (+) or negative (–)

Fragmentary description	Influence on degradability	
	Linear	Non-linear model
Nitroso [-N-N=O]	–	–
Aliphatic alcohol [-OH]	+	+
Aromatic alcohol [-OH]	+	+
Aliphatic acid [-C(=O)-OH]	+	+
Aromatic acid [-C(=O)-OH]	+	+
Aldehydes [-CHO]	+	+
Ester [-C(=O)-O-C]	+	+
Amide [-C(=O)-N or -C(=S)-N]	+	+
Triazine ring (symmetrical)	+	–
Aliphatic chlorides [-Cl]	+	–
Aromatic chlorides [-Cl]	+	–
Aliphatic bromides [-Br]	+	–
Aromatic bromides [-Br]	+	+
Aromatic iodides [-I]	–	–
C with 4 single bonds & no H	+	+
Aromatic nitro [$-NO_2$]	–	–
Aliphatic amines [$-NH_2$ or -NH-]	+	–
Aromatic amines [$-NH_2$ or -NH-]	–	–
Cyanide/Nitrile [-C ≡ N]	+	+
Sulfonic acids/salts → on the aromatic ring	+	+
Pyridine ring	–	–
Aromatic ethers [aromatic-O-aromatic]	+	+
Aliphatic ethers [C-O-C]	+	–
Ketones [-C-C(=O)-C-]	+	+
Tertiary amines	–	–
Phosphate ester	+	+
Azo groups [-N=N-]	–	–
Carbamates or thiocarbamates	–	+
Fluoro [-F]	+	–
Aromatic-CH_3	+	+
Aromatic-CH_2	–	–

(continued)

Table 18.3 (continued)

Fragmentary description	Influence on degradability	
	Linear	Non-linear model
Aromatic-CH	–	+
Aromatic-H	+	+
Methyl [-CH_3]	+	+
-CH_2- [linear]	+	+
-CH- [linear]	–	–
-CH_2- [cyclic]	+	+
-CH- [cyclic]	+	–
-C=CH [alkenyl hydrogen]	+	+
Hydrazines [-N-NH-]	–	–
Quaternary amines	–	+
Tin [Sn]	+	–
Molecular weight parameter Equation constant	–	–

are very common in metabolites of normal metabolism.

In contrast, some structural features slow down the degradation considerably. Chlorine and nitro groups, especially on aromatic rings, are considered to be the cause of chemical persistence. Furthermore, structural features such as quaternary C atoms (CR R R_{123} R_4 without R = H) and tertiary nitrogen atoms (NR_1 R_2 R_3 without R = H) appear to impede degradability.

Overall, it should be noted that the model analyses provide important insights, **but** the **reductionist approach should be used with caution**. As shown in the box "Estimation of a reductionist assessment", exceptions always occur that do not obey QSAR rules. **Ideas introduced by statistics** can stimulate a different view of a problem, but definitive answers **need experimental investigation**. However, QSAR is already an important method in chemical registration in the United States. To better assess chemicals, improvements are needed not only in the development of meaningful testing systems but, more importantly, advances in the assessment itself.

The evaluation of a chemical by the different models is shown as a result sheet for the known chemical benzene in ◘ Fig. 18.4. In addition to the results, the fragment criteria used are also listed there (◘ Fig. 18.5).

The respective models were tested for accuracy using 295 chemicals: Biowin1 (Linear Regression Model) showed an 89.5%, Biowin2 (Non-Linear Regression Model) a 93.2%, Biowin5 an 81.3% and Biowin6 an 80.7% correct assignment to "not readily degradable" or "readily degradable". The use of the combination of the two models 3&5 is advisable, as expert knowledge is also included here.

Estimation of a Reductionist Assessment

The BIOWIN approaches, which only use the involved fragments of the respective molecule, generally lack subtleties, which are, however, needed to take into account the effects of the substitution position. This can be illustrated by comparing calculated data

Fig. 18.4 Result sheet of the determination of the aerobic degradability for benzene with the program *BIOWIN* and the model calculations Biowin1–6. #The values in Biowin1 are calculated as: Σ fragments + molecular weight parameter + equation constant. ##The values in Biowin2 are calculated as: $e^{\Sigma \text{ fragments + molecular weight parameter + equation constant}}/1 + e^{\Sigma \text{ fragments + molecular weight parameter + equation constant}}$. ###"Ready Biodegradability Prediction" is a combination of the information from two model calculations. A classification of "YES" is present when Biowin3 evaluates (weeks or faster) and Biowin5 reports (>0.5). The combination should allow a higher accuracy of the prediction

```
SMILES : c(cccc1)c1
CHEM   : benzene
MOL FOR: C6 H6
MOL WT : 78.11
-------------------------- BIOWIN v4.02 Results ----------------------------

   Biowin1 (Linear Model Prediction)     :  Biodegrades Fast
   Biowin2 (Non-Linear Model Prediction):  Biodegrades Fast
   Biowin3 (Ultimate Biodegradation Timeframe):  Weeks-Months
   Biowin4 (Primary  Biodegradation Timeframe):  Days-Weeks
   Biowin5 (MITI Linear Model Prediction)    :  Readily Degradable
   Biowin6 (MITI Non-Linear Model Prediction):  Readily Degradable
   Ready Biodegradability Prediction:  NO ###

------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin1 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  1  |  Unsubstituted aromatic (3 or less rings)  |  0.3192 |  0.3192
 MolWt|  *  |  Molecular Weight Parameter             |         | -0.0372
 Const|  *  |  Equation Constant                      |         |  0.7475
============+=========================================+=========+=========
   RESULT   |    Biowin1 (Linear Biodeg Probability)  |         |  1.0296 #
============+=========================================+=========+=========

------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin2 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  1  |  Unsubstituted aromatic (3 or less rings)  |  7.1908 |  7.1908
 MolWt|  *  |  Molecular Weight Parameter             |         | -1.1092
 Const|  *  |  Equation Constant                      |         |  3.0087
============+=========================================+=========+=========
   RESULT   |  Biowin2 (Non-Linear Biodeg Probability)|         |  0.9999 ##
============+=========================================+=========+=========

 A Probability Greater Than or Equal to 0.5 indicates --> Biodegrades Fast
 A Probability Less Than 0.5 indicates --> Does NOT Biodegrade Fast



------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin3 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  1  |  Unsubstituted aromatic (3 or less rings)  | -0.5859 | -0.5859
 MolWt|  *  |  Molecular Weight Parameter             |         | -0.1726
 Const|  *  |  Equation Constant                      |         |  3.1992
============+=========================================+=========+=========
   RESULT   |  Biowin3 (Survey Model - Ultimate Biodeg)  |         |  2.4406
============+=========================================+=========+=========

------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin4 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  1  |  Unsubstituted aromatic (3 or less rings)  | -0.3428 | -0.3428
 MolWt|  *  |  Molecular Weight Parameter             |         | -0.1127
 Const|  *  |  Equation Constant                      |         |  3.8477
============+=========================================+=========+=========
   RESULT   |   Biowin4 (Survey Model - Primary Biodeg)  |         |  3.3922
============+=========================================+=========+=========

 Result Classification:   5.00 -> hours     4.00 -> days    3.00 -> weeks
  (Primary & Ultimate)    2.00 -> months    1.00 -> longer

------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin5 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  6  |  Aromatic-H                             |  0.0082 |  0.0493
 MolWt|  *  |  Molecular Weight Parameter             |         | -0.2324
 Const|  *  |  Equation Constant                      |         |  0.7121
============+=========================================+=========+=========
   RESULT   |  Biowin5 (MITI Linear Biodeg Probability)  |         |  0.5291
============+=========================================+=========+=========

------+-----+-----------------------------------------+---------+---------
 TYPE | NUM |       Biowin6 FRAGMENT DESCRIPTION      |  COEFF  |  VALUE
------+-----+-----------------------------------------+---------+---------
 Frag |  6  |  Aromatic-H                             |  0.1201 |  0.7208
 MolWt|  *  |  Molecular Weight Parameter             |         | -2.2551
 Const|  *  |  Equation Constant                      |         |  0.7475
============+=========================================+=========+=========
   RESULT   |Biowin6 (MITI Non-Linear Biodeg Probability)|         |  2.5257
============+=========================================+=========+=========

 A Probability Greater Than or Equal to 0.5 indicates --> Biodegrades Fast
 A Probability Less Than 0.5 indicates --> Does NOT Biodegrade Fast
```

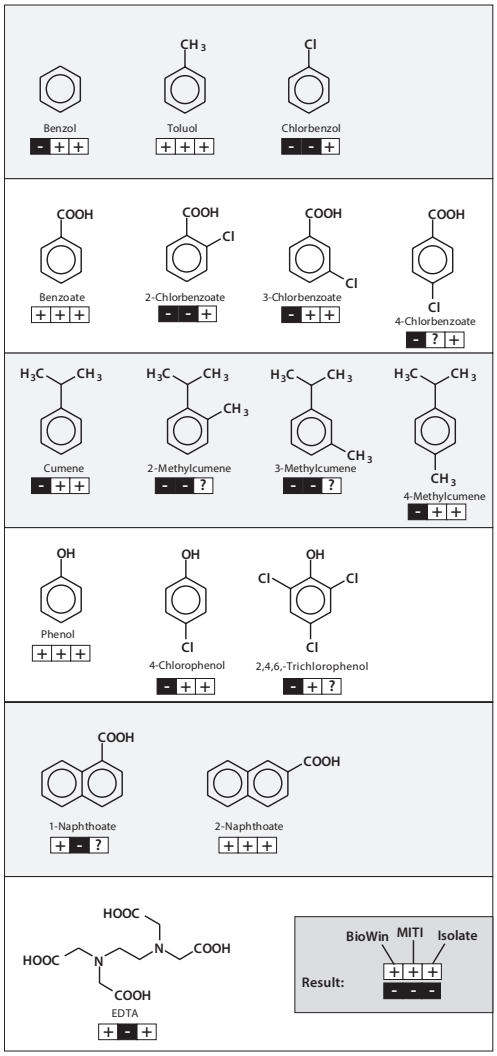


Fig. 18.5 Comparison of assessment of rapid biodegradation by QSAR (Biowin) and experimental data from MITI and pure bacterial cultures described using the substance as a C and E source; +: "readily degradable"; –: "not readily degradable"; ?: no information available

In addition to the partly contradictory results, the data listed make it clear that pure cultures can be isolated in the laboratory under optimal conditions even for substances that have been assessed as non-degradable and determined by MITI tests.

with data obtained by MITI test systems, using substituted aromatics such as cumene and chlorinated phenols as examples (see Fig. 18.5). Both cumene (isopropylbenzene) and 4-methylcumene (*p*-cymene) have been judged to be "readily degradable" in the MITI test. 2-Methyl- and 3-methylcumene have been found experimentally to be "not readily degradable". However, the Biowin models predict that neither cumene nor any of the methylcumoles can be judged as "readily degradable".

A similar picture is shown for the chlorinated phenols. Phenol and 2,4,6-trichlorophenol have been shown to be "readily degradable" in the MITI test. On the other hand, 2-chloro-, 3-chloro-, 4-chloro-, 2,3-dichloro-, 2,4-dichloro-, 2,5-dichloro-, 2,6-dichloro-, 3,4-dichloro-, 3,5-dichloro- and 2,4,5-trichlorophenol are "not readily degradable". But only phenol is predicted to be degradable by Biowin model calculations: "**False negative result**" of prediction. The example of chlorine-substituted benzoates shows a comparably contradictory result.

Another example shows that the predictions made by the model calculations can also turn out to be "**false positives**". A carboxyl group on an aromatic ring is estimated to be positive for degradability. Why then is 1-naphthoate negative in the MITI test, while 2-naphthoate tests as degradable? The Biowin models make no distinction between the isomers. In this particular case, it is probably trivial reasons for the different result in the practical degradation tests. One cannot always assume that isomers are degraded by the same degradation pathway.

The degradability of EDTA is estimated very differently in the various Biowin model calculations: the combination of the mathematical models Biowin3&5 says that degradation should occur. The experimental MITI tests, on the other hand, say that EDTA is "not degradable".

