Benthic Diatom Monitoring and Assessment of Freshwater Environments: Standard Methods and Future Challenges

6

Soizic Morin, Nora Gómez, Elisabet Tornés, Magdalena Licursi and Juliette Rosebery

Abstract

Since biofilms integrate the environmental effects of water chemistry, along with the physical and geomorphological characteristics of rivers and lakes, they have been widely applied in biomonitoring. In particular, diatoms are extensively used as reliable environmental indicators. Diatoms are microscopic, unicellular brown algae, which often dominate the algal biomass of biofilms. The shape and morphology of the siliceous skeleton, the frustules, unique to each taxon are used for taxonomical identification. The floras are diverse, in relation to their geographical location (climate, geology, relief) and to the quality of the aquatic environments they inhabit. Indeed, species are sensitive to the water physicochemical parameters and their presence/abundance is therefore correlated to water quality.

Diatom sensitivity or tolerance towards different environmental parameters has long been studied and used to implement bioassessment methods. Such methods evolved from indices of saprobity designed first for European streams, to developments of various diatom indicators worldwide, able to highlight different types of pollution (pH, salinity, nutrients, toxicants).

The objective of this chapter is to provide scientists and water managers with a broad overview of diatom tools helpful to monitor the ecological status of freshwater environments. We describe the applicability range and the limitations of the main existing methods, metrics (indices, traits) and types of surveys used, as well as the challenges faced by scientists to improve routine biomonitoring.

Introduction

Various biological metrics have been developed to assess the health of freshwater ecosystems. They reflect integrative effects of present and past conditions, whereas traditional chemical and physical measurements only apply to the current situation. Several studies have shown that community-based indicators are needed to evaluate water quality. Diatom biomonitoring has benefited, over other existing periphytic bioindicators, from a long research history. Indeed, typical water quality assessment in flowing watercourses is mainly based on benthic diatoms, i.e. diatoms growing on diverse natural (pebbles, macrophytes) or artificial (glass slides, ceramic tiles) substrates immerged in the water. However, biofilms are composed of diverse components other than diatoms. Although the planktonic microflora is used widely as biological quality element (especially in lacustrine environments), benthic green algae and cyanobacteria are poorly known and no consensual bioindicator exists.

Diatoms are a siliceous class of unicellular algae known to be very diverse (Guiry, 2012) and sensitive to chemical conditions. They are excellent bioindicators due to their short generation time and their varying ecological preferences. Moreover, the structural elements in their siliceous cell walls allow reliable taxonomic

determination at specific and subspecific levels. They are distributed throughout the world in nearly all types of aquatic systems and usually account for the highest number of species among the primary producers in aquatic systems. They respond to environmental disturbances not only at the community level through changes in diversity, but also by shifts in dominant taxa. Over the last decades, a great number of diatom-based methods (reviewed in Prygiel *et al.*, 1999) have been proposed to assess water quality. No one single metric is applied worldwide, but most of the diatom indices used are minor adjustments of a common approach based on the knowledge of the ecological spectrum of species, combining sensitivity to pollution and indicator value.

This chapter aims to review the main characteristics of diatoms which have been used to develop bioassessment methods in freshwater environments. Various indicators are described, with respect to the types of pollution that can be addressed, and to the kinds of approach implemented. The pros and cons of existing systems, as well as future challenges in biomonitoring are presented.

Potential of diatoms for water quality assessment

Diatoms (Bacillariophyceae) are particularly interesting as indicators of water quality (Table 6.1). These unicellular brown microalgae (size ranging from < 10 to $> 500\,\mu m$) occur in all aquatic environments and are found at almost all levels of pollution. Diatoms can account for up to 80% of the taxa present in streams, rivers, lakes and wetlands (McIntire *et al.*, 1996). They are used worldwide as biological markers to assess water quality: in Europe to support the implementation of the Water Framework Directive (Kelly *et al.*, 1998), but also in routine monitoring surveys in Canada, USA, Japan, Australia, South America, etc. (e.g. Lobo *et al.*, 2004; Gómez and Licursi, 2001; UNESCO-WHO-UNEP, 1996).

Diatoms occupy a diverse range of habitats: they can be planktonic (freely living in the water column) or fixed on diverse substrata (periphytic on hard surfaces, epipsammic on sand, epiphytic on macrophytes, see Chapter 1). Periphytic diatoms are relatively sedentary, and live their whole life cycle in the water implying that their community structure is tightly associated with local environmental conditions at the sample site. Thus, diatoms are generally collected from hard surfaces immerged (natural or introduced, like glass slides or ceramic tiles; e.g. Sekar *et al.*, 2004). Moreover, their sampling requires minimal effort (scraping or pipetting the surface, using corers), causes minimal disturbance to the sampling sites and it is possible at sites where other bioindicators, e.g. benthic invertebrates and fish are absent (Lear *et al.*, 2012).

Besides being easy to sample, diatoms have the great advantage that their frustules provide detailed features enabling reliable identification at species and subspecies levels. These siliceous walls are easily preserved and cleaned of organic material (with hydrogen peroxide, hydrochloric acid; AFNOR, 2003), to be permanently mounted on microscope slides in refringent resin for identification under light microscopy at high magnification (x1000), with oil immersion.

On the other hand, microscopic determination of diatom taxa requires precise identification than can only be reached by trained operators. Among the main diatom features to be taken into account when doing microscopic identification are: the general shape of the valve (the kind of symmetry), cell dimensions, length–to-breadth ratio, and ornamentations (presence or absence of one or two raphes, i.e. longitudinal slits on the valve; orientation of the striae; presence of specific features; stria density).

Diatom community structure reflects a gradient of general pollution in water quality (although causes are not always identifiable) integrated over time on a relatively short timescale, in relation to high sensitivity of individual species towards different levels of pollutions (Lowe and Pan, 1996; McCormick and Cairns, 1994; Stevenson and Pan, 1999; Whitton and Rott, 1996). In order to determine species sensitivity, the methodology most frequently used is a direct comparison between the physicochemical water quality and the species composition of the corresponding records in relative abundances of the taxa using appropriate multivariate analyses. Results are generally presented as species optima towards

Table 6.1 Advantages and drawbacks of diatoms as bioindicators of water quality

Advantages	Drawbacks
Sampling	
Biofilm presence generally visible to the naked eye on the substrata	
Quick and easy collection (scraping, pipetting, using corers for soft sediments and sand)	
Possible use of artificial substrates	Risks of vandalism or loss (e.g. floods) of artificial substrates
Taxon identification	
Numerous identification resources (books, articles, web)	Difficult and ever-changing systematics
	Time-consuming counting/identification of samples
	High quality microscope necessary
Bioassessment power	
Widespread distribution, even in hostile environments	Geographically specific distributions (endemism)
High species diversity (about 10 000 species known)	Heterogeneous biomass distribution (e.g. light dependent)
Sensitive to numerous kinds of pollution	Poorly sensitive to habitat disturbances
Conservation of the frustules (paleolimnological applications) and integrative power variable depending on the species	Integrative power lower than for higher organisms
Index calculation	
Dedicated software (e.g. Omnidia)	Applicability of indices generated for other geographic regions

different environmental parameters (Charles *et al.*, 2006; Ponader *et al.*, 2008), or species ecological profiles expressed as species probability of presence along ecological gradients (Potapova *et al.*, 2004).

Once the polluo-sensitivity or resistance of species to defined classes of quality are hierarchized (e.g. Van Dam *et al.*, 1994), species can be grouped and used as indicators for saprobic conditions (Sládeček, 1986), salinity (Ziemann, 1991), acidification (Birks *et al.*, 1990) and eutrophication in lakes and rivers (Steinberg and Schiefele, 1988; Hofmann, 1994). The relative abundances of the different species identified are reported and used, e.g. for calculation of water quality indices. Depending on the methods, a minimum of 400 individuals identified is required for reliable assessment (AFNOR 2000). Diatomists have created practical tools such as the software Ominida (Lecointe *et al.*, 1993). This software allows to efficiently compute diatom inventories from research and monitoring programmes, manage data and calculate indices.

Towards a harmonized way of using diatoms for biomonitoring

The need to monitor water quality led to the development of standardized sampling protocols and assessment methods, through single, simplified indices. Such indices were created by adapting the formula of Zelinka and Marvan (1961), basically combining the abundances of species and their individual ecological preferences, into a single score of water quality. Among them, DAipo (Diatom Assemblage Index to organic pollution; Watanabe *et al.*, 1986), IBD (Indice Biologique Diatomées, Coste *et al.*, 2009), IDEC (Indice Diatomées de l'Est du Canada; Lavoie *et al.*, 2006), IPS (Indice de Polluosensibilité Spécifique; Coste *in* Cemagref,

1982), TDI (Trophic Diatom Index; Kelly and Whitton, 1995), and IDP (Pampean Diatom Index; Gómez and Licursi, 2001) are successfully used in many monitoring programmes.

Recently, limitations in these approaches have been identified, linked to the importance of reliable autoecological taxon information and to the potential general application of indices developed in a particular geographical area (e.g. application of European indices to USA; Potapova and Charles, 2007). To overcome critical limitations concerning pertinent biomonitoring irrespective of regional differences in taxa, climate and other local constraints, approaches such as the use of a common metric for the different European countries (intercalibration exercises, Kelly *et al.*, 2009; Almeida *et al.*, 2014) have been developed.

Diatom assemblages respond rapidly (weeks) and sensitively to environmental change and provide highly informative assessment of the biotic integrity of aquatic ecosystems. To limit the biases linked to regional distribution of species, different characteristics can also be used in the monitoring such as taxa richness, diversity, biomass, autoecology of individual species, biotic indices, percentages of aberrant diatoms, percentages of motile diatoms, mortality, among others descriptors (Stevenson and Bahls, 1999). Other approaches are complementarily employed, based on non-taxonomical indicators, assuming that a given pressure selects for certain characteristics, whatever the location/site studied. The diagnosis therefore relies on the proportion of ecological characteristics derived from species distributions and on their classification in terms of ecological preferences (as described previously) or 'traits' (e.g. postures, growth forms, motility, or the ecological guilds defined by Passy, 2007).

Indices have been developed mainly to assess organic pollution, eutrophication or pH. But they fail when there is low nutrient enrichment or several superimposed anthropogenic influences. Therefore, attempts are made to develop diatom descriptors more specific to toxic pollution (see Chapter 8). They generally require a combination of structural and morphological descriptors (e.g. cell sizes, deformities).

Implementation of diatom-based assessment in rivers and streams: main approaches, advantages and limitations

Assessment of lotic systems has received more attention than lakes (King *et al.*, 2005), although the dynamics of phytoplankton as well as paleoecological reconstructions have been extensively investigated in these systems. However, lacustrine biomonitoring of contemporary water quality is increasingly applied, and is based on diatoms sampled in the littoral zone and with methods similar to those used in rivers and streams.

Diatom monitoring is mainly used for regulatory purposes, with very large scale programmes (from basin to country) and allows mapping water quality using simple biological indices. This approach gives information about the current state of rivers, and can also be used to monitor recovery processes following rehabilitation programmes. More specific approaches to determine the role of certain pressures in modifying the community *in situ* can also be performed, such as before/after impact studies (that can be studied in space or time), or the use of translocation experiments. Applied examples of these three complementary approaches are provided below.

Large scale monitoring programmes and data integration

In the last decades, numerous countries from the northern hemisphere have included diatoms in their biomonitoring programmes, while in the southern hemisphere their use is less frequent (Rimet, 2012).

Communicating the conditions of biological systems, and the impact of human activities on aquatic ecosystems, is the ultimate purpose of biological monitoring. As an example, the biomonitoring programme of the Matanza-Riachuelo River, a highly polluted Argentinean hydrographic system (Fig. 6.1) is presented. This basin has a surface area of 2240 km^2, and is inhabited by more than five million people. The deterioration of the water quality of the main watercourse and of most of its tributaries highlights a strong polluting load from household and industrial sewage waters. The urban pollution widely exceeds the diluting and self-depuration capacity of the river, as well

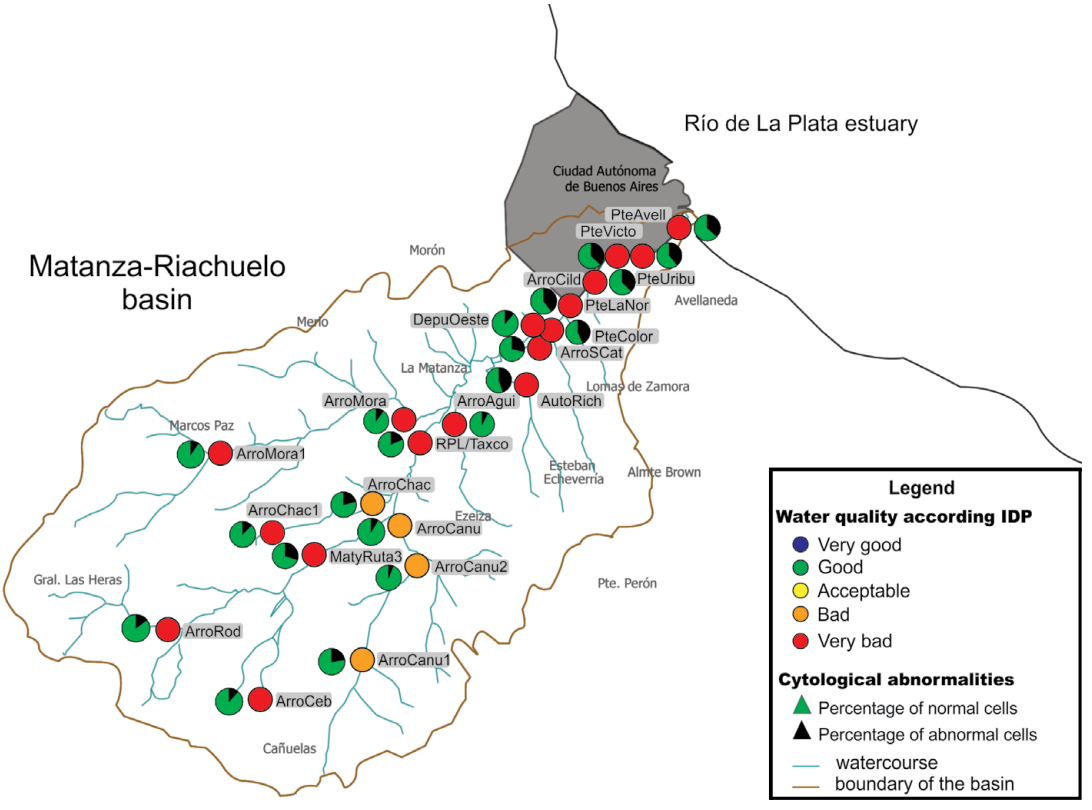


Figure 6.1 Water quality map corresponding to the 2010 monitoring results of Matanza-Riachuelo basin employing the Pampean Diatom Index (IDP) and percentage of cytologic abnormalities.

as toxic contaminants, chromium, copper, lead and cadmium concentrations above the guideline levels of water quality for protection of aquatic life. In this case, a regional index, the Pampean Diatom Index (IDP) (Gómez and Licursi, 2001), is employed to describe the different environmental conditions related to the eutrophication and the enrichment with organic matter through five water qualities codified with different colours (Fig. 6.1). Biomonitoring results expressed on maps constitute a useful tool for stakeholders as they provide quick visualization, through the use of different graphic codes and colours, of the evolution of the water quality in hydrographic basins through time and space. Likewise, the evaluation of cytological abnormalities (percentage of aberrant frustules and cytoplasmic content impaired) also contributes to the detection of changes of the water quality mainly associated to toxic pollution (Figs. 6.1 and 6.2). So, in the lower basin, strong symptoms of eutrophication and high amounts of organic matter and heavy metals can be diagnosed through high values of IDP, but also high percentages of cytological abnormalities.

Before/after impact studies (in space or time)

Diatoms have been used in experimental designs for comparing time series or differences in a treated area (or impacted) and control area, before or after the intervention or experimental treatment. The before/after impact assessment is a very suitable methodology for assessing whether or not a stress has changed the environment, to determine which components are adversely affected, and to estimate the magnitude of the effects. The simplest approach, referred to as the before–after design (BA), considers the time scale and involves collection of data prior to the beginning of activity and compares it with data recorded after the start of the activity (Smith, 2002). On the other hand the before-after-control-impact-paired

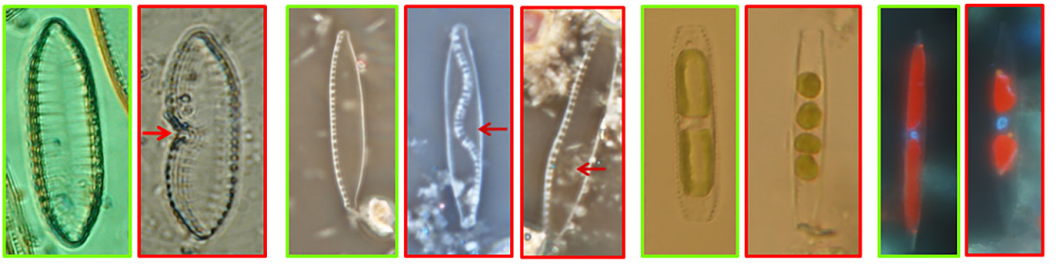


Figure 6.2 Normal cells (framed in green) and specimens with a deformed frustule or modified ornamentation (*Surirella angusta* and *Nitzschia palea*) and impaired cytoplasmic content (*Nitzschia* sp.) (framed in red, the arrows indicate the alterations) recorded in the Matanza-Riachuelo basin.

(BACIP) approach is a proper method, involving as well space scale, to evaluate the effects of the stressor being assessed; in this approach the 'impact area' is paired to another area referred to as 'control area' (Stewart-Oaten, 1996). Paired samples are collected a number of times, both before and after the perturbation, simultaneously (or nearly so) at both a 'Control' and 'Impact' location. The standard analytical approach, using the resulting BACIPS data, is to calculate the difference between 'Control' and 'Impact' values on each date (termed delta), and test whether the mean of these deltas changes from before to after the perturbation (Bence *et al.*, 1996). This type of approach allows the elucidation of potential effects that may occur in aquatic environments as a result of a stressor.

A study conducted in a Pampean watercourse (La Choza stream) is an example of application of this methodology in assessing the impact of a stressor (Artigas *et al.*, 2013). The diatom assemblages inhabiting the epipelic biofilm of this stream were exposed to a continuous surplus of inorganic nutrients; increasing concentrations of nitrogen and phosphorus in water 3-fold the basal concentration. Nutrient enrichment was achieved by the use of fertilizer bags distributed along the reach; the period of exposure was of 14 months. The changes in nutrient concentration were associated with a significant increase (BACIPS, $P < 0.001$) in diatom density and a decrease in species richness and diversity (Fig. 6.3). Changes in the relative proportions of the diatom taxa were also observed; while some taxa showed moderate to high variations (*Nitzschia palea*, *N. frustulum*, *Melosira varians*, *N. supralitorea* and *Caloneis bacillum*) others showed minor changes. Furthermore nutrient enrichment favoured shifting the proportion of stalked or filamentous to more motile growth forms (Fig. 6.4). The fertilization in La Choza caused a mild to moderate effect, not immediately felt, on the diatom assemblages. Taking into account the increase in urbanization and agricultural activity in the Pampean plain, it is likely that biodiversity can be seriously impaired if the entrance of nutrients to these ecosystems is not mitigated. A similar study carried out in an oligotrophic Mediterranean forest stream (Veraart *et al.*, 2008) reported that long-term nutrient addition has significant effects on the algal biomass and community composition, and this was detectable despite the low light availability resulting from the dense tree canopy. Results obtained from field experiments are extremely valuable, illustrating the expected effects of different pollution scenarios.

Active biomonitoring: use of translocation experiments

Over the last decades, artificial substrates (e.g. glass slides or plastic sheets for periphyton, plastic trays for epipelon) have been increasingly used for collecting diatom communities; consequently promoting the development of translocation experiments (Ivorra *et al.*, 1999; Morin *et al.*, 2010; Tolcach and Gómez, 2002; Sierra and Gómez, 2010). Basically, artificial substrates are immersed in a river site, and then transferred elsewhere to build scenarios concerning the responses of the community to changes in environmental conditions. Diatom communities from a reference (unpolluted) site can be transferred to an

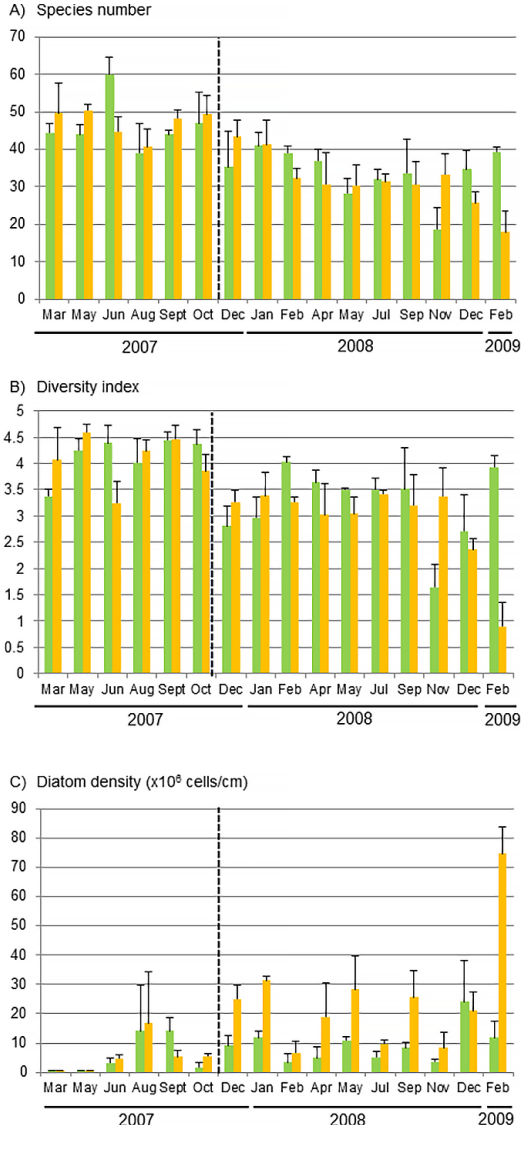


Figure 6.3 (A) Richness, (B) diversity and (C) densities in the control (green bars) and enriched (orange bars) reaches in La Choza stream. The dotted line indicates the onset of the fertilization period.

impacted location, to explore the effects of water quality degradation. Opposite translocations (polluted to unpolluted site) can be performed to study simulated improvement of water quality. Fig. 6.5 illustrates the recovery of community structure and water quality indices (using the IPS; Coste *in* Cemagref, 1982) in diatom communities sampled along a gradient of orthophosphates. Diatom composition differed significantly between upstream, intermediate and downstream sites. According to the gradient of eutrophication, IPS values decreased going downstream. Communities translocated from the two contaminated sites recovered a taxonomic composition closer to the reference (upstream) within 1 month, as well as increasing IPS values.

The use of translocation to assess the recovery potential of diatom communities has been recently questioned due to the fact that it is impossible to discriminate between the sole effect of water quality improvement and recolonization by immigrants from the upstream pool (Morin *et al.*, 2012a). However, translocation experiments remain powerful monitoring tools to assess potential gains in ecological health after remediation, especially in the case of connected hydrosystems.

Diatom-based assessment shifts towards fundamental ecology

The effects of anthropogenic modification of abiotic factors on natural variations in water quality variables and biotic relationships should be addressed. In contrast to studies on biofilm bacterial communities that have focused on large-scale biogeography (Martiny *et al.*, 2006; Battin *et al.*, 2007), information on the relative importance of natural and anthropogenically driven variations in diatom communities is limited to recent ecoregional approaches (see below). Recent works also aim at integrating the role of biotic factors, i.e. competition or facilitation within the community, in structuring the assemblages. Together, these works provide information that may be implemented in multimetric approaches. Finally, molecular approaches have also been developed to study the phylogeny of diatoms and more precisely define the concept of species in diatoms, and shifting towards molecular biomonitoring.

Spatial distributions

Many diatom species are known to have broad distributions; others seem limited to specific climatic zones or geographical regions, or are endemic to a particular habitat. Despite the importance of benthic diatoms as biomonitors, large and

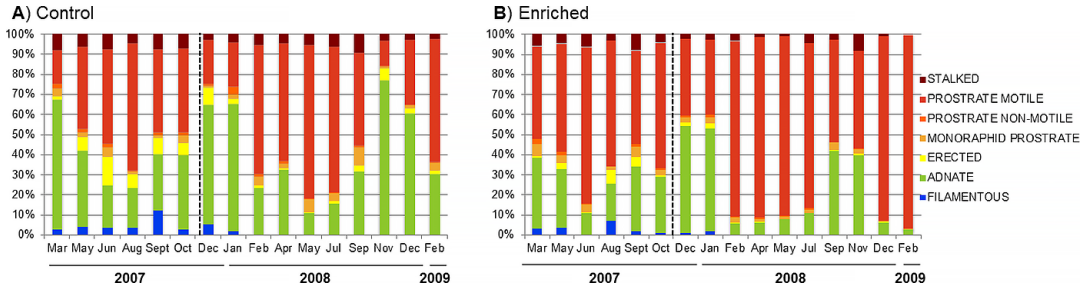


Figure 6.4 Relative abundance of diatom growth forms in the Control (A) and Enriched (B) reaches in La Choza stream. The dotted line indicates the onset of the fertilization period.

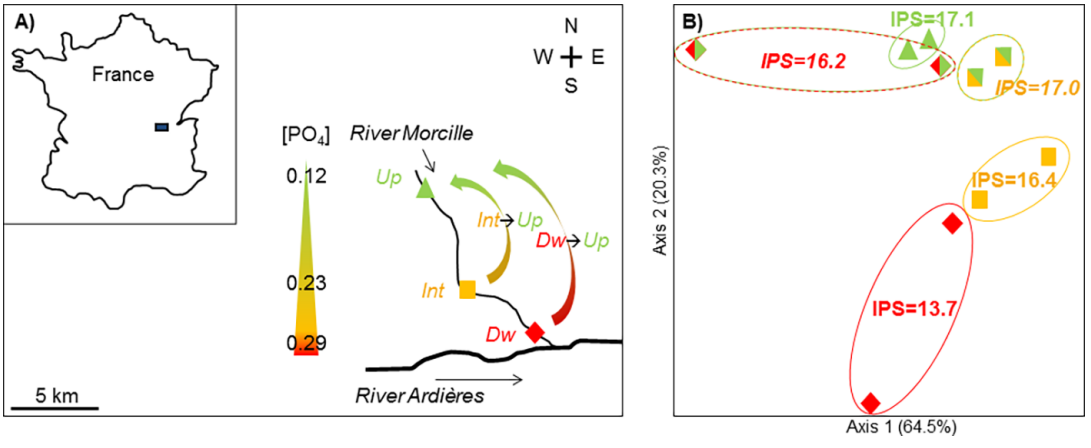


Figure 6.5 Translocation along a gradient of orthophosphates. (A) Location of the sample sites along the River Morcille. Diatom communities are sampled after 2 months at the three sites (Up: upstream, Int: intermediate, Dw: downstream), or after 1 month in their original site followed by 1 month upstream (Int → Up and Dw → Up). Average orthophosphate concentrations are shown in mg/l. (B) Principal component analysis based on relative abundances of the 40 dominant diatom taxa showing the discrimination between sampled communities, and corresponding average IPS values.

regional scale knowledge of their structure and function is still scarce. Their distributional patterns respond to a multitude of different factors, from the biogeochemical characteristics of water and its nutrient content, to geomorphological and physiographical features, but also to biotic interactions, that operate over a wide range of spatial and temporal scales (Menge and Olson, 1990). Water chemistry, light availability, variations in temperature, water velocity, substrata type and grazing are among the factors that potentially affect benthic diatoms (Stevenson *et al.*, 1996). The respective relevance of water quality variation and physiographical processes in a particular geographical area are expressed in a complex gradient, in which the interaction between local and broader-scale factors determines the composition of diatom communities (Leira and Sabater, 2005). In addition, the relative effects of anthropogenic variation over natural variation in determining the distribution of a given community should also be considered. Nutrient enrichment and human disturbances act to change local and large-scale factors reducing the regional differences (Tornés *et al.*, 2007). An obvious consequence of the overriding effect of human activities is that differences in relative abundance and composition of diatom communities are clearer among relatively undisturbed sites than among sites severely affected by nutrient enrichment. It has been proved then that this knowledge is crucial in the assessment of biological quality, which is based on the degree of

deviation between expected and observed conditions (Tornés *et al.*, 2012).

Recently, an increasing body of research supports the idea that microorganisms exhibit biogeographical patterns with no strict evidence of ubiquitous and global distribution (Hillebrand *et al.*, 2001; Heino and Soininen, 2005). At the landscape scale, local species belong to broader metacommunities, shaped by dispersion, connectivity, biotic interactions and habitat area (Altermatt *et al.*, 2013; Chapter 4). In particular, historical processes (i.e. colonization, extinction, dispersion, migration) may determine global diversity patterns of diatoms (Vyverman *et al.*, 2007). Potapova and Charles (2002) demonstrated that large-scale spatial patterns of species dispersal, independent of local environmental characteristics, cannot be neglected in broad-scale studies. In their study almost one-third of the explainable variation in diatom species composition at the USA national scale was attributed to spatial factors. Similarly, another recent spatial concept applied to diatoms is nestedness (Soininen, 2008; Tornés and Ruhí, 2013). Nestedness is a metacommunity-based concept which quantifies the overlap in species composition between high and low diversity sites (Atmar and Patterson, 1993). Nested structures occur when assemblages of species-poor sites are subsets of the assemblages of species-rich sites. The extent to which environmental variables determine nestedness is still poorly understood. For example, Tornés and Ruhí (2013) identified hydrological stability as the main driver of nestedness of diatom communities in Mediterranean rivers.