18.2.2 Degradable Alternatives to Current Chemicals

In the search for antibiotics, natural substances used to be looked at as **lead structures in order** to derive new substances with a specific effect. In the production of chemicals, the focus was solely on the technological property. Today, environmental suitability must also be taken into account. So a compromise is attempted: substances with the **desired efficacy** are **copied from nature**. However, the **aspect of degradability** is also taken into account, taking into account the above-mentioned general knowledge.

Aminopolycarboxylates have been developed as alternatives for EDTA: EDTA is a highly efficient chelator, but is rated as not degrading in the MITI assay (tertiary amine functions linked by an ethylene bridge). This contrasts with strombin (◘ Fig. 18.6), a natural product that degrades well but has poor technical performance in terms of complexing properties. The search for a compromise should take into account the following facts: EDTA, which complexed heavy metals, is not taken up into the cell. Only the uptake of the free acid takes place and only alkaline earth EDTA complexes with correspondingly low complexation constants allow the uptake and thus the degradation. This means that complexing agents with less good complexing properties are needed when it comes to degradation.

A combination of strombin and EDTA is iminodisuccinate (IDS). IDS can form a fivefold coordinated complex with metal ions. For the octahedral form of the complete complex structure, an additional water molecule is required in the sixth coordination site (◘ Fig. 18.7). The five ligands of the IDS that can act as electron donors are the four carboxylate groups and the nitrogen. The metal ion and the ligands of the IDS form 4 chelate rings.

EDTA is the strongest complexing agent irrespective of the metal ions, whereas IDS, ethylenediaminodisuccinate and NTA have lower but comparable complexing properties. The complexation constants pK with Ca^{2+} make this clear: EDTA 10.7; IDS 6.7; ethylenediaminodisuccinate 4.2; NTA 6.4. IDS can therefore only be used as an EDTA substitute if the weaker binding properties towards metal ions can be accepted.

The field of application of IDS as a medium strength chelator is extensive. It is used in everyday products such as surface and sanitary cleaners, laundry and dishwashing detergents, shampoos, shower gels and creams, as well as in large-scale processes such as textile and paper production, the photographic industry and for the production of trace nutrient fertilizers for agricultural use.

The IDS shows a low remobilisation rate for heavy metals and a good overall ecotoxicological profile. Biodegradation tests with technical IDS showed a DOC removal of 89% in the OECD 302B test and 79% in the OECD 301E test (for the tests see ► Sect. 5.2.1).

Technical mixtures used consist of enantiomers in the following composition: 50% R,S-IDS, 25% S,S-IDS and 25% R,R-IDS (◘ Fig. 18.6). They meet the criterion of "ready biodegradability". However, the individual IDS enatiomers are degraded differently: R,S- and S,S-IDS are readily degradable. The enantiomer R,R-IDS, on the other hand, is not degraded with activated sludge from a municipal wastewater treatment plant, but is degraded with activated sludge from an industrial chemical plant. For a final assessment of the degradation behaviour in nature, the IDS metal complexes have to be considered, as they are predominantly present. Fe^{2+}- and Ca^{2+}-IDS fulfil the criterion of easy degradability in the MITI test, while Mn^{2+}- and Cu^{2+}-IDS only showed an oxygen depletion of 55% and 40% respectively, i.e. must be assessed as not easily degradable.

"Natural degradation" is therefore not necessarily sufficient to achieve the desired goal, since enatiomers can show different degradation behavior and since the binding of the metal ion to chelators can lead to different degradation behavior.

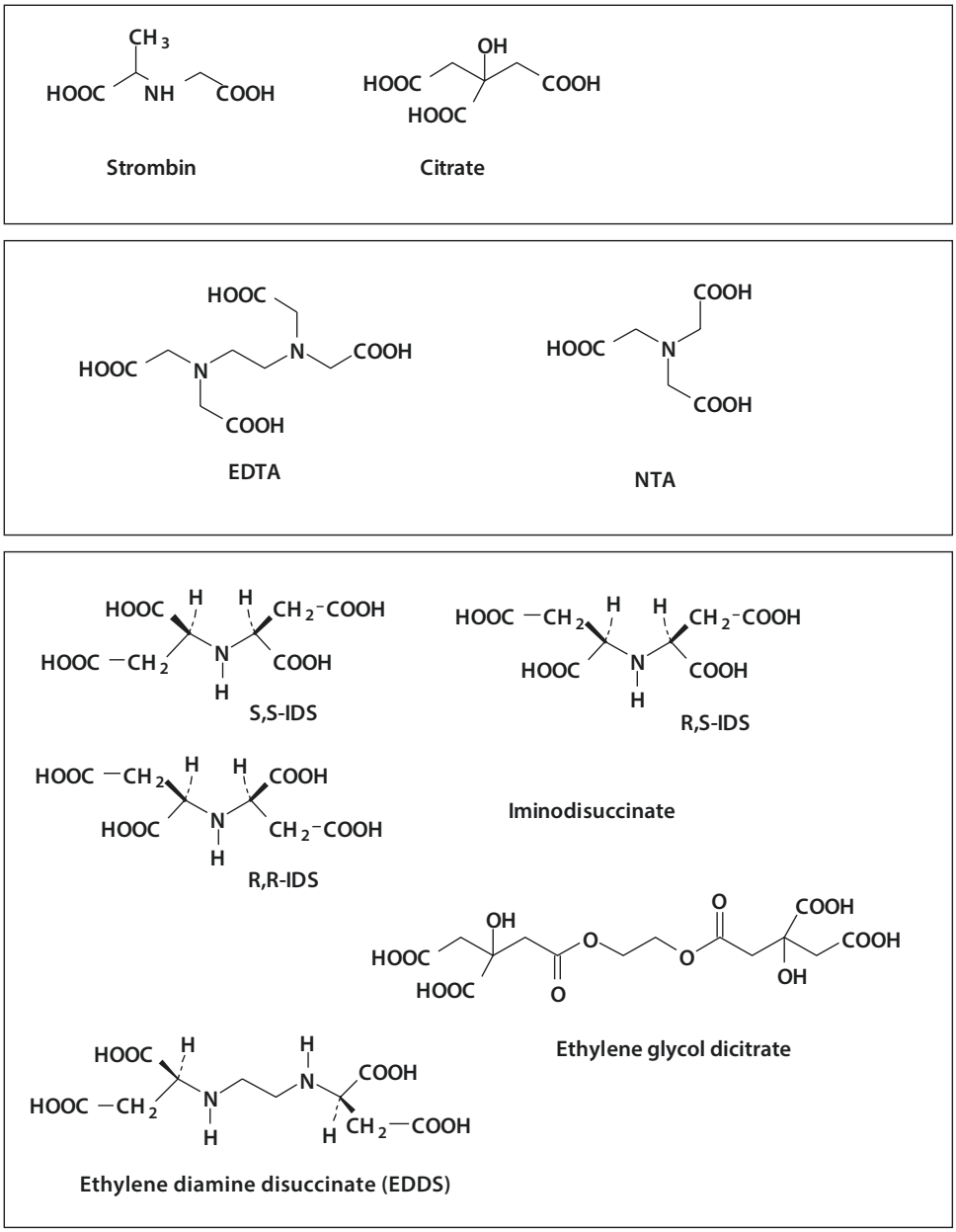


Fig. 18.6 Comparison of natural products, synthetic, classical products and "compromise products" with chelating properties

Another example of a degradable complexing agent is citrate. In order to improve its technological properties, two molecules of citrate were linked together by means of ethylene glycol via hydrolyzable ester bonds: The chelator now has good technical properties for use in the textile bleaching process and at the same time shows easy degradability.

Examples from the same field of technology are dispersants for dyestuffs used in textile processing. Dispersants must have hydrophilic and lipophilic properties. They

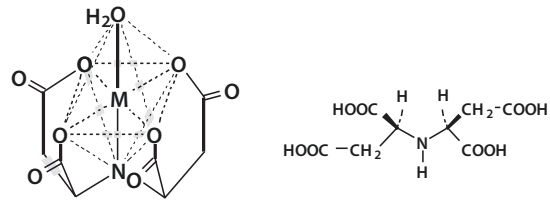


Fig. 18.7 Iminodisuccinate (IDS), as metal complex (left), without complexation (right)

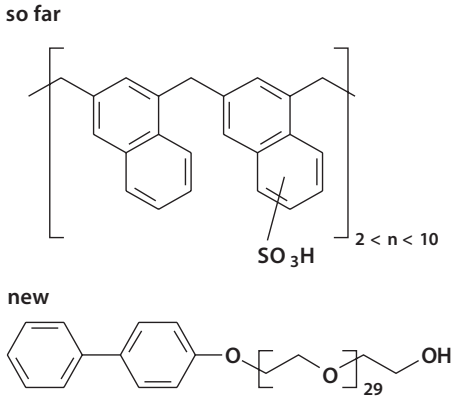


Fig. 18.8 Dispersants for dyestuffs used in textile processing: naphthalenesulfonic acids and ethoxylated hydroxybiphenyls, respectively

are therefore composed of aromatics, the lipophilic part, and they have sulfonic acid groups to ensure water solubility. The naphthalenesulfonic acids used so far are known for their poor degradability. The "new" dispersants consist of biphenyl (lipophilic) to which polyethoxy groups (hydrophilic) are coupled. The ethoxylated hydroxybiphenyls are **readily degradable** (Fig. 18.8).

18.3 Product-Integrated Environmental Protection Through Biotechnology

Chemical and mechanical process steps are increasingly being replaced by efficient biotechnological processes. Ultimately, this not only benefits the environment, but also pays off. Successful applications of biotechnology in production-integrated environmental protection (PIUS) are listed below:

- Textile industry: "Biostoning" of jeans (cellulases), desizing (starch degradation: amylases) and bleach removal (catalases, peroxidases).
- Leather industry: globulin and fat removal (proteases), pickling (last operation before tanning: loosening of collagen by proteases).
- Food industry: sweetener production (phenylalanine for aspartame), degradation of turbid substances and digestion of fruit fibres (pectinases).
- Paper and pulp industry: pulping of wood fibres (xylanases), dewatering of paper (pectinases, cellulases), bio-bleaching, bio-pulping, lipase for ink removal, paper coating with modified starch from potatoes or corn (amylase).
- Chemical industry: production of special and fine chemicals, use of enzymes in detergents, production of amino acids, organic acids, alcohols, carbohydrates and vitamins.
- Metal industry: cleaning and degreasing, biological ore leaching, for example for copper extraction.

Biotechnical production processes are not *per se* environmentally compatible. Therefore, they must also be subjected to an ecological assessment in order to justify their use.

In a research project of the Federal Environment Agency, (a) the biotechnical production of vitamin B_2, (b) a process step in leather production and (c) the use of enzymes in detergents were compared with the respective non-biotechnical alternative. The comparative assessment was based on the standard procedures for conducting life cycle assessments.