Integrating the role of biotic interactions

Assembly rules, through general principles, try to explain the different processes leading to the presence of a particular species at a given site (Weiher and Keddy, 1999). The species sensitivity towards the environmental conditions and their ability to participate in spatial dispersion represent two important processes (see above). A third major process concerns biotic interactions, which have been very poorly studied to date. This consists in studying species co-occurrence in biofilms through the concepts of competition, passive coexistence and facilitation. But how to disentangle the relative importance of this biotic process in comparison to the others? In reality two types of approaches exist to unravel the causes: mathematically by comparing real communities to virtual ones with no biotic interactions, and practically by conducting laboratory experiments.

As diatoms are part of a complex three-dimensional matrix, we can assume that competition plays a significant role in community structure. However, to our knowledge, there is no strict evidence of competitive exclusion between diatom species from natural stream ecosystems. To date, only Heino and Soininen (2005) have tried to highlight such patterns for stream diatom communities, by a mathematical approach. From Finish data they calculated an index of species pairs that do not co-occur, i.e. chequerboard pairs (C-score, Stone and Roberts, 1990), which is a measure of the exclusion rate between species. They compared the results to those obtained from random communities, with no biotic interactions (Gotelli, 2000). Despite the higher number of chequerboard pairs in the real dataset, the particular structure of the environmental dataset used did not allow interpretation of the results as evidence of competitive exclusion.

Concerning positive biotic relationships, such as niche complementarity, Burkholder *et al.* (1990) by laboratory autoradiographic techniques, reported a direct comparison of phosphate uptake by adnate and by loosely attached diatoms in an intact biofilm matrix. They highlighted the fact that loosely attached cells took up significantly more radiolabel than did the underlying adnate cells, which were more isolated from the water column nutrient source. The results gave evidence of a physiological assimilation gradient among diatoms, where loosely attached cells can form a significant barrier to nutrient entry. It can then be concluded from those seminal works that such species could facilitate the persistence of underlying sensitive species. Moreover, in an experimental investigation of periphytic succession in recirculating laboratory streams, Passy and Larson (2011) examined the density and the relative abundance of diatoms across gradients of low to high nutrient supply and low to intermediate current velocity. They concluded that the

mechanism of species succession, especially at a functional level, was a neutral coexistence where sensitive species were neither facilitated nor outcompeted by tolerant species but controlled by the environment.

Multimetric developments to better diagnose the type of stressors

Analysis of diatom assemblages can be based on taxonomic (e.g. composition and abundance, richness, diversity, species sensitivity, growth form, motility) or non-taxonomic measures (e.g. enzyme activity, chlorophyll content). The most common taxonomic-based techniques used for monitoring rivers worldwide belong to three basic approaches: indicator species or traits towards different environmental parameters, indices of community structure (e.g. diversity, richness, evenness), or biotic indices. Multimetric indices can combine these three types of approaches into a unitless measure, to be able to respond both to specific sources of stress and to general perturbations (Karr and Chu, 1997). Ideally, the series of indicators should represent key information about structure, function and composition (Dale and Beyeler, 2001). Then, such indices integrate metrics that must show clear relationships with different types of stressors, and should reduce uncertainty and increase robustness of assessment in comparison to single metrics by combining different types of metrics indicative of different environmental conditions and community features. Several studies in Europe (Hering *et al.*, 2006) and in USA (Barbour *et al.*, 1999; Karr and Chu, 1999) have proved the multimetric index to be a valuable approach for assessing the ecological status of water bodies. The first attempt to settle a diatom-based multimetric index was that of Hill *et al.* (2000) who proposed a Periphytic Index of Biotic Integrity (PIBI) for the Mid-Appalachian region (USA). PIBI includes diatom and non-diatom criteria, linked to the relative abundance of some genera, the percentage of acidophilic or eutraphentic diatoms, the percentage of motile diatoms. These metrics were well correlated with both water quality and stream depth and width. Then Griffith *et al.* (2002) proposed for the Southern Rockies ecoregion of Colorado (USA) diatom metrics rather linked to riparian disturbances (percentage of the species *A. minutissimum* for example) or metal pollution (number of diatom cells). More recently Delgado *et al.* (2010) developed a diatom multimetric index (MDIAT) as a combination of metric values (existing European diatom-based indices and percentage of local reference taxa) responding to organic and nutrient stressors.

Progress in taxonomic identification using molecular tools

Molecular biology is increasingly used for diatom phylogenetic analyses (Bruder and Medlin, 2007; Medlin *et al.*, 2008; Medlin, 2010; Medlin and Kaczmarska, 2004), but classifications based on molecular studies and current systematics based on morphological features rarely cross (Cox, 2009). Resulting identifications are difficult to compare, with in some cases generic boundaries incorrectly drawn (Medlin *et al.*, 2008). Challenge in correct identification concerns species with very subtle morphological differences (Behnke *et al.*, 2004) or original species descriptions covering more than one genotype (Evans *et al.*, 2008; Mann *et al.*, 2008; Sarno *et al.*, 2005) and vice-versa with species of large phenotypic plasticity, e.g. leading to identification of the extremes as different taxa (Mann *et al.*, 2010). Primers specific to diatoms have been proposed by Valiente Moro *et al.* (2009) but they are not specific enough to characterize the real diversity of samples (Morin *et al.*, 2012b). Next-generation sequencing was recently attempted to inventory taxonomic diversity in diatom communities (Kermarrec *et al.*, 2013a). Although taxonomic assignation was not always stringent, barcoding approaches offer promising perspectives for high throughput screening of diatom diversity, and may represent a powerful tool for biomonitoring in the future.

Today, molecular tools are rather used to support taxonomy, particularly in the case of cryptic species where they are needed to refine identifications (e.g. Evans *et al.*, 2008; Mann *et al.*, 2008). The combination of molecular techniques with microscopic observations allows progress in classification, especially when dealing with complicated species complexes (Kermarrec *et al.*, 2013b).

Conclusions

Diatom-based biomonitoring is widely used for regulatory purposes, at large scales or for many local applications. Here we reviewed methods differing in their objectives (evaluation of the general water quality, diagnostic of local impacts) and highlighted the recent inflexions in diatom research, towards increasing consideration of fundamental ecology and multi-proxy approaches. Molecular tools are also considered to potentially contribute to a better assessment of water quality, as they may improve our knowledge of diatom taxonomy and help to refine taxa ecological requirements.

Besides diatoms, diverse other periphytic components are likely to be employed, although no consensual bioindicator exists. Classifications have been proposed for monitoring eutrophication based on non-diatom benthic algae in Europe (Rott *et al.*, 1999; Whitton, 1999). The Periphyton Index of Trophic status (PIT: Schneider and Lindstrøm, 2011) developed in Norway combines non-diatom indicator species with non-autotrophic taxa and provides accurate eutrophic assessment in waters where diatoms are hardly found. Recent developments in molecular approaches also offer promising perspectives, e.g. for the use of benthic cyanobacteria as powerful bioindicators (Loza *et al.*, 2013).

References

AFNOR (2000). Qualité de l'Eau. Détermination de l'indice biologique diatomées (IBD) – Norme NF T90–354 –63p.

AFNOR (2003). Qualité de l'Eau. Guide pour l'échantillonnage en routine et le pré-traitement des diatomées benthiques de rivières – Norme NF EN 13946 –16p.

Almeida, S.F.P., Elias, C., Ferreira, J., Tornés, E., Puccinelli, C., Delmas, F., Dörflinger, G., Urbanič, G., Marcheggiani, S., Rosebery, J., *et al.* (2014). Water quality assessment of rivers using diatom metrics across Mediterranean Europe: a methods intercalibration exercise. Sci. Total Environ. *476–477*, 768–776.

Altermatt; F., Seymour, M., and Martinez, N. (2013). River network properties shape α-diversity and community similarity patterns of aquatic insect communities across major drainage basins. J. Biogeogr. *40*, 2249–2260.

Artigas, J., García-Berthou, E., Bauer, D.E., Castro, M.I., Cochero, J., Colautti, D., Cortelezzi, A., Donato, J.C., Elosegi, A., Feijoó, C., *et al.* (2013). Global pressures, specific responses: effects of nutrient enrichment in streams from different biomes. Environ. Res. Lett. *8*, 014002.

Atmar, W., and Patterson, B. (1993). The measure of order and disorder in the distribution of species in fragmented habitat. Oecologia. *96*, 373–382.

Battin, T.J., Sloan, W.T., Kjelleberg, S., Daims, H., Head, I.M., Curtis, T.P., and Eberl, L. (2007). Microbial landscapes: new paths to biofilm research. Nat. Rev. Microbiol. *5*, 76–81.

Barbour, M.T., Gerritsen, J., Snyder, B.D., and Stribling, J.B. (1999). Rapid bioassessment protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates and fish, second edition. EPA-841-B-99-002. (US Environmental Protection Agency, Office of Water, Washington, DC, USA).

Behnke, A., Friedl, T., Chepurnov, V.A., and Mann, D.G. (2004). Reproductive compatibility and rDNA sequence analyses in the *Sellaphora pupula* species complex (Bacillariophyta). J. Phycol. *40*, 193–208.

Bence, J.R., Stewart-Oaten, A., and Schoeter, S.C. (1996). Estimating the size of an effect from a before-after-control-impact paired series design: the predictive approach applied to a power plant study. In Detecting Ecological Impacts: Concepts and Application in Coastal Habitats, Schmitt, R.J., and Osenberg, C.W., eds. (Academic Press Inc., San Diego, CA, USA), pp. 133–150.

Birks, H.J.B., Une, J.M., Juggins, S., Stevenson, A.C., and Braack, C.J.F.T. (1990). Diatom pH reconstruction. Phil. Trans. R. Soc. Lond. B, 263–278.

Bruder, K., and Medlin, L.K. (2007). Molecular assessment of phylogenetic relationships in selected species/genera in the naviculoid diatoms (Bacillariophyta). I. The genus *Placoneis*. Nova Hedw. *85*, 331–352.

Burkholder, J.M., Wetzel, R.G., and Klomparens, K.L. (1990). Direct comparison of phosphate uptake by adnate and loosely attached microalgae within an intact biofilm matrix. Appl. Environ. Microbiol. *56*, 2882–2890.

Cemagref (1982). Etude des méthodes biologiques d'appréciation quantitative de la qualité des eaux (Rapport Q.E. Lyon A.F. – Bassin Rhône-Méditerranée-Corse).

Charles, D.F., Acker, F.W., Hart, D.D., Reimer, C.W., and Cotter, P.B. (2006). Large-scale regional variation in diatom-water chemistry relationships: Rivers of the eastern United States. Hydrobiologia *561*, 27–57.

Coste, M., Boutry, S., Tison-Rosebery, J., and Delmas, F. (2009). Improvements of the Biological Diatom Index (BDI): Description and efficiency of the new version (BDI-2006). Ecol. Indic. *9*, 621–650.

Cox, E.J. (2009). What's in a name? – Diatom classification should reflect systematic relationships. Acta Bot. Croat. *68*, 443–454.

Dale, V.H., and Beyeler, S.C. (2001). Challenges in the development and use of ecological indicators. Ecol. Indic. *1*, 3–10.

Delgado, C., Pardo, I., and García, L. (2010). A multimetric diatom index to assess the ecological status of coastal Galician rivers (NW Spain). Hydrobiologia. *644*, 371–384.

Evans, K.M., Wortley, A.H., Simpson, G.E., Chepurnov, V.A., and Mann, D.G. (2008). A molecular systematic approach to explore diversity within the *Sellaphora pupula* species complex (Bacillariophyta) J. Phycol. *44*, 215–231.

Gómez, N., and Licursi, M. (2001). The Pampean Diatom Index (IDP) for assessment of rivers and streams in Argentina. Aquat. Ecol. *35*, 173–181.

Gotelli, N.J. (2000). Null model analysis of species co-occurrence patterns. Ecology. *81*, 2606–2621.

Griffith, M.B., Hill, B.H., Herlihy, A.T., and Kaufmann, P.R. (2002). Multivariate analysis of periphyton assemblages in relation to environmental gradients in Colorado rocky mountain streams. J. Phycol. *38*, 83–95.

Guiry, M.D. (2012). How many species of algae are there? J. Phycol. *48*, 1057–1063.

Heino, J., and Soininen, J. (2005). Assembly rules and community models for unicellular organisms: patterns in diatoms of boreal streams. Freshwat. Biol. *50*, 567–577.

Hering, D., Feld, C.K., Moog, O., and Ofenböck, T. (2006). Cook book for the development of a multimetric Index for biological condition of aquatic ecosystems: experiences from the European AQEM and STAR projects and related initiatives. Hydrobiologia. *566*, 311–324.

Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H., and Johnson, C.B. (2000). Use of periphyton assemblage data as an index of biotic integrity. J. N. Am. Benthol. Soc. *19*, 50–67.

Hillebrand, H., Watermann, F., Karez, R., and Berninger, U.G. (2001). Differences in species richness patterns between unicellular and multicellular organisms. Oecologia. *126*, 114–124.

Hofmann, G. (1994). Aufwuchs diatoms in Seen und ihre Eignung als Indikatoren der Trophie (Bibliotheca Diatomologica 30).

Ivorra, N., Hettelaar, J., Tubbing, G.M.J., Kraak, M.H.S., Sabater, S., and Admiraal, W. (1999). Translocation of microbenthic algal assemblages used for *in situ* analysis of metal pollution in rivers. Arch. Environ. Contam. Toxicol. *37*, 19–28.

Karr, J.R., and Chu, E.W. (1997). Biological monitoring and assessment: using multimetric indexes effectively (University of Washington, EPA 235-R97–001).

Karr, J.R., and Chu, E.W. (1999). Restoring life in Running Waters: Better Biological Monitoring (Island Press, Washington, DC, USA).

Kelly, M., Bennett, C., Coste, M., Delgado, C., Delmas, F., Denys, L., Ector, L., Fauville, C., Ferréol, M., Golub, M., *et al.* (2009). A comparison of national approaches to setting ecological status boundaries in phytobenthos assessment for the European Water Framework Directive: results of an intercalibration exercise. Hydrobiologia *621*, 169–182.

Kelly, M., Cazaubon, A., Coring, E., Dell'Uomo, A., Ector, L., Goldsmith, B., Guasch, H., Hürlimann, J., Jarlman, A., Kawecka, B., *et al.* (1998). Recommendations for the routine sampling of diatoms for water quality assessments in Europe. J. Appl. Phycol. *10*, 215–224.

Kelly, M.G., and Whitton, B.A. (1995). The Trophic Diatom Index: a new index for monitoring eutrophication in rivers. J. Appl. Phycol. *7*, 433–444.

Kermarrec, L., Franc, A., Rimet, F., Chaumeil, P., Humbert, J.F., and Bouchez, A. (2013a). Next-generation sequencing to inventory taxonomic diversity in eukaryotic communities: a test for freshwater diatoms. Mol. Ecol. Resour. *13*, 607–619.

Kermarrec, L., Bouchez, A., Rimet, F., and Humbert, J.-F. (2013b). First Evidence of the Existence of semi-cryptic species and of a phylogeographic structure in the Gomphonema parvulum (Kützing) Kützing complex (Bacillariophyta). Protist. *164*, 686–705.

King, L., Clarke, G., Bennion, H., Kelly, M., and Yallop, M. (2005). Sampling littoral diatoms in lakes for ecological status assessments: a literature review (Science Report SC030103/SR1, Environment Agency, Bristol, UK).

Lavoie, I., Campeau, S., Grenier, M., and Dillon, P.J. (2006). A diatom-based index for the biological assessment of eastern Canadian rivers: an application of correspondence analysis (CA). Can. J. Fish. Aquat. Sci. *63*, 1793–1811.

Lear, G., Dopheide, A., Ancion, P., Roberts, K., Washington, V., Smith, J., and Lewis, G.D. (2012). Biofilms in freshwater: their importance for the maintenance and monitoring of freshwater health. In Microbial Biofilms: Current Research and Applications, Lear, G., and Lewis, G.D., eds. (Caister Academic Press, Norfolk, UK), pp. 129–152.

Lecointe, C., Coste, M., and Prygiel, J. (1993). Omnidia – Software for taxonomy, calculation of diatom indices and inventories management. Hydrobiologia. *269/270*, 509–513.

Leira, M., and Sabater, S. (2005). Diatom assemblages distribution in catalan rivers, NE Spain, in relation to chemical and physiographical factors. Wat. Res. *39*, 73–82.

Lobo, E.A., Callegaro, V.L.M., Hermany, G., Bes, D., Wetzel, C.A., and Oliveira, M.A. (2004). Use of epilithic diatoms as bioindicators from lotic systems in southern Brazil, with special emphasis on eutrophication. Acta Limnol. Bras. *16*, 25–40.

Lowe, R.L., and Pan, Y. (1996). Benthic algal communities as biological monitors. In Algal Ecology: Freshwater Benthic Ecosystems, R.J. Stevenson, M.L. Bothwell, and R.L. Lowe, eds. (Aquatic Ecology Series, Academic Press Inc., San Diego, CA, USA), pp. 705–739.

Loza, V., Perona, E., and Mateo, P. (2013). Molecular fingerprinting of cyanobacteria from river biofilms as a water quality monitoring tool. Appl. Environ. Microbiol. *79*, 1459–1472.

Mann, D.G., Thomas, S.J., and Evans, K.M. (2008). Revision of the diatom genus *Sellaphora*: a first account of larger species in the British Isles. Fottea. *8*, 15–78.

Mann, D.G., Williams, D.W., Kociolek, J.P., and Cox, E.J. (2010). Discovering diatom species: is a long history of disagreements about species-level taxonomy now at an end? Plant Ecol. Evol. *143*, 251–264.

Martiny, J.B., Bohannan, B.J., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine M.C., Kane, M., Krumins, J.A., Kuske, C.R., *et al.* (2006).

Microbial biogeography: putting microorganisms on the map. Nat. Rev. *4*, 102–112.

McCormick, P.V., and Cairns, J. (1994). Algae as indicators of environmental change. J. Appl. Phycol. *6*, 509–526.

McIntire, C.D., Gregory, S.V., Steinman, A.D., and Lamberti, G.A. (1996). Modelling algal communities: an example from stream ecology. In Algal Ecology: Freshwater Benthic Ecosystems, Stevenson, R.J., Bothwell, M.L., and Lowe, R.L., eds. (Aquatic Ecology Series, Academic Press Inc., San Diego, CA, USA), pp. 669–704.

Medlin, L., Jung, I., Bahulikar, R., Mendgen, K., P., K., and Kooistra, W.H.C.F. (2008). Evolution of the Diatoms. VI. Assessment of the new genera in the araphids using molecular data. Nova Hedw. *133*, 81–100.

Medlin, L.K. (2010). Pursuit of a natural classification of diatoms: An incorrect comparison of published data. Eur. J. Phycol. *45*, 155–166.

Medlin, L.K., and Kaczmarska, I. (2004). Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision. Phycologia *43*, 245–270.

Menge, B.A., and Olson, A.M. (1990). Role of scale and environmental factors in regulation of community structure. Trends Ecol. Evol. *5*, 52–57.

Morin, S., Lambert, A.-S., Artigas, J., Coquery, M., and Pesce, S. (2012a). Diatom immigration drives biofilm recovery after chronic copper exposure. Freshwat. Biol. *57*, 1658–1666.

Morin, S., Roubeix, V., Batisson, I., Winterton, P., and Pesce, S. (2012b). Characterization of freshwater diatom communities: Comparing taxonomic and genetic-fingerprinting approaches. J. Phycol. *48*, 1458–1464.

Morin, S., Pesce, S., Tlili, A., Coste, M., and Montuelle, B. (2010). Recovery potential of periphytic communities in a river impacted by a vineyard watershed. Ecol. Indic. *10*, 419–426.

Passy, S.I. (2007). Diatom ecological guilds display distinct and predictable behavior along nutrient and disturbance gradients in running waters. Aquat. Bot. *86*, 171–178.

Passy, S., and Larson, C. (2011). Succession in stream biofilms is an environmentally driven gradient of stress tolerance. Microb. Ecol. *62*, 414–424.

Ponader, K.C., Charles, D.F., Belton, T.J., and Winter, D.M. (2008). Total phosphorus inference models and indices for coastal plain streams based on benthic diatom assemblages from artificial substrates. Hydrobiologia *610*, 139–152.

Potapova, M.G., and Charles, D.F. (2002). Benthic diatoms in USA rivers: distributions along spatial and environmental gradients. J. Biogeogr. *29*, 167–187.

Potapova, M., and Charles, D.F. (2004). Potential use of rare diatoms as environmental indicators in USA rivers, in: Proceedings of the 17th International Diatom Symposium (Biopress, Bristol, UK), pp. 281–295.

Potapova, M., and Charles, D.F. (2007). Diatom metrics for monitoring eutrophication in rivers of the United States. Ecol. Indic. *7*, 48–70.

Prygiel, J., Coste, M., and Bukowska, J. (1999). Review of the major diatom-based techniques for the quality assessment of rivers – State of the art in Europe. In Use of Algae for Monitoring Rivers III, J. Prygiel, B.A. Whitton, and J. Bukowska, eds. (Agence de l'Eau Artois Picardie, Douai), p224–238.

Rimet, F. (2012). Recent views on river pollution and diatoms. Hydrobiologia. *683*, 1–24.

Rott, E., Pipp, E., Pfister, P., van Dam, H., Orther, K., Binder, N., and Pall, K. (1999). Indikationslisten für Aufwuchsalgen in Österreichischen Fliessgewassern. Teil 2: Trophieindikation. vol 2. Bundesministerium für Land- und Forstwirtschaft. Wien.

Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I., and Zingone, A. (2005). Diversity in the genus *Skeletonema* (Bacillariophyceae), 2. An assessment of the taxonomy of *S. costatum*-like species, with the description of four new species. J. Phycol. *41*, 151–176.

Schneider, S., and Lindstrøm, E.-A. (2011). The periphyton index of trophic status PIT: a new eutrophication metric based on non-diatomaceous benthic algae in Nordic rivers Hydrobiologia. *665*, 143–155.

Sekar, R., Venugopalan, V.P., Satpathy, K.K., Nair, K.V.K., and Rao, V.N.R. (2004). Laboratory studies on adhesion of microalgae to hard substrates Hydrobiologia. *512*, 109–116.

Sládeček, V. (1986). Diatoms as indicators of organic pollution. Acta Hydroch. Hydrob. *14*, 555–566.

Smith, E.P. (2002). BACI design. In Encyclopedia of Environmetrics, A.H. El-Shaarawi and W.W. Piegorsch, eds. (John Wiley & Sons Ltd, Chichester, UK), pp. 141–148.

Soininen, J. (2008). The ecological characteristics of idiosyncratic and nested diatoms. Protist. *159*, 65–72.

Steinberg, C., and Schiefele, S. (1988). Biological indication of trophy and pollution of running waters. Z. Wasser Abwass. For. *21*, 227–234.

Stevenson, R.J., and Bahls, L. (1999). Periphyton protocols. In Rapid Bioassessment Protocols for use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, 2nd edn, M.T. Barbour, J. Gerritsen, B.D. Snyder, and J.B. Stribling, eds. (US Environmental Protection Agency, Office of Water, Washington, DC, USA), p1–22.

Stevenson, R.J., Bothwell, M.L., and Lowe, R.L. (1996). Algal ecology: Freshwater Benthic Ecosystems (Academic Press Inc., San Diego, CA, USA).

Stevenson, R.J., and Pan, Y. (1999). Assessing environmental conditions in rivers and streams with diatoms. In The Diatoms – Applications for the Environmental and Earth Sciences, E.F. Stoermer and J.P. Smol, eds. (Cambridge University Press, Cambridge, UK), pp. 11–40.

Stewart-Oaten, A. (1996). Problems in the analysis of environmental monitoring data. In Detecting Ecological Impacts: Concepts and Application in Coastal Habitats, Schmitt, R.J., and Osenberg, C.W., eds. (Academic Press Inc., San Diego, CA, USA), pp. 109–132.

Stone, L., and Roberts, A. (1990). The checkerboard score and species distribution. Oecologia *85*, 74–79.

Tolcach, E.R., and Gómez, N. (2002). The effect of translocation of microbenthic communities in a

polluted lowland stream. Ver. Int. Verein. Limnol. *28*, 254–258.

Tornés, E., Cambra, J., Gomà, J., Leira, M., Ortiz, R., and Sabater, S. (2007). Indicator taxa of benthic diatom communities: a case study in Mediterranean streams. Ann. Limnol. *43*, 1–11.

Tornés, E., Leira, M., and Sabater, S. (2012). Is the biological classification of benthic diatom communities concordant with ecotypes? Hydrobiologia *695*, 43–55.

Tornés, E., and Ruhí, A. (2013). Flow intermittency decreases nestedness and specialisation of diatom communities in Mediterranean rivers. Freshwat. Biol. *58*, 2555–2566.

UNESCO-WHO-UNEP (1996). Water Quality Assessments (Chapman & Hall, Cambridge, UK).

Valiente Moro, C., Crouzet, O., Rasconi, S., Thouvenot, A., Coffe, G., Batisson, I., and Bohatier, J. (2009). New design strategy for development of specific primer sets for PCR-based detection of Chlorophyceae and Bacillariophyceae in environmental samples. Appl. Environ. Microbiol. *75*, 5729–5733.

van Dam, H., Mertens, A., and Sinkeldam, J. (1994). A coded checklist and ecological indicator values of freshwater diatoms from the Netherlands. Neth. J. Aquat. Ecol. *28*, 117–133.

Veraart, A.J., Romaní, A.M., Tornés, E., and Sabater, S. (2008). Algal response to nutrient enrichment in forested oligotrophic stream. J. Phycol. *44*, 564–572.

Sierra, M.V., and Gómez, N. (2010). Assessing the disturbance caused by an industrial discharge using field transfer of epipelic biofilm. Sci. Total Environ. *408*, 2696–2705.

Vyverman, W., Verleyen, E., Sabbe, K., Vanhoutte, K., Sterken, M., Hodgson, D.A., Mann, D.G., Juggins, S., Vijver, B.V.D., and Jones, V. (2007). Historical processes constrain patterns in global diatom diversity. Ecology. *88*, 1924–1931.

Watanabe, T., Asai, K., Houki, A., Tanaka, S., and Hizuka, T. (1986). Saprophilous and eurysaprobic diatom taxa to organic water pollution and Diatom Assemblage Index (DAIpo). Diatom. *2*, 23–73.

Weiher, E., and Keddy, P. (1999). Ecological assembly rules: perspectives, advances, retreats (Cambridge University Press, Cambridge, UK).

Whitton, B.A., and Rott, E. (1996). Use of Algae for monitoring rivers II (Studia Student. G.m.b.H).

Whitton, B.A. (1999). Perspective on the use of phototrophs to monitor nutrients in running waters Aquat. Conserv.: Mar. Freshwat. Ecosyst. *9*, 545–549.

Zelinka, M., and Marvan, P. (1961). Zurprazisierung der biologischen Klassifikation des Reinheitfliessendergewasser. Arch. Hydrobiol. *57*, 389–407.

Ziemann, H. (1991). Veränderungen der Diatomeenflora der Werra unter dem Einfluß des Salzgehaltes. Acta Hydroch. Hydrob. *19*, 159–174.

The Use of Biofilms to Assess the Effects of Chemicals on Freshwater Ecosystems

7

Helena Guasch, Joan Artigas, Berta Bonet, Chloe Bonnineau, Oriol Canals, Natàlia Corcoll, Arnaud Foulquier, Julio López-Doval, Sandra Kim Tiam, Soizic Morin, Enrique Navarro, Stephane Pesce, Lorenzo Proia, Humbert Salvadó and Alexandra Serra

Abstract

Nowadays, biofilms are one of the principal targets of community ecotoxicology in aquatic ecosystems with a high potential for future use in ecotoxicology. A large set of methods derived from biofilm ecology has successfully been applied in ecotoxicology providing a diverse and comprehensive toolbox. Our ability to quantify the effects of pollution on different biofilm components, allows the direct effects of pollutants on the most sensitive community and their indirect effects on the rest of biofilm components to be evaluated. Biofilms are also a site for biotransfomation and/or transfer of chemicals to other aquatic organisms, supporting a more generalized use of biofilms in environmental chemistry. Investigations aiming to describe processes at biofilm scale, like nutrient dynamics and those including simple food chains, have recently been applied, providing the opportunity of upscaling the effects of pollutants on biofilms to food webs and ecosystems. Finally, biofilm ecotoxicology should now focus on providing the theoretical background for understanding the complex set of responses of natural communities to pollution. This knowledge should also be the basis for guiding the selection of the most appropriate tools and the development of new approaches for a better detection of the impact of pollution on aquatic life.

Introduction

The increasing worldwide contamination of freshwater systems with thousands of industrial and natural chemical compounds is one of the key environmental problems facing humanity. Developing and refining tools to assess the impact of these pollutants on aquatic life is still a challenging issue (Hering *et al.*, 2010). In spite of the inherent complexity of natural systems, the basis for using natural biofilms to assess acute and chronic effects of pollution is rather simple. It is expected that the effects of toxicity will first trigger a biochemical response, e.g. by the activation of detoxification mechanisms, causing thereafter physiological alterations, such as a reduction in photosynthetic activity and respiration, and leading finally to a reduction in the growth of the most sensitive species and the selection of the most tolerant species causing a shift in the structure (i.e. species composition) of the biofilm community. Together with the analysis of water chemistry, and the prevailing environmental conditions, a set of biofilm parameters (i.e. endpoints) may be used to assess the effects of pollutions under real-exposure scenarios (Fig. 7.1).

It has been shown that the use of biofilms in ecotoxicology is rather common, either in field or laboratory investigations. The majority of studies deal with metals and pesticides, but several investigations have recently been focused on emerging compounds (Guasch *et al.*, 2012). Here we aim to update previous reviews and to provide a critical overview of the most common endpoints used to

assess the biological and ecological effects of pollution, the results obtained in different exposure scenarios, and future trends in the use of biofilms in ecotoxicology.

Biofilm ecotoxicology – a multi-component approach

The biological composition of biofilms is very broad, including several types of communities: algal, bacterial, fungal, protozoan and microinvertebrate communities, each of them including a large list of species involved in many ecological processes.

Owing to the prominent role that algae play in biofilms growing in illuminated surfaces, the majority of ecotoxicological investigations have focused on the algal component of biofilms (reviewed in Corcoll *et al.*, 2012a), while fewer studies have focused on the bacterial component (reviewed in Proia *et al.*, 2012a). Studies dealing with other biofilm components, such as fungi or protozoa, in spite of their important role, have received little attention.

Effects of pollutants on the autotrophic component of biofilms

If light is available, algae and other phototrophic organisms become the main component of biofilms. Effects of pollutants have been investigated on both the function and structure of the autotrophic component of biofilms (Table 7.1).

Among the functional descriptors used, photosynthesis-related parameters are some of the most relevant endpoints for assessing toxicity towards algae. Pulse amplitude modulated (PAM) fluorescence techniques were developed to measure among other parameters, photosynthetic capacity and efficiency, and non-photochemical photosynthetic processes. These functional endpoints are largely applied to evaluate the effects of chemicals on biofilms for their sensitivity to a large panel of chemicals, especially those targeting photosystem II, like herbicides or certain metals (e.g. copper) (Serra *et al.*, 2009; Ricart *et al.*, 2009; Laviale *et al.*, 2011). Fluorescence techniques are easy to apply (for more details, see review by Corcoll *et al.*, 2012a) and are even useful for assessing the impact of physical stressors, such as ultraviolet radiation (Navarro *et al.*, 2008). The analysis of accessory pigments (e.g. β-carotene, diatoxanthin, diadinoxanthin, pheophytin, etc.) has also been shown to suitably detect early toxicity of compounds targeting directly and/or indirectly the photosynthetic apparatus (Laviale *et al.*, 2010; Corcoll *et al.*, 2012b,c; Bonnineau *et al.*, 2013).

Chronic exposure to contaminants exerts a selection pressure on the community that may be reflected by physiological changes at species level (modifying its tolerance against contaminants) or by changes in the abundance and composition of algal communities (i.e. biomass, species composition). PAM fluorescence, or high-performance liquid chromatography (HPLC), is used to quantify the relative distribution of algal groups (green, blue and brown algae) within a biofilm based on photosynthetic pigments (e.g. Corcoll *et al.*, 2012a,b). In biofilms, taxonomic community identification is generally performed for diatoms (Chapter 6), a highly diverse, cosmopolitan class of brown algae. Shifts in structure have led to classifications based on species sensitivity/tolerance to contaminants (Morin *et al.*, 2009, 2012, 2014; Ricart *et al.*, 2009). Specific morphological endpoints, e.g. teratologies (Falasco *et al.*, 2009) or cell sizes (Luís *et al.*, 2011), have also proved to detect metal pollution successfully. Algal taxonomy has been largely used to study toxicant-induced selection in biofilm communities, due to its tradition but also its high sensitivity. More recently, molecular tools using DNA sequences have been described as promising tools to assess the prevalence of specific gene sequences in tolerant communities and their taxonomic affinities in natural biofilms (Eriksson *et al.*, 2009).

Quantitative real-time polymerase chain reaction (qPCR) techniques have been used with success in the field of ecotoxicology in order to assess the effects of various contaminants on different diatom species (planktonic and benthic). For instance, after the exposure of *Thalassiosira pseudonana* to PAHs, Bopp and Lettleri (2007) observed strong up-regulation of *lacsA*, which is involved in the fatty acid metabolism and repression of *sil3*, contributing to the formation of the silica shell; highlighting then a possible impact of such compounds on these functions. In a different study, Guo *et al.* (2013) reported up-regulation of

Table 7.1 Summary of biofilm endpoints (in bold) and methods (in italics) in ecotoxicology

Functional responses at molecular, cell, community or ecosystem level	Changes in biomass	Effects on the structure and architecture of the community
Autotrophic organisms		
Photosynthesis: *PAM, ^{14}C-HCO_3 uptake*	**Chlorophyll concentration**: *spectrophotometry*	**Algal groups**: *microscope, HPLC*
Tolerance induction: *toxicity assays, DNA sequences*	**Algal density**: *microscope, flow cytometry*	**Species composition**: *microscope*
		Diatom cell size, teratoforms: *microscope, flow cytometry*
		Genetic diversity: *fingerprinting techniques*
Bacteria		
C uptake: *^3H-thymidne incorporation*	**Bacterial density**: *microscope, flow cytometry*	**Genetic diversity**: *fingerprint, FISH, CARD-FISH, NGS*
Respiration: *substrate-induced respiration*		
Physiological profile: *MicroRespTM*		
Denitrification		
Antibiotic resistance genes		
Fungi		
Respiration: *substrate- induced respiration*	**Fungal density**: *microscope (mycelium growth)*	**Species composition**: *microscope*
Reproduction: *sporulation*	**Biomass**: *ergosterol concentration*	**Genetic diversity**: *fingerprint, NGS*
Extracellular degradation of organic matter: *EEA by enzymatic assays or qPCR*		
Protozoa		
Duplication rate: *dynamics of cell density*	**Cell density**: *microscope*	**Cell damage**: *lysosomal membrane stability, cytoplasmatic vacuolization, etc.*
Grazing activity and endocytotic rate: *clearance assays, intake of particles*		**Species composition**: *microscope*
		Genetic diversity: *fingerprint*
Whole biofilm		
CR, GPP and NPP: *O_2 change, MicroResp*	**AFDM**: *weight of organic material after burning biomass*	**3D structure**: *confocal microscopy*
PO4 and NH4 uptake: *nutrient addition*	**DW**: *weight of the whole biofilm after drying biomass*	**Accumulation and bio accumulation**: *intracellular/total metal concentration, total concentration of chemicals*
Antioxidant response: *antioxidant enzyme activities (AEA)*		**Contaminant transfer**: *food web experiments*
Extracellular degradation of organic matter: *EEA by enzymatic assays*		
Leaf litter breakdown: *biomass changes*		

PAM, pulse amplitude modulated fluorescence; HPLC, high-performance liquid chromatography; fingerprint, DGGE (denaturing gradient gel electorphoresis); T-RFLP, terminal restriction fragment length polymorphism; FISH, fluorescence *in situ* hybridization; NGS, next-generation sequencing; MicroResp, basal/substrate induced respiration; EEA, extracellular enzyme activity; AEA, antioxidant enzyme activity; qPCR, quantitative polymerization chain reaction; AFDM, ash-free dry mass; CR, community respiration; GPP, gross primary production; NPP, net primary production; DW, dry weight.

The endpoints and methods used to assess the effects of chemicals can be specific to the different biofilm communities: phototrophic organisms; bacteria; fungi and protozoa, or affect the whole biofilm. These methods provide information about functional attributes (from molecular and physiological responses to biofilm-mediated ecosystem functions), changes in biomass, effects on the community structure (e.g. community composition) and architecture (3D structure) of biofilms or accumulation and trophic transfer of chemicals.

heat shock protein 70/90 (HSP70 and HSP90) on the diatom *Ditylum brightwellii* after copper and nickel exposure but not after exposure to endocrine-disrupting chemicals (BPA, PCB, and endosulfan), revealing that these genes are differentially involved in the defence response against various environmental stressors. Moreover, gene expression is an early and sensitive biomarker of toxicant exposure. Actually, qPCR tools are able to reveal toxic effects, whereas other endpoints like growth inhibition are not (Bopp and Lettleri, 2007; Kim Tiam *et al.*, 2012). They were also shown to respond at environmental concentrations. Indeed Kim Tiam *et al.* (2012) observed early differential expression of genes involved in regulation of mitochondrial metabolism (*cox1*, *nad5*, 12S) and photosynthesis (*psaA*, *d1*) on the diatom *Eolimna minima* after exposure to cadmium concentrations of 10µg/l.

Effects of pollutants on bacteria

Given the generally close link between bacterial and algal production in stream biofilms (Scott *et al.*, 2008), effects of toxicants on biofilm bacterial communities can be either direct, or indirect by following changes in the autotrophic component (Ricart *et al.*, 2009; Proia *et al.*, 2011). The functional response of biofilm bacteria to environmental stressors can be evaluated using a large set of global descriptors, including bacterial growth (Lawrence *et al.*, 2007), bacterial production, by measuring incorporation of radiolabelled thymidine (Paulson *et al.*, 2000; Blanck *et al.*, 2003), and bacterial survival rates (Ricart *et al.*, 2010) (Table 7.1). Toxicants can also affect biogeochemical processes associated with bacterial metabolism, such as organic matter decomposition and nutrient cycling. Such effects on biofilm bacterial communities can be assessed through the measurement of extracellular enzyme activities (EEA) involved in carbon, nitrogen or phosphorus acquisition (Ricart *et al.*, 2009; Tlili *et al.*, 2010; Fechner *et al.*, 2012), or through the measurement of gas production to evaluate basal or substrate-induced respiration (Tlili *et al.*, 2011a,b), denitrification (Chénier *et al.*, 2006; Wang *et al.*, 2014) or community-level physiological profile (Lawrence *et al.*, 2004, 2007; Boivin *et al.*, 2006; Tlili *et al.*, 2011b). The potential of biofilm bacterial communities to degrade or mineralize organic compounds (e.g. pesticides, pharmaceuticals or endocrine disruptors) can also be viewed as a promising ecotoxicological tool (Paje *et al.*, 2002; Pesce *et al.*, 2009; Writer *et al.*, 2011a, 2011b). In addition to their functional impact, toxicants may affect the structure and diversity of biofilm bacteria. Those effects can be assessed quantitatively, by determining bacterial cell densities using microscopy (Proia *et al.*, 2011, 2012b) or flow cytometry (Villeneuve *et al.*, 2011), and semi-quantitatively, by using fluorescence *in situ* hybridization (FISH) and catalysed reported deposition-fluorescence *in situ* hybridization (CARD-FISH) to detect the impact of toxicants on community composition at a broad phylogenetic level (Brummer *et al.*, 2000; Lawrence *et al.*, 2007; Proia *et al.*, 2013a). Toxicant effects on the bacterial community composition can also be evaluated by using molecular fingerprint techniques (Dorigo *et al.*, 2010; Tlili *et al.*, 2010). New perspectives are now given by next-generation sequencing (NGS) that provide a more detailed characterization of community composition and allow taxonomic identification of bacterial community members, as shown by recent studies aimed at assessing bacterial diversity on river biofilms using NGS-based approaches (Besemer *et al.*, 2012; Hall *et al.*, 2012; Bricheux *et al.*, 2013) (Table 7.1).

In the last decade, ecotoxicology has also been focused on investigating the fate and effects of antibiotics in nature. As an example, the prevalence of antibiotic resistance genes in bacteria of stream biofilms has recently been demonstrated (e.g. Dutour *et al.*, 2002; Fox *et al.*, 2008; Marti *et al.*, 2013), as well as the effects of real mixtures of antibiotics detected in the bacterial compartment of highly impacted river biofilm (Proia *et al.*, 2013a).

Effects of pollutants on fungi

Evaluation of chemical stress in aquatic fungal communities has been mostly performed in leaf biofilms, because of the great fungal biomass accrual (ca. 98% of total microbial biomass) and strong toxicant adsorption potential in this substratum. Responses of leaf fungal communities to toxicants are mostly evaluated through the litter breakdown (Moreirinha *et al.*, 2011; Artigas *et al.*,

2012; Flores *et al.*, 2014), a key ecosystem process used as an indicator of functional stream integrity (Gessner and Chauvet, 2002). Metals (e.g. copper and zinc) and organic pesticides (e.g. azole fungicides) can depress litter decomposition (Duarte *et al.*, 2008; Artigas *et al.*, 2012) above a certain threshold concentration. Toxicant effects may be based on the respiration (substrate-induced respiration) and reproduction (sporulation) activities of the fungal community (Tlili *et al.*, 2010; Moreirinha *et al.*, 2011). Functional descriptors, such as cellulolytic (cellobiohydrolase), hemicellulolytic (β-xylosidase) and ligninolytic (phenol oxidase) extracellular enzyme activities, have been used to determine toxicant impairment on fungal capacities to degrade organic matter and alter carbon cycling in rivers (Artigas *et al.*, 2012). Methodological approaches based on gene regulation encoding for extracellular enzymes (e.g. quantitative real-time PCR, Solé *et al.*, 2012) have become promising tools to advance in the understanding of molecular mechanisms controlling microbial activities involved in carbon cycling and mitigation of environmental pollution (e.g. pesticide degradation). From a structural point of view, the density and taxonomic composition of aquatic hyphomycete communities (dominant in submerged leaves) are shown to be sensitive to heavy metals (Duarte *et al.*, 2008) and organic pesticides (Bundschuh *et al.*, 2011). Genetic approaches (including fingerprint, and NGS-techniques) are considered as useful tools to identify toxicant effects in aquatic hyphomycete communities (Moreirinha *et al.*, 2011; Artigas *et al.*, 2012; Tolkkinen *et al.*, 2013; Flores *et al.*, 2014), but *in situ* approaches are lacking regarding the literature. In parallel, the use of stable isotope probing techniques (optimized for soil microbial communities, Park *et al.*, 2006) are promising tools to identify populations capable of degrading pollutants and, therefore, of comprehending the adaptation potential of fungal communities in contaminated ecosystems including their use in bioremediation (Table 7.1).

Effects of pollutants on protozoa

As unicellular organisms associated to biofilms, protozoa are closely in contact with the surrounding environment and show high sensitivity to aquatic pollution. Compared to other aquatic consumers, protozoa communities have a faster physiological response and succession process (i.e. the replacement of species over time) due to their higher growth rate (Salvadó *et al.*, 1995; Nicolau *et al.*, 2001; Zhou *et al.*, 2008; Madoni, 2011). Indeed, protozoa are also affected by pollutants. Heavy metals (Niederlehner and Cairns, 1992; Madoni, 2000; Holtze *et al.*, 2003; Díaz *et al.*, 2006; Martín-González *et al.*, 2005; Rico *et al.*, 2009; Ancion *et al.*, 2013), ammonia (Niederlehner and Cairns, 1990), pesticides (Shi *et al.*, 2013), polycyclic aromatic hydrocarbons, PAHs (Lara *et al.*, 2007) and nanoparticles (Mortimer *et al.*, 2010) among other pollutants (Bringmann and Kühn, 1980; Nalecz-Jawecki *et al.*, 1993; Selivanovskaya *et al.*, 1997) have been demonstrated to affect protozoa. The effects of each pollutant vary depending on its concentration and its exposure time (Cairns and Pratt, 1993) and by the specific capability of each species to acclimatize, to recover its population and to bioaccumulate the pollutant (Martín-González *et al.*, 2006). In that sense, the study of structural and functional attributes of the protozoa community provides several useful endpoints for assessing pollution in aquatic ecosystems. Effects of pollutants have been observed on protozoa richness (Gracia *et al.*, 1994; Fernandez-Leborans and Novillo, 1995; Nicolau *et al.*, 2005) or species composition (Fernandez-Leborans and Novillo, 1995; Canals *et al.*, 2013), e.g. the stalked ciliate *Opercularia* spp is normally associated to stressed or polluted ecosystems. In addition to classical endpoints, such as mortality (Bergquist and Bovee, 1976; Salvadó *et al.*, 1997) or duplication rate (Salvadó *et al.*, 1997; Gomiero *et al.*, 2012), effects of pollutants on cell viability (e.g. Nalecz-Jawecki *et al.*, 1993; Salvadó *et al.*, 1997; Mortimer *et al.*, 2010), grazing activity or endocytotic rate (K_e) (Nicolau *et al.*, 2001; Gomiero *et al.*, 2012) have also been measured. Finally, effects of toxicity at cellular level, such as lysosomal membrane stability (Gomiero *et al.*, 2012), cytoplasmatic vacuolization and mitocondrial degeneration have also been observed (Martín-Gonzalez *et al.*, 2006). In addition to classical methods based on microscopic analyses, fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE)

and terminal restriction fragment length polymorphism (T-RFLP), are gaining greater prominence, as these approaches are increasing our knowledge of the complexity of biofilm protozoa communities (Dopheide *et al.*, 2008, 2009). Nevertheless, combining microscopic and molecular analyses is recommended to obtain further information.

Ecotoxicological responses of the whole biofilm

Biofilms are not only an assemblage of aquatic organisms but ubiquitous complex structures with a large proportion of non-living organic and inorganic matter with a high adsorption capacity. While many ecotoxicological investigations focus on the effects of chemicals on specific compartments of the biofilm, endpoints providing information about the effects of chemical exposure on the whole biofilm, such as bioaccumulation, oxidative stress or nanoparticle toxicity, are also interesting (Table 7.1). Investigations describing processes at biofilm scale, like primary production and nutrient dynamics, provide the opportunity of upscaling the effects of pollutants on biofilms to ecosystem functioning.

Accumulation of pollutants in natural biofilms

Total concentrations of chemicals in water fluctuate in time, and do not always reflect the integrated exposure to water chemicals of organisms living in that environment, thus complicating the establishment of direct relationships to toxicity. Monitoring chemical bioaccumulation may overcome this problem because it can represent real bioavailability and exposure. Thus, the accumulation of pollutants in biofilms can be considered the first step in the exposure of microbial organisms living in the biofilm matrix and of those placed at higher trophic levels. In addition, it can also be considered as a detoxification pathway (see Chapter 10).

Bioaccumulation of chemicals in biofilms is influenced by several interacting physical and chemical parameters of the environment like current velocity, temperature, pH, nutrients and organic matter concentration in water or the hydrophobicity of each compound (Headley *et al.*, 1998; Sabater *et al.*, 2002; Meylan *et al.*, 2004; Lundqvist *et al.*, 2012), but also by biological proprieties of the biofilm, such as its age, thickness or EPS composition (Headley *et al.*, 1998; Lawrence *et al.*, 2001). Bioaccumulation kinetics of chemicals are rather complex and depend on the substance's chemical properties, as well as on uptake mechanisms that may be passive and/or active. Metal bioaccumulation in biofilms has been studied extensively, and is described as a two-step process. Metals are first adsorbed extracellularly (in the EPS or onto cell surfaces), before being absorbed into cells by uptake mechanisms (Holding *et al.*, 2003). Intracellular and total metal content in biofilms can be measured easily, to improve the description of exposure (Meylan *et al.*, 2003; Morin *et al.*, 2008a; Serra *et al.*, 2009). In spite of the expected variability in bioaccumulation capacity of biofilms, a large number of studies reported a strong relation between metal bioaccumulation and changes in the structure, composition and function of algal and bacterial communities living in biofilms (Duong *et al.*, 2008; Morin *et al.*, 2008b; Ancion *et al.*, 2010; Bonet *et al.*, 2012; Corcoll *et al.*, 2012c). Studies reporting herbicide bioaccumulation in biofilms are rather numerous (Headley *et al.*, 1998; Lawrence *et al.*, 2001). However, the investigations of the link to toxicity are scarce, probably because of the highly complex and diverse toxicokinetics of these compounds and the impossibility to separate between intracellular and extracellular accumulation. More recently, several authors have reported the bioaccumulation of pharmaceuticals and endocrine disruptors in biofilms (Writer *et al.*, 2011a, 2013; Wunder *et al.*, 2011). However, their link to toxicity on biofilm is still not confirmed. As many compounds susceptible to provoking deleterious impacts on the biota are likely to be accumulated in biofilms (e.g. Lawrence *et al.*, 2001; Sabater, 2003), measuring toxicant concentrations in this 'natural passive sampler' – the biofilm – may be a valuable alternative to traditional chemical monitoring. This measure would provide ecologically relevant information about the potential risk of contaminants for the aquatic ecosystem and may be especially useful and reliable for those compounds not undergoing metabolization into the biofilm (e.g. metals).

Detecting biofilm under oxidative stress

Chemical contamination in biofilm is likely to induce direct or indirect oxidative stress by enhancing reactive oxygen species (ROS) production or impairing cellular antioxidant responses. The resulting excess in ROS can provoke lipid peroxidation, membrane disruption, alteration in cell structures and mutagenesis (Scandalios, 1993; Mittler, 2002; Edreva, 2005; Wolfe-Simon *et al.*, 2005; Lesser, 2006). Though oxidative stress can be specifically induced by some toxicant (e.g. copper), it can also result from general metabolism alteration and thus indicates a low 'health' status of biofilm. Therefore, the detection of oxidative stress damage and response within the whole biofilm community is expected to provide information on biofilm stress status and its ability to cope with further oxidative stress (Bonnineau *et al.*, 2013)

Lipid peroxide quantification is a common measure of cellular oxidative damage that can be estimated at community level. For instance, Vera *et al.* (2012) used the thiobarbituric acid-reactive substances (TBARS) assay to show how exposure to an environmentally relevant concentration of a glyphosate formulation provoked oxidative damage in the biofilm community.

Nevertheless, most of the recent work has been focused on biofilm antioxidant capacity, rather than on oxidative damage. In fact, to keep the oxidative balance under control, organisms have non-enzymatic mechanisms (e.g. glutathione, carotenoids and phenolics; Okamoto *et al.*, 2001) as well as enzymatic mechanisms (e.g. glutathione-S-transferase: GST, catalase: CAT, ascorbate peroxidase: APX, glutathione reductase: GR and superoxide dismutase: SOD activities). In particular, several authors have proposed using antioxidant enzyme activities (AEAs) as biomarkers of pollution due to their capacity to respond to both organic and inorganic pollutants (Valavanidis *et al.*, 2006; Guasch *et al.*, 2010a,b; Maharana *et al.*, 2010; Bonnineau *et al.*, 2011; Bonet *et al.*, 2012, 2013, 2014). In biofilms, AEAs are defined as a global indicator of the 'health' status of the whole biofilm, then considered as a black box. AEA measurement at community level is expected to reflect the tendency (activation or inhibition) observed in the majority of individuals and species within the community (Bonnineau *et al.*, 2012).

Biofilm AEAs have been used at different scales in both laboratory and field studies, mainly to determine the antioxidant response of the community to a specific chemical. For instance, in several studies, AEAs have been found to be more sensitive to contaminant than traditional biomarkers such as photosynthetic parameters (Dewez *et al.*, 2005; Guasch *et al.*, 2010b; Bonet *et al.*, 2013, 2014). Measuring AEA response throughout a gradient of oxidative stress can also provide information on the antioxidant capacity of the community. Indeed, AEAs are expected to increase with increasing oxidative stress until ROS overcomes the cell defence system and AEAs eventually decrease due to cellular damage. From this unimodal (bell shape) pattern of response, a range of oxidative stress levels by which AEAs increased can be defined; within this range the community is expected to be able to alleviate oxidative stress. This range defines the antioxidant capacity of a community and is influenced by various parameters (e.g. biofilm age, pre-exposure to contamination). For instance, chronic exposure of biofilm to the herbicide oxyfluorfen led to an increase in biofilm CAT capacity. Biofilms chronically exposed to oxyfluorfen were able to respond to higher concentrations of oxyfluorfen by an increase in CAT activity while in non-adapted biofilms (those not previously exposed), CAT activity decreased in response to acute exposure to high levels of oxyfluorfen, probably because of oxidative damage (Bonnineau *et al.*, 2013).

Since oxidative stress can be greatly influenced by environmental parameters, such as light or temperature (Butow *et al.*, 1997; Aguilera *et al.*, 2002; Li *et al.*, 2010), both laboratory and field studies are needed to better understand AEAs responses and interpret their variations (Bonnineau *et al.*, 2013). For instance, Bonet *et al.* (2012) showed that, under controlled conditions (microcosm study), APX clearly decreased due to Zn exposure while, in the field, the inhibition of GST was shown to be a biomarker of Zn exposure (Bonet *et al.*, 2013, 2014). These differences were attributed to variations in environmental parameters and a specific effort has been made to better understand

field variability. In an annual monitoring, Bonet *et al.* (2013) observed that AEA followed the seasonality of the system, changing as a response to light and water temperature fluctuations. However, seasonality was not observed in the polluted site, where Zn masked this pattern of variation.

Detecting oxidative stress in biofilms provides information on oxidative damage (e.g. Lipid peroxidation), antioxidant responses and antioxidant capacity of the community. These markers of oxidative stress can be used to detect alteration within biofilm community due to pollutant exposure but also to environmental variations (e.g. climate change) (Bonet *et al.*, 2013).

Differential biofilm gene expression

In aquatic ecosystems, biofilm ecotoxicology has been used to investigate contaminant effects at different levels of biological organization, from species composition to biogeochemical processes. New approaches suggest going even deeper within biofilm and investigating structure and function at molecular level. Differential gene expression has been studied until now on single species (i.e. diatom cultures). Nevertheless, the tools developed for diatoms are extremely promising and in the future could be expanded to the whole biofilm. The qPCR tools have been tested with less success at the community level (i.e. a biofilm composed of different diatom species and other organisms), using these specific gene sequences principally because of the lack of available nucleotide sequences of such organisms in genomic databases (Tiam, unpublished data).

Biofilms in nanoparticle ecotoxicology

Experiments with biofilms are an optimal target for assessing the environmental risks related to new emerging toxicants such as nanoparticles. As biofilms grow on submerged surfaces, they are especially exposed to engineered nanoparticles (ENPs). Nanotechnology development is leading to a proliferation of products that are likely to become a source of many different engineered nanoparticles (ENP) in the environment, where their fate, behaviour and effects are mostly unknown. Their nano-size allows these materials to interact at molecular scale with organisms present in the environment. Among other effects in rivers, ENPs may impact on photosynthetic organisms. The ENPs have direct and indirect toxicological effects on different organisms present in biofilms (Navarro *et al.*, 2008). The physical characteristics of ENPs facilitate their transport in suspension. In addition, their large density, as that of metallic nanomaterials and their surface properties, which may enhance agglomeration processes, may provoke sedimentation under reduced hydrodynamics. This process will deposit ENPs in biofilms where biouptake may take place, thus leading to toxic effects. In addition, since biofilm ecotoxicological testing can be done under the controlled conditions of micro or mesocosms (artificial channels), certain methodological problems associated to ENP experimentation, mostly related to the lack of control and characterization of ENPs and the environmental conditions prevailing during the exposure of the organisms, will be avoided (Handy *et al.*, 2012).

Biofilm ecotoxicology – link between pollution and ecosystem health

Biofilms are considered biological entities which play a key role in ecosystem functioning, and are in turn very sensitive to chemical exposure. Investigations aiming to describe processes at biofilm scale like nutrient dynamics and those including simple food chains, are common in ecological research but less used in ecotoxicology. These approaches have recently been applied, providing the opportunity of upscaling the effects of pollutants on biofilms to food webs and ecosystems.

Upscaling biofilm responses to ecosystem processes

Biofilm communities are composed of many microbial species with a key role in ecosystem functioning offering important insights regarding mechanisms occurring from the single cell level to biogeochemical processes at a larger scale by mediating processes, such as oxygen production, nutrient uptake and organic matter transformation (Battin *et al.*, 2003; see Chapter 5). In fluvial systems, for example, combining community-scale (such as mesocosm experiments) and

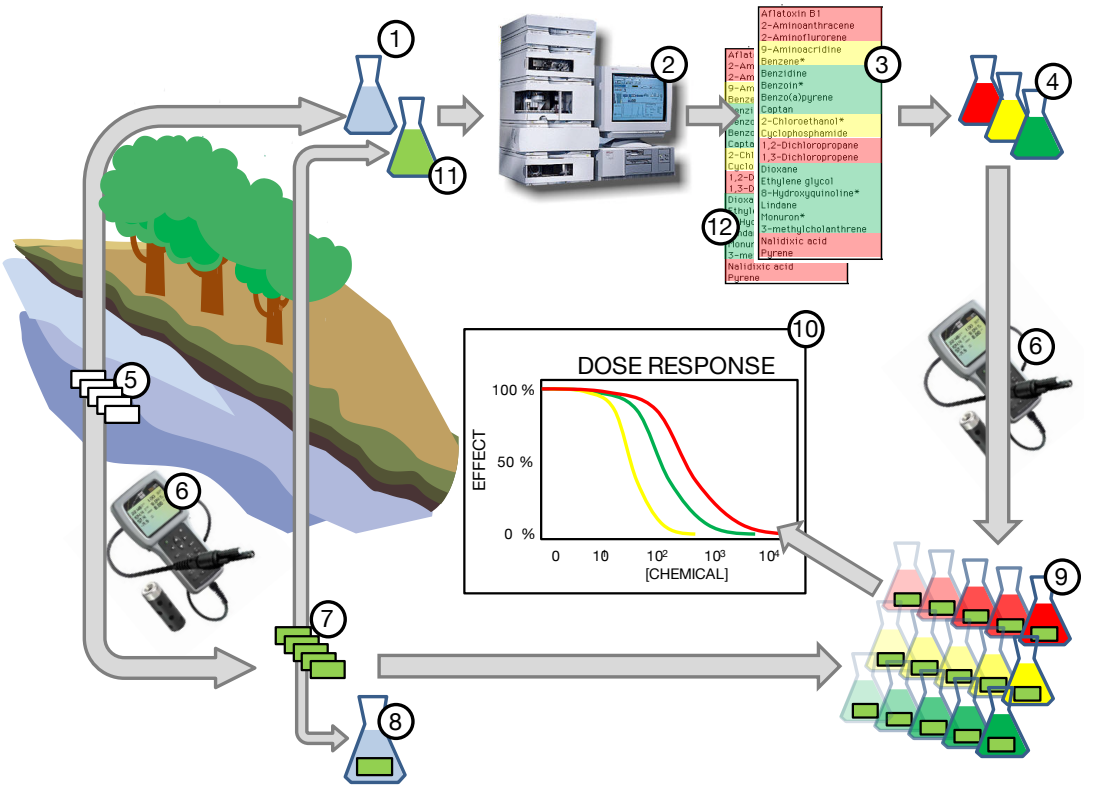


Figure 7.1 Conceptual framework for the use of freshwater biofilms in aquatic ecotoxicology. Water samples (1) from a river affected by chemical pollution are analysed (2). The chemicals present in the water (3) are classified according to their mode of action (different shaded colours), and a reduced number of chemicals representing the different modes of action (represented by different colours) are selected (4). In parallel, artificial substrata (5) are placed at the sampling points, and during a few weeks or months (depending on the type of community and local conditions), the environmental variables are monitored (6). This information can be used later during the experimental design, to study their role as modulating factors of the measured ecotoxicity. After the colonization time, the substrata containing the freshwater biofilms are sampled (7), some are preserved for analytical purposes (8), and others are used for dose–response experiments (9). Results are plotted and modelled (10) and the relevant concentrations measured (as the Effective Concentration reducing by 50% the biological parameter measured: EC_{50}). In addition, biofilms can be processed to extract (11) and analyse (2) the chemicals adsorbed and/or bioaccumulated. The list of the detected chemicals (12) can complement the information obtained from water samples (3).

whole-reach scale (field or ecosystem) measurements together with the characterization of the structure and function of biofilms contribute to a better understanding of the effects of human activities on ecosystem processes.