Background

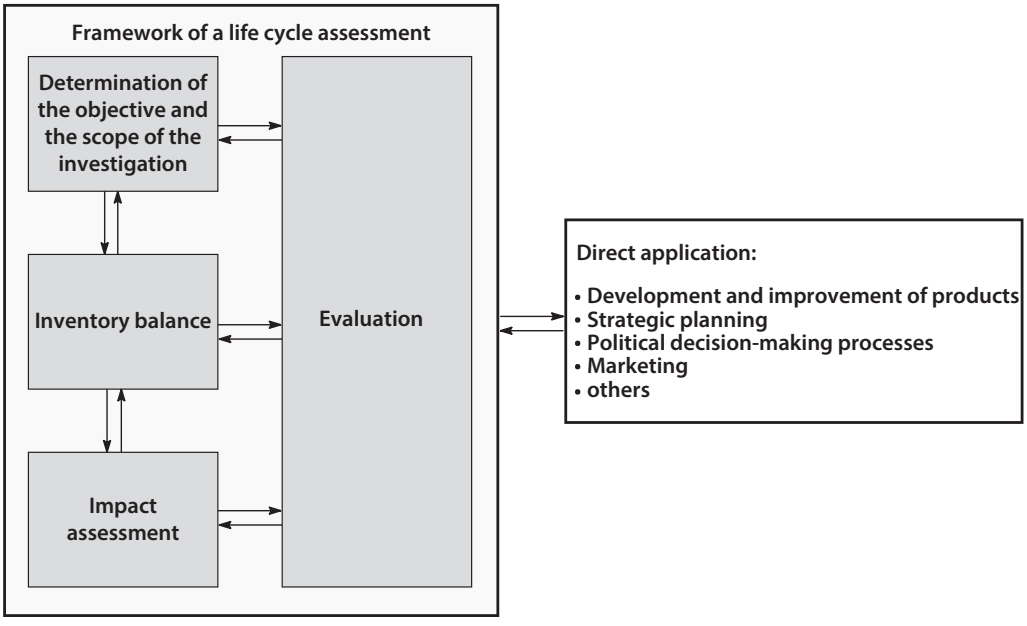
Life Cycle Assessment (LCA) according to DIN EN ISO 14040 and 14044 is a procedure for recording and evaluating the environmental impact of products, processes and services over their entire life cycle, i.e. from raw material extraction through production and use to disposal. The term "*cradle-to-grave*" is often used. Today, life cycle assessment according to DIN EN ISO 14040 and 14044 can be described as the main form of product-related assessment. In 1997, the International Organization for Standardization (ISO) standardized the procedure for preparing product life cycle assessments, which has since been revised several times.

DIN EN ISO 14040 ("Environmental management—Life cycle assessment—Principles and framework") contains principles and framework conditions for the preparation of product-related life cycle assessments. DIN EN ISO 14044 ("Environmental management—Life cycle assessment—Requirements and guidelines") regulates the requirements and instructions in detail.

The preparation of a life cycle assessment according to DIN EN ISO 14040 and DIN EN ISO 14044 is basically a relative approach.

Life cycle assessment studies consist of four components. The relationship between the components is shown in ◘ Fig. 18.9.

Results of life cycle assessments can provide useful information for a variety of decision-making processes. The impact categories and the respective specific parameters of the life cycle inventory are not prescribed in a binding manner, but are selected individually. The Federal Environment Agency (UBA), for example, uses the following impact categories compiled in ◘ Table 18.4 when preparing life cycle assessments.



◘ **Fig. 18.9** Components of a life cycle assessment according to DIN EN ISO 14040

Table 18.4 Impact categories considered

Impact category	Balance sheet parameters	Unit/indicator results
Resource use, cumulative energy use	KEA: fossil, nuclear, other, renewable, hydropower	kJ KEA total
Greenhouse effect	CO_2, CH_4, N_2O, CF_4	kg CO_2 equivalent
Acidification	NO_x, SO_2, H_2S, HCl, HF, NH_3	kg SO_2 equivalent
Terrestrial eutrophication	NO_x, NH_3	kg PO_4^{3-} equivalent
Aquatic eutrophication	P_{total}, CSB, N_{total}, NH_4^+, NO_3^-	kg PO_4^{3-} equivalent
Photochemical oxidation	Benzene, CH_4, NMVOC, VOX, formaldehyde	kg ethene equivalent
Human toxicity	Individual parameters	No aggregation; Indication in kg in each case
Ecotoxicity	Individual parameters	No aggregation; Indication in kg in each case

NMVOC non methane volatile organic compounds; *KEA* cumulative energy input

18.3.1 Process Comparison: Biotechnical and Chemical-Technical Processes

The **comparison of biotechnological and chemical processes is** explained using the example of vitamin B production$_2$ and a process step in leather production.

18.3.1.1 Biotechnological and Chemical-Technical Production of Vitamin B_2

The chemical-technical production of vitamin B_2 uses a multi-stage synthesis process for which, in addition to renewable raw materials, various environmentally relevant chemicals are also used (see Fig. 18.10, left). The biotechnological production process, on the other hand, requires only a single-stage fermentation, for which only small quantities of chemical auxiliaries with low environmental relevance are needed in addition to renewable raw materials. The biomass, which is produced in comparatively large quantities as waste, can be biologically recycled so that it has no negative influence on the overall balance of the process.

Table 18.5 summarizes the results of the comparative impact assessment. Significant environmental reductions for the aggregated impact categories of cumulative energy input (CED resource use), greenhouse gas, acidification, terrestrial eutrophication and ozone formation potential were determined for the biotechnical process compared to the chemical-technical process. However, the aquatic eutrophication potential was higher for the biotechnical process than for the chemical-technical process. The comparison of the processes for the 5 individual substances classified as toxic to humans and evaluated showed only minor differences for the parameters benzo(a) pyrene and lead with a generally low pollution level. The relatively low cadmium emissions were higher in the biotechnical process than in the chemical-technical process. In the case of the sulfur dioxide and dust

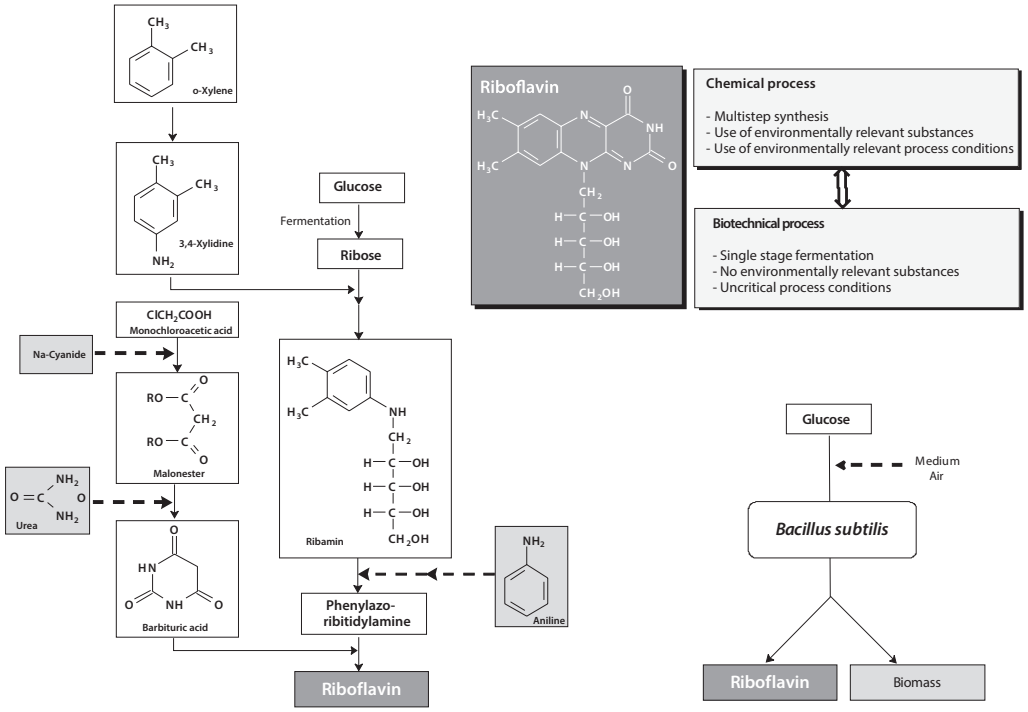


Fig. 18.10 Schematic comparison of the chemical-technical and biotechnical production of vitamin B_2

Table 18.5 Effect estimate comparing biotechnological and chemical-technical vitamin B_2 production

	Units	Environmental relief through biotechnical process[a]
Impact categories, aggregated		
KEA	GJ	+
Global warming potential	mg CO_2 equivalent	+
Acidification potential	kg SO_2 equivalent	+
Eutrophication potential (terrestrial)	kg PO_4 equivalent	+
Eutrophication potential (aquatic)	kg PO_4 equivalent	–
Ozone creation potential	kg ethene equivalent	+
Individual substances toxic to humans		
Benzo(a)pyrene (L)	g	±
Lead (L)	g	±
Cadmium (L)	g	–
Sulfur dioxide (L)	kg	+

(continued)

Table 18.5 (continued)

	Units	Environmental relief through biotechnical process[a]
Dust (L)	kg	+
Ecotoxic individual substances		
Ammonia (L)	kg	–
Hydrogen fluoride (L)	kg	±
Sulfur dioxide (L)	kg	+
Hydrogen sulfide (L)	g	+
Nitrogen oxides (L)	kg	+
Ammonium (W)	kg	–
AOX (W)	g	+
Chloride (W)	kg	+
Hydrocarbons (W)	kg	+

(L): Air; (W): Water
[a] + Environmental relief compared to conventional process; – Additional load

emitted in relevant quantities, the comparison showed a pronounced environmental relief for the application of the biotechnical process. For the 9 individual substances classified and evaluated as ecotoxic, the biotechnical process showed in some cases significant environmental relief for 6 parameters in comparison with the chemical-technical process. For the parameters ammonia in air and water the biotechnical process showed higher emission values. The emissions were at a low level for both processes for the parameter hydrogen fluoride.

As early as 1990, the development and implementation of a fermentative single-stage process for the production of vitamin B_2 at BASF led to a 95% reduction in waste, 30% in CO_2 emissions and 60% in resource consumption compared with the old petrochemical process. Production costs were thus reduced by a total of 40% through the use of the fungus *Ashbya gossypii.*

18.3.1.2 Biotechnological and Chemical-Technical Production of Leather

Leather production consists of a chain of individual steps which can differ depending on the process used, the type of raw material and the product to be manufactured. For the process comparison, the partial step of **soaking** and **liming** was considered. Steeping has the task of freeing the rawhide from adhering impurities, removing preservatives and restoring the swelling condition as on the body of the living animal. Liming is the process step in which hides are dehaired and the fibrous structure of the hide is broken down. The evaluations include a comparison of a "hair-destroying chemical process" and a "hair-preserving enzymatic process". In the hair-destroying process, the hair is largely hydrolytically decomposed by the use of chemicals and discharged into the wastewater. In the

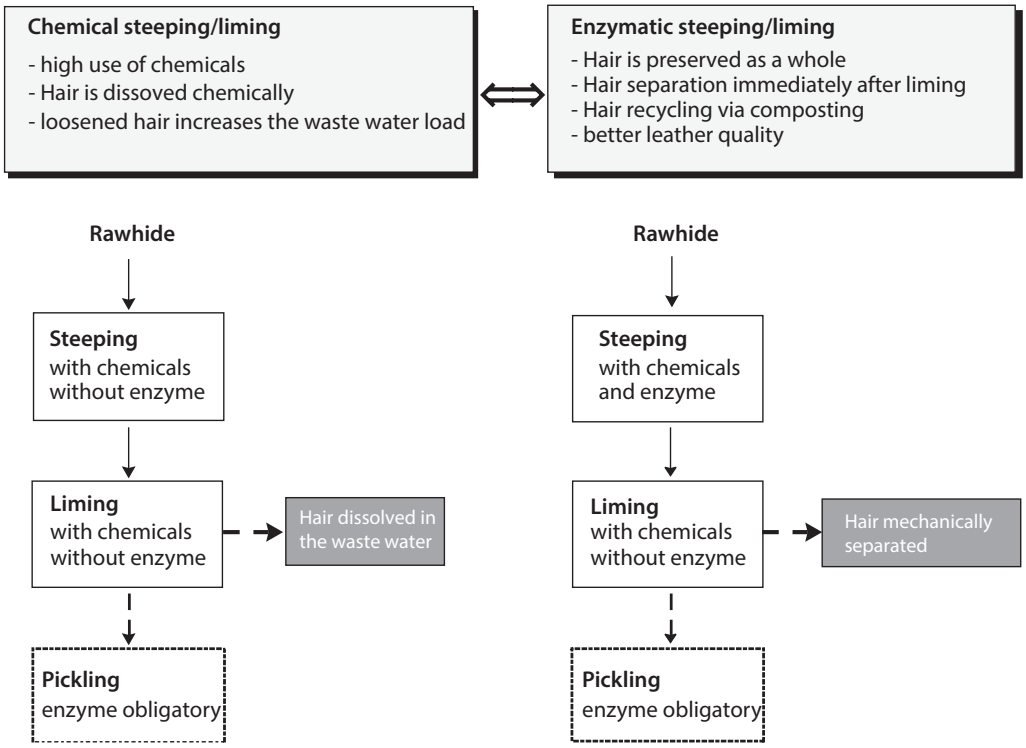


Fig. 18.11 Leather production: comparison of the chemical-technical and biotechnical processes of soaking/liming

hair-preserving process, the hairs are merely loosened and then mechanically removed by whaling or mechanical depilation. They can then be separated and sent for recycling. Worldwide, about 15% of the processes practised for steeping and liming are said to make use of biocatalysts (Fig. 18.11).

The results of the impact assessment for the comparison of enzymatic and chemical process control during steeping and liming are summarised in Table 18.6.

Compared to the chemical process, the enzymatic soaking/laundering showed environmental relief potentials for all aggregated impact categories.

For the five individual substances classified and evaluated as toxic to humans, the emissions for the parameters cadmium, sulfur dioxide and dust were significantly lower in the enzymatic than in the chemical soaking/etching process.

In the case of the nine individual substances classified and evaluated as ecotoxic, the results document significant reduced emissions for seven substances when enzymatic soaking/acidification is applied.

18.3.2 Environmental Relief Effects Through Product Substitution

Analogous products with functional equivalence were compared with regard to raw material supply, manufacturing processes for the chemical-technical and the biotechnical product as well as their subsequent use phase (product use + waste disposal).

Table 18.6 Impact assessment of the use of enzymes for soaking/liming in leather production

	Units	Environmental relief through the use of enzymes[a]
Impact categories, aggregated		
KEA	GJ	+
Global warming potential	kg CO_2 equivalent	+
Acidification potential	kg SO_2 equivalent	+
Eutrophication potential (terrestrial)	kg PO_4 equivalent	+
Eutrophication potential (aquatic)	kg PO_4 equivalent	+
Ozone creation potential	kg ethene equivalent	±
Individual substances toxic to humans		
Benzo(a)pyrene (L)	mg	±
Lead (L)	kg	±
Cadmium (L)	kg	+
Sulfur dioxide (L)	kg	+
Dust (L)	kg	+
Ecotoxic individual substances		
Ammonia (L)	kg	+
Hydrogen fluoride (L)	kg	±
Sulfur dioxide (L)	kg	+
Hydrogen sulfide (L)	kg	±
Nitrogen oxides (L)	kg	+
Ammonium (W)	kg	+
AOX (W)	kg	+
Chloride (W)	kg	+
Hydrocarbons (W)	kg	+

(L): Air; (W): Water
[a] + Environmental relief compared to conventional process

18.3.2.1 Product Comparison: Enzyme Use in Heavy-Duty Detergents

Today, enzymes are an integral part of the market-leading laundry detergents, as their use enables savings to be made on other detergent ingredients in some cases. The aim of this product comparison was therefore to find out whether the production and use of an enzyme-containing detergent can reduce the environmental impact (Fig. 18.12).

The results of the impact assessment for the comparison of enzyme-free and enzyme-containing detergents by the scenarios **Modern Detergent** and **Traditional Detergent** are summarized in Table 18.7.

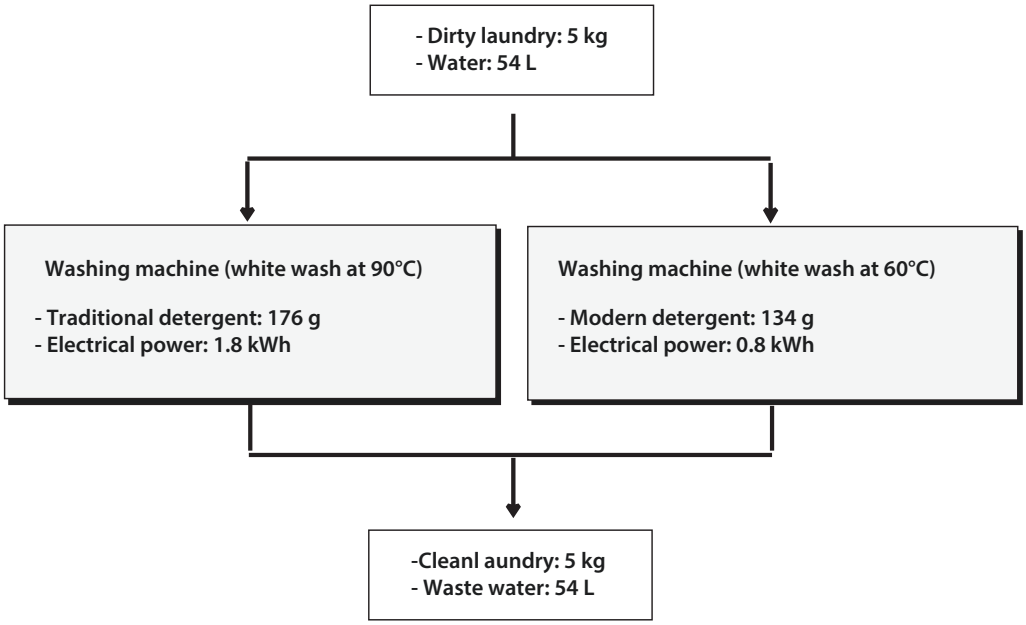


Fig. 18.12 Schematic comparison of the washing process with enzyme-free and enzyme-containing detergent

Table 18.7 Effect estimation when comparing **modern detergents** and **traditional detergents**

	Units	Environmental relief through modern detergent[a]
Impact categories, aggregated		
KEA	MJ	+
Global warming potential	kg CO_2 equivalent	+
Acidification potential	g SO_2-equivalent	+
Eutrophication potential (terrestrial)	g PO_4-equivalent	+
Eutrophication potential (aquatic)	g PO_4-equivalent	+
Ozone creation potential	g ethene equivalent	+
Individual substances toxic to humans		
Benzo(a)pyrene (L)	mg	±
Lead (L)	mg	+
Cadmium (L)	mg	+
Sulfur dioxide (L)	g	+
Dust (L)	g	+

(continued)

Table 18.7 (continued)

	Units	Environmental relief through modern detergent[a]
Ecotoxic individual substances		
Ammonia (L)	g	+
Hydrogen fluoride (L)	g	+
Sulfur dioxide (L)	g	+
Hydrogen sulfide (L)	g	±
Nitrogen oxides (L)	g	+
Ammonium (W)	g	+
AOX (W)	g	+
Chloride (W)	g	+
Hydrocarbons (W)	g	±

(L): Air; (W): Water
[a] + Environmental relief compared to enzyme-free detergent

It can be seen that the Modern Detergent showed in part significant environmental relief potentials for all impact categories considered and almost all individual parameters.

18.3.3 Summary PIUS

It is now impossible to imagine environmental protection without biotechnology. However, advanced PIUS concepts in particular are increasingly benefiting from its almost inexhaustible potential. The advantages are obvious:

- Saving energy and valuable resources (for example, reduced wastewater volumes in the paper industry).
- Optimization of efficiency (less raw material required, better yield, less waste).
- Reduction of unwanted by-products and waste (mostly biodegradable and ecologically harmless) or conversion into biodegradable substances.
- Increase in quality due to gentler treatment of the raw materials.
- Less need for chemicals.
- Improvement of product quality.
- Reduction of process times.

In contrast to many mechanical or chemical processes, biotechnological processes are therefore referred to as "gentle" processes.

Safety-Related System Comparison

The **comparison of biotechnological and chemical plants, processes and products** carried out in the research project of **the** Federal Environment Agency shows that **biotechnology** can have **clear ecological advantages over the chemical alternative**.

If one analyses biotechnical and chemical plants with regard to the process- and substance-related as well as the biological hazard potential, the following facts become clear: While biotechnical

processes take place at normal pressure and room temperature, chemical processes often take place at high pressures and, in some cases, high temperatures. Chemical processes also frequently involve the use of a significantly higher number of hazardous substances. The biological agents used in biotechnical processes usually have only a low to negligible safety risk. Thus, biotechnical processes used on a large scale can also be considered significantly more advantageous in terms of safety.

The OECD has also been able to demonstrate in 21 case studies that the **application of modern biotechnological processes** can lead to **a reduction in environmental pollution and operating costs**.

18.4 Biofuels

As shown in ▶ Sect. 14.5, biogas can be produced from biomass. In addition, the production of basically three different types of liquid fuel from biomass is possible: bioethanol, biodiesel and biomass-to-liquid (BTL) fuel (◘ Fig. 18.13).

OECD-FAO *Agricultural Outlook Biofuel production 2010–2019*

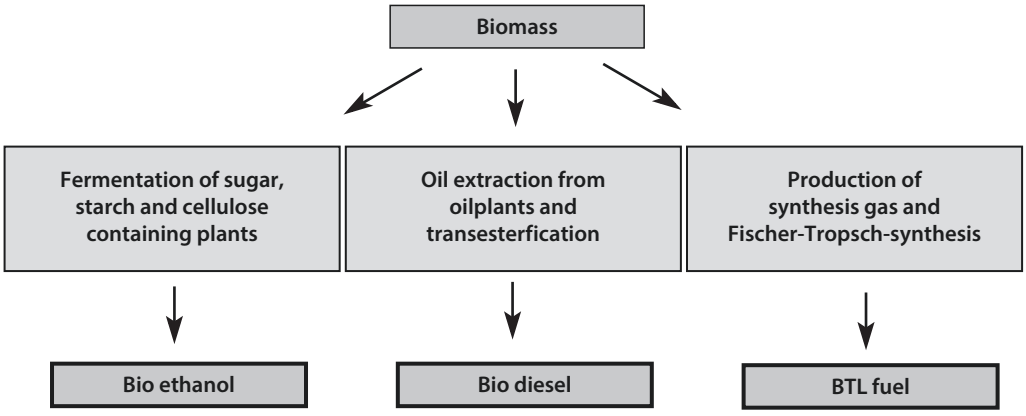
In 2011, around 100 billion litres of bioethanol were already produced worldwide. Production is to be increased to 160 billion litres by 2019.

The development of ethanol production from feedstocks in the period presented by the OECD shows that the main feedstock for ethanol production remains coarse grain (corn) (◘ Fig. 18.14). Consumption of coarse grains for ethanol production grows relatively little after 2015, when the *Conventional Renewable Fuels* programme in the USA reaches its maximum.

Nearly 40% of the increase in global ethanol production is the result of increased production from cane sugar, mainly from Brazil, to meet domestic and US demand.

Ethanol production from second generation biomass is not expected to develop until the end of the period, when it will account for about 7% of total ethanol production.

Roots and tubers as well as molasses will play a role as raw materials for ethanol production in developing countries.



◘ **Fig. 18.13** Alternative fuels from biomass

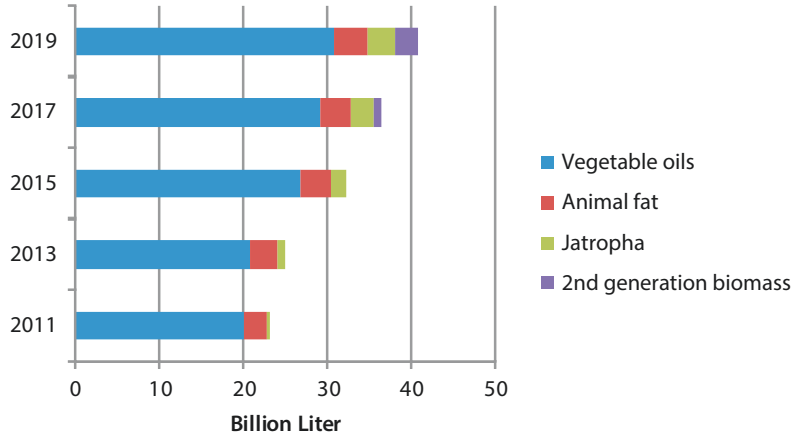


Fig. 18.14 Global ethanol production from various feedstocks. (Second generation biomass: all biomass except foodstuffs [wood, straw, industrial waste wood, energy crops, residues])

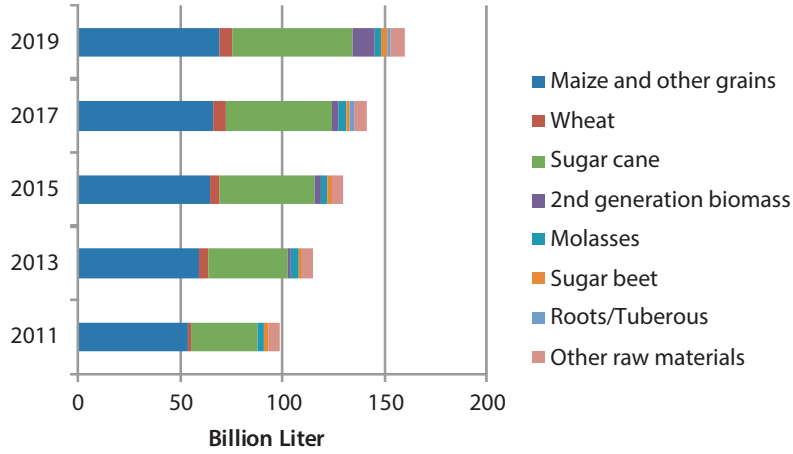


Fig. 18.15 Global biodiesel production from various feedstocks. (Second generation biomass: all biomass except foodstuffs [wood, straw, industrial waste wood, energy crops, residues])

Wheat, coarse grains and sugar beet are used in the EU to produce ethanol.