Rosi-Marshall *et al.* (2013) in a field study, using Nutrient Diffusing Substrata (NDS) found that a mix of pharmaceuticals produced changes in biofilm metabolism parameters, consisting of a decrease in community respiration (CR) and gross primary production (GPP). Another study used field translocation between impacted and non-impacted sites to assess the effects of industrial discharge on biofilm functioning. These authors found a rapid shift (one week) from autotrophic- to heterotrophic-dominated metabolism when the communities from the non-impacted site were translocated to impacted sites. In contrast, when communities were transferred back to the reference site, the recovery to autotrophy took up to four weeks (Sierra and Gómez, 2010).

Hill *et al.* (1997) used chambers to measure benthic metabolism in a rocky mountain stream which had elevated metal pollution and found that GPP and net primary productivity (NPP)

decreased with increasing metal concentrations by one order of magnitude from the reference site to the most impacted site. The effects of different pharmaceuticals on biofilm metabolism and nutrient uptake were assessed *in vitro* and *in situ* (using NDS in the field) in a central Indiana river (USA). The *in vitro* experiments showed that ammonium uptake was reduced after exposure to nicotine and caffeine, and nitrate uptake was increased by nicotine exposure, while no effects were observed on microbial metabolism. On the other hand, an *in situ* experiment showed that nicotine increased microbial respiration (Bunch and Bernot, 2011). Nutrient uptake was also used to assess the effects of metals on fluvial biofilms. Serra *et al.* (2009) found a slight decrease in phosphate uptake after chronic copper exposure of the biofilm to 26 µg/l in artificial channels. In another mesocosm study, Proia *et al.* (2011) showed that triclosan (60 µg/l) inhibited biofilm phosphate uptake up to 71% and uptake rates did not recover until two weeks after the end of exposure. The negative effect of triclosan on biofilm capacity to uptake phosphate was confirmed in other investigations using microcosms and revealed the persistence of this effect over time (Proia *et al.*, 2013b; Guasch *et al.*, *in press*).

These studies exemplify how classical biofilm processes, such as nutrient uptake and community metabolism commonly investigated in ecosystem ecology, are sensitive tools for assessing the ecotoxicological effects of pollutants on freshwater communities and ecosystems. In addition, addressing these endpoints allows the ecological relevance of the observed effects at different levels, from community to whole ecosystem scales, to be increased.

Biofilms in ecotoxicological food web studies

Studies based on food-web relationships between biofilms and their grazers provide a high degree of environmental realism. Due to the increased complexity, these studies allow us to assess the responses of communities and within communities and the evaluation of the direct and indirect effects of pollutants at different trophic levels (Culp *et al.*, 2000; Geiszinger *et al.*, 2009).

These investigations are mainly performed in experimental conditions in order to ensure control of environmental variables (Fig. 7.2), but some field studies also exist. Literature reviews provide interesting experimental models (Culp *et al.*, 1996; Ledger *et al.*, 2009). In most cases,

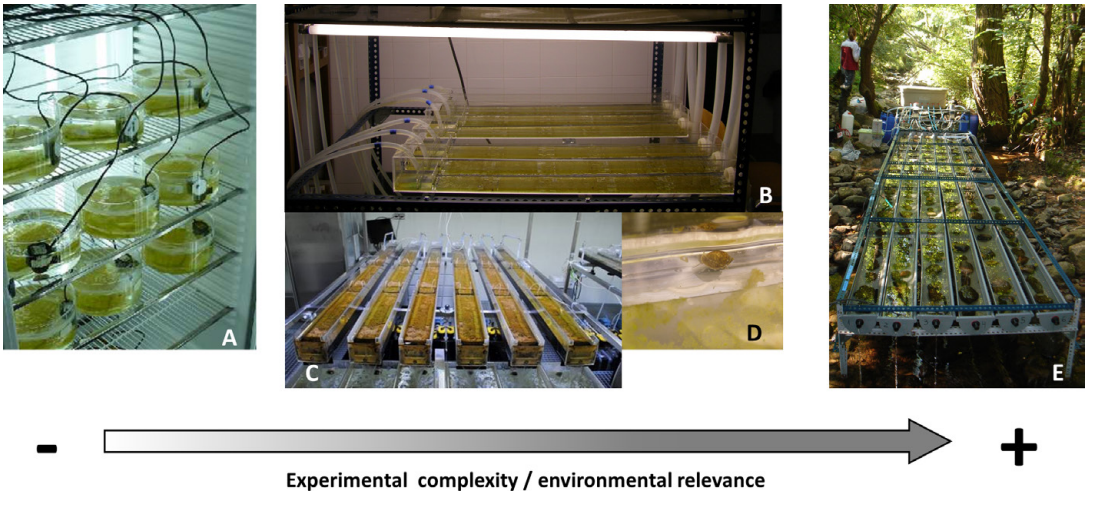


Figure 7.2 Experimental settings of biofilm ecotoxicology. Biofilm communities growing on artificial substrata (e.g. sandblasted glass substrata) are exposed to chemicals under controlled conditions. Exposure can be done in (A) crystallizing dishes (1.5 l volume) with recirculating water; (B) recirculating indoor channels (1 m long); (C) one flow through indoor channels (2 m long). (D) detail of a snail (grazer) placed on top of biofilms in a grazing experiment. (E) one flow through outdoor channels (5 m long).

food-web experiments involve biofilms and a grazer in order to study biomagnification and transfer of the test substance from primary producer to consumer. Other experiments address the possible additional effects of grazing pressure on a chemically stressed biofilm or the possible indirect effects of pollutants on grazers or biofilms due to toxicant-induced alterations of ecological relevance. Generally speaking, insects and molluscs have been used as grazers. Food-web experiments with biofilms have been applied in order to study the ecotoxicological effect of pesticides (Muñoz *et al.*, 2001; Real *et al.*, 2003; López-Doval *et al.*, 2010; Lundqvist *et al.*, 2012), metals (Irwing *et al.*, 2003; Conley *et al.*, 2011; Xie and Buchwalter, 2011; Kim *et al.*, 2012; Li *et al.*, 2012), nanoparticles (Kulacki *et al.*, 2012) and emerging pollutants (Evans-White and Lamberti, 2009), among other compounds.

Several authors demonstrated the importance of biofilms in the introduction of toxicants in the food web by means of food-web experiments. In the case of zinc, bioaccumulation in biofilm, metal transfer and bioaccumulation in the grazer *Centroptilum triangulifer* were shown (Kim *et al.*, 2012). Irwing *et al.* (2003) demonstrated that mayflies grazing on biofilms contaminated with cadmium showed significant inhibition in growth and feeding in comparison to those exposed to contaminated water. Xie and Buchwalter (2011), using biochemical responses in the mayfly *C. triangulifer*, confirmed that cadmium is more toxic by ingestion of contaminated biofilm than by direct exposure to contaminated water. Experiments with food webs demonstrated that high nutritional quality and quantity of available biofilm diminish the toxicological response of mayflies to selenium (Conley *et al.*, 2011). Bioavailability of pollutants is modulated by the influence of environmental factors on biofilm, as demonstrated with food-web experiments. Increasing levels of phosphate enhanced bioaccumulation of copper in biofilms and dietary toxicity to the amphipod *Hyalella azteca* (Li *et al.*, 2012). In an experiment with freshwater snails and biofilms, Lundqvist *et al.* (2012) reported that dissolved organic matter in water interferes in the sorption of pesticides (carbofuran, lindane and chlorpyrifos) to biofilms and is, therefore, a factor that can modulate bioavailability and bioaccumulation of insecticides.

The presence or absence of grazers can interfere in the effects of toxicants on functional or structural characteristics of biofilms. Muñoz *et al.* (2001) studied the effects of atrazine in a single food web and described reduction of carbon incorporation and algal diversity in biofilm due to the interaction of grazers (*Physa acuta*) with the herbicide. Evans-White and Lamberti (2009) observed that toxicants in combination with grazers increased chlorophyll concentration and algal diversity. On the contrary, similar experiments with food webs did not find interactive effects of grazing and toxicants on biofilm (Real *et al.*, 2003; López-Doval *et al.*, 2010). Indirect effects on the structure and function of biofilms have been observed as a consequence of the changes in the physiology and behaviour of *P. acuta* induced by the toxicant (Evans-White and Lamberti, 2009).

Overall, it is reasonable to expect that grazing may influence the response of biofilms to toxic exposure. Communities suffering both grazing pressure and the effects of toxic substances will have less ability to overcome grazing effects than non-exposed communities, because toxicity will limit algae regrowth and facilitate the extinction of the less abundant species after grazing. This interaction may have remarkable ecological implications since grazing pressure will magnify the negative effects that toxicants exert on ecosystem processes, such as primary production and nutrient cycling (Fig. 7.3).

Environmental factors modulating biofilm response to pollutants

In the field, environmental conditions are highly variable and organisms are rarely under optimal conditions. There is growing awareness that these abiotic parameters can strongly constrain ecosystem responses to anthropogenic contamination (Fisher *et al.*, 2013). Nevertheless, their influence is rarely taken into account in single-species ecotoxicological tests. Indeed, single species have a limited range of acclimation to environmental parameters and studies performed at community level appear to be better suited for investigating the influence of environmental factors on contamination effects (Clements *et al.*, 2009). In

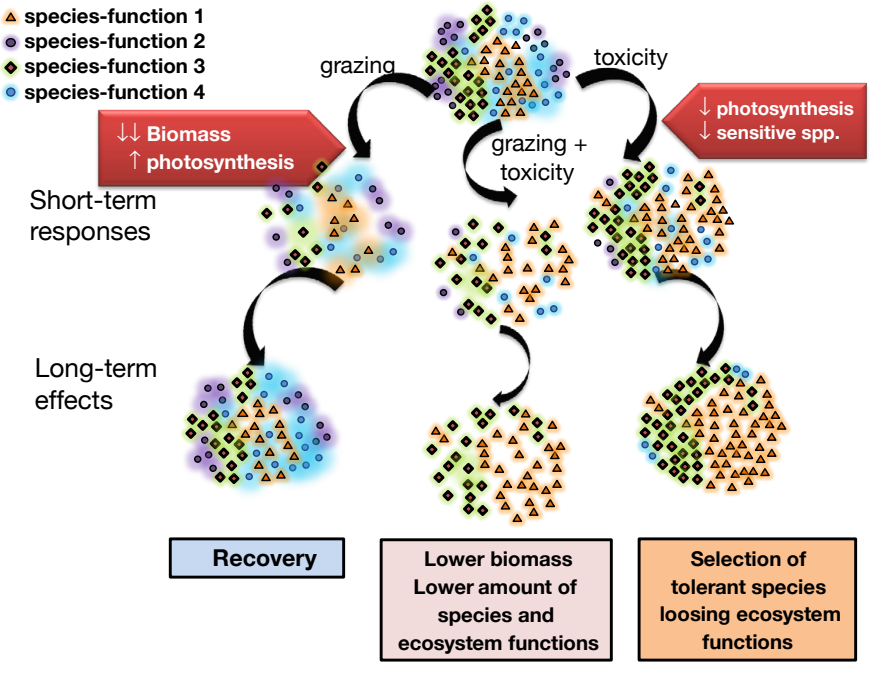


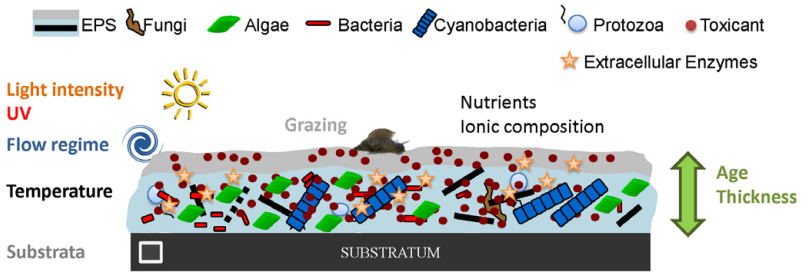
Figure 7.3 General model of the individual and combined effects of grazing and toxicity on biofilms. Based on a simple four-species biofilm model, it is expected that toxicity will constrain the ability of the community to recover from grazing pressure. In addition to the selection pressure exerted by toxicity, causing a reduction in activity (i.e. photosynthesis) and an increase in the relative abundance of the most tolerant species (sp1 and sp3), the reduction in population size caused by the non-selective effect of grazing on biofilms, will increase the risk of extinction for the less abundant species. Overall, grazing and toxicity will have cumulative negative effects on biofilms causing a reduction in the number of species, the biomass and ecosystem functions.

aquatic ecosystems, environmental parameters, such as light intensity, flow regime or temperature, strongly influence biofilm structure and function (Chapter 1) and these factors can have a critical effect on biofilm community response to contamination (Fig. 7.4).

Light intensity and regime is highly variable in the field due to seasonal variations and/or changes in riparian vegetation. Nevertheless, light is the first energy source for the autotrophic component of biofilm and therefore modulates not only biofilm structure and function but also biofilm response to herbicides and metals, as shown by several authors at both laboratory and field scale (Guasch *et al.*, 2003; Laviale *et al.*, 2010; Bonnineau *et al.*, 2012; Bonet *et al.*, 2013). Not only was biofilm grown under high light intensity more sensitive to the herbicide atrazine (field study, Guasch *et al.*, 2003), but it was also more tolerant to glyphosate (laboratory study, Bonnineau *et al.*, 2012).

While flow regime can affect chemical bioavailability (Osorio *et al.*, 2014), this highly variable abiotic factor can also modulate biofilm structure and function (Graba *et al.*, 2013). Therefore, the flow regime under which biofilm is grown is also susceptible to alter the capacity of biofilm to cope with chemical toxicity. For instance, a simulated drought event in artificial streams reduced biofilm capacity to recover from a subsequent 48 hour exposure to a bactericide (87 µg/l of triclosan) at both structural (high bacterial mortality) and functional level (reduced phosphate uptake) (Proia *et al.*, 2013b). Villeneuve *et al.* (2011) also showed that biofilms grown under a turbulent flow regime have a higher sensitivity to pesticides than biofilms grown under a laminar flow regime.

The influence of other factors like sediment deposition (Magbanua *et al.*, 2013), temperature (Larras *et al.*, 2013), nutrient concentration (Tlili *et al.*, 2010) or salinization (Rotter *et al.*, 2013)



Modulating environmental factors	Biofilm	Toxicant effect
↕	Mortality	⇑
↕	Biomass	⇓
■ ↕	Species composition	Change
	Photosynthesis	⇓
	Oxidative stress	⇑
↕	Food quality for grazers	⇓

Figure 7.4 Interactions between environmental factors and contamination in river biofilms. The main abiotic factors modulating biofilm structure and function are indicated in a schematic view of a biofilm (adapted from Romani, 2010). Environmental parameters and toxicants are likely to affect similar biofilm parameters, as indicated in the table; the expected negative impact of a toxicant is indicated by an arrow.

on biofilm response to pollutants has also been investigated (Fig 7.4).

These previous studies have shown how environmental parameters can constrain community capacity to respond to a pollutant but also to recover from contamination exposure. To better understand ecosystem responses to contamination, it is essential to take into account these parameters in toxicity assessment. Since the influence of abiotic factors on biofilm structure and function has been intensively investigated in ecology, the use of biofilm in ecotoxicology appears then as a realistic approach, in which environmental parameters can be integrated into toxicity assessment.

Conclusions and future recommendations

Biofilms are nowadays one of the principal targets of community ecotoxicology with a high potential for future uses in ecotoxicology. A large set of methods derived from biofilm ecology have successfully been applied in ecotoxicology providing a diverse and comprehensive toolbox. On the one hand, our ability to quantify the effects of pollution on different biofilm components, allows us to evaluate the direct effects of pollutants on the most sensitive community (e.g. algae in the case of herbicides or bacteria for antibiotics) and also their indirect effects on the rest of biofilm components and on higher trophic levels because all of them are closely related through biological interactions. For example, the model presented for biofilms exposed to toxicants under grazing pressure exemplifies the advantage of using complex biological models like biofilms and their grazers to improve our ability to predict the effects of pollution in multiple-stress scenarios (Fig. 7.3). On the other hand, enormous progress has been made regarding sensitivity. The application of early warning systems, for example the study of AEAs in whole biofilms, may allow us to detect early responses of the community by the activation of mechanisms of defence towards toxicity. In terms of analytical chemistry, different methods have been refined to quantify low concentrations of a large panel of chemicals in biota, including biofilm samples. In addition to metals, recent

investigations have shown that many organic pollutants have a tendency to adsorb and/or be uptaken in biofilms, acting as 'natural passive samplers'. Biofilms are also a site for biotransfomation and/or transfer of chemicals to other aquatic organisms, supporting a more generalized use of biofilm samples in environmental chemistry. This methodological progress is also visible in terms of new applications like the use of biofilms to investigate nanoparticle toxicity.

The set of biofilm endpoints described (Table 7.1) provides a powerful toolbox covering the expected responses of biofilms to pollution at different temporal scales: from early responses to acute exposure (e.g. by the activation of mechanisms of detoxification, the inhibition of photosynthesis or respiration), to long-term effects after chronic exposure (e.g. extinction of the most sensitive species and changes in the whole community structure). It is important to highlight the potential that different molecular approaches may have on our ability to detect the effects of pollution on the diversity of species of the different biofilm components (Table 7.1). In contrast to the study of some of the biofilm components, such as algae, with a long tradition in taxonomy (i.e. the use of diatom species composition as biological indicators; see Chapter 6), assessing the effects of toxicity on the species composition of other biofilm components is less common (i.e. bacteria). In this regard, the application of molecular tools may contribute to overcome this limitation, understood, however, as a complement of rather than a substitute for microscope observation classical taxonomy.

Based on the principles of ecotoxicology and their progress as a scientific discipline, there has been an increasing interest in linking chemical pollution with ecosystem health. In addition to biofilm endpoints, which are biomarkers of exposure, biofilm ecology provides an opportunity to link exposure with ecosystem functioning. Classical biofilm processes, such as nutrient uptake and community metabolism commonly investigated in ecosystem ecology, allows the ecological relevance of the observed effects from community to whole ecosystem integrity to be increased.

It is also important to point out that biofilm ecotoxicology has also benefited from the fast progress of genetics. As an example, metagenomics is envisaged as a promising approach for targeting the effect of contaminants on specific biofilm functions, and the microorganism responsible behind them.

While biofilm ecotoxicology studies have inherited the methods and basics of biofilm ecology and community ecotoxicology, a general framework to formulate a hypothesis about the response of this model of an aquatic community to human perturbations is still lacking. As shown in this review, a large set of methods has been refined and validated. Bearing this in mind, biofilm ecotoxicology should now focus on providing the theoretical background for understanding the complex set of responses of natural communities to pollution. This knowledge should also be the basis to guide the selection of the most appropriate tools and the development of new approaches for a better detection of the impact of pollution on aquatic life.

Acknowledgements

This study has been carried out with financial support from the French National Research Agency (ANR) in the framework of the Investments for the Future Programme, within the Cluster of Excellence COTE (ANR-10-LABX-45) and ANR CESA PoToMAC (ANR-11-CESA-0022). Financial support was also provided by the Spanish Science and Education Ministry (project CTM2009-14111-CO2–01) and the Spanish Economy and Competitiveness Ministry (project CGL2013–43822-R).

References

Aguilera, J., Bischof, K., Karsten, U., Hanelt, D., and Wiencke, C. (2002). Seasonal variation in ecophysiological patterns in macroalgae from an Arctic fjord. II. Pigment accumulation and biochemical defence systems against high light stress. Mar. Biol. *140*, 1087–1095.

Ancion, P.Y., Lear, G., and Lewis, G.D. (2010). Three common metal contaminants of urban runoff (Zn, Cu and Pb) accumulate in freshwater biofilm and modify embedded bacterial communities. Environ. Pollut. *158*, 2738–2745.

Ancion, P.Y., Lear, G., Dopheide, A., and Lewis, G.D. (2013). Metal concentrations in stream biofilm and sediments and their potential to explain biofilm microbial community structure. Environ. Pollut. *173*, 117–124.

Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., and Pesce, S. (2012). Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. Aquat. Toxicol. *122–123*, 197–205.

Battin, T.J., Kaplan, L.A., Newbold, J.D., and Hansen, C.M.E. (2003). Contributions of microbial biofilms to ecosystem processes in stream mesocosms. Nature *426*, 439–442.

Bergquist, B.L., and Bovee, E.C. (1976). Cadmium: quantitative methodology and study of its effect upon the locomotor rate of Tetrahymena pyriformis. Acta Protozool. *15*, 47.

Besemer, K., Peter, H., Logue, J.B., Langenheder, S., Lindström, E.S., Tranvik, L.J., and Battin, T.J. (2012). Unraveling assembly of stream biofilm communities. ISME J. *6*, 1459–1468.

Blanck, H., Admiraal, W., Cleven, R.F.M.J., Guasch, H., Van den Hoop, M.A.G.T., Ivorra, N., Nyström, B., Paulsson, M., Petterson, R.P., Sabater, S., and Tubbing, G.M.J. (2003). Variability in zinc tolerance, measured as incorporation of radio-labeled carbon dioxide and thymidine, in periphyton communities sampled from 15 European river stretches. Arch. Environ. Contam. Toxicol. *44*, 17–29.

Boivin, M.E.Y., Massieux, B., Breure, A.M., Greve, G.D., Rutgers, M., and Admiraal, W. (2006). Functional recovery of biofilm bacterial communities after copper exposure. Environ. Pollut. *140*, 239–246.

Bonet, B., Corcoll, N., and Guasch, H. (2012). Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms. Ecotox. Environ. Safe. *80*, 172–178.

Bonet, B., Corcoll, N., Acuña, V., Sigg, L., Behra, R., and Guasch, H. (2013). Seasonal changes in antioxidant enzyme activities of freshwater biofilms in a metal polluted Mediterranean stream. STOTEN. *444*, 60–72.

Bonet, B., Corcoll, N., Tlili, A., Morin, S., and Guasch, H. (2014). Antioxidant enzyme activities in biofilms as biomarker of Zn pollution in a natural system: an active bio-monitoring study. Ecotox. Environ. Safe. *103*, 82–90.

Bonnineau, C., Bonet, B., Corcoll, N., and Guasch, H. (2011). Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. Ecotoxicology *20*, 293–303.

Bonnineau, C., Moeller, A., Barata, C., Bonet, B., Proia, L., Sans-Piché, F., Schmitt-Jansen, M., Guasch, H., and Segner, H. (2012). Advances in the Multibiomarker Approach for Risk Assessment in Aquatic Ecosystems. In Emerging and Priority Pollutants in Rivers Bringing Science into River Management Plans, Guasch, H., Ginebreda, A., and Geiszinger, A. eds. The Handbook of Environmental Chemistry. (Springer, Berlin/ Heidelberg, Germany), pp. 147–179.

Bonnineau, C., Tlili, A., Faggiano, L., Montuelle, B., and Guasch, H. (2013). The use of antioxidant enzymes in freshwater biofilms: Temporal variability vs. toxicological responses. Aquat. Toxicol. *136–137*, 60–71.

Bopp, S.K., and Lettleri, T. (2007). Gene regulation in the marine diatom Thalassiosira pseudonana upon exposure to polycyclic aromatic hydrocarbons (PAHs). Gene. *396*, 293–302.

Bricheux, G., Morin, L., Le Moal, G., Coffe, G., Balestrino, D., Charbonnel, N., Bohatier, J., and Forestier, C. (2013). Pyrosequencing assessment of prokaryotic and eukaryotic diversity in biofilm communities from a French river. Microbiologyopen. *2*, 402–414.

Bringmann, G., and Kühn, R. (1980). Comparison of the toxicity thresholds of water pollutants to bacteria, algae and protozoa in the cell multiplication inhibition test. Water Res. *14*, 231–241.

Brummer, I.H., Fehr, W., and Wagner-Dobler, I. (2000). Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl. Environ. Microbiol. *66*, 3078–3082.

Bunch, A.R., and Bernot, M.J. (2011). Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. Ecotoxicology *20*, 97 – 109.

Bundschuh, M., Zubrod, J.P., Kosol, S., Maltby, L., Stang, C., Duester, L., and Schulz, R. (2011). Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquat. Toxicol. *104*, 32–37.

Butow, B.J., Wynne, D., and Tel-Or, E. (1997). Antioxidative protection of Peridinium gatunense in Lake Kinneret: seasonal and daily variation. J. Phycol. *33*, 780–786.

Cairns, Jr.J., and Pratt, J.R. (1993). Trends in ecotoxicology. STOTEN (Proceedings of the 2nd European Conference on Ecotoxicology) *134*, 7–22.

Canals, O., Salvadó, H., Auset, M., Hernández, C., and Malfeito, J.J. (2013). Microfauna communities as performance indicators for an A/O Shortcut Biological Nitrogen Removal moving-bed biofilm reactor. Water Res. *47*, 3141–3150.

Chénier, M.R., Beaumier, D., Fortin, N., Roy, R., Driscoll, B.T., Lawrence, J.R., and Greer, C.W. (2006). Influence of nutrient inputs, hexadecane and temporal variations on denitrification and community composition of river biofilms. Appl. Environ. Microbiol. *72*, 575–584.

Clements, W.H., and Rohr, J.R. (2009). Community responses to contaminants: Using basic ecological principles to predict ecotoxicological effects. Environ. Toxicol. Chem. *28*, 1789–1800.

Conley, J.M., Funk, D.H., Cariello, N.J., and Buchwalter, D.B. (2011). Food rationing affects dietary selenium bioaccumulation and life cycle performance in the mayfly Centroptilum triangulifer. Ecotoxicology *20*, 1840–1851.

Corcoll, N., Ricart, M., Franz, S., Sans-Piché, F., Schmitt-Jansen, M., and Guasch, H. (2012a). The Use of Photosynthetic Fluorescence Parameters from Autotrophic Biofilms for Monitoring the Effect of Chemicals in River Ecosystems. In Emerging and Priority Pollutants in Rivers, Guasch, H., Ginebreda, A., and Geiszinger, A. eds. (Springer, Berlin/ Heidelberg, Germany), pp. 85–115.

Corcoll, N., Bonet, B., Leira, M., Montuelle, B., Tlili, A., and Guasch, H. (2012b). Light History Influences the Response of Fluvial Biofilms to Zn Exposure. J. Phycol. *48*, 1411–1423.

Corcoll, N., Bonet, B., Morin, S., Tlili, A., Leira, M., and Guasch, H. (2012c). The effect of metals on photosynthesis processes and diatom metrics of biofilm from a metal-contaminated river: A translocation experiment. Ecol. Indic. *18*, 620–631.

Culp, J.M., Podemski, C.L., Cash, K.J., and Lowell, R.B. (1996). Utility of field-based artificial streams for assessing effluent effects on riverine ecosystems. J. Aquat. Ecosyst. Health. *5*, 117–124.

Culp, J.M., Lowell, R.B., and Cash, K.J. (2000). Integrating mesocosm experiments with field and laboratory studies to generate weight-of-evidence risk assessments for large rivers. Environ. Toxicol. Chem. *19*, 1167–1173.

Dewez, D., Geoffroy, L., Vernet, G., and Popovic, R. (2005). Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga Scenedesmus obliquus. Aquat. Toxicol. *74*, 150–159.

Díaz, S., Martín-González, A., and Gutiérrez, J.C. (2006). Evaluation of heavy metal acute toxicity and bioaccumulation in soil ciliated protozoa. Environ. Int. *32*, 711–717.

Dopheide, A., Lear, G., Stott, R., and Lewis, G. (2008). Molecular characterization of ciliate diversity in stream biofilms. Appl. Environ. Microbiol. *74*, 1740–1747.

Dopheide, A., Lear, G., Stott, R., and Lewis, G. (2009). Relative diversity and community structure of ciliates in stream biofilms according to molecular and microscopy methods. Appl. Environ. Microbiol. *75*, 5261–5272.

Dorigo, U., Bérard, A., Rimet, F., Bouchez, A., and Montuelle, B. (2010). In situ assessment of periphyton recovery in a river contaminated by pesticides. Aquat. Toxicol. *98*, 396–406.

Duarte, S., Pascoal, C., Alves, A., Correia, A., and Cássio, F. (2008). Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. Freshwater Biol. *53*, 91–101.

Duong, T.T., Morin, S., Herlory, O., Feurtet-Mazel, A., Coste, M., and Boudou, A. (2008). Seasonal effects of cadmium accumulation in periphytic diatom communities of freshwater biofilms. Aquat. Toxicol. *90*, 19–28.

Dutour, C., Bonnet, R., Marchandin, H., Boyer, M., Chanal, C., Sirot, D., and Sirot, J. (2002). CTX-M-1, CTX-M-3 and CTX-M-14 beta-lactamases from Enterobacteriaceae isolated in France. Antimicrob. Agents. Chemother. *46*, 534–537.