The situation with biodiesel production is similar to that with ethanol. Worldwide, about 23 billion liters were produced in 2011, which is expected to increase to 40 billion liters by 2019 (Fig. 18.15).

Oil from food crops is expected to remain the main feedstock to produce biodiesel. But its share of total biodiesel production is expected to fall from nearly 90% to about 75% in 2019. This trend will be mainly due to the production of biodiesel from jatropha in India. Furthermore, biodiesel production is expected to increase due to the use of animal fat in the US and the availability of second generation biomass (all non-food biomass: wood, straw, industrial waste wood, energy crops, residues) in recent years by 2019. Biodiesel produced from second

generation biomass is then expected to account for nearly 6.5% of total biodiesel production in 2019.

Overall, a significant proportion of global production of cereals, sugar and vegetable oils is consumed in the production of biofuels.

In 2019, about 13% of global production of coarse grains will be used to produce ethanol, compared to 9% today. 16% of global production of vegetable oil will then be consumed for biodiesel production compared to 9% today.

The share of cane sugar to be used for global ethanol production in 2019 will reach nearly 35%.

► http://www.oecd.org/site/oecd-fao-agriculturaloutlook/biofuelproduction2010-19.htm

18.4.1 Bioethanol

Bioethanol is produced by fermenting plants containing carbohydrates. Besides starch-containing plants such as wheat, rye or corn, sugar cane and sugar beet are the most commonly used feedstocks. While sugar-containing plants can be fermented directly, the starch in cereals and potatoes must first be broken down enzymatically into sugar (see ► Sect. 4.4.4).

For bioethanol, a conversion factor of three is assumed for the most efficient raw materials, i.e. 3 kg of biomass produces 1 kg of bioethanol. An annual yield of 2560 L of bioethanol per hectare based on grain is calculated.

The production costs in Europe are around 60 cents per litre.

In the USA and other European countries, ethanol is mainly produced from corn, while Brazil relies on the fermentation of sugar from sugar cane.

In Europe, Sweden is the pioneer with E85 fuel, a fuel blend containing up to 85% ethanol. Only so-called **flexible fuel vehicles** can use E85 fuel. Due to the low energy density of ethanol, they consume about 50% more fuel by volume than cars running on gasoline.

1 L ethanol replaces approx. 0.66 L petrol and can be blended with up to 5%. Ethanol has properties that improve the quality of petrol fuels. For example, the alcohol has a higher octane number than conventional petrol fuels (◘ Table 18.8).

◘ **Table 18.8** Comparison of energy contents and chemical properties of different fuels

Fuel	Energy density per mass (MJ/kg)	Energy density per volume (MJ/L)	Average octane number
Otto fuel	42.7	32.0	90
Diesel	45.5	38.7	
Ethanol	29.7	20.8	116

Li et al. (2010)

18.4.2 Biodiesel

Biodiesel is obtained by transesterification, for example with methanol, of vegetable oil (often rapeseed oil with an oil content of 40–45%) or animal fats (◘ Fig. 18.16).

The transesterification product of rapeseed oil acquires the properties that largely correspond to those of mineral oil-based diesel fuel. The disadvantage of biodiesel is its aggressiveness: it easily causes corrosion and leaking seals, and filters also clog. Rubbers and plastics previously used in engines dissolve. In addition, the injection pump in the car may suffer.

For the use of biodiesel in pure form, the approval of the engine manufacturer is

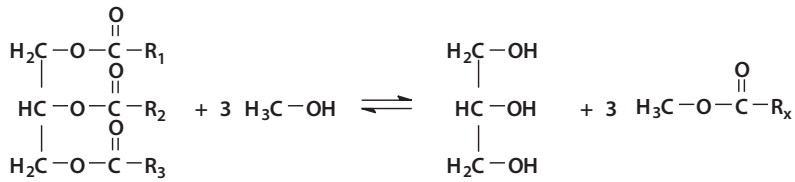


Fig. 18.16 Reaction sequence in the catalytic transesterification of triglyceride rapeseed oil to the corresponding fatty acid methyl esters

Table 18.9 Comparison of conventional and alternative fuels

Fuel	Infrastructure available	Conversion of the engines	Sustainability
Petrol	Yes	Not necessary	None
Diesel	Yes	Not necessary	None
Bioethanol	Yes/no	Necessary	Yes
Biodiesel	Yes/no	Necessary	Yes
BTL fuel	No	Not necessary	Yes

required. In blends, biodiesel up to 5% can be used without adaptation of the engine.

The conversion factor for rapeseed is about three: 3 kg of rapeseed produces 1 kg of biodiesel. The production costs in Germany are around 75 cents per litre.

18.4.3 Biomass-to-Liquid Fuel

Biomass-to-liquid (BTL) fuel is created through three process steps: First, biomass is converted to biocoke and high energy density gas containing tar in a low temperature process. Tar-free synthesis gas (CO and H_2) is produced from these starting raw materials. In a third step, liquid hydrocarbons are produced from the synthesis gas using the Fischer-Tropsch process on iron and cobalt catalysts.

Production costs are estimated to be around 60 cents per litre in the future.

BTL fuel has numerous advantages: It can be used in conventional gasoline and diesel engines without having to convert them.

For BTL fuel, a conversion factor of five is assumed for the most efficient feedstocks; 5 kg of biomass produces 1 kg of BTL fuel. Since the whole plant can be used, the field yield (fuel per hectare) is up to three times higher than for biodiesel or bioethanol (Table 18.9).

18.5 Electricity from Microorganisms

Fuel cells and, in connection with them, the hydrogen economy are generally predicted to have a great future. Renewable energy sources in particular could play a central role in this area. Completely different paths are being taken to convert chemical energy into electrical energy without combustion, analogous to the fuel cell.

The so-called "bio fuel cell" uses microorganisms from whose metabolism electrons are separated and fed to an anode.

Biofuel cells for the generation of electrical energy from abundant organic substrates

(including waste or sedimentary material) can involve a variety of approaches.

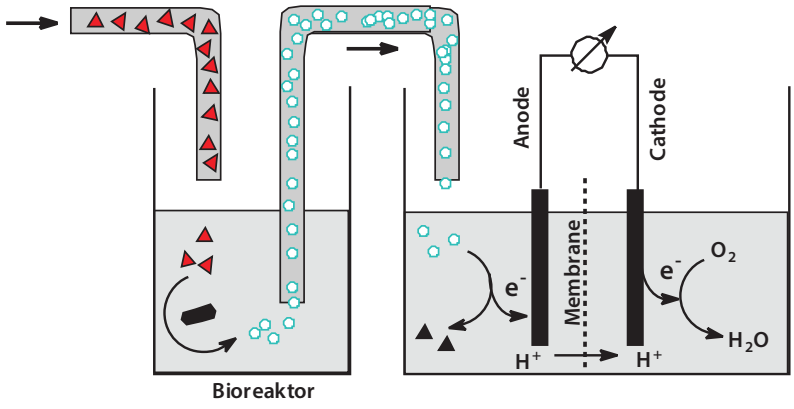
18.5.1 Hydrogen Production in Bioreactors for Conventional Fuel Cells

Microorganisms have the ability to produce electrochemically active substances that are metabolic intermediates or end products of anaerobic respiration.

For the purposes of energy formation, these fuel substances are produced at a separate location and then transported to the fuel cell to be consumed as fuel. In this case, the bioreactor produces the fuel, which means that the biological part of the system is not directly integrated into the electrochemical part (◘ Fig. 18.17). This setup allows the electrochemical part to run under conditions that are not compatible with the biological part of the plant. The two parts can even be operated independently of each other in time. The fuel most commonly used in such a setup is hydrogen, which is fed to the advanced and highly efficient H_2/O_2 fuel cells from the bioreactor.

18.5.2 Microbial Production of Fuel in the Anode Compartment of the Fuel Cell

In another setup, the fermentation process takes place directly in the anodic compartment of the fuel cell, with the anode being supplied *in situ* with the fermentation product produced (◘ Fig. 18.18). In this case, the conditions in the anodic compartment are determined by the biological system, so they are considerably different from those in conventional fuel cells. It is therefore a true biofuel cell and not a combination of bioreactor and conventional fuel cell. This design is also often based on the biological production of H_2, but the electrochemical oxidation of the H_2 occurs in the presence of the biological components under mild conditions. To ensure anaerobic conditions, cells are immobilized on the anode in gels, which means that the fermentation of *Clostridium butyricum* takes place directly on the surface of the electrode, so that the anode is supplied with H_2. An O_2 electrode is often used as the cathode.



◘ **Fig. 18.17** Schematic diagram of a bio-fuel cell with separate bioreactor for fuel production and conventional fuel cell. Pictorial parts: Primary substrate (glucose or sewage waste: triangle empty); fuel (circle empty); oxidized, spent fuel (triangle full)

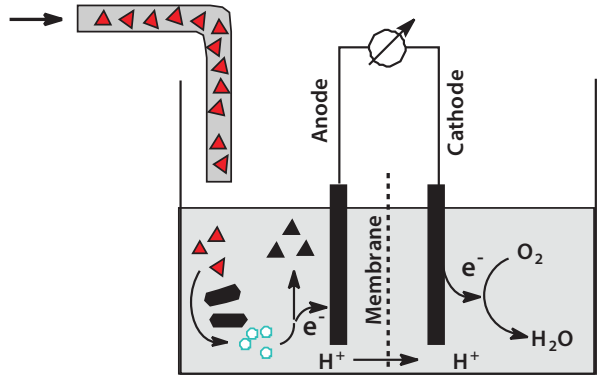


Fig. 18.18 Schematic diagram of a biofuel cell with integrated production of the fuel in the anode compartment. Image parts: Primary substrate (glucose: triangle empty); fuel (circle empty); oxidized, spent fuel (triangle full)

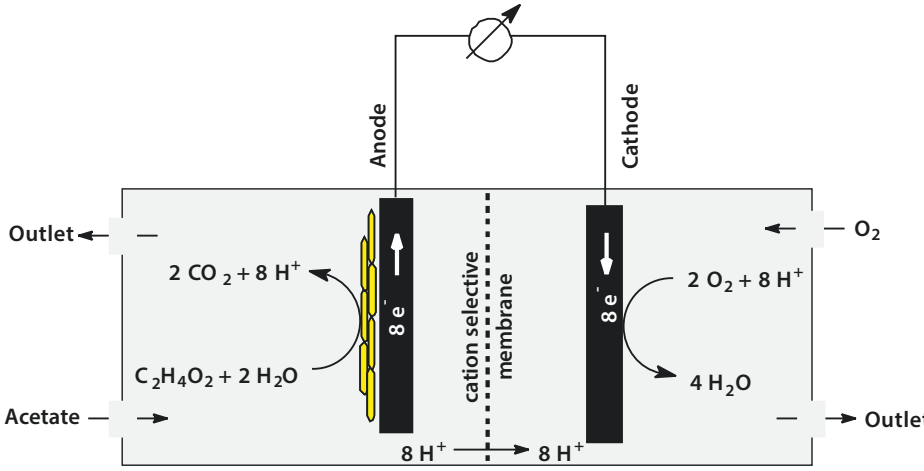


Fig. 18.19 Structure and process in a biofuel cell with the electricigen, *Geobacter,* as electron supplier

18.5.3 Direct Electron Transport from the Cell to the Electrode

The metal-reducing bacterium *Shewanella putrefaciens* has cytochromes in the outer membrane. These electron carriers are able to generate an anodic current in the absence of a terminal electron acceptor (under anaerobic conditions). This is an example of a so-called mediator-free electron transport.