Edreva, A. (2005). Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach. Agr. Ecosyst. Environ. *106*, 119–133.

Evans-White, M.A., and Lamberti, G.A. (2009). Direct and indirect effects of a potential aquatic contaminant on grazer–algae interactions. Environ. Toxicol. Chem. *28*, 418–426.

Eriksson, K.M., Antonelli, A., Nilsson, R.H., Clarke, A.K., and Blanck, H. (2009). A phylogenetic approach to detect selection on the target site of the antifouling compound irgarol intolerant periphyton communities. Environ. Microbiol. *11*, 2065–2077.

Falasco, E., Bona, F., Badino, G., Hoffmann, L., and Ector, L. (2009). Diatom teratological forms and environmental alterations: a review. Hydrobiologia *623*, 1–35.

Fechner, L.C., Dufour, M., and Gourlay-Francé, C. (2012). Pollution-induced community tolerance of freshwater biofilms: measuring heterotrophic tolerance to Pb using an enzymatic toxicity test. Ecotoxicology *21*, 2123–2131.

Feng, H., Huang, X., Zhang, Q., Wei, G., Wang, X., and Kang, Z. (2012). Selection of suitable inner reference genes for relative quantification expression of microRNA in wheat. Plant Physiol. Biochem. *51*, 116–122.

Fernandez-Leborans, G., and Novillo, A. (1995). The effects of cadmium on the successional stages of a freshwater protozoa community. Ecotox. Environ. Safe. *31*, 29–36.

Fischer, B.B., Pomati, F., and Eggen, R.I.L. (2013). The toxicity of chemical pollutants in dynamic natural systems: The challenge of integrating environmental factors and biological complexity. STOTEN. *449*, 253–259.

Flores, L., Banjac, Z., Farré, M., Larrañaga, A., Mas-Martí, E., Muñoz, I., Barceló, D., and Elosegi, A. (2014). Effects of a fungicide (imazalil) and an insecticide (diazinon) on stream fungi and invertebrates associated with litter breakdown. STOTEN. *476–477*, 532–541.

Fox, R.E., Zhong, Z., Krone, S., and Top, E.M. (2008). Spatial structure and nutrients promote invasion of IncP-1 plasmids in bacterial populations. ISME J. *2*, 1024–1039.

Geiszinger, A., Bonnineau, C., Faggiano, L., Guasch, H., Lopez-Doval, J.C., Proia, L., Ricart, M., Ricciardi, F., Romani, A., Rotter, S., *et al.* (2009). The relevance of the community approach linking chemical and biological analyses in pollution assessment. Trends Anal. Chem. (TrAC). *28*, 619–626.

Gessner, M.O., and Chauvet, E. (2002). A case for using litter breakdown to assess functional stream integrity. Ecol. Appl. *12*, 498–510.

Gomiero, A., Sforzini, S., Dagnino, A., Nasci, C., and Viarengo, A. (2012). The use of multiple endpoints to assess cellular responses to environmental contaminants in the interstitial marine ciliate Euplotes crassus. Aquat. Toxicol. *114–115*, 206–216.

Gracia, M.P., Salvadó, H., Rius, M., and Amigó, J.M. (1994). Effects of copper on ciliate communities from activated sludge plants. Acta Protozool. *33*, 219–226.

Guasch, H., Admiraal, W., and Sabater, S. (2003). Contrasting effects of organic and inorganic toxicants on freshwater periphyton. Aquat. Toxicol. *64*, 165–175.

Guasch, H., Serra, A., Corcoll, N., Bonet, B., and Leira, M. (2010a). Metal ecotoxicology in fluvial biofilms: potential influence of water scarcity. In Sabater, S., and

Barceló, D. (eds), Water Scarcity in the Mediterranean: Perspectives Under Global Change (Springer, Berlin, Germany), pp. 41–54.

Guasch, H., Atli, G., Bonet, B., Corcoll, N., Leira, M., and Serra, A. (2010b). Discharge and the response of biofilms to metal exposure in Mediterranean rivers. Hydrobiologia *657*, 143–157.

Guasch, H., Bonet, B., Bonnineau, C., Corcoll, N., López-Doval, J., Muñoz, I., Ricart, M., Serra, A., and Clements, W. (2012). How to link field observations with causality? Field and experimental approaches linking chemical pollution with ecological alterations. In Emerging and Priority Pollutants in Rivers. Guasch, H., Ginebreda, A., and Geiszinger, A., eds (Springer, Berlin/Heidelberg, Germany), pp. 181–218.

Guasch, H., Ricart, M., López-Doval, J., Bonnineau, C., Proia, L., Morin, S., Muñoz, I., Romaní, A.M., and Sabater, S. (2015). Interactive effects of natural biotic stressors and chemicals on periphyton communities: the influence of grazing on triclosan toxicity. Freshwater Biol. In press.

Guo, R., Lee, M.A., and Ki, J.S. (2013). Different transcriptional responses of heat shock protein 70/90 in the marine diatom Ditylum brightwellii exposed to metal compounds and endocrine-disrupting chemicals. Chemosphere. *92*, 535–543.

Hall, E.K., Besemer, K., Kohl, L., Preiler, C., Scheider, T., Riedel, K., Wanek, W., and Battin, T. (2012). Effects of resource chemistry on the composition and function of stream hyporheic biofilms. Front. Microbiol. *14*, 3–35.

Handy, R.D., van den Brink, N., Chappell, M., Muhling, M., Behra, R., Dusinska, M., Simpson, P., Ahtiainen, J., Jha, A.N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T.F., and Riediker, M. (2012). Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? Ecotoxicology *21*, 933–972.

Headley, J.V., Gandrass, J., Kuballa, J., Peru, K.M., and Gong, Y. (1998). Rates of sorption and artitioning of contaminants in river biofilm. Environ. Sci. Technol. *32*, 3968–3973.

Hering, D., Borja, A., Carstensen, J., Carvalho, L., Elliott, M., Feld, C.K., Heiskanen, A.S., Johnson, R.K., Moe, J., Pont, D., Solheim, A.L., and van de Bund, W. (2010). The European Water Framework Directive at the age of 10: A critical review of the achievements with recommendations for the future. STOTEN. *19*, 4007–4019.

Hill, B.H., Lazorchak, J.M., McCormick, F.H., and Willingham, W.T. (1997). The effects of elevated metals on benthic community metabolism in a rocky mountain stream. Environ. Pollut. *95*, 183–190.

Holding, K.L., Gill, R.A., and Carter, J. (2003). The relationship between epilithic periphyton (Biofilm) bound metals and metals bound to sediments in freshwater systems. Environ. Geochem. Hlth. *25*, 87–93.

Holtze, M.S., Ekelund, F., Rasmussen, L.D., Jacobsen, C.S., and Johnsen, K. (2003). Prey-predator dynamics in communities of culturable soil bacteria and protozoa: differential effects of mercury. Soil Biol. Biochem. *35*, 1175–1181.

Irwing, E.C., Baird, D.J., and Culp, J.M. (2003). Ecotoxicological responses of the Mayfly *Baetis tricaudatus* to dietary and waterborne cadmium: implications for toxicity testing. Environ. Toxicol. Chem. *22*, 1058–1064.

Kim, K.S., Funk, D.H., and Buchwalter, D.B. (2012). Dietary (periphyton) and aqueous Zn bioaccumulation dynamics in the mayfly Centroptilum triangulifer. Ecotoxicology *21*, 2288–2296.

Kim Tiam, S., Feurtet-Mazel, A., Delmas, F., Mazzella, N., Morin, S., Daffe, G., and Gonzalez, P. (2012). Development of q-PCR approaches to assess water quality: Effects of cadmium on gene expression of the diatom Eolimna minima. Water Res. *46*, 934–942.

Kulacki, K.J., Cardinale, B.J., Keller, A.A., Bier, R., and Dickson, H. (2012). How do stream organisms respond to, and influence, the concentration of Titanium dioxide nanoparticles? A mesocosm study with algae and herbivores. Environ. Toxicol. Chem. *31*, 2414–2422.

Lara, E., Berney, C., Ekelund, F., Harms, H., and Chatzinotas, A. (2007). Molecular comparison of cultivable protozoa from a pristine and a polycyclic aromatic hydrocarbon polluted site. Soil Biol. Biochem. *39*, 139–148.

Larras, F., Lambert, A.-S., Pesce, S., Rimet, F., Bouchez, A., and Montuelle, B. (2013). The effect of temperature and a herbicide mixture on freshwater periphytic algae. Ecotox. Environ. Safe. *98*, 162–170.

Laviale, M., Prygiel, J., and Créach, A. (2010). Light modulated toxicity of isoproturon toward natural stream periphyton photosynthesis: a comparison between constant and dynamic light conditions. Aquat. Toxicol. *97*, 334–342.

Laviale, M., Morin, S., and Créach, A. (2011). Short term recovery of periphyton photosynthesis after pulse exposition to the PSII inhibitors atrazine and isoproturon. Chemosphere. *84*, 731–734.

Lawrence, J.R., Kopf, G., Headley, J.V., and Neu, T.R. (2001). Sorption and metabolism of selected herbicides in river biofilm communities. Can. J. Microbiol. *47*, 634–641.

Lawrence, J.R., Chénier, M., Roy, R., Beaumier, D., Fortin, N., Swerhone, G.D.W., Neu, T.R., and Greer, C.W. (2004). Microscale and molecular assessment of the impacts of nickel, nutrients, and oxygen level on river biofilm communities. Appl. Environ. Microbiol. *70*, 4326–4339.

Lawrence, J.R., Swerhone, G.D.W., Topp, E., Korber, D.R., Neu, T.R., and Wassenaar, L.I. (2007). Structural and functional responses of river biofilm communities to the nonsteroidal anti-inflammatory diclofenac. Environ. Toxicol. Chem. *26*, 573–582.

Ledger, M.E., Harris, R.M.L., Armitage, P.D., and Milner, A.M. (2009). Realism of model ecosystems: an evaluation of physicochemistry and macroinvertebrate assemblages in artificial streams. Hydrobiologia *617*, 91–99.

Lesser, M.P. (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. *68*, 253–278.

Li, L., Zhao, J., and Tang, X. (2010). Ultraviolet irradiation induced oxidative stress and response of antioxidant system in an intertidal macroalgae *Corallina officinalis* L.J. Environ. Sci. *22*, 716–722.

Li, M., Costello, D.M., and Burton, G.M. (2012). Interactive effects of phosphorus and copper on Hyalella azteca via periphyton in aquatic ecosystems. Ecotox. Environ. Safe. *83*, 41–46.

López-Doval, J.C., Ricart, R., Guasch, H., Romaní, A.M., Sabater, S., and Muñoz, I. (2010). Does Grazing Pressure Modify Diuron Toxicity in a Biofilm Community? Arch. Environ. Con. Tox. *58*, 955–962.

Luís, A.T., Teixeira, P., Almeida, S.F.P., Matos, J.X., and da Silva, E.F. (2011). Environmental impact of mining activities in the Lousal area (Portugal): Chemical and diatom characterization of metal-contaminated stream sediments and surface water of Corona stream. STOTEN, *409*, 4312–4325.

Lundqvist, A., Bertilsson, S., and Goedkoop, W. (2012). Interactions with DOM and biofilms affect the fate and bioavailability of insecticides to invertebrate grazers. Ecotoxicology *21*, 2398–2408.

Madoni, P. (2000). The acute toxicity of nickel to freshwater ciliates. Environ. Pollut. *109*, 53–59.

Madoni, P. (2011). Protozoa in wastewater treatment processes: A minireview. Ital. J. Zool. *78*, 3–11.

Magbanua, F.S., Townsend, C.R., Hageman, K.J., Lange, K., Lear, G., and Lewis, G.D., and Matthaei, C.D. (2013). Understanding the combined influence of fine sediment and glyphosate herbicide on stream periphyton communities. Water Res. *47*, 5110–5120.

Maharana, D., Jena, K., Pise, N.M., and Jagtap, T.G. (2010). Assessment of oxidative stress indices in a marine macro brown alga Padina tetrastromatica (Hauck) from comparable polluted coastal regions of the Arabian Sea, West coast of India. J. Environ. Sci. *22*, 1413–1417.

Marti, E., Jofre, J., and Balcazar, J.L. (2013). Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS One *8*, e78906.

Martín-González, A., Borniquel, S., Díaz, S., Ortega, R., and Gutiérrez, J.C. (2005). Ultrastructural alterations in ciliated protozoa under heavy metal exposure. Cell Biol. Int. *29*, 119–126.

Martín-González, A., Díaz, S., Borniquel, S., Gallego, A., and Gutiérrez, J.C. (2006). Cytotoxicity and bioaccumulation of heavy metals by ciliated protozoa isolated from urban wastewater treatment plants. Res. Microbiol. *157*, 108–118.

Meylan, S., Behra, R., and Sigg, L. (2003). Accumulation of copper and zinc in periphyton in response to dynamic variations of metal speciation in freshwater. Environ. Sci. Technol. *37*, 5204–5212.

Meylan, S., Behra, R., and Sigg, L. (2004). Influence of metal speciation in natural freshwater on bioaccumulation of copper and zinc in periphyton: a microcosm study. Environ. Sci. Technol. *38*, 3104–3111.

Mittler, R. (2002). Oxidative stress, antioxidant and stress tolerance. Plant. Sci. *7*, 405–410.

Moreirinha, C., Duarte, S., Pascoal, C., and Cássio, F. (2011). Effects of cadmium and phenanthrene mixtures on aquatic fungi and microbially mediated leaf litter decomposition. Arch. Environ. Contam. Toxicol. *61*, 211–219.

Morin, S., Duong, T.T., Dabrin, A., Coynel, A., Herlory, O., Baudrimont, M., Delmas, F., Durrieu, G., Schäfer, J., Winterton, P., Blanc, G., and Coste, M. (2008a). Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. Environ. Pollut. *151*, 532–542.

Morin, S., Duong, T.T., Herlory, O., Feurtet-Mazel, A., and Coste, M. (2008b). Cadmium toxicity and bioaccumulation in freshwater biofilms. Arch. Environ. Contam. Toxicol. *54*, 173–186.

Morin, S., Bottin, M., Mazzella, N., Macary, F., Delmas, F., Winterton, P., and Coste, M. (2009). Linking diatom community structure to pesticide input as evaluated through a spatial contamination potential (Phytopixal): A case study in the Neste river system (South-West France). Aquat. Toxicol. *94*, 28–39.

Morin, S., Cordonier, A., Lavoie, I., Arini, A., Blanco, S., Duong, T.T., Tornés, E., Bonet, B., Corcoll, N., Faggiano, L., *et al.* (2012). Consistency in diatom response to metal-contaminated environments. In Emerging and Priority Pollutants in Rivers: Bringing Science into River Management Plans (Handbook of Environmental Chemistry), Guasch, H., Ginebreda. A., and Geiszinger, A., eds. (Springer, Heidelberg, Germany), pp. 117–146.

Morin, S., Corcoll, N., Bonet, B., Tlili, A., and Guasch, H. 2014. Diatom responses to zinc contamination along a Mediterranean river. Plant Ecol. Evol. *147*, 325–332.

Mortimer, M., Kasemets, K., and Kahru, A. (2010). Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology *269*, 182–189.

Muñoz, I., Real, M., Guasch, H., Navarro, E., and Sabater, S. (2001). Effects of atrazine on periphyton under grazing pressure. Aquat. Toxicol. *55*, 239–249.

Nalecz-Jawecki, G., Demkowicz-Dobrzahski, K., and Sawicki, J. (1993). Protozoan *Spirostomum ambiguum* as a highly sensitive bioindicator for rapid and easy determination of water quality. STOTEN. *134*, 1227–1234.

Navarro, E., Robinson, C.T., and Behra, R. (2008). Increased tolerance to ultraviolet radiation (UVR) and cotolerance to cadmium in UVR-acclimatized freshwater periphyton. Limnol. Oceanogr. *53*, 1149–1158.

Nicolau, A., Dias, N., Mota, M., and Lima, N. (2001). Trends in the use of protozoa in the assessment of wastewater treatment. Res. Microbiol. *152*, 621–630.

Nicolau, A., Martins, M.J., Mota, M., and Lima, N.l. (2005). Effect of copper in the protistan community of activated sludge. Chemosphere. *58*, 605–614.

Niederlehner, B.R., and Cairns, J. Jr. (1990). Effects of ammonia on periphytic communities. Environ. Pollut. *66*, 207–221.

Niederlehner, B.R., and Cairns, J. Jr. (1992). Community response to cumulative toxic impact: effects of acclimation on Zinc tolerance of Aufwuchs. Can. J. Fish. Aquat. Sci. *49*, 2155–2163.

Okamoto, O.K., Pinto, E., Latorre, L.R., Bechara, E.J.H., and Colepicolo, P. (2001). Antioxidant Modulation in Response to Metal-Induced Oxidative Stress in Algal Chloroplasts. Arch. Environ. Contam. Toxicol. *40*, 18–24.

Osorio, V., Proia, L., Ricart, M., Pérez, S., Ginebreda, A., Cortina, J.L., Sabater, S., and Barceló, D. (2014). Hydrological variation modulates pharmaceutical levels and biofilm responses in a Mediterranean river. STOTEN. *472*, 1052–1061.

Paje, M.L.F., Kuhlicke, U., Winkler, M., and Neu, T.R. (2002). Inhibition of lotic biofilms by Diclofenac. Appl. Microbiol. Biotechnol. *59*, 488–492.

Park, J., Congeevaram, S., Ki, D.W., and Tiedje, J.M. (2006). Use of stable isotope probing in selectively isolating target microbial community genomes from environmental samples for enhancing resolution in ecotoxicological assessment. Mol. Cell. Toxicol. *2*, 11–14.

Paulsson, M., Nyström, B., and Blanck, H. (2000). Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosm study. Aquat. Toxicol. *47*, 243–257.

Pesce, S., Martin-Laurent, F., Rouard, N., and Montuelle, B. (2009). Potential for microbial diuron mineralisation in a small wine-growing watershed: from treated plots to lotic receiver hydrosystem. Pest. Manag. Sci. *65*, 651–657.

Proia, L., Morin, S., Peipoch, M., Romaní, A.M., and Sabater, S. (2011). Resistance and recovery of river biofilms receiving short pulses of Triclosan and Diuron. Sci. Total Environ. *409*, 3129–3137.

Proia, L., Cassiò, F., Pascoal, C., Tlili, A., and Romaní, A.M. (2012a). The use of attached microbial communities to assess ecological risks of pollutants in river ecosystems. The role of heterotrophs. In Emerging and Priority Pollutants in Rivers: Bringing Science into River Management Plans, Guasch, H., Ginebreda, A., and Geiszinger, A. eds (Springer Verlag, Berlin/Heidelberg, Germany) pp. 55–83.

Proia, L., Romaní, A.M., and Sabater, S. (2012b). Nutrients and light effects on stream biofilms: a combined assessment with CLSM, structural and functional parameters. Hydrobiologia *695*, 281–291.

Proia, L., Lupini, G., Osorio, V., Pérez, S., Barceló, D., Schwartz, T., Amalfitano, S., Fazi, S., Romaní, A.M., and Sabater, S. (2013a). Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river. Chemosphere *92*, 1126–1135.

Proia, L., Vilches, C., Boninneau, C., Kantiani, L., Farré, M., Romaní, A.M., Sabater, S., and Guasch, H. (2013b). Drought episode modulates the response of river biofilm to triclosan. Aquat. Toxicol. *127*, 36–45.

Real, M., Muñoz, I., Guasch, G., Navarro, E., and Sabater, S. (2003). The effect of copper exposure on a simple aquatic food chain. Aquat. Toxicol. *63*, 283–291.

Ricart, M., Barceló, D., Geiszinger, A., Guasch, H., de Alda, M.L., Romaní, A.M., Vidal, G., Villagrasa, M., and Sabater, S. (2009). Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. Chemosphere. *76*, 1392–1401.

Ricart, M., Guasch, H., Alberch, M., Barceló, D., Bonnineau, C., Geiszinger, A., Farré, M.L., Ferrer, J., Ricciardi, F., Romaní, A.M., Morin, S., Proia, L., Sala, L., Sureda, D., and Sabater, S. (2010). Triclosan persistence trough wastewater treatment plants and its potential toxic effects on river biofilms. Aquat. Toxicol. *100*, 346–353.

Rico, D., Martín-González, A., Díaz, S., de Lucas, P., and Gutiérrez, J.C. (2009). Heavy metals generate reactive oxygen species in terrestrial and aquatic ciliated protozoa. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. *149*, 90–96.

Romani, A.M. (2010). Freshwater biofilms. In Biofouling, Durr, S., and Thomason, J.C., eds. (Blackwell Publishing Ltd., Oxford, UK), pp. 137–153.

Rosi-Marshall, E.J., Kincaid, D.W., Bechtold, H.A., Royer, T.V., Rojas, M., and Kelly, J.J. (2013). Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilm. Ecol. Appl. *23*, 583–593.

Rotter, S., Heilmeier, H., Altenburger, R., and Schmitt-Jansen, M. (2013). Multiple stressors in periphyton – comparison of observed and predicted tolerance responses to high ionic loads and herbicide exposure. J. Appl. Ecol. *50*, 1459–1468.

Sabater, S., Navarro, E., and Guasch, H. (2002). Effects of copper on algal communities at different current velocities. J. Appl. Phycol. *14*, 391–398.

Sabater, S., Buchaca, T., Cambra, J., Catalan, J., Guasch, H., Ivorra, N., Munoz, I., Navarro, E., Real, M., and Romani, A.M. (2003). Structure and function of benthic algal communities in an extremely acid river. J. Phycol. *39*, 481–489.

Salvadó, H., Gracia, M.P., and Amigó, J.M. (1995). Capability of ciliated protozoa as indicators of effluent quality in activated sludge plants. Water Res. *29*, 1041–1050.

Salvadó, H., Gracia, M.P., Amigó, J.M., and Rius, M. (1997). Effects of Cadmium on growth and motility in *Euplotes aediculatus* isolated from Activated Sludge. Bull. Environ, Contam. Toxicol. *58*, 838–844.

Scandalios, J.G. (1993). Oxygen Stress and Superoxide Dismutases. Plant. Physiol. *101*, 7–12.

Scott, J.T., Back, J.A., Taylor, J.M., and King, R.S. (2008). Does nutrient enrichment decouple algal–bacterial production in periphyton? J. N. Am. Benthol. Soc. *27*, 332–344.

Selivanovskaya, S.Y., Petrov, A.M., Egorova, K.V., and Naumova, R.P. (1997). Protozoan and metazoan communities treating a simulated petrochemical industry wastewater in a rotating disc biological reactor. World J. Microb. Biot. *13*, 511–517.

Serra, A., Corcoll, N., and Guasch, H. (2009). Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history. Chemosphere. *74*, 633–641.

Shi, Y., Lu, Y., Meng, F., Guo, F., and Zheng, X. (2013). Occurrence of organic chlorinated pesticides and their ecological effects on soil protozoa in the agricultural

soils of North Western Beijing, China. Ecotoxicol. Environ. Safe. *92*, 123–128.

Sierra, V.M., and Gómez, N. (2010). Assessing the disturbance caused by an industrial discharge using field transfer of epipelic biofilm. STOTEN. *408*, 2696–2705.

Solé, M., Müller, I., Pecyna, M.J., Fetzer, I., Harms, H., and Schlosser, D. (2012). Differential Regulation by Organic Compounds and Heavy Metals of Multiple Laccase Genes in the Aquatic Hyphomycete Clavariopsis aquatica. Appl. Environ. Microb. *78*, 4732–4739.

Tlili, A., Bérard, A., Roulier, J.L., Volat, B., and Montuelle, B. (2010). PO43- dependence of the tolerance of autotrophic and heterotrophic biofilm communities to copper and diuron. Aquat. Toxicol. *98*, 165–177.

Tlili, A., Marechal, M., Montuelle, B., Volat, B., Dorigo, U., and Bérard, A. (2011a). Use of the MicroRespTM method to assess pollution induced community tolerance to metals for lotic biofilms. Environ. Pollut. *159*, 18–24.

Tlili, A., Corcoll, N., Bonet, B., Morin, M., Montuelle, B., Bérard, A., and Guasch, H. (2011b). In situ spatio-temporal changes in pollution-induced community tolerance to zinc in autotrophic and heterotrophic biofilm communities. Ecotoxicology *20*, 1823–1839.

Tolkkinen, M., Mykrä, H., Markkola, A.M., Aisala, H., Vuori, K.M., Lumme, J., Pirttilä, and A.M., and Muotka, T. (2013). Decomposer communities in human-impacted streams: species dominance rather than richness affects leaf decomposition. J. Appl. Ecol. *50*, 1142–1151.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., and Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. *64*, 178–189.

Vera, M.S., Fiori, E.D., Lagomarsino, L., Sinistro, R., Escaray, R., Iummato, M.M., Juárez, A., Molina, M. del C.R. de, Tell, G., and Pizarro, H. (2012). Direct and indirect effects of the glyphosate formulation Glifosato Atanor® on freshwater microbial communities. Ecotoxicology *21*, 1805–1816.

Villeneuve, A., Montuelle, B., and Bouchez, A. (2011). Effects of flow regime and pesticides on periphytic communities: evolution and role of biodiversity. Freshwater Biol. *56*, 2245–2259.

Wang, S.Y., Bernhardt, E.S., and Wright, J.P. (2014). Urban stream denitrifier communities are linked to lower functional resistance to multiple stressors associated with urbanization. Hydrobiologia *726*, 13–23.

Wolfe-Simon, F., Grzebyk, D., Schofield, O., and Falkowski, P.O. (2005). The role and evolution of Superoxide dismutases in algae. J. Phycol. *41*, 453–465.

Writer, J.H., Ryan, J.N., and Barber, L.B. (2011a). Role of biofilms in sorptive removal of steroidal hormones and 4-nonylphenol compounds from streams. Environ. Sci. Technol. *45*, 7275–7283.

Writer, J.H., Barber, L.B., Ryan, J.N., and Bradley, P.M. (2011b). Biodegradation and attenuation of steroidal hormones and alkylphenols by stream biofilms and sediments. Environ. Sci. Technol. *45*, 4370–4376.

Writer, J.H., Antweiler, R.C., Ferrer, I., Ryan, J.N., and Thurman, E.M. (2013). In-stream attenuation of neuro-active pharmaceuticals and their metabolites. Environ. Sci. Technol. *47*, 9781–9790.

Wunder, D.B., Bosscher, V.A., Cok, R.C., and Hozalski, R.M. (2011). Sorption of antibiotics to biofilm. Water Res. *45*, 2270–2280.

Xie, L., and Buchwalter, D.B. (2011). Cadmium exposure route affects antioxidant responses in the mayfly Centroptilum triangulifer. Aquat. Toxicol. *105*, 199–205.

Zhou, Q., Zhang, J., Fu, J., Shi, J., and Jiang, G. (2008). Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal. Chim. Acta *606*, 135–150.

Biofilm Development in Sewer Networks

8

Oriol Gutierrez, Guangming Jiang, Keshab Sharma and Zhiguo Yuan

Abstract

Wastewater collection systems, or sewers, are crucial sections of the urban water cycle where complex microbial, chemical and physicochemical processes take place. This chapter aims to give an overview of the diversity and importance of biofilms and bioreactions occurring in sewers, paying special attention to its detrimental effects. Sewer biofilms can be divided in two main classes:

1 Submerged biofilms: including activities of sulfate-reducing bacteria (SRB) responsible for the formation of sulfide (H_2S, an odorous, toxic and corrosion-inducer compound), Methanogenic Archaea (MA) responsible for the formation of methane (CH_4, an explosive and potent greenhouse gas) and the Fermentation processes that increase the two previous biofilms metabolism.
2 Unsubmerged biofilms: activities of biofilms growing on the gas phase of sewers that causes loss of concrete mass, cracking of the sewer pipes and ultimately, structural collapse. This process is known as microbially induced concrete corrosion (MICC).

The structure of sewer-biofilms and mechanisms for the control of its harmful effects are described.

Introduction

Wastewater collection systems, or sewers, consist of an underground network of physical structures-installations composed of pipelines, pump stations, manholes and channels that convey the wastewater from its source to the point where it is discharged. The discharge point is usually a Wastewater Treatment Plant (WWTP) but may also be natural environments (Fig. 8.1). Sewer systems are crucial in protecting public health as these prevent the spread of diseases by avoiding population exposure to the contaminated wastewater. By definition, wastewater is water that has been adversely affected in quality by anthropogenic influence, either from domestic households (such as showers, toilets and washing machines) or from industrial processes (Metcalf and Eddy, 2003). Thus composition of wastewater, although contains more than 99% of water, varies widely depending upon the source. Sewage can contain suspended solids (that can create sludge deposits and anaerobic conditions in sewers), biodegradable organics (proteins, carbohydrates and fats that can lead to the depletion of oxygen in water and develop septic conditions), pathogens, nutrients (nitrogen and phosphorus), priority pollutants (organic and inorganic compounds with acute toxicity, and heavy metals from industrial processes) and dissolved inorganics (such as calcium, sodium and sulfate).

Sewers are very important assets of the urban water systems. For instance, in Spain only, the sewer networks have an extension of around 89,900 kilometres (equivalent to twice the equator distance) that collect the wastewater generated from 8110 municipalities, covering 86% of the total population. Similarly, the length of sewer pipes are 1,200,000 and 117,000 km in the USA and Australia, respectively (AWA, 2011; US EPA, 1991). The total asset value of these networks is estimated to be about one trillion dollars in the

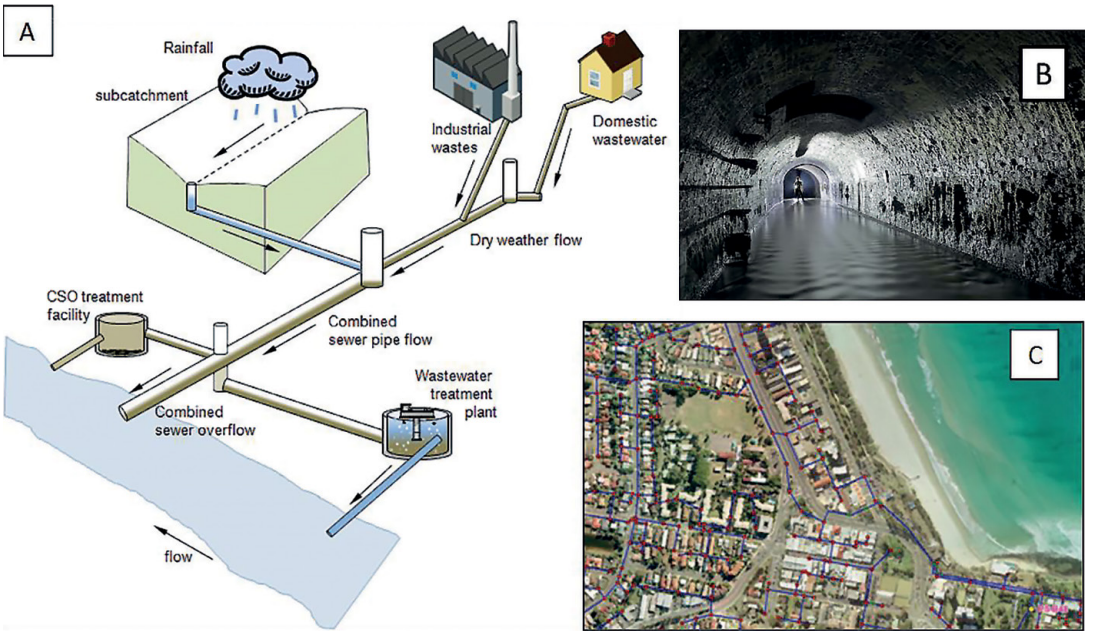


Figure 8.1 (A) Scheme of the urban sewer system; (B) picture of sewer trunk main in Paris (France), courtesy of sub-urban.com; and (C) distribution of a sewer network in the domestic suburb of Burleigh Heads, courtesy of Gold Coast City Council (Australia).

USA and $100 billion in Australia (Brongers, 2001).

Depending on the topography, sewers are classified to include two types of pipes: gravity sections and pressure mains (Fig. 8.2). Gravity sewers are used to collect wastewater from multiple sources and convey the wastewater by gravity to a central location. Gravity pipes have sufficient slopes to keep the wastewater flowing naturally through the system without having excessive solid deposition. Whenever wastewater has to be transported to a higher location and flow under gravity is not possible, pump stations need to be employed. Pump stations are normally installed at low elevation points of the sewer network in order to pump the sewage up through a pressure main to another gravity pipe, to convey wastewater over a hill, and/or in case of nearly flat terrains up to a treatment facility. Depending on the topography of each catchment, gravity pipes or pressure mains may be predominant in a sewer network.

Sewers have been traditionally considered only as a system for hydraulic transport of sewage. However processes occurring in sewer systems are

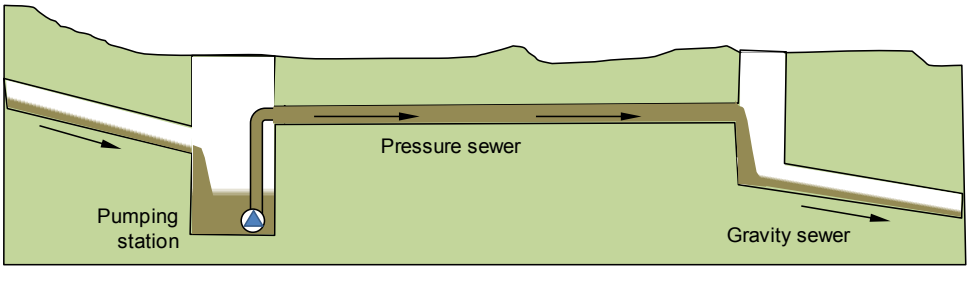


Figure 8.2 Sewer network types of pipe depending on topography.

much broader than solely the hydraulic processes. Sewers are 'reactors' where complex microbial, chemical and physicochemical processes take place. The complexity of those reactions depends very much on the inherent sewer features such as:

1 Wastewater matrix, which includes a diversity of microorganisms and pollutants (such as organic matter, nutrients and particles) varying with the location and time within the network.
2 Presence of different sewer phases such as suspended wastewater phase, biofilms, sediments and surface of the sewer in contact with the sewer atmosphere (Fig. 8.3).
3 Microbial processes that occur under changing environmental conditions such as aerobic (presence of dissolved oxygen in wastewater), anoxic (presence of nitrate or nitrite), anaerobic (absence of oxidant compounds in the wastewater) and at different level of redox potentials.

Fig. 8.3 presents the most important processes occurring in sewer systems. It can be seen that some processes are biologically mediated by biofilms. The diversity and importance of bioreactions in sewers is going to be described in sections below.

Biofilms and biological reactions have important impacts on sewers functioning. Microorganisms are widely present in the wastewater and are exposed to a range of substrates that contain fractions both of organic matter and inorganic compounds (Metcalf and Eddy, 2003). Sewer biofilms grow attached to sewer surfaces including walls, sediments and other physical supports (Hvitved-Jacobsen, 2002). They take the form of a concentrated layer of microorganisms with self-imbedded matrix of extracellular polymeric substances that hold together in a shape of slime. According to Hvitved-Jacobsen (2002), sewer biofilms typically have a water content of 70–90%, 50–90% of organic matter and a relatively high content of carbohydrates and proteins. Different types of microorganisms will prevail in particular sections of sewers depending on hydraulic features and wastewater composition. Hydraulic features are related to the reaeration zones of the pipes and include turbulence flow of sewage, ventilation of the systems and depth of the wastewater column. Wastewater composition characteristics are related to substrate availability, dissolved organic matter, temperature, pH and redox of sewage.

This chapter aims to give an overview of the most common biofilms found in sewers, paying special attention to its detrimental effects.

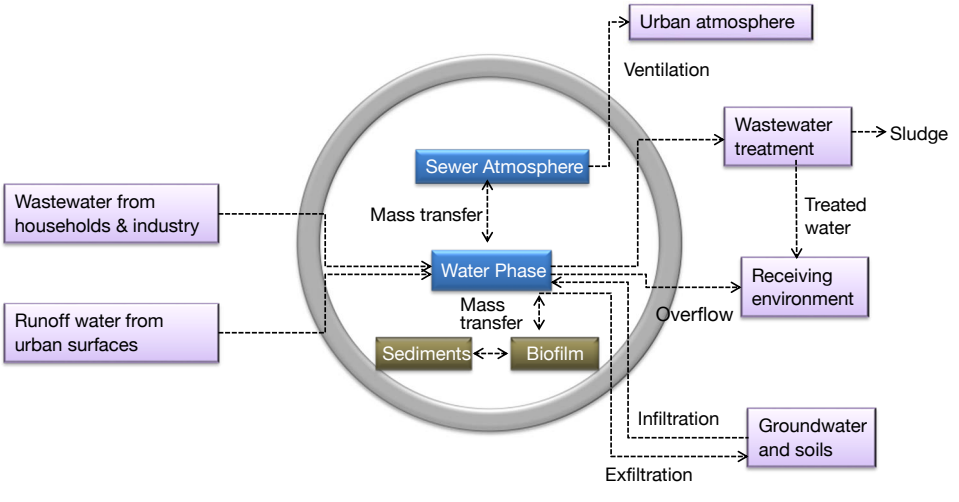


Figure 8.3 Wastewater flows and processes occurring in sewers.

Submerged sewer biofilms dynamics

As widely explained in this book, biofilms need the presence of water, therefore sewer biofilms develop mainly in submerged conditions. Anaerobic sewer bioprocesses, related to both the sulfur and the carbon cycles, are very important in sewers. The interaction of those processes and the aerobic transformation of wastewater are crucial for the performance of urban wastewater systems. To alleviate and control the sewer corrosion and odour problems, various liquid-phase technologies have been used to reduce the formation/emission of H_2S or CH_4 (WERF, 2007). The most commonly used chemicals for liquid-phase technologies include oxygen, nitrate, magnesium hydroxide, iron salts, and caustic shocking to deactivate sewer biofilms (Gutierrez *et al.*, 2008, 2009, 2014; Jiang and Yuan, 2013a; Jiang *et al.*, 2011a; Zhang *et al.*, 2009). The dynamics of submerged sewer biofilms under different environmental conditions are presented in this section.

Anaerobic sewer biofilms

Anaerobic conditions develop under the absence of dissolved oxygen leading to sewage septicity. Anaerobic conditions are very common in sewers and promote the development of biofilms which are the cause of the majority of process-related problems. The main anaerobic bioprocesses in sewers are:

- anaerobic sulfate respiration
- methane generation
- fermentation.

Anaerobic sulfate respiration

In situations where there is lack of oxygen and nitrate, sulfate (SO_4^{2-}) becomes the most thermodynamically efficient electron acceptor. The anaerobic sulfate respiration is carried out by a functional group of bacteria commonly called sulfate-reducing bacteria (SRB) under anaerobic conditions as a dissimilatory reduction reaction (equations 8.1–8.5). SRB obtain energy by oxidizing organic compounds and/or molecular hydrogen (H_2) while reducing sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S-HS^-) during its metabolism. *Desulfovibrio* and *Desulfotomaculum* are the dominant genera of SRB (Muyzer and Stams, 2008).

SRB grow inside the biofilm in sewer walls and take the sulfate and organic matter present in sewage for their metabolism (Fig. 8.4). Sulfate concentrations between 15–30 mg $S\text{-}SO_4^{2-}$/l are typical in domestic sewage but those can go higher depending on the source of drinking water or if the water comes from mineral sources with strong presences of salts like sulfate (Pikaar *et al.*, 2014).

Within sewer networks, sulfide is formed mainly in completely filled rising main sections, more prone to septicity than partially filled gravity sections where reareation from the gas phase can occur (Hvitved-Jacobsen, 2002). Sulfide concentration in rising mains depends upon the sewer features such as the hydraulic retention time (HRT, time that sewage remains in a certain pipe) and the area/volume ratio (A/V ratio, the inner surface area to volume ratio, i.e. 2/r, where r is the radius of the pipe, relative surface of sewer innerwalls to the volume of sewage that contains). The production and accumulation of hydrogen sulfide is a major concern for wastewater utilities and health of residents nearby the sewers. The problems related with sulfide depend upon the extent by which H_2S escapes from the liquid phase of sewers (where is formed) to the sewer

Sulfate-reducing reactions	ΔGo' (kJ/reaction)*	Equation no.
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	–151.9	8.1
$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	–47.6	8.2
$Propionate^- + 0.75SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75HS^- + 0.25H^+$	–37.7	8.3
$Butyrate^- + 0.5SO_4^{2-} \rightarrow 2\ Acetate^- + 0.5HS^- + 0.5H^+$	–27.8	8.4
$Lactate^- + 0.5SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.5HS^-$	–80.2	8.5

*Thauer *et al*. (1977).

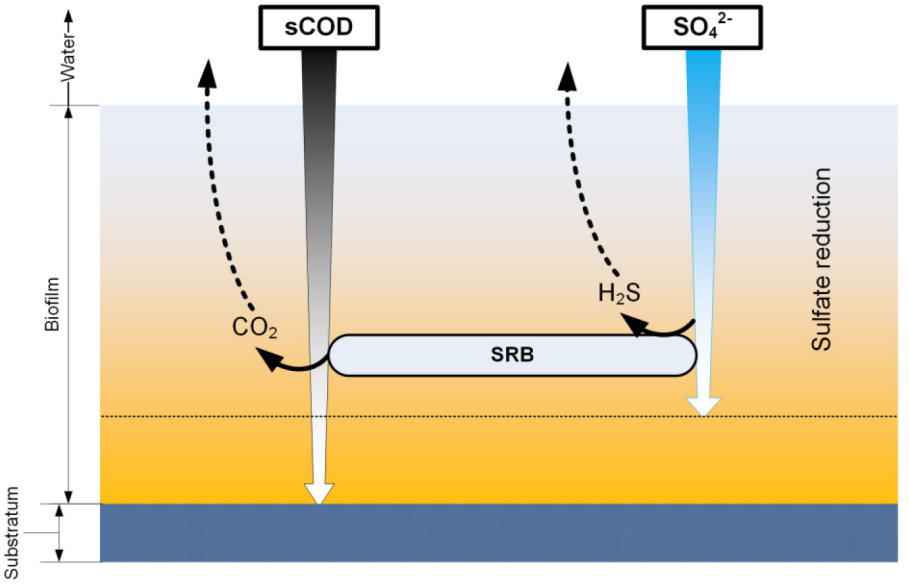


Figure 8.4 Conceptual representation of the anaerobic sulfate respiration of sewer biofilms. sCOD: Organic matter dissolved in wastewater expressed as soluble chemical oxygen demand.

atmosphere, the rate of H_2S stripping or transfer to the sewer atmosphere that takes place under turbulent conditions (drop structures, manholes, wetwells) and in gravity sewers. The extent of transfer depends mainly on the pH of sewage. H_2S is a weak diabasic acid which dissociates As Per the equation 8.6.

$$H_2S \leftrightarrow H^+ + HS^-$$
$$pK_a = -\log K_a = 7.1 \qquad (8.6)$$

As long as H_2S remains dissolved in wastewater, it does not present any harm and will be eventually oxidized chemical or biochemically. The ionic form (HS^-) is soluble in water and remains dissolved. On the other hand the molecular form (H_2S) is more volatile, and can be easily released from the sewer liquid phase. When H_2S is released to the sewer headspace and build-up there, it causes major detrimental effects including odour nuisance and toxicity. H_2S is a flammable and poisonous gas with a characteristic odour of rotten eggs. Its odour concentration threshold is very low, 0.0047 ppmv in gas, and is potentially dangerous because its smell is quickly lost as the concentration increases. Exposure to lower concentrations (10–100 ppmv) can result in eye irritation, a sore throat and cough, nausea, shortness of breath, and fluid in the lungs. These effects are believed to be due to the fact that hydrogen sulfide combines with alkali present in moist surface tissues to form sodium sulfide. Exposure to concentrations higher than 300 ppmv in air can cause death by pulmonar oedema and over 1000 ppmv cause immediate collapse with loss of breathing, even after inhalation of a single breath.

A third major effect of biogenic H_2S consists of the induced corrosion of sewer walls and infrastructures. Biogenic sulfide corrosion is a bacterially mediated process in which hydrogen sulfide gas is subsequently converted to sulfuric acid that attacks concrete and steel within wastewater environments. The hydrogen sulfide gas is biochemically oxidized in the presence of moisture to form sulfuric acid. This process causes critical problems to wastewater managers in terms of repairing and rehabilitation expenses. Sewer assets are under serious threat with an estimated annual asset loss of around $14 billion in the USA alone (Brongers *et al.*, 2002). Sulfide induced concrete corrosion is recognized as a main cause in most cases (US EPA, 1991). For instance, in Australia alone the costs of infrastructure depreciation due to sulfide-induced corrosion are estimated to

be in the order of 100 million dollars per year. Full details of this process are presented below.

Methane generation

Hydrogen sulfide is not the only detrimental compound produced in anaerobic sewer systems. Recent field studies showed that a significant amount of methane (CH_4) is formed in sewers, particularly in pressure pipes (Foley *et al.*, 2009). Methanogenesis is carried out by Methanogenic Archaea (MA), a domain distinct from bacteria. Bacteria and archaea differ in cell wall characteristics, membrane lipid composition, in RNA polymerase structure and, therefore, protein synthesis (Gantner *et al.*, 2011). Biogenic formation of methane is a form of anaerobic respiration in which the terminal electron acceptor is not oxygen but carbon compounds of low molecular weight. In contrast to sulfate reducers, methanogens use a limited number of substrates for growth and energy production. Quantitatively, hydrogen, carbon dioxide and acetate are the most important and best-known substrates for methanogens (Muyzer and Stams, 2008). Equations 8.7–8.9 represent the process of methane production.

Ongoing studies in Australia, USA and Spain are addressing this lack of knowledge on the CH_4 formation from sewers. Methane formation in sewers has recently been reported. For instance, Guisasola and co-workers (2008) measured two rising mains in Gold Coast (Australia) where dissolved methane concentrations ranging between 5 and 30 mg/l, equivalent to 20–120 mg COD/l, were measured. Gutierrez and co-workers detected CH_4 production in pressure sewers of la Costa Brava (Spain) (Gutierrez *et al.*, 2012). Measurements at multiple locations of a rising main have shown methane at concentrations between 1.5 and 9 mg COD/l. Similarly to H_2S, methane concentration in sewers has shown to be dependent on the HRT of wastewater and the A/V ratio of the pipe.

Methane production and emission from sewers induce serious concerns as it is a potent greenhouse gas with a global warming potential 21 times that of carbon dioxide over a 100 year horizon. To date, methane production from sewer systems has been largely overlooked in the GHG inventories. The latest report from the Intergovernmental Panel on Climate Change (IPCC) concerning greenhouse emissions did not consider methane production from closed or underground sewer systems (IPCC *et al.*, 2013), despite some previous indications that domestic sewage could be one of the anthropogenic methane sources (Minami and Takata, 1997). Methane is explosive at low concentrations, thus represents a safety risk in confined spaces like sewer manholes because of its low explosion limit (lower explosive level is approximately 5% mix in air) (Spencer *et al.*, 2006). Further, methane production in sewers inevitably consumes the soluble organic compounds (sCOD) including volatile fatty acids (VFAs), which may be required in the receiving wastewater treatment plant for biological nutrient removal.

In anaerobic environments with low redox potentials (< -200 mV), SRB compete with other anaerobes, including fermentative bacteria, proton-reducing acetogenic bacteria, homoacetogens and methanogens, for the available common substrates. In the presence of sulfate in excess, sulfate reducers compete with methanogens for the common substrates hydrogen and acetate and with syntrophic methanogenic communities (Dar *et al.*, 2008). However Guisasola *et al.* (2009) showed that methane and sulfide are simultaneously produced in sewer systems, which implies the coexistence of MA and SRB in sewers biofilms and that these bacteria function simultaneously. The simultaneous functioning of the SRB and MA is related to the spatial arrangement of these bacteria in sewer biofilms. Sewer biofilms are relatively thick (several hundred micrometres;

Methanogenic reactions	ΔGo' (kJ/reaction)	Equation no.
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	–135.6	8.7
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O^-$	–130.7	8.8
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	–31.0	8.9

Mohanakrishnan *et al.*, 2007) and the Sulfate/Organic Matter ratio (S/COD) shows spatial variation inside the biofilm, being relatively high near the surface in contact with the bulk liquid and close to zero in the inner zone adjacent to the pipe surface (Sun *et al.*, 2014). Fig. 8.5 depicts a schematic view of this hypothesis, which is supported by the sulfide profile measured in sewer biofilms by Mohanakrishnan *et al.* (2007). In contrast, the supply of methanogenesis precursors (VFA) is unlikely to be limiting within the biofilm. For this reason, the lower affinity of MA for these precursors is not a handicap to the growth of methanogens deeper within the biofilm. With sulfate most likely only partially penetrating the biofilm, conceptually two different zones may appear in the biofilm: a sulfate-reducing anaerobic zone (nearer the surface, dominated by SRB) and a deeper anaerobic zone dominated by MA Thus, the extent of methanogenesis in a sewer system is inversely proportional to the sulfate penetration length into the biofilm.

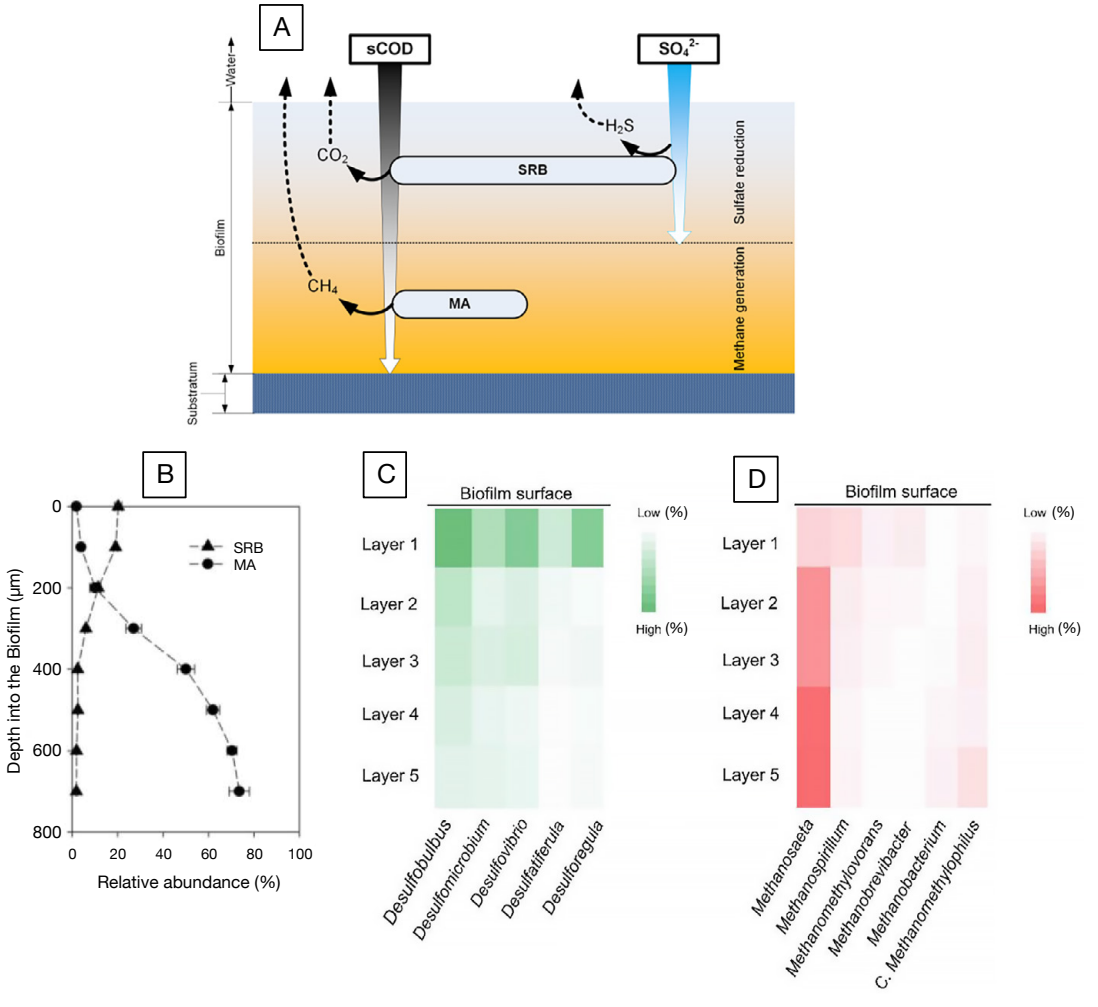


Figure 8.5 (A) Conceptual stratified biofilm model under anaerobic conditions including SRB (sulfate-reducing bacteria) and MA (methanogenic archaea); (B) The SRB and MA proportions of total microorganisms (bacteria and archaea) detected by FISH within the sewer biofilms; (C) Heatmap displaying the abundance and distribution of the predominant SRB genera in different sewer biofilm layers from the biofilm surface to the bottom (Layer 1 to Layer 5). (D) Heatmap displaying the abundance and distribution of the predominant MA genera in different sewer biofilm layers from the biofilm surface to the bottom (layer 1 to layer 5) (Sun *et al.*, 2014).

Fermentation/acetogenic reactions	ΔGo' (kJ/reaction)	Equation no.
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.1	8.10
$Butyrate^- + 2H_2O \rightarrow 2\ Acetate^- + H^+ + 2H_2$	+48.3	8.11
$Lactate^- + 2H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	–4.2	8.12

In fact, the competition between SRB and MAis not unique to sewer systems. Environmental microbiologists have devoted much attention to such competition in natural anaerobic environments, for example aquatic sediments or paddy rice soils (Abram and Nedwell, 1978; Sørensen *et al.*, 1981; Lovley *et al.*, 1982; Oremland and Polcin, 1982; van Bodegom and Stams, 1999; Abram and Nedwell, 1978; Bodegom and Stams, 1999; Lovley *et al.*, 1982; Oremland and Polcin, 1982; Sørensen *et al.*, 1981) and anaerobic digesters (Bhattacharya *et al.*, 1996; Gupta, 1994; Kalyuzhnyi and Fedorovich, 1998).

Fermentation

Fermentation consists of the partial breakdown of dissolved organic matter that yields organic by-products of low molecular weight. Under anaerobic conditions, degradation of easily biodegradable organic matter is the dominating process. Although fermentation processes do not present direct harmful effects in sewers, its by-products are essential to SRB and MA activities since anaerobic fermentation is the source of H_2 and VFAs. Fermenters create a syntrophic relation with SRB and MA populations in which H_2 and acetate (preferred electron donors) are rapidly scavenged while being produced. Fermentation takes place in the suspended water phase, biofilms and sewer sediments. The number of fermentation reactions occurring in sewers is quite extensive. The more relevant ones are presented in equations 8.10–8.14.

In addition, fermentation also plays a role in the formation of odorous compounds under strictly anaerobic conditions. Therefore there is a strong interaction between the carbon and sulfur cycles in anaerobic sewers that promote the effects described above.

Anoxic sewer microbial processes

Nitrate salts (sodium and calcium nitrate) are sometimes artificially added in to pressure sewers to avoid anaerobic conditions and prevent sulfide formation (Jiang *et al.*, 2009; Mohanakrishnan *et al.*, 2009; Okabe *et al.*, 2007; Zhang *et al.*, 2008). In the last 70 years, nitrate dosing has been used by water industry to control hydrogen sulfide production in sewers (Ganigue *et al.*, 2011). The addition of a thermodynamically favourable electron acceptor to anaerobic sewers aims to shift redox conditions and prevent the sulfate respiration. Anoxic sulfide oxidation by sewer biofilms using nitrate can be described as a two-step process, namely the oxidation of sulfide to elemental sulfur, and oxidation of elemental sulfur to sulfate (equations 8.15 and 8.16, respectively) (Jiang *et al.*, 2009).

Saracevic *et al.* (2006) applied 40 mgN-NO_3^-/l nitrate to a 5.0 km long rising main sewer, and discovered that after a lag time of 3–4 days, the nitrate application reduced sulfide concentrations from 10–20 mg S-S^{2-}/l to below 2–3 mg S-S^{2-}/l. Nitrate concentration of 5 mg N-NO_3^-/l in wastewater were reported to be sufficient to inhibit sulfide production in a 61-km-long gravity sewer (Rodríguez-Gómez *et al.*, 2005).

Nitrate does not have an immediate or long-lasting inhibitory/toxic effect on sulfate reduction on sewer biofilms. Biofilms in a nitrate-receiving laboratory sewer system were found to fully maintain their sulfidogenic activity (sulfide production

Homoacetogenic reactions	ΔGo' (kJ/reaction)	Equation no.
$4H_2 + 2HCO_3^- + H^+ \rightarrow Acetate^- + 4H_2O$	–104.6	8.13
$Lactate^- \rightarrow 1.5\ Acetate^- + 0.5H^+$	–56.5	8.14

Nitrate denitrification reactions	ΔGo' (kJ/reaction)	Equation no.
$5S^{2-} + 2NO_3^- + 12H^+ \rightarrow 5S^0 + N_2 + 6H_2O$	–955	8.15
$5SO + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$	–2738	8.16

rate in the absence of nitrate) during several months of nitrate addition (Auguet *et al.*, 2014; Mohanakrishnan *et al.*, 2009). Two main mechanisms have been suggested to control sulfide production by nitrate addition in sewers: anoxic sulfide oxidation and competitive exclusion of SRB. The first involves the growth of a chemolithotrophic sulfide-oxidizing nitrate-reducing community, able to oxidize sulfide to elemental sulfur as a major intermediate coupled to nitrate reduction. The latter triggers the development of a heterotrophic, nitrate reducing bacteria (hNRB) community, competing with SRB for organic electron donors. Jiang *et al.* (2013) proposed a conceptual biofilm model with competitive and synergistic interactions among hNRB, sulfide-oxidizing nitrate-reducing bacteria (soNRB), SRB and MA occurring in anoxic sewer biofilms (Fig. 8.6). Similarly as presented in sections before, microbial stratification within the biofilm plays a major role and H_2S and CH_4 control are related to penetration of nitrate into the biofilm. Sulfate-reducing activity and methanogenesis would persist respectively in the deeper parts of the biofilm where soluble chemical oxygen demand would still be able to penetrate but not nitrate.

These sulfide-oxidizing bacteria (SOB), e.g. *Thiobacillus denitrificans*, are mostly chemoautotrophic and have been extensively studied (Sublette and Sylvester, 1987; Cadenhead and Sublette, 1990; Chazal and Lens, 2000; Yang *et al.*, 2005).