In *Geobacter* species, further metal reducers, electron-conducting pili, so-called nanowires, were found to be produced by the bacteria. The bacterium forms a biofilm on graphite electrodes, and mediator-free electron transport takes place, which can be used in a biofuel cell. The setup is shown in ◘ Fig. 18.19 for *Geobacter* and the fuel acetate.

18.5.4 Mediators for Electron Transport

Chemical mediators are often used to accept electrons from the electron transport chain in order to transport them to the anode of the fuel cell. In this case, the process taking place in the organisms is clearly different from the natural one, since the electron flow is to the electrode and not to the natural electron acceptor.

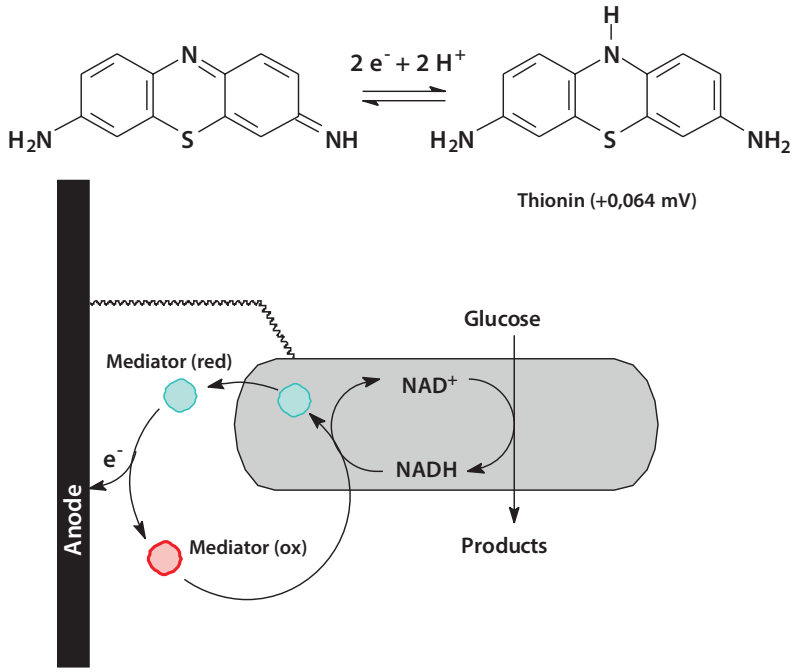


Fig. 18.20 Electron transport from a cell to the anode with a low molecular weight, diffusible mediator molecule. The organism, here *Proteus vulgaris,* is covalently bound to the electrode

Since the natural electron acceptors are usually much more efficient than these mediators, they must be removed from the system.

The *in situ* electrical coupling of the metabolites produced in the microbial cell with the electrode system can also be ensured by diffusible electron mediators.

These small molecular weight redox species can serve as transport vehicles for electrons between the interior of the bacteria and the electrode. Thionine has been tested particularly frequently as a mediator of electron transport with *Proteus vulgaris* and *Escherichia coli* (Fig. 18.20).

Producing Electricity with Cyanobacteria

In the search for sustainable ways to produce energy, microorganisms or biomolecules extracted from them are constantly being tried out. Photosynthetic systems have been widely used to transfer electrons using solar energy (McCormick et al., 2015, Larom et al., 2015, Pinhassi et al., 2016; Zhao et al., 2015; Efrati et al., 2016; Gizzie et al., 2015; Sekar et al., 2014, 2016; Sawa et al., 2017). Isolated photo-systems have been used for this purpose within a bio-photoelectrochemical cell to produce electrical energy (Mershin et al., 2012; Yehezkeli et al., 2012; Efrati et al., 2013; Li et al., 2016; Zhao et al., 2014). An immobilized mediator as an exogenous electron acceptor has been widely used in this regard (Zhao et al., 2015; Efrati et al., 2016; Yehezkeli et al., 2012; Zhao et al., 2014). However, one of the main problems with the use of isolated photosynthetic complexes is the relatively short lifetime of the biological components of the system due to damage from radicals formed in the reaction cen-

ters (Yao et al., 2012; McCormick et al., 2013). The use of living cells could address this problem due to the presence of repair systems that can replace proteins of photo-damaged photosynthesis. However, extracting electrons from living organisms is complicated because it requires exogenous electron carriers (mediators) that can cross the membrane, such as ferricyanide, cytochrome, or 2,6-dichloro-1,4-benzoquinone (Pinhassi et al., 2016; Sekar et al., 2014; McCormick et al., 2013; Pinhassi et al., 2015; Larom et al., 2010; Calkins et al., 2013).

To overcome the aforementioned difficulty regarding isolated systems, a harmless gentle physical treatment was used to move cyanobacterial cells to a graphite electrode and allow light-driven electron transfer using a cell's own endogenous mediator to the electrode in a biophotoelectrochemistry cell (Saper et al., 2018). The addition of exogenous electron donors or acceptors was not necessary (McCormick et al., 2011; Sekar et al., 2016; Cereda et al., 2014; Wei et al., 2016).

The photocurrent is thereby derived from the photosystem. The current is finally used for hydrogen evolution at the cathode at a bias voltage of 0.65 V. Thus, a setup of a biophotoelectrochemical system with living cyanobacteria was shown, in which a stable photocurrent can be used for hydrogen generation.

Test Your Knowledge

- Which factors influence the degradability?
- You want to use baculoviruses against insect pests. How narrow is the time window of application?
- Compare the different (bio)insecticides with each other in terms of speed of action.
- Why are the very high complexation constants of EDTA problematic when you think about degradability?
- What potential problems arise from the use of fungal preparations against insects, as food crops could also be inoculated at the same time?
- What do you understand by PIUS?
- Name bioinsecticides against Anopheles and the blackfly.
- What events first brought *Bacillus thuringiensis* to attention?
- What is the advantage of Bt endotoxins over β-exotoxin? Describe the mechanism of action of both toxins.
- Name Bt plants.
- Which secondary metabolites can be used as bioinsecticides?
- What is your opinion of QSAR in assessing chemicals for biodegradability? List any unresolved problems with the use of mathematical estimates of degradability.
- Which impact categories are considered in a life cycle assessment?
- Which form of biofuel can be produced from the rape seed, which from the whole plant?
- Describe types of bio-fuel cells. What is the function of mediators? What are nanowires? Inform yourself in the given literature regarding market maturity of such power generators.

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Studie zu Biokraftstoffen von Festel Capital: gunter.festel@festel.com.
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Food for Thought

Contents

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19.1 Sustainability, the Concept

The preservation and production of a safe and clean environment are today associated with the concept of **sustainability**. It implies a concept of using a system that should be preserved in its essential characteristics and whose stock can be regenerated in a natural way. The term sustainability should be questioned, however, as it is used in very different ways. Interests do not seem to be irrelevant in the manifold uses. What all users have in common is that sustainability is used to imply, or is intended to imply, something positive. Some authors state that due to the diverse definition **sustainability** has become a rubber word. In the original literal sense ("lasting for a long time") and in the figurative, originally silvicultural sense ("silvicultural principle according to which no more wood may be felled than can grow back at any one time"), the word comes from "to keep up" with the meaning "to last or remain for a long time". In today's usage, the term implies that in other areas, too, something can or should last, remain, have an aftereffect or be—long after it has been built or set in motion.

Hans Carl von Carlowitz

"We cannot act against nature, but only with it."

Hardly anyone has ever heard the name of the nobleman Hans Carl von Carlowitz, who was born in 1645 and rose to become a powerful official of Kursachsen (Electoral Saxony). Von Carlowitz worked in Freiberg, the old Saxon silver city at the foothills of the Ore Mountains.

Von Carlowitz published his *Sylvicultura Oeconomica* in a thick folio volume. The "Naturmäßige Anweisung zur Wilden Baum-Zucht" (Natural Instruction for Wild Tree Breeding)—as the subtitle reads—is considered the first work of forestry science. It revolves around the idea and concept of sustainability. Wherever it appears in history, the idea of sustainability is a child of crisis. Around 1700, the existence of silver mining in Saxony was threatened. It was not the exhaustion of the deposits that was feared. The problem that was predicted was the lack of wood.

In his book, von Carlowitz advocates a number of measures to counter the problem: Improving thermal insulation in house construction and the use of energy-saving smelting furnaces and kitchen stoves, planned reforestation through sowing and planting, and last but not least, finding substitutes for wood. Von Carlowitz recommends the use of peat.

19.2 Sustainability, Environmental Microbiology a Contribution

As early as 1994, environmental biotechnology was considered by the OECD to be a key technology for the prevention, detection and elimination of environmental pollution, as it offers a promising potential for environmentally relevant problem solutions due to its broad range of applications and diverse methods.

At that time, the OECD listed the following **five application perspectives of environmental biotechnology**, whereby future priority should be given to the prevention of environmental damage, although post-cautionary environmental protection measures are also important.

- Analytics and Monitoring;
- Treatment of pollutants or materials with the aim of recycling or reuse (value-added processes);

- Purification of water, soil and air by biotechnical processes (end-of-pipe processes);
- Production-integrated biotechnical processes to reduce the amount of waste/emissions or to improve the treatment possibilities of the generated waste;
- Development of biomaterials with lower environmental impact during the manufacturing process.

The need for aftercare environmental protection measures using biotechnological processes is obvious, as there are more than 300,000 suspected contaminated sites in Germany alone. The remediation and reuse of thousands of brownfield sites in industrial focus regions is of fundamental importance for economic development and land use.

Through the integrated use of biotechnological processes and products, environmental protection in the sense of resource conservation or environmental relief can also be achieved in areas other than the classical ones (biotechnological processes for exhaust air and wastewater purification and soil remediation), for example in the fields of health, nutrition, chemicals and agriculture.

Sustainable development presents all scientific disciplines with a task of a new dimension. For environmental microbiology, this means that the field should not be understood solely as a sub-discipline of microbiology, but that the findings of microbiology should contribute to sustainable development. A transdisciplinary view of environmental problems is necessary.

The problems in the application of environmental microbiology, which are often very complex in detail, can only be solved by research activities of different orientation and different disciplines. The tension between basic and application-oriented research will remain. It is becoming apparent that new interdisciplinary perspectives on environmental research are being opened up via interest in microbial pollutant degradation. The OECD (1994) pointed out the need for long-term oriented basic research in the environmental field. Only in this way can it be prevented that a too close linking of research to "immediate practice" has an obstructive effect.

19.3 Environment and Environmental Microbiology, Reflection

In conclusion, using some of the critical questions posed in lectures and seminars, comments made and arguments advanced on environmental chemicals, the reader should answer for themselves the question: What does environmental microbiology have to offer in addressing environmental problems?

Since When Environmental Chemicals?
The substance DDT, known since the nineteenth century, was identified as a highly active insecticide by Paul Müller in 1994. Müller proved that it acted on mosquitoes and other harmful insects. DDT appeared to be non-toxic to humans and was also cheap and easy to produce. DDT was successfully used in World War II to combat various species of mosquitoes that spread malaria. Müller received the Nobel Prize for Medicine in 1948.