More recently, nitrite (NO_2^-) has also been used for sulfide and methane control in sewer pipes (Jiang *et al.*, 2010; Mohanakrishnan *et al.*, 2008; see equations 8.17 and 8.18 as reaction examples). In addition to the oxidizing capacity as NO_3^-, NO_2^- has a toxic effect on microorganisms thanks to its metabolic inhibitor properties. Nitrite blocks sulfate reduction in SRB by inhibiting the

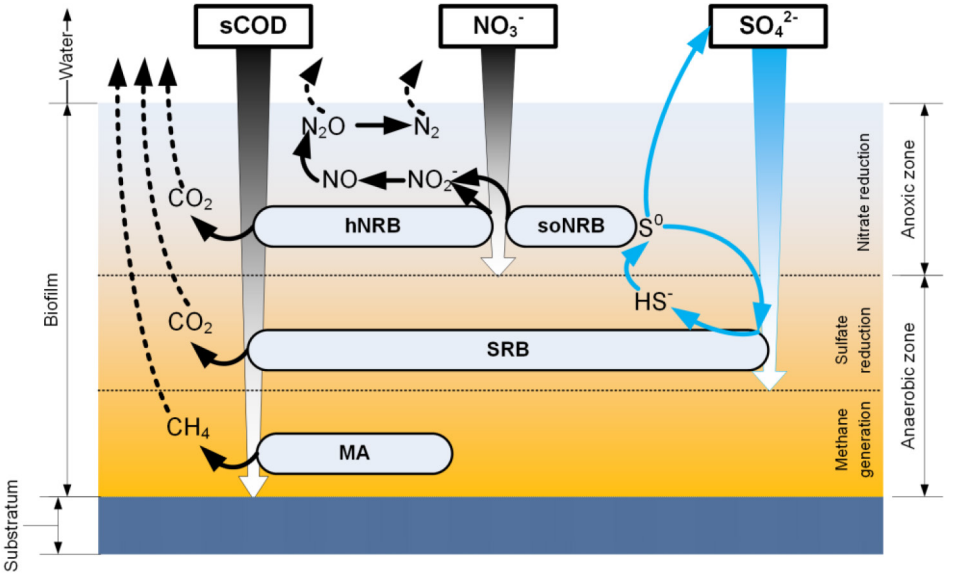


Figure 8.6 Conceptual stratified biofilm model under anoxic conditions: Competitive and synergistic interactions among heterotrophic nitrate reducing bacteria (hNRB), sulfide-oxidizing nitrate-reducing bacteria (soNRB), sulfate-reducing bacteria (SRB), and methanogenic archaea (MA). Blue arrows indicate the sulfur cycling between soNRB and SRB.

Nitrite denitrification reactions	ΔGo' (kJ/reaction)	Equation no.
$3HS^- + 8NO_2^- + 5H^+ \rightarrow 3SO_4^{2-} + 4N_2 + 4H_2O$	–2944	8.17
$3HS^- + 2NO_2^- + 5H^+ \rightarrow 3S^0 + N_2 + 4H_2O$	–917	8.18

dissimilatory sulfite reductase gene that catalyses the conversion of sulfite to sulfide (Hubert *et al.*, 2005; Nemati *et al.*, 2001). Some SRB possess a nitrite reductase gene (which prevents the inhibition of SRB by nitrite), but the function of the enzyme is purely for detoxification, and energy is not generated by the reduction (Greene *et al.*, 2003). Denitrifying bacterial species like *Thiobacillus denitrificans* can oxidize sulfide to elemental sulfur simultaneously reducing nitrogenous species to dinitrogen (Mahmood *et al.*, 2007). Nitrite toxicity produces a long-lasting inhibitory effect for H_2S and CH_4 even after the termination of the nitrite addition Conversely, other oxidants (such as nitrate or oxygen) would need to be present in the bulk at all times and in all sections of the sewer in order to effectively prevent sulfide accumulation.

Jiang and coauthors (2011) revealed that the protonated form of nitrite, HNO_2, free nitrous acid (FNA) is responsible of its biocidal effect. Sewer biofilms in lab reactor exposed to FNA concentrations above 0.2 mgN-NO_2^-/l decreased its viable fraction by 80% only with 6 hours of contact time. The recovery of methane production was about seven times slower compared to the recovery of sulfide production. The biocidal effect was found to be strongly dependent on the FNA concentration rather than the nitrite concentration or the pH level separately (for the pH range of 6.0–7.6). Based on the biocidal effects of FNA, intermittent dosing of nitrite with acid (to form FNA) is potentially a cost-effective strategy to control sulfide and methane production in sewers. Several studies have been confirmed this hypothesis (Jiang *et al.*, 2011a, 2013a).

Aerobic sewer microbial processes

Aerobic microorganisms are commonly found in sewer systems especially in the gravity sections where turbulence and reaeration promote conditions optimal to their growth. These organisms

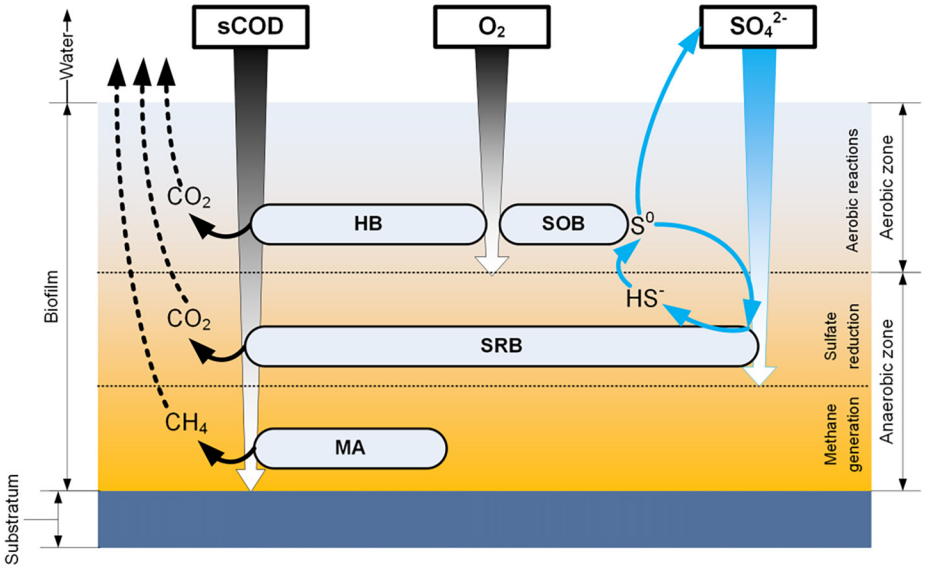


Figure 8.7 Conceptual stratified biofilm model under aerobic conditions: competitive and synergistic interactions among heterotrophic bacteria (HB), sulfide-oxidizing bacteria (SOB), sulfate-reducing bacteria (SRB), and methanogenic archaea (MA). Blue arrows indicate the sulfur cycling between SOB and SRB.

use oxygen as a terminal electron acceptor and consume dissolved organic matter in the sewage in their respiration process (Metcalf and Eddy, 2003). Aerobic processes consist of a major sink of sulfide in sewer systems (Fig. 8.7). The shift between aerobic and anaerobic conditions in wastewater of a sewer system can be very dynamic. Solubility of oxygen depends on the temperature (8.3 mg O_2/l at 25°C, and this increases with the decrease in the temperature) and the respiration rate depends on the availability of degradable organic matter and temperature and may reach values as high as 20–40 mg O_2/l·h (Hvitved-Jacobsen, 2002). Thus the reaeration rate in gravity sewers could be insufficient to maintain the oxic conditions. Thus, the gravity sewers with low slopes and in warm climate are likely to develop anaerobic conditions.

Artificial injection of oxygen to sewers is widely used by wastewater authorities to mitigate the sulfide problems (Boon *et al.*, 1998; Ganigue *et al.*, 2011; Hvitved-Jacobsen, 2002; Zhang *et al.*, 2008). A laboratory study carried out in lab-sewer simulating system showed that oxygen is an effective chemical and biological oxidant of sulfide but it did not prevent the sulfide and methane production in the biofilm, which continued in the deeper layers of biofilm irrespective of the oxygen concentration in the bulk (Ganigué and Yuan, 2014; Gutierrez *et al.*, 2008). This is in agreement with the stratification theory proposed by Jiang and co-authors for oxidants addition to sewers (Jiang *et al.*, 2013a). Both studies reported that sulfide and methane accumulation resumed on complete depletion of oxygen at a certain depth of biofilm. Oxygen did not exhibit any toxic effect on sulfate-reducing bacteria (SRB) in the biofilm. With regards to the SRB, it further stimulated its growth and increased its activity in biofilms in downstream sewer sections due to increased availability of sulfate at these locations as the result of oxic conditions upstream. Furthermore the oxygen uptake rate of the system increased with repeated exposure to oxygen, with concomitant increase in the consumption of organic carbon in the wastewater due to the development of an heterotrophic community within the sewer (Ganigué and Yuan, 2014; Gutierrez *et al.*, 2008).

Unsubmerged sewer biofilms

Microbially induced concrete sewer corrosion

Concrete corrosion in sewers is primarily a result of biological processes occurring in the biofilms

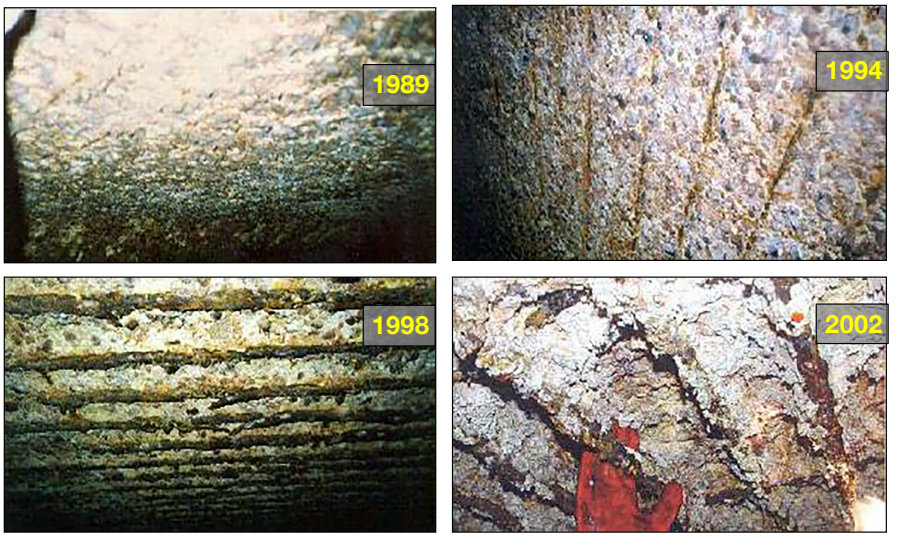


Figure 8.8 Rapid corrosion of a large gravity sewer pipe: 10 km length, 2 m width and 3 m height. The rehabilitation cost of the pipe was 100 Million Australian $. Courtesy of Sydney Water Corporation.

growing on the sewer pipe surface exposed to the gas phase. Due to the significant role played by microorganisms in sewer corrosion, it is termed as microbially induced concrete corrosion (MICC). Corrosion causes loss of concrete mass, cracking of the sewer pipes and ultimately, structural collapse (Fig. 8.8). The rehabilitation and replacement of corrosion damaged sewers involves very high costs. It is estimated that the annual cost of concrete corrosion within the water and wastewater infrastructure is about US$36 billion in USA (Koch *et al.*, 2002). This cost is expected to increase as the ageing infrastructure continues to fail (Sydney *et al.*, 1996; US EPA, 1991).

As shown in Fig. 8.9, sulfide production by sulfate-reducing bacteria and the subsequent emission to the sewer headspace are the primary causes for concrete sewer corrosion. The biological production of sulfuric acid from oxidation of hydrogen sulfide with oxygen (Joseph *et al.*, 2012a; Parker, 1945) causes mass loss of concrete (Islander *et al.*, 1991; Ismail *et al.*, 1993). The corrosion-causing biofilms grow on the surface of corroding concrete (Okabe *et al.*, 2007).

A range of corrosion products is formed during various stages of the corrosion process. The components of uncorroded cement are mainly hydrated calcium silicate ($CaO{\cdot}SiO_2{\cdot}2H_2O$) and portlandite ($Ca(OH)_2$). Abiotic processes that include carbonation and H_2S acidification, result in $CaCO_3$, and $Ca(HS)_2$ and S^0 as the main products (Wei *et al.*, 2014). During active concrete corrosion, biologically produced sulfuric acid leads to the formation of two important corrosion products: gypsum and ettringite, according to the reactions below (equations 8.19–8.22):

$$CaO{\cdot}SiO_2{\cdot}2H_2O + H_2SO_4 \rightarrow CaSO_4 + Si(OH)_4 + H_2O \quad (8.19)$$

$$H_2SO_4 + CaCO_3 \rightarrow CaSO_4 + H_2CO_3 \quad (8.20)$$

$$H_2SO_4 + Ca(OH)_2 \rightarrow CaSO_4{\cdot}2H_2O \text{ (gypsum)} \quad (8.21)$$

$$3CaSO_4{\cdot}2H_2O + 4CaO{\cdot}Al_2O_3{\cdot}13H_2O + 14H_2O \rightarrow (CaO)_3{\cdot}Al_2O_3{\cdot}(CaSO_4)_3{\cdot}32H_2O \text{ (ettringite)} \quad (8.22)$$

Both gypsum and ettringite have significantly higher volumes than intact cement, estimated to range from 124% to 700% (Monteny *et al.*, 2000; Parande *et al.*, 2006). The expansion is believed to cause internal cracking and pitting, which in turn, exposes more surface area for acid attack. However, recent findings revealed that micro-cracking at the corrosion front is more likely caused by the iron rust deposition (Jiang *et al.*, 2014).

Microbial structure and populations of corrosion biofilms

Fresh concrete in sewer pipes immediately after construction is usually immune to biological attack because of its high alkalinity (pH around 12), a result of the formation of calcium hydroxide ($Ca(OH)_2$) as the byproduct of cement

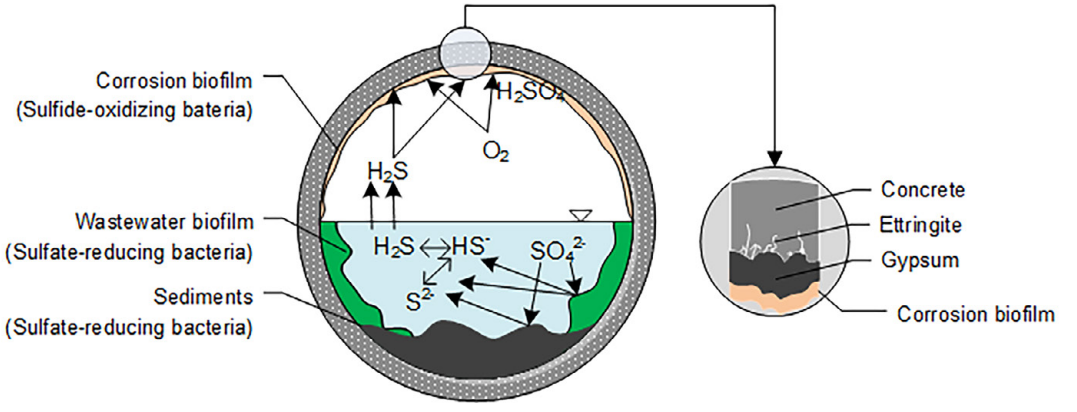


Figure 8.9 Processes involved in the microbially induced concrete corrosion in sewers.

hydration. Anaerobic conditions in wastewater favour the production of hydrogen sulfide and carbon dioxide, which builds up in the sewer air (Guisasola *et al.*, 2009; Lahav *et al.*, 2004; Nielsen *et al.*, 2005). Over time, the pH of the alkaline concrete surface is gradually reduced by the carbonation (equations 8.23–8.24) and neutralization of hydrogen sulfide (equations 8.25–8.27) (Bagreev and Bandosz, 2004, 2005; Joseph *et al.*, 2012b). It was also found that the attachment and colonization of some pioneer microorganisms (mainly heterotrophic, halotolerant, and neutrophilc bacteria) on the concrete surface could have a great impact on the initial pH decrease (Okabe *et al.*, 2007). The initial acidification processes can reduce the pH of concrete sewer from around 12 to 9, this can establish suitable growth conditions for the subsequent emergence and propagation of sulfur and sulfide oxidizing microorganisms on the concrete surface (Joseph *et al.*, 2012a).

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O \quad (8.23)$$

$$CaO{\cdot}SiO_2{\cdot}2H_2O + CO_2 \rightarrow CaCO_3 + \text{Silica} + H_2O \quad (8.24)$$

$$CaCO_3 + H_2S \rightarrow Ca(HCO_3)_2 + Ca(HS)_2 \quad (8.25)$$

$$Ca(OH)_{2+}2H_2S \rightarrow Ca(HS)_2 + 2H_2O \quad (8.26)$$

$$Ca(HS)_2 + O_2 \rightarrow 2S + Ca(OH)_2 \quad (8.27)$$

When the surface pH reaches 9, various microorganisms can colonize on the concrete surface with the availability of moisture and nutrients (i.e. stage 2 of corrosion development shown in Fig. 8.10). Due to the abundance of hydrogen sulfide and oxygen, some neutrophilic sulfide-oxidizing bacteria (SOB) start to colonize and grow on the concrete (Mori *et al.*, 1992). The dominant SOB species of the second stage of corrosion include *Thiothrix* sp., *Thiobacillus plumbophilus*, *Thiomonas* sp., and *Halothiobacillus neapolitanus* (Okabe *et al.*, 2007). These SOB species were probably responsible for the production of sulfuric acid from the gaseous hydrogen sulfide (Vollertsen *et al.*, 2008; Zhang *et al.*, 2008; Zivica and Bajza, 2001). The sulfide-oxidizing bacteria, along with some fungi, algae, and lichens form biofilms (Fig. 8.10), which further reduce down the surface pH of concrete (Domingo *et al.*, 2011; Nica *et al.*, 2000).

In the third stage, at which the pH remains around 2, the acidophilic SOB *Acidithiobacillus thiooxidans* appeared and became the most dominant microbial species (Okabe *et al.*, 2007;

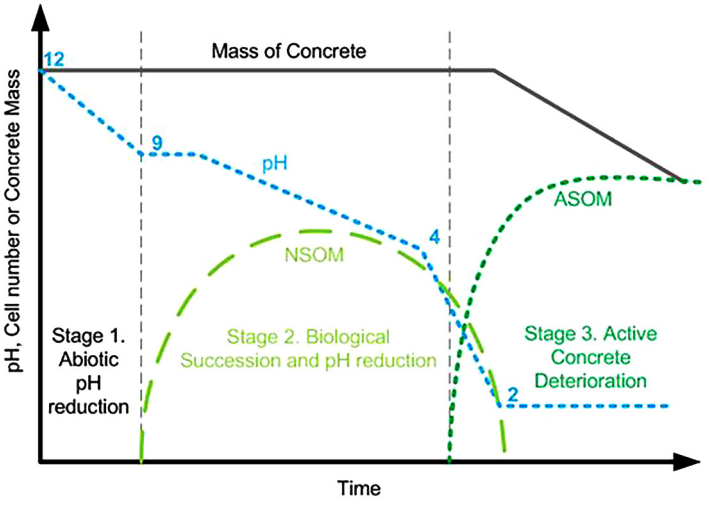


Figure 8.10 The succession of different sulfide-oxidizing bacteria with the changes of surface pH due to the development of concrete corrosion in sewers. Adapted from Islander *et al*. (1991). Neutrophilic sulfide-oxidizing microorganisms (NSOM); acidophilic sulfide-oxidizing microorganism (ASOM).

Table 8.1 Sulfide-oxidizing bacteria (*Thiobacillus*) species involved in the microbially induced concrete corrosion in sewers, and their characteristics (Okabe *et al.*, 2007; Roberts *et al.*, 2002)

Species	Growth pH	Temperature (°C)	Lifestyle	Sulfur substrates
*Thiobacillus thiop*arus	4.5–10	15–42	Autotrophic aerobe	Thiosulfate, sulfide, thiocyanate
Starkeya novella (Thiobacillus novellus)	5–9.2	10–37	Mixotroph	Thiosulfate
Halothiobacillus neapolitanus	4.5–8.5	8–39	Autotroph	Sulfur, sulfide, thiosulfate
Thiomonas intermedia	5–7.5	15–37	Mixotroph	Sulfur, thiosulfate
Thiobacillus plumbophilus	4–6.5	9–41	Autotroph	Sulfide
Thiothrix spp.	Neutral pH	10–30	Mixotroph	Sulfide, thiosulfate
Thiobacillus intermedius (Thiomonas intermedia)	1.7–9	15–37	Mixotroph	Thiosulfate
Acidithiobacillus thiooxidans	0.5–5.5	10–37	Autotroph	Thiosulfate, sulfur
Acidiphilum acidophilum	1.5–6.0	10–35	Heterotroph	Sulfur, thiosulfate

Wei *et al.*, 2014), although there are some conflicting findings about the dominant species in different environments (Cayford *et al.*, 2012; Parker, 1951; Sand and Bock, 1984). The high amount of sulfuric acid produced by corrosion biofilm leads to significant loss of concrete mass at this stage.

The important species like *Thiobacillus* sp. (syn. *Acidithiobacillus* sp.) and other SOB species found in corrosion biofilms are listed in Table 8.1. The first seven species are neutrophilic sulfide-oxidizing microorganisms (NSOM), while the last three are acidophilic sulfide-oxidizing microorganism (ASOM). The NSOM, and probably some fungi, further reduce the concrete pH to 4–5. At the end of stage 2, ASOM starts to grow in the sewer corrosion biofilm due to both the low pH and also the sulfur products produced by NSOM. Sulfur generated by NSOM is an essential substrate for ASOM, which produce significant amounts of sulfuric acid (Jensen *et al.*, 2008; Kelly and Wood, 2000). The optimum growth pH, trophic property (e.g. autotrophic or mixotrophic), and ability to utilize different sulfur compounds (e.g. H_2S, S^0, and $S_2O_3^{2-}$) by SOB determine the order of appearance and the dominance of different SOB species on corroding concrete surfaces in sewer systems (Okabe *et al.*, 2007).

In addition to the important SOB species in the corrosion biofilm, there are many other microorganisms being identified that are able to oxidize sulfur compounds, most of which

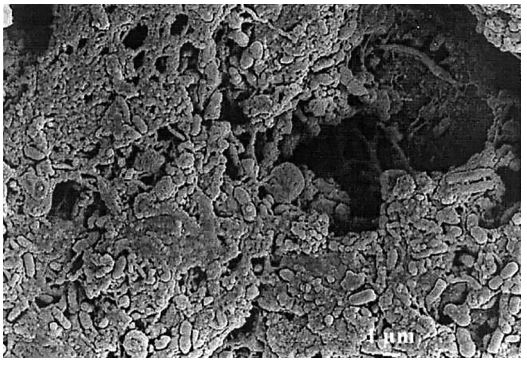


Figure 8.11 Concrete surface (scanning electron microscopy image, 4000×) covered with a layer of biofilms containing many 1–3 mm-sized rods of thiobacilli (Monteny *et al.*, 2000).

are heterotrophic bacteria (*Pseudomonas, Streptomyces, Arthrobacter, Bacillus, Flavobacter, Micromonas*, etc.) and fungi which may grow as co-cultures with SOB (Coleman and Gaudet, 1993; Nica *et al.*, 2000). It was suggested that fungus, e.g. *Fusarium* sp., act synergistically with thiobacilli by either oxidizing sulfide to thiosulfate which can then be used by thiobacilli, or excreting extracellular polymeric substances (EPS) that facilitates thiobacilli to attach to elemental sulfur (Cho and Mori, 1995; Gu *et al.*, 1998) (Fig. 8.11). It was found that over 50% of the corrosion biofilm community was heterotrophic bacteria other than SOB (Okabe *et al.*, 2007). The variety of microorganisms, both heterotrophic and autotrophic

bacteria and fungi, isolated from corroding sewers shows the complexity of the microbiological population in the corrosion biofilms.

Factors impacting the corrosion biofilm activities

The H_2S concentration in real sewers varies greatly due to the different hydraulic retention time, flow velocity and wastewater characteristics. In addition to high relative humidity (see below), a H_2S level >2 ppm was suggested to be required for the sulfide oxidation to proceed on concrete sewers (O'Dea, 2007). It is traditionally assumed that corrosion biofilm activity (i.e. biological sulfide oxidation) is directly proportional to H_2S emission rate (De Belie *et al.*, 2004). The well-known Pomeroy model can be used to calculate the deterioration rate of concrete sewer pipes (Pomeroy, 1990).

$$C_r = \frac{11.5k\phi_{sw}}{alk} \tag{8.28}$$

where C_r = corrosion rate (mm/year); k = factor related to the acid formation, based on climate conditions, 0.8 in moderate climates; f_{sw} = sulfide flux at the air–wall interface [g $H_2S/(m^2 \cdot h)$]; and alk = alkalinity of the pipe material (g $CaCO_3$/g concrete).

A recent report established a relationship between corrosion rate and controlling factors including H_2S gaseous concentration, relative humidity and temperature for concrete sewers either exposed to air or near the wastewater surface (Jiang *et al.*, 2014). The corrosion rate is proportional to the biofilm activity that is generally formulated as a power function of H_2S concentration (Nielsen *et al.*, 2005).

In sewers, water and nutrients provided by sewage are found to promote the microbial corrosion, especially for the area close to the wastewater level in a sewer pipe (Mori *et al.*, 1992). For the pipe surface further away from wastewater level, the relative humidity of the sewer air and the condensation process on the concrete surface would generate a water film for microbial growth. It was reported that humidity plays a role in surface neutralization at the early stage of sewer concrete corrosion (Joseph *et al.*, 2012a). During a long-term investigation of sewer corrosion, it was found that humidity is important for the corrosion biofilms which are far from the water level in sewer pipes (Jiang *et al.*, 2014). This is because high humidity leads to high moisture content in concrete that facilitates increased biological H_2S oxidation and sulfate production.

Short-term changes of temperature are typical for sewer systems and the interactions between sewer systems and sewage (Vollertsen *et al.*, 1999). One important process for sewer concrete corrosion is the air-water transfer of hydrogen sulfide, which was found to increase with increasing temperature at a constant turbulence level (Yongsiri *et al.*, 2004). It was widely accepted that sulfide oxidation rate, both chemically and biologically, increases with temperature, which can be described with an Arrhenius relationship (Nielsen *et al.*, 2004, 2006). The sulfide oxidation rate was reported to be doubled for a temperature increase of 7–9°C. In addition, sewer systems located in different climates would have been acclimated to different temperatures. The sulfide oxidation rates, and accordingly corrosion rates, would thus be very different for different climatic regions. However, no clear effects of temperature (5–17°C) was found for the hydrogen sulfide oxidation kinetics (Nielsen *et al.*, 2012). This was attributed to the population dynamics of SOB in the corrosion layers. Another study also reported non-significant effects of temperature between 18–35°C on the sulfide oxidation and corrosion rates (Jiang *et al.*, 2014). Further studies are needed to clarify the effects of temperature on the actual corrosion rates in sewers.

Control of concrete sewer corrosion

To alleviate and control the concrete sewer corrosion problems, various gas-phase technologies have been used to reduce or remove H_2S from sewer air. The sewer air treatment technologies include activated carbon adsorption, chemical scrubbing, and biotrickling filters for the biological oxidation of H_2S (Sivret and Stuetz, 2010). In addition to removing H_2S from sewer air, the ventilation also reduce the humidity in the sewer gas phase, which can significantly reduce the sewer concrete corrosion (Jiang *et al.*, 2014).

However, it is difficult to control sulfide in the entire sewer network using chemical dosing or

sewer air treatment due to costs and site restrictions. It is thus relevant and essential to make the sewer itself resistant to corrosion by concrete-based technologies that construct new sewers with corrosion-resistant concrete (proactive prevention) or repair, coat and line the corroded concrete surfaces (passive rehabilitation) (Haile *et al.*, 2010; Hewayde *et al.*, 2007). This approach includes applications of admixtures, protective coatings, and acid-resistant cement to prevent chemical attack by sulfuric acid. Antimicrobial coatings and admixtures, such as silver/copper zeolites (Zeomic®) and water-stabilized silicone quaternary ammonium salt (Conshield®) among a few other commercialized products, were also applied to reduce or eliminate the microbial activity (De Muynck *et al.*, 2009; Rivera-Garza *et al.*, 2000; Yamanaka *et al.*, 2002).

Recently, it was reported that *Escherichia coli* DH5*a* biofilm showed great potential to control and minimize microbially induced concrete corrosion by *Thiobacillus neapolitanus* and *Thiobacillus thiooxidans* (Soleimani *et al.*, 2013a). A protective biofilm layer with a depth of 20–40 mm was successfully grown on the surface of cement mortars (Soleimani *et al.*, 2013b). However, due to the highly oligotrophic condition on the sewer surface exposed to air, it is unlikely this technology can be implemented in real gravity sewer.

For new sewers, it is preferable to be constructed with corrosion-resistant concrete because the rehabilitation after corrosion damage is usually difficult and expensive. This can be achieved by using cementitious materials containing the corrosion inhibitors as an admixture (Saraswathy and Song, 2007). Different cements or cements with admixtures have been trialed with limited success. Current admixtures mainly focus on changing the physiochemical properties of concrete, such as reducing permeability or increasing buffering capacity. Concrete with a lower content of tricalcium aluminate (C_3A) was shown to have better resistance to the sulfate attack (Monteny *et al.*, 2000). Polymer additions are also used to modify concrete, with its corrosion resistance to acid attack being improved or worsen for different polymers (Vincke *et al.*, 2001).

Conclusions and perspectives

Biofilms are naturally present in urban sewer systems. Sewer biofilms can grow under different environments (anaerobic/aerobic) leading to the growth of different types of microorganisms. The release of detrimental compounds from sewer biofilms, such as H_2S or CH_4, has been thoroughly described in this chapter. A significant amount of knowledge has been generated in recent years but further investigations are still required to fully understand sewer biofilms and control its impacts on urban water systems.

It is important to have a good understanding of the biofilm-functioning under the specific sewer conditions in order to develop appropriate control strategies. In this regard, new methods for the control of the production of detrimental compounds in sewer biofilms are currently being developed and tested. For instance, free nitrous acid, an emerging chemical, has been shown to be cost-effective and environment-friendly in both lab studies and full scale scenarios (Jiang and Yuan, 2013a,b; Jiang *et al.*, 2011b, 2013b).

Integration of sewers with different subsystems of the urban water cycle is also receiving a most deserved attention. Recent studies have demonstrated that using sulfate-based coagulants in drinking water can have a major impact in sulfate content of sewer downstream, enhancing the development of sewer biofilms (Pikaar *et al.*, 2014).

Moreover further research is required to understand how the sewer biofilms contribute to the in-sewer biotransformation of various chemicals of different interests. Very little is known about the role of biofilms in the transformation of many micro pollutants detected in sewers, including pharmaceuticals and personal care products. A limited number of recent studies have shown sewer biofilms to play an important role in the degradation of illicit drugs and residues, pharmaceuticals and health-related compounds (Jelic *et al.*, 2014; Thai *et al.*, 2013, 2014).

With regards to the microbially induced corrosion problem, microbial communities in corrosion biofilms and their biological processes are still not fully delineated. A better understanding of the microorganisms involved and their metabolisms

will lead to more efficient control strategies and optimal sewer management.

Acknowledgements

Oriol Gutierrez would like to acknowledge the project CTM 2011-27163 funded by the Spanish Government Ministerio de Economía y Competitividad, the European Commission project 2010-RG277050 and the support from the Economy and Knowledge Department of the Catalan Government through a Consolidated Research Group (2014 SGR 291)-Catalan Institute for Water Research. Dr Guangming Jiang is the recipient of a Queensland State Government's Early Career Accelerate Fellowship (Australia).

References

Abram, J., and Nedwell, D. (1978). Inhibition of methanogenesis by sulfate reducing bacteria competing for transferred hydrogen. Arch. Microbiol. *117*, 89–92.

Auguet, O., Pijuan, M., Guasch-Balcells, H., Borrego, C., and Gutierrez, O. (2015). Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions. Water Res. *68*, 522–532.

Bagreev, A., and Bandosz, T. (2004). Carbonaceous materials for gas phase desulphurization: role of surface. Am. Chem. Soc. Div. Fuel Chem. *49*, 817–821.

Bagreev, A., and Bandosz, T.J. (2005). On the Mechanism of Hydrogen Sulfide Removal from Moist Air on Catalytic Carbonaceous Adsorbents. Ind. Eng. Chem. Res. *44*, 530–538.

De Belie, N., Monteny, J., Beeldens, A., Vincke, E., Van Gemert, D., and Verstraete, W. (2004). Experimental research and prediction of the effect of chemical and biogenic sulfuric acid on different types of commercially produced concrete sewer pipes. Cem. Concr. Res. *34*, 2223–2236.

Bhattacharya, S.K., Uberoi, V., and Dronamraju, M.M. (1996). Interaction between acetate fed sulfate reducers and methanogens. Water Res. *30*, 2239–2246.

Bodegom, P.M. va., and Stams, A.J.M. (1999). Effects of alternative electron acceptors and temperature on methanogenesis in rice paddy soils. Chemosphere *39*, 167–182.

Boon, A.G., Vincent, A.J., and Boon, K.G. (1998). Avoiding the problems of septic sewage. Water Sci. Technol. *37*, 223–231.

Brongers, M.P.H. (2001). Drinking water and sewer systems. In Cost of Corrosion Study. (NACE, USA), pp. 1–11.

Brongers, M., Virmani, P.Y., and Payer, J.H. (2002). Drinking water and sewer systems. In Corrosion Costs and Preventive Strategies in the United States. (United States Department of Transportation Federal Highway Administration Publication No FHWA-RD-01–156), pp. 1–16.

Cayford, B.I., Dennis, P.G., Keller, J., Tyson, G.W., and Bond, P.L. (2012). High-throughput amplicon sequencing reveals distinct communities within a corroding concrete sewer system. Appl. Environ. Microbiol. *78*, 7160–7162.

Cho, K.-S., and Mori, T. (1995). A newly isolated fungus participates in the corrosion of concrete sewer pipes. Water Sci. Technol. *31*, 263–271.

Coleman, R.N., and Gaudet, I.D. (1993). Thiobacillus neopolitanus implicated in the degradation of concrete tanks used for potable water storage. Water Res. *27*, 413–418.

Dar, S.A., Kleerebezem, R., Stams, A.J.M., Kuenen, J.G., and Muyzer, G. (2008). Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl. Microbiol. Biotechnol. *78*, 1045–1055.

Domingo, J.W.S., Revetta, R.P., Iker, B., Gomez-Alvarez, V., Garcia, J., Sullivan, J., and Weast, J. (2011). Molecular survey of concrete sewer biofilm microbial communities. Biofouling *27*, 993–1001.

Foley, J., Yuan, Z., and Lant, P. (2009). Dissolved methane in rising main sewer systems: field measurements and simple model development for estimating greenhouse gas emissions. Water Sci. Technol. *60*, 2963–2971.

Ganigue, R., Gutierrez, O., Rootsey, R., and Yuan, Z. (2011). Chemical dosing for sulfide control in Australia: an industry survey. Water Res. *45*, 6564–6574.

Ganigué, R., and Yuan, Z. (2014). Impact of oxygen injection on CH4 and N2O emissions from rising main sewers. J. Environ. Manage. *144*, 279–285.

Gantner, S., Andersson, A.F., Alonso-Sáez, L., and Bertilsson, S. (2011). Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. J. Microbiol. Meth. *84*, 12–18.

Greene, E.A., Hubert, C., Nemati, M., Jenneman, G.E., and Voordouw, G. (2003). Nitrite reductase activity of sulfate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. Environ. Microbiol. *5*, 607–617.

Gu, J.D., Ford, T.E., Berke, N.S., and Mitchell, R. (1998). Biodeterioration of concrete by the fungus Fusarium. Int. Biodeterior. Biodegradation *41*, 101–109.

Guisasola, A., Sharma, K.R., Keller, J., Yuan, Z., and Jiang, G. (2009). Development of a model for assessing methane formation in rising main sewers. Water Res. *43*, 2874–2884.

Gupta, A. (1994). Methanogenesis and sulfate reduction in chemostats – I. Kinetic studies and experiments. Water Res. *28*, 781–793.

Gutierrez, O., Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Keller, J., and Yuan, Z. (2008). Evaluation of oxygen injection as a means of controlling sulfide production in a sewer system. Water Res. *42*, 4549–4561.

Gutierrez, O., Park, D., Sharma, K.R., and Yuan, Z. (2009). Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms. Water Res. *43*, 2549–2557.

Gutierrez, O., Sharma, K.R., Batista, J., Pijuan, M., and Poch, M. (2012). Monitoring and prediction of sulfide and methane emisions from sewer in mediterreanean cities. In META 2012, X Mesa Española de Tratamiento de Aguas.

Gutierrez, O., Sudarjanto, G., Ren, G., Ganigué, R., Jiang, G., and Yuan, Z. (2014). Assessment of pH shock as a method for controlling sulfide and methane formation in pressure main sewer systems. Water Res. *48*, 569–578.

Haile, T., Nakhla, G., Allouche, E., and Vaidya, S. (2010). Evaluation of the bactericidal characteristics of nano-copper oxide or functionalized zeolite coating for bio-corrosion control in concrete sewer pipes. Corros. Sci. *52*, 45–53.

Hewayde, E.H., Nakhla, G.F., Allouche, E.N., and Mohan, P.K. (2007). Beneficial impact of coatings on biological generation of sulfide in concrete sewer pipes. Struct. Infrastruct. Eng. *3*, 267–277.

Hubert, C., Nemati, M., Jenneman, G., and Voordouw, G. (2005). Corrosion risk associated with microbial souring control using nitrate or nitrite. Appl. Microbiol. Biotechnol. *68*, 272–282.

Hvitved-Jacobsen, T. (2002). Sewer systems and processes. In Sewer Processes: Microbial and Chemical Process Engineering of Sewer Networks. Hvitved-Jacobsen, T., Vollertsen, J., and Nielsen, A.H., eds (CRC Press, Taylor and Francis Group LLC, Boca Raton, FL, USA), pp. 1–10.

Islander, R.L., Devinny, J.S., Mansfeld, F., Postyn, A., and Shih, H. (1991). Microbial ecology of crown corrosion in sewers. J. Environ. Eng. *117*, 751–770.

Ismail, N., Nonaka, T., Noda, S., and Mori, T. (1993). Effects of carbonation on microbial corrosion of concretes. J. Constr. Manag. Eng. *20*, 11–138.

Jelic, A., Rodriguez-Mozaz, S., Barcelo, D., and Gutierrez, O. (2014). Impact of in-sewer transformation on 43 pharmaceuticals in a pressurized sewer under anaerobic conditions. Water Res. *68*, 98–108.

Jensen, H.S., Nielsen, A.H., Hvitved-Jacobsen, T., and Vollertsen, J. (2008). Survival of hydrogen sulfide oxidizing bacteria on corroded concrete surfaces of sewer systems. Water Sci. Technol. *57*, 1721–1726.

Jiang, G., and Yuan, Z. (2013a). Synergistic inactivation of anaerobic wastewater biofilm by free nitrous acid and hydrogen peroxide. J. Hazard. Mater. *250–251*, 91–98.

Jiang, G., and Yuan, Z. (2013b). Inactivation kinetics of anaerobic wastewater biofilms by free nitrous acid. Appl. Microbiol. Biotechnol. *98*, 1367–1376.

Jiang, G., Sharma, K.R., Guisasola, A., Keller, J., and Yuan, Z. (2009). Sulfur transformation in rising main sewers receiving nitrate dosage. Water Res. *43*, 4430–4440.

Jiang, G., Gutierrez, O., Sharma, K.R., and Yuan, Z. (2010). Effects of nitrite concentration and exposure time on sulfide and methane production in sewer systems. Water Res. *44*, 4241–4251.

Jiang, G., Gutierrez, O., and Yuan, Z. (2011a). The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res. *45*, 3735–3743.

Jiang, G., Gutierrez, O., and Yuan, Z. (2011b). The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res. *45*, 3735–3743.

Jiang, G., Sharma, K.R., and Yuan, Z. (2013a). Effects of nitrate dosing on methanogenic activity in a sulfide-producing sewer biofilm reactor. Water Res. *47*, 1783–1792.

Jiang, G., Keating, A., Corrie, S., O'Halloran, K., Nguyen, L., and Yuan, Z. (2013b). Dosing free nitrous acid for sulfide control in sewers: Results of field trials in Australia. Water Res. *47*, 4331–4339.

Jiang, G., Wightman, E., Donose, B.C., Yuan, Z., Bond, P.L., and Keller, J. (2014). The role of iron in sulfide induced corrosion of sewer concrete. Water Res. *49*, 166–174.

Joseph, A.P., Keller, J., Bustamante, H., and Bond, P.L. (2012a). Surface neutralization and H(2)S oxidation at early stages of sewer corrosion: influence of temperature, relative humidity and H(2)S concentration. Water Res. *46*, 4235–4245.

Joseph, A.P., Keller, J., Bustamante, H., and Bond, P.L. (2012b). Surface neutralization and H2S oxidation at early stages of sewer corrosion: Influence of temperature, relative humidity and H2S concentration. Water Res. *46*, 4235–4245.

Kalyuzhnyi, S.V., and Fedorovich, V.V. (1998). Mathematical modelling of competition between sulfate reduction and methanogenesis in anaerobic reactors. Bioresour. Technol. *65*, 227–242.

Kelly, D.P., and Wood, A.P. (2000). Reclassification of some species of Thiobacillus to the newly designated genera Acidithiobacillus gen. nov., Halothiobacillus gen. nov and Thermithiobacillus gen. nov. Int. J. Syst. Evol. Microbiol. *50*, 511–516.

Koch, G.H., Brongers, M.P.H., Thompson, N.G., Virmani, Y.P., and Payer, J. (2002). Corrosion costs and preventive strategies in the United States (NACE International, Houston, TX, USA).

Lahav, O., Lu, Y., Shavit, U., and Loewenthal, R. (2004). Modeling hydrogen sulfide emission rates in gravity sewage collection systems. J. Environ. Eng. *130*, 1382–1389.

Lovley, D.R., Dwyer, D.F., and Klug, M.J. (1982). Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol. *43*, 1373–1379.

Mahmood, Q., Zheng, P., Cai, J., Wu, D., Hu, B., and Li, J. (2007). Anoxic sulfide biooxidation using nitrite as electron acceptor. J. Hazard. Mater. *147*, 249–256.

Metcalf and Eddy, I. (2003). Wastewater Engineering, Treatment and Reuse, 4th edn (McGraw-Hill, New York, NY, USA).

Mohanakrishnan, J., Gutierrez, O., Meyer, R.L., and Yuan, Z. (2008). Nitrite effectively inhibits sulfide and methane production in a laboratory scale sewer reactor. Water Res. *42*, 3961–3971.

Mohanakrishnan, J., Gutierrez, O., Sharma, K.R., Guisasola, A., Werner, U., Meyer, R.L., Keller, J., and Yuan, Z. (2009). Impact of nitrate addition on biofilm properties and activities in rising main sewers. Water Res. *43*, 4225–4237.

Monteny, J., Vincke, E., Beeldens, A., De Belie, N., Taerwe, L., Van Gemert, D., and Verstraete, W. (2000). Chemical, microbiological, and *in situ* test methods

for biogenic sulfuric acid corrosion of concrete. Cem. Concr. Res. *30*, 623–634.

Mori, T., Nonaka, T., Tazaki, K., Koga, M., Hikosaka, Y., and Noda, S. (1992). Interactions of nutrients, moisture and pH on microbial corrosion of concrete sewer pipes. Water Res. *26*, 29–37.

De Muynck, W., De Belie, N., and Verstraete, W. (2009). Effectiveness of admixtures, surface treatments and antimicrobial compounds against biogenic sulfuric acid corrosion of concrete. Cem. Concr. Compos. *31*, 163–170.

Muyzer, G., and Stams, A.J.M. (2008). The ecology and biotechnology of sulfate-reducing bacteria. Nat. Rev. Microbiol. *6*, 441–454.

Nemati, M., Mazutinec, T.J., Jenneman, G.E., and Voordouw, G. (2001). Control of biogenic H2S production with nitrite and molybdate. J. Ind. Microbiol. Biotechnol. *26*, 350–355.

Nica, D., Davis, J.L., Kirby, L., Zuo, G., and Roberts, D.J. (2000). Isolation and characterization of microorganisms involved in the biodeterioration of concrete in sewers. Int. Biodeterior. Biodegradation *46*, 61–68.

Nielsen, A.H., Vollertsen, J., and Hvitved-Jacobsen, T. (2004). Chemical sulfide oxidation of wastewater – effects of pH and temperature. Water Sci. Technol. *50*, 185–192.

Nielsen, A.H., Yongsiri, C., Hvitved-Jacobsen, T., and Vollertsen, J. (2005). Simulation of sulfide buildup in wastewater and atmosphere of sewer networks. Water Sci. Technol. *52*, 201–208.

Nielsen, A.H., Vollertsen, J., and Hvitved-Jacobsen, T. (2006). Kinetics and stoichiometry of aerobic sulfide oxidation in wastewater from sewers-effects of pH and temperature. Water Environ. Res. *78*, 275–283.

Nielsen, A.H., Hvitved-Jacobsen, T., and Vollertsen, J. (2012). Effect of sewer headspace air-flow on hydrogen sulfide removal by corroding concrete surfaces. Water Environ. Res. *84*, 265–273.

O'Dea, V. (2007). Understanding biogenic sulfide corrosion. Mater. Perform. *46*, 36–39.

Okabe, S., Odagiri, M., Ito, T., and Satoh, H. (2007). Succession of sulfur-oxidizing bacteria in the microbial community on corroding concrete in sewer systems. Appl. Environ. Microbiol. *73*, 971–980.

Oremland, R.S., and Polcin, S. (1982). Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl. Environ. Microbiol. *44*, 1270–1276.

Parande, A.K., Ramsamy, P.L., Ethirajan, S., Rao, C.R.K., and Palanisamy, N. (2006). Deterioration of reinforced concrete in sewer environments. Proc. Inst. Civ. Eng. Eng. *159*, 11–20.

Parker, C.D. (1951). Mechanics of corrosion of concrete sewers by hydrogen sulfide. Sewage Ind. Waste. *23*, 1477–1485.

Parker, D. (1945). The corrosion of concrete. 2. The fuction of *Thiobacillus concretivorus* (Nov-spec) in the corrosion of concrete exposed to atmospheres containing hydrogen sulfide. Aust. J. Exp. Biol. Med. Sci. *23*, 91–98.

Pikaar, I., Sharma, K.R., Hu, S., Gernjak, W., Keller, J., and Yuan, Z. (2014). Reducing sewer corrosion through integrated urban water management. Science *345*, 812–814.

Pomeroy, R.D. (1990). The Problem of Hydrogen Sulphide in Sewers (Clay Pipe Development Association Limited, London).

Rivera-Garza, M., Olguín, M.T., García-Sosa, I., Alcántara, D., and Rodríguez-Fuentes, G. (2000). Silver supported on natural Mexican zeolite as an antibacterial material. Microporous Mesoporous Mater. *39*, 431–444.

Roberts, D.J., Nica, D., Zuo, G., and Davis, J.L. (2002). Quantifying microbially induced deterioration of concrete: initial studies. Int. Biodeterior. Biodegradation *49*, 227–234.

Rodríguez-Gómez, L.E., Delgado, S., Álvarez, M., and Elmaleh, S. (2005). Inhibition of Sulfide Generation in a Reclaimed Wastewater Pipe by Nitrate Dosage and Denitrification Kinetics. Water Environ. Res. *77*, 193–198.

Sand, W., and Bock, E. (1984). Concrete corrosion in the Hamburg Sewer system. Environ. Technol. Lett. *5*, 517–528.

Saraswathy, V., and Song, H.-W. (2007). Improving the durability of concrete by using inhibitors. Build. Environ. *42*, 464–472.

Sivret, E., and Stuetz, R.M. (2010). Sewer odour abatement practices – an Australian survey. Water *37*, 77–81.

Soleimani, S., Ormeci, B., and Isgor, O.B. (2013a). Growth and characterization of Escherichia coli DH5α biofilm on concrete surfaces as a protective layer against microbiologically influenced concrete deterioration (MICD). Appl. Microbiol. Biotechnol. *97*, 1093–1102.

Soleimani, S., Ormeci, B., and Isgor, O.B. (2013b). Evaluation of *E. coli* biofilm as a protective barrier against microbiologically influenced deterioration of concrete (MICD) under mesophilic temperatures. Water Sci. Technol. *68*, 303–310.

Sørensen, J., Christensen, D., and Jørgensen, B.B. (1981). Volatile fatty acids and hydrogen as substrates for sulfate-reducing bacteria in anaerobic marine sediment. Appl. Environ. Microbiol. *42*, 5–11.

Sun, J., Hu, S., Sharma, K.R., Ni, B.-J., and Yuan, Z. (2014). Stratified microbial structure and activity in sulfide- and methane- producing anaerobic sewer biofilms. Appl. Environ. Microbiol. *80*, 7042–7052.

Sydney, R., Esfandi, E., and Surapaneni, S. (1996). Control concrete sewer corrosion via the crown spray process. Water Environ. Res. *68*, 338–347.

Thai, P.K., O'Briena, J., Jiang, G., Gernjak, W., Yuan, Z., Eaglesham, G., and Mueller, J.F. (2014). Degradability of creatinine under sewer conditions affects its potential to be used as biomarker in sewage epidemiology. Water Res. *55*, 272–279.

Thai, P.K., Jiang, G., Gernjak, W., Yuan, Z., Lai, F.Y., and Mueller, J.F. (2014). Effects of sewer conditions on the degradation of selected illicit drug residues in wastewater. Water Res. *48*, 538–547.

Thauer, R.K., Jungermann, K., Decker, K., and Pi, P.P.H. (1977). Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. *41*, 809.

US EPA (1991). Hydrogen sulphide corrosion in wastewater collection and treatment system. Technical report (Office of Water WH595, Washington, DC, USA).

Vincke, E., Boon, N., and Verstraete, W. (2001). Analysis of the microbial communities on corroded concrete sewer pipes – a case study. Appl. Microbiol. Biotechnol. *57*, 776–785.

Vollertsen, J., Almeida, M. d C., and Hvitved-Jacobsen, T. (1999). Effects of temperature and dissolved oxygen on hydrolysis of sewer solids. Water Res. *33*, 3119–3126.

Vollertsen, J., Nielsen, A.H., Jensen, H.S., Wium-Andersen, T., and Hvitved-Jacobsen, T. (2008). Corrosion of concrete sewers – the kinetics of hydrogen sulfide oxidation. Sci. Total Environ. *394*, 162–170.

Wei, S., Jiang, Z., Liu, H., Zhou, D., and Sanchez-Silva, M. (2014). Microbiologically induced deterioration of concrete: a review. Braz. J. Microbiol. *44*, 1001–1007.

Water Environ. Res. Foundation (2007). Minimization of odor and corrosion in collection systems: phase I (WERF, Alexandria, VA and IWA Publishing, London).

Yamanaka, T., Aso, I., Togashi, S., Tanigawa, M., Shoji, K., Watanabe, T., Watanabe, N., Maki, K., and Suzuki, H. (2002). Corrosion by bacteria of concrete in sewerage systems and inhibitory effects of formates on their growth. Water Res. *36*, 2636–2642.

Yang, W., Vollertsen, J., and Hvitved-Jacobsen, T. (2005). Anoxic sulfide oxidation in wastewater of sewer networks. Water Sci. Technol. *52*, 191–199.

Yongsiri, C., Vollertsen, J., Rasmussen, M., and Hvitved-Jacobsen, T. (2004). Air-water transfer of hydrogen sulfide: an approach for application in sewer networks. Water Environ. Res. *76*, 81–88.

Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., and Verstraete, W. (2008). Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: A review. Water Res. *42*, 1–12.

Zhang, L., Keller, J., and Yuan, Z. (2009). Inhibition of sulfate-reducing and methanogenic activities of anaerobic sewer biofilms by ferric iron dosing. Water Res. *43*, 4123–4132.

Zivica, V., and Bajza, A. (2001). Acidic attack of cement based materials – a review. Part 1. Principle of acidic attack. Constr. Build. Mater. *15*, 331–340.

Part III

New Technologies Using Biofilms

Biofilm Biodegradation Potential

9

Freshta Akbari, Natasha Andrade, Merily Horwat and Birthe V. Kjellerup

Abstract

Persistent organic pollutants (POPs) are present in the environment after decades of industrial activity and have contaminated soils and sediments worldwide. The group of contaminants described as POPs includes toxic compounds such as polychlorinated biphenyls (PCBs), dioxins, chlorinated ethenes and polycyclic aromatic hydrocarbons (PAHs) and brominated flame retardants. Bioremediation of POPs utilizing microbial communities in the biofilm mode of growth has enhanced the removal of POPs from the environments most often converting the organic pollutants to harmless materials. The state-of-the-art for biofilm based solutions for biodegradation of POPs is primarily based on laboratory experiments often performed at optimal conditions. Thus the influence of natural conditions such as nutrient requirement, bioavailability, life style and physico-chemical conditions might vary depending on the POP in question and the environment such as co-contaminants. Field studies of biofilm based solutions are becoming more frequent and most seem promising. Along with these studies knowledge about the mechanisms by which either indigenous or bioaugmented microorganisms forming biofilms enhancing bioremediation is increasingly being expanded.

Biofilms and environmental pollutants

In natural environments, microorganisms mainly exist in biofilms, which can be defined as communities of microorganisms that are set in a self-produced extracellular polymeric substance (EPS) attached to a surface (Costerton, 1999). This mode of life is advantageous for the individual cells compared to a planktonic mode as they are able to communicate and protect each other from the outside environment, while exchanging nutrients and genetic materials among one another and their environment (Costerton, 1999; Davies *et al.*, 1998). Additionally, biofilms are steady structures, since EPS gives the biofilm a dynamic three-dimensional structure consisting of water channels and voids allowing the transport of nutrients and electron acceptors (Ferrera *et al.*, 2004; Gross *et al.*, 2007). Biofilm can be formed of one bacterial species. However, in most cases they are heterogeneous structures containing a multitude of bacterial species as well as fungi, yeast, protozoa, algae, and other microorganisms (Ferrera *et al.*, 2004; Baker *et al.*, 2009).

Biofilms impact natural and industrial systems as well as human health (Hall-Stoodley *et al.*, 2004; Wu *et al.*, 2013). Although biofilms can cause damages to the equipment, impair human health, and contaminate products, they can provide extensive benefits in the treatment of drinking water and wastewater, detoxification, and biodegradation of hazardous contaminants (Bertin *et al.*, 2007; Accinelli *et al.*, 2012). Thus knowledge of their potential benefits to the environment and human health is important and requires a multidisciplinary effort.

Persistent organic pollutants in the environment

Owing to increasing industrial activity the past two centuries, persistent organic pollutants

(POPs) have accumulated in the environment and contaminated many aquatic sites (Lucas *et al.*, 1993; Hu *et al.*, 2014; Kumar *et al.*, 2014). POPs are a group of synthetic compounds such as polychlorinated biphenyls (PCBs), dioxins, chlorinated ethenes and polycyclic aromatic hydrocarbons (PAHs) that were extensively produced during the industrial revolution (Hu *et al.*, 2014; Kumar *et al.*, 2014). Once they are released into the air, water, and sediment, they greatly impact the human health and natural environments due to their high persistency, toxicity, and chemical stability making POPs persist for many decades (Hu *et al.*, 2014; Kumar *et al.*, 2014). After their production stopped, they still were exposed to humans through contaminated food sources such as seafood and dairy products. Their lipophilic nature allows them to persist in the adipose tissues of animals and humans and thus bio-accumulate in the food chain (Kumar *et al.*, 2014). Elimination of POPs from contaminated sites is of utmost importance as they remain in the environment and eventually enter the food chain causing adverse effects in humans (Lucas *et al.*, 1993; Kumar *et al.*, 2014). Commonly applied remediation methods for POPs include dredging and capping (USEPA, 2005). Not only are these techniques expensive, but they can result in high concentrations in the water environment as contaminated sediment is resuspended (Cho *et al.*, 2009). Therefore, less invasive and more effective bioremediation methods are being explored, which will be covered later in this chapter.

Polychlorinated biphenyls (PCBs)

Among the organic pollutants, polychlorinated biphenyls (PCBs) are the most toxic and persistent contaminants. They were applied in multiple industrial products including coolants, transformer oils, flame retardants from the 1930s until their ban in the 1970s (Kimbrough, 1995; Payne *et al.*, 2013). Their low flammability, high stability, and high vaporization temperature made them optimal for industrial purposes (Martinez *et al.*, 2010; Payne *et al.*, 2013). Despite their ban over four decades ago, weathered remains of PCBs are still present in aquatic sediments (Fagervold *et al.*, 2007; Martinez *et al.*, 2010; Payne *et al.*, 2013). PCBs are not easily broken in nature; thus they contaminate soil and sediment, and pose toxicity risks for organisms as they bio-accumulate in the food chain (Vater *et al.*, 1995; Safe *et al.*, 1997; Payne *et al.*, 2013). They are carcinogenic, toxic, and harmful to reproductive and endocrine systems as well as other organs and systems. Therefore, remediation of PCBs is crucial due to their potential carcinogenicity and ability to accumulate to toxic degrees in higher organisms (Kannan *et al.*, 1998).

Current sources are still causing PCBs to enter the environment such as left over industrial installations and accidental synthesis through combustion that enters via atmospheric deposition (Alcock and Behnisch, 1998; Kim, 2004). These new PCB releases can be transported in stormwater and might also be found in waste water treatment plants. Previous research has indicated that bacterial isolates from PCB contaminated sediments can dechlorinate the biphenyl ring structure in both aerobic and anaerobic environments (Kjellerup *et al.*, 2012). However, the performance of native microorganisms is rarely sufficient to degrade PCBs to a required cleanup level due to limited abundance. For these reasons, developing effective and efficient remediation tools is sought after and the application of biofilm based methods would be advantageous.

Dioxins

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/Fs) are aromatic chemicals that originate as a result of industrial and combustion by-products (Buser *et al.*, 1985; Cormier *et al.*, 2006). In preparing the industrial chemicals, chlorinated phenols are the starting material, mainly in pesticides. As a result of heating commercial chlorinated phenols, dioxins are released into the environment (Buser *et al.*, 1985; Lohman and Seigneur, 2001; Cormier *et al.*, 2006). Dioxins are found around the world in air, soils, sediment, and food products. Upon their release into the environment, a range of developmental, immunological, and neurological toxic risks are exposed to humans and animals (Buser *et al.*, 1985; Cormier *et al.*, 2006). Due to their chemical stability, high dielectric constants, and non-flammability, they tolerate extreme conditions and are a serious

threat to natural systems due to accumulation in the food chain (Lohman and Seigneur, 2001).

Chlorinated ethenes

Chlorinated ethenes are among the most widespread organochlorine water pollutants and they are formed from mainly from emission of burning biomass and volcanoes (Lohner *et al.*, 2011; Tobiszewski and Namiesnik, 2012). Chloroethenes are utilized as cleaning materials, paint removers, industrial solvents, and pesticides and due to their often improper disposal, they can be found primarily in groundwater (Lohner *et al.*, 2011;, Tobiszewski and Namiesnik, 2012). One of their properties is low solubility in water, which enables them to form plumes of dense non-aqueous phase liquid giving them the ability to migrate and making their degradation challenging (Tobiszewski and Namiesnik, 2012). TCE is also a volatile compound and can enter the gas phase and penetrate the pores of surrounding soils (USEPA, 1992). Among them, tricholorethenes (TCE) and perchloroethenes (PCE) are commonly found in contaminated sites (Lohner *et al.*, 2011). These compounds can be reductively dechlorinated by natural processes, which can result in the creation of vinyl chloride, which is extremely toxic to humans (USEPA, 1992).

Anaerobically, the bacterial cultures of *Dehalococcoides* class have shown to reductively dechlorinate TCE and PCE (Maymo-Gatell *et al.*, 1999; Krajmalnik-Brown *et al.*, 2007). Table 9.1

Table 9.1 Bacterial species that have been applied in biofilm based bioremediation approaches

Contaminant	Organisms involved	Process	Reference
Polychlorinated biphenyls (PCBs),	*Dehalobium chlororcoceae* DF1	Anaerobic	May *et al*. (2008)
	Burkholderia xenovorans strain LB400	Aerobic	Bartels *et al*. (1999), Chain