Rachel Carson published her book "**Silent Spring**" in 1962, in which she warned of a world in which birds would no longer sing. Carson drew attention to the fact that the use of chemicals containing chlorine, such as DDT, was damaging the environment as well as humans. The chemical stability of DDT, which had initially been considered desirable, allowed the compound to persist in soil and water for a long time. DDT was shown to accu-

mulate in animal and human fatty tissue over decades. Seabirds and birds of prey, which are at the bottom of the food chain and feed on heavily contaminated rodents and fish, particularly suffered from the effects of the insecticide, Carson wrote. The birds produced such thin-shelled eggs because of the poisoning that offspring were increasingly absent. Some species were on the verge of extinction as a result.

The substance was banned in the USA and other industrialised countries from 1972.

This was the beginning of a critical assessment of chemicals in the environment. Chloraromatics, with the examples of DDT and PCBs, were for a long time the environmental chemicals par excellence. These chemicals provided the impetus for critical thinking about chemistry.

Does what was said then still apply today?

DDT is still used for malaria control in developing countries. What do you think about it?

Weigh up the risks and different interests!

Which chemicals are in focus in the current discussion?

Chlorine Chemicals

Generally, chlorine chemicals are considered the "devilish" chemicals. What does the chemist say?

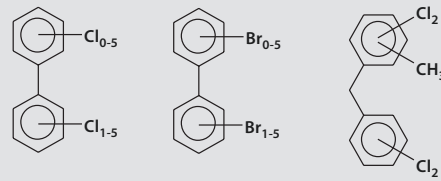
Chlorine substituents are an important element in synthetic chemistry.

A distinction must be made between unconscious, accidental release, as can occur with intermediates in synthetic chemistry, and deliberate, intentional release of chemicals, as with herbicides and insecticides. In the case of the latter, rapid degradation is required. The unintentional release of chemicals must be avoided.

The criteria listed apply to all chemicals. Chemicals with chlorine substituents are therefore not a critical exception.

The Call for Alternatives

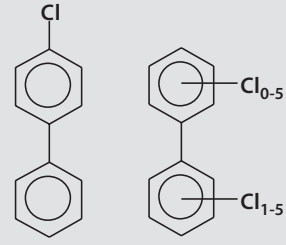
Are there safe alternatives to known environmental chemicals? Has ignorance been exploited as in the replacement of PCBs by PBBs? Is Ugilec a useful substitute for PCBs?



PCBs PBBs (FireMaster) Ugilec141

Misunderstanding

Is it true that microbial PCB degradation has been misunderstood as PCB degradation? The pCB is *para*-chlorobiphenyl and not, as one might think, the environmentally relevant polychlorinated biphenyls, the mixture of up to 70 congeners.



*p*CB PCBs

Superbugs

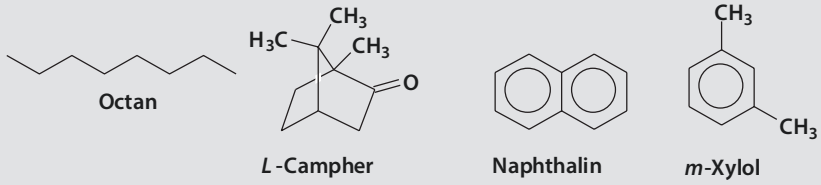
In 1981, a patent was granted in the USA for a "superbug" that would eat oil. This was a strain of *Pseudomonas* in which plasmids encoding the degradation of octane, camphor, naphthalene and xylene had been collected.

It was claimed that this superbug could "pounce with ravenous hunger" on toxic petroleum residues, but was never used in the environment because the release of genetically modified bacteria was not allowed.

Crude oil consists of a cocktail of hundreds of individual substances.

Can the organism cope with the given task with its degradation potential? Does the restrictive argument regarding genetic engineering apply if the degradation properties have entered the host naturally by means of conjugation?

Recently, the marine bacterium *Alcanivorax borkumnensis* was presented to the public as a petroleum eater. Does a similar problem with the mixture of compounds also apply here, if its degradation potential is limited to alkanes?



Renewable Raw Materials: Climate-Neutral?

Renewable raw materials are CO_2-neutral. Are they therefore also climate-neutral? In order to produce a sufficient amount of biomass, fertilizer must be used.

The denitrification that begins with waterlogging when fertilizer is applied can lead to N_2O formation. N_2O is a greenhouse gas about 300 times more potent than CO_2. Is there a complete life cycle assessment that presents an objective evaluation of the benefits of renewable raw materials, including the N_2O problem?

Renewable Raw Materials: Tank or Plate?

In Germany, renewable raw materials were cultivated on an estimated area of approx. 2.3 million ha in 2011. Considering the total agricultural area in Germany of approx. 17 million ha, this is a considerable part that is no longer available for food or feed production.

Is the arable land required for the production of biomass for energy production sufficient in Germany?

Bio-fuel

The different aspects of biotechnology with regard to environmental relevance are obvious for the example of bioethanol as a fuel: In Brazil, it was shown that environmental pollution through wastewater and soil erosion can be possible consequences of production. The organic pollution load of the wastewater per liter of ethanol produced was equivalent to about 4 population equivalents.

If economic aspects are brought into the discussion, a market study from 2006 shows the following: Taking the German mineral oil tax of 65.4 EUR cents per liter of gasoline and 47 EUR cents per

liter of diesel as well as a price of US$ 60 per barrel of crude oil for gasoline and diesel production, the production of biodiesel or bioethanol from wheat is not profitable in Europe. At present, BTL fuel cannot be produced competitively either.

At the current oil price, only large-scale bioethanol and biobutanol production from lignocellulosic feedstock has the potential to be competitive. In the medium term, biobutanol produced from straw can provide a cost-efficient biofuel in Europe, with a reasonable profit margin even without tax exemption.

From 1 January 2007, binding biofuel quotas will apply in Germany for petrol and diesel. Every litre of petrol and diesel must then contain a minimum proportion of biofuel, initially 1.2% for petrol. The total proportion of biofuel in petrol and diesel is to rise to 8% by 2015.

Prevention or Elimination of Hazards from Chemicals by Means of Microorganisms

The short-term goal is to avert danger in the wastewater sector through sewage treatment plants. This prevents the eutrophication of water bodies. This prevents chemicals from contaminating the ecosystem of water bodies. The cleaning of soils contaminated by oil spills is another established method, which, however, is rather medium-term with a time requirement of months.

If there is no immediate danger from contamination, one can limit oneself to observation. The goal with old contamination is to restore the natural state. Questions need to be answered as to why Natural Attenuation has not occurred to date. What was missing for the microbial population to function in the groundwater, in the soil, so that the contamination still exists?

Does it always have to be total dismantling to eliminate problems caused by environmental chemicals?

Persistence and Bioavailability

Why does a substance show persistence?

Attention to the physico-chemical properties of the compounds causing contamination should be placed at the beginning of the assessment of microbial processes. Thus, it is often already possible to answer the question whether the inability of the microorganisms is the sole cause of lack of degradation. In the case of PAHs, poor bioavailability is the cause of the chemicals remaining at the site.

With the limited bioavailability of PAHs in soil contamination, the question is whether the toxicity potential for humans, the tendency to harm from such sources is present. Is a reduction of the amounts in groundwater/soil by microbiological systems possible or even necessary?

It is often physico-chemical properties, rather than structural features (as originally assumed), that are responsible for the long persistence of a chemical in the environment.

Thoughts on Degradability

It is often the circumstances, such as the absence of nitrogen, phosphate or oxygen, that are responsible for the fate of a chemical in the environment. The important question is therefore: where do the chemicals migrate to, where do they stay, do they leave the ecosystems that are actually favourable for degradation?

It is important to note that chemical groups can affect degradability differently under aerobic and anaerobic conditions.

Environmental chemicals can get into the wrong, anaerobic environmental areas, so that the aerobic degradation, which is possible in principle, cannot take place.

What do you think of the frequently expressed suggestion of using nitrate as an "oxygen substitute" in *in situ* soil remediation?

We generally lack data on the degradation of environmental chemicals in anoxic environments, as well as the test systems necessary to determine it.

An important observation is that degradability is not an absolute! Limits are defined/fixed by committees. Thus, a statement about degradation is determined in a short time horizon.

The question is: Are the effects that negatively affect ecosystems to be expected during the fate of a chemical? Is the substance present in the environment in this way and are the concentrations high enough for such effects to be feared?

What does the knowledge that "there are organisms that have been isolated from environmental media in the laboratory" say about degradability in the environment? It only says: There are microbial catalysts that could be used at higher concentrations, for example for bioaugmentation in soil remediation or in a sewage treatment plant.

Methane: A Greenhouse Gas That Can Be Influenced?

Is it possible to change or control the influence of microbial systems on global processes?

If more swamps are created in the world, for example due to the thawing of permafrost areas, there will be an increased release of methane that cannot be influenced. The increase in the area of rice paddies as well as cattle herds, on the other hand, is within human influence. Recently, the possibility that plants also emit methane has been reported.

If this is true, is there any possibility to influence the methane-induced greenhouse effect?

With Regard to the Release of Pollutants, Much Has Changed for the Better

The Chemicals Act has required degradability tests for years.

A number of persistent chemicals have been phased out.

The unintentional release of chemicals in factories or at petrol stations through leaks, spills or "disposal" has been drastically reduced.

A good indicator of environmental exposure to chemicals is breast milk. It is noted that "not only the exposure to pesticides, but also to persistent substances such as PCBs is declining: today's mothers and fathers are considerably less exposed to them than their parents' generation".

Does this mean that almost everything is in order with the measures listed? Can the rest be regulated with product- or production-integrated environmental protection?

Is the required effect (duration of effect) of products always compatible with good degradability?

Is degradability an important criterion for drugs?

Processes as a Black Box

Is environmental microbiology still needed in times of tight budgets? Is it not possible without a "more thorough understanding" of the processes?

Haven't engineers been treating sewage properly for 100 years without know-

ing exactly what microorganisms are in the treatment plant?

Doesn't "Natural Attenuation" do the "rest" in eliminating contamination? Can't environmental scientists just make their models based on sum parameters?

Researchers Give Clear Rejection to Bioenergy

Biofuel has once again come under criticism from scientists: Environmental groups have commented on a study by the London-based Institute for European Environmental Policy (IEEP). Biofuel is "more harmful to the climate than the fossil fuels it is supposed to replace". According to the study, the increasing use of biofuel in Europe will lead to a rise in climate-damaging CO_2 emissions because huge areas of land worldwide would have to be converted into additional arable land for the production of agrofuels. The IEEP researchers examined the official plans of 23 EU member states for the expansion of renewable energies by 2020. Germany would then add 5.5 million tonnes of biofuel per year to petrol and diesel, making it the front-runner in terms of consumption, ahead of the UK, France and Spain.

In total, 9.5% of the energy used for transport in Europe in 2020 should come from biofuel, which would be produced almost entirely from oilseed, palm oil, cane and beet sugar and wheat. According to the study, up to 69,000 square kilometres of forests, pastures and wetlands worldwide would have to be cultivated as arable land—an area more than twice the size of Belgium.

As a result, up to 56 million tonnes of CO_2 would be released annually. This would correspond to an additional 12–26 million cars on Europe's roads.

The Rise in Temperature Has Paused for 15 years

The climate has recently developed differently than predicted: warming has been stalling for 15 years, and the upward trend in average global temperatures has not continued since 1998.

It has even been recently predicted that despite the rapid increase in greenhouse gas emissions, the pause in temperatures could continue at a high level until the end of 2017.

Until now, scientists thought 14 years without further warming was consistent with their projections—but not "15 years or more."

Several possible causes of the temperature standstill are discussed:

- **Oceans swallow more heat than thought.**
- **Dry stratosphere:** Since the year 2000, the stratosphere has become significantly drier. Water at altitude warms the ground indirectly, as water droplets cause heat to re-radiate. It is calculated that the dryness has caused the temperature near the ground to rise a quarter slower.
- **Exhaust gases in Asia:** Sulfur-containing exhaust gases from the emerging industrial countries of China and India act as a sunshade. The sulfur bell slows warming by a tenth of a watt per square metre. This would reduce the CO_2-driven warming by one third.
- **Cold-water tide in the Pacific:** Years with La Niña are responsible for the largest downward swings in global temperature. La Niña is a weather event that usually occurs every few years following an El Niño event. During this event, a tide of cool water rises to the surface in the Pacific Ocean. Freshening trade winds drive the cold deep water rising off the

coast of Peru, whose temperature is up to 3 °C below average, up to the surface from the east to Southeast Asia. Three La Niñas have risen since 1998 and have undoubtedly slowed the warming.

The National Academy of Sciences Leopoldina Presents Critical Statement on the Use of Bioenergy

In a statement on the limits and possibilities of the use of bioenergy, the Leopoldina working group "Bioenergy" concludes that bioenergy cannot make a quantitatively important contribution to the energy turnaround as a sustainable energy source for Germany today or in the future.

They criticise that the cultivation of oil plants in Germany consumes considerably more land than other renewable energy sources. They also point out that the production of biofuels produces gases that are harmful to the climate and that the cultivation of the plants promotes nutrient pollution of the soil and water.

The Leopoldina researchers believe that plants for bioenergy should only be cultivated in this country if food and bioenergy production can be combined.

Compared to other renewable energy resources such as photovoltaic, solar thermal and wind energy, bioenergy consumes more land and is often associated with higher greenhouse gas emissions and environmental impacts. In addition, bioenergy potentially competes with food production. Priority should be given to saving energy and improving energy efficiency.

The opinion explains under which conditions the use of bioenergy can make limited sense. It addresses which conversion options from biomass to biofuels, such as bioethanol and biodiesel, exist and are under development. It also highlights research avenues aimed at using solar energy to produce hydrogen from water in an environmentally friendly and sustainable way.

The Bioeconomy Council, which advises the German government, recently stated that 23% of the energy consumed in Germany could be covered by bioenergy in 2050, but primarily through imports. The European Union also aims to provide 10% of fuel for transport purposes from renewable energy sources by 2020.

ISAAA Report 2012: The Cultivation of Genetically Modified Crops Is Growing Everywhere, Including in Europe

Due to a genetic modification, the newly developed potato variety Amflora® produces a starch that consists entirely of amylopectin. This enables an optimized material use for the production of paper, textiles or adhesives. In the case of conventional starch without genetic engineering, a complex removal of the second starch polymer, amylose, is required.

The advantages of the Amflora potato lie in the additional value created for farmers and the starch industry. The use of Amflora—for example in the production of paper—is **expected to save energy, water and raw materials**.

Question: What reasons were given for withdrawing permission to grow these plants in Europe? (◘ Table 19.1).

An Act of Creation: Artificial Life—Life from a Test Tube?

One of the goals of synthetic biology is to create synthetic cells whose genome sequence is designed on the computer,

Table 19.1 Countries growing genetically modified crops in 2011

Rank	Country	Area in million hectares	Cultivated plants
1	USA	69.0	Maize, soya, cotton, rape, pumpkin, papaya, alfalfa, sugar beet
2	Brazil	30.3	Corn, soy, cotton
3	Argentina	23.7	Corn, soy, cotton
4	India	10.6	Cotton
5	Canada	10.4	Maize, soya, rape, sugar beet
6	China	3.9	Cotton, poplars, tomatoes, petunias, peppers, papaya
7	Paraguay	2.8	Soy
8	Pakistan	2.6	Cotton
9	South Africa	2.3	Corn, soy, cotton
10	Uruguay	1.3	Corn, soy
29	Germany	<0.1 (2 ha)	Potato

Clive James, ISAAA Report (2012)

recreated by chemical synthesis, and brought to life in a cellular environment.

In the laboratories of the Venter Institute in Rockville and San Diego, USA, scientists created a microorganism that is completely controlled by artificially synthesized genetic material. The research groups reported the chemical synthesis of a 1.08 megabase pair *Mycoplasma mycoides* genome. The chemically synthesized genome was transplanted into a *Mycoplasma capricolum* cell, where it induced the formation of a new *Mycoplasma mycoides cell.* In the new *M. mycoides cell,* like a prosthesis, only the synthesized genome is present, which contains recognition sequences and minor intentional and non-intentional deviations from the original genome sequence. The new cells are self-replicating and show the expected phenotypic properties (briefly described and commented by Pühler, 2010).

In a gimmicky manner, practically all major international media picked up on the publication by Craig Venter and his team. "Artificially programmed cells", Venter declared, "could make the principles of life clear and, **quite incidentally**, free the world from energy problems, produce medicines or **clean polluted soil**" (from DIE ZEIT 27.05.2010).

Why are the following problems encountered in a soil cleanup eliminated by the use of artificial DNA? (1) Chemicals occur in the organic soil matrix and not in the water phase due to physicochemical properties. Thus, they are removed from microbial access. (2) Environmental conditions run counter to microbial degradation, such as the absence of oxygen, which is essential for a reaction.

Does Venter, do his computer scientists know anything about the chemistry of degradation of chemicals? Does Venter know anything about the living conditions of special microorganisms without which the degradation cannot exist?

Environmental Awareness

No sooner had the UN agreed at the Earth Summit in Rio in 1992 on the claim to operate within the limits of growth and with consideration for future generations than the term sustainability was watered down, bent, shortened or misused for **greenwashing**. The term greenwashing encompasses the totality of all advertising-relevant measures within the framework of a marketing campaign or the public relations work of a company, which is intended to suggest to consumers and shareholders that an ecological philosophy is taken into account, promoted and perceived in the daily work.

"If you can't think of anything to say about global crises, talk about sustainability," Klaus Töpfer, the long-serving head of the UN Environment Organisation, once said with bitter derision.

Bacterial Microbiome of Kitchen Sponges

Kitchen sponges are predominantly made of foam, such as polyurethane. Their huge inner surface due to numerous pores offers microorganisms plenty of room to grow.

Studies conducted to date on kitchen sponges have largely used cultivation-dependent approaches, often focusing on specific microbial targets. Often, growth on selective media has not been further confirmed, for example by 16S rRNA gene sequencing.

The bacterial microbiome of used domestic kitchen sponges was extensively analyzed by high-speed 16S rRNA gene sequencing to decipher the actual taxonomic assemblage and diversity, and to assess the influence of selected intrinsic (sponge-specific) and extrinsic (sponge-use-specific) factors on microbiome structure. The pathogenic potential of the sponge microbiota should be estimated. The spatial distribution pattern of bacteria in kitchen sponge tissue should be investigated by 3D microscopy using fluorescence in situ hybridization in combination with confocal laser scanning microscopy (FISH-CLSM) to complement and validate the sequence data.

The work shows that kitchen sponges have higher bacterial diversity than previously thought, based on cultivation-based studies, and that human pathogens represent only a minority of their microbiome.

362 different species of bacteria were discovered. Five of the ten most frequently found species belong to the so-called risk group 2, which means they are potentially pathogenic. These are environmental and aquatic bacteria, but also bacteria that are typical of human skin. Faecal bacteria and food poisoners or diarrhoeal pathogens, on the other hand, were hardly detected.

In some cases, the bacteria reached densities of more than 5×10^{10} cells per cm^3. These are concentrations that are otherwise only found in faecal samples. The high densities can be explained by the optimal living conditions that bacteria find in the sponge: in addition to the large surface area for growth, plenty of moisture and many nutrients, something from food residues and dirt.

Of particular concern was that sponges which, according to their users, had been cleaned regularly, for example in the microwave or by washing out, showed significantly higher levels of the potentially pathogenic bacteria, presumably due to a higher stress tolerance.

Plastic in the Sea

Synthetic organic polymers did not come into widespread use until the 1950s. By 2015, global production had risen to 322 million tonnes (Mt) per year.

About half is used for packaging and other disposable items. 40% of plastic waste does not end up in managed landfills or recycling facilities. 4.8–12.7 Mt per year ends up as macroscopic waste and microplastic particles in the ocean, where the plastic is very difficult to remove. It accumulates in organisms and sediments and remains there much longer than on land.

More than 140 Mt of plastic waste is now drifting through the oceans in five giant whirlpools. Much of the plastic waste in the oceans comes from rivers. By far the most plastic waste has been flushed into the oceans by the Yangtze—China's longest river—with an estimated 333,000 tonnes. The Ganges follows in second place with about 115,000 t. According to estimates, 86% of the plastic that enters the oceans via rivers comes from Asia—primarily from China. By comparison, rivers in Africa contributed just under 8% to the pollution of the world's oceans with plastic, Europeans 0.28% (Lebreton et al., 2017).

But plastic waste is also part of the everyday life of the rinse line on almost all beaches in the world. Even worse than old water bottles or bags are the things you don't see. A beautiful sandy beach nowadays is unfortunately no longer just sand. It's riddled with tiny little bits of plastic. Where does the **microplastic** come from that pollutes the food chain in the sea?

According to a study by Browne et al. (2011), a large proportion of microplastic fibres could enter the sea through the washing of textiles and thus via wastewater.

Problems from microplastics may also be of concern to freshwater environments. Microplastics are released from a variety of sources, including emissions from wastewater treatment plants and from the degradation of larger plastic wastes. Due to chemical composition, freshwater ecosystems are exposed to a mixture of micro- and nano-sized particles, leached additives, and subsequent degradation products (Lambert & Wagner, 2018).

Hot Time

The earth will become four to five degrees Celcius warmer than before the beginning of industrialization—with such a scenario, scientists from the Potsdam Institute for Climate Impact Research warn of a "hot age" on earth. Currently, the planet is already a good one degree warmer on average than in pre-industrial times. Even if the Paris climate agreement is adhered to, decisive tipping processes could be triggered in the global environmental system. Feedback processes would become self-perpetuating as a critical point is crossed. Factors driving this development are reported to include thawing permafrost in Russia, Canada and northern Europe, ice melt in Antarctica and rainforest dieback. All of these processes are interconnected, they say, and the collapse of one can trigger that of another.

Mosquito Control with *Bacillus thuringiensis* Really Environmentally Friendly?

Mosquitoes are controlled in wetlands all over the world, including those that are protected and considered important for Europe, such as those on the Upper

Rhine. As an alternative to other insecticides, the biological biocide *Bacillus thuringiensis israelensis* (Bti) has been applied several times a year for decades. It is considered to be an environmentally friendly control method because it has no lethal effects on organisms other than mosquitoes. However, Bti also has effects on the closely related twitch mosquitoes, also known as dancing mosquitoes, which occur in high species numbers and densities in wetlands. Chironomid mosquitoes are a key food source in wetland food webs due to their large biomass and high protein content. Suckermidge larvae are eaten by other insects and fish in the water, while the hatched midges are used as food by birds, bats or dragonflies.

The sensitivity of the chironomid *Chironomus riparius* to Bti was investigated, following the complete life cycle with all four larval stages.

Larvae from the first larval stage are up to 100 times more sensitive than the older fourth larval stages. Their sensitivity was lower by a factor of 200 than the amount regularly applied for mosquito control in the Upper Rhine. A risk factor applied in the approval is thus exceeded by a factor of 2000 (Kästel et al., 2017).

Microorganisms and Climate Change. An Explanation

To understand how humans and other life forms on Earth can withstand anthropogenic climate change, it is important to consider the knowledge of the microbial "invisible majority". It is necessary to learn not only how microorganisms affect climate change (including greenhouse gas production and consumption), but also how they are affected by climate change and other human activities. The central role and global importance of microorganisms in the biology of climate change is documented. Attention is drawn to the fact that the impacts of climate change will depend to a large extent on the responses of microorganisms, which are essential for an environmentally sustainable future (Cavicchioli et al., 2019).

Role of Microbial Interactions with Microplastics in Marine Ecosystems

A review study of biofilms on microplastics can be summarized as follows (Oberbeckmann & Labrenz, 2019):

1. The frequently postulated assumption that pathogenic bacteria accumulate specifically on microplastics and are thus spread particularly rapidly and widely could not be substantiated. The majority of microorganisms growing on microplastics appear to belong to opportunistic colonists that do not differentiate between natural and artificial surfaces. Microplastics therefore do not pose a higher risk than natural particles of being colonized by pathogenic bacteria.
2. Bacteria colonize the particles, but they are not capable of decomposing the plastic that enters the marine environment. Nor are they likely to acquire this ability in a period of time relevant to human society.

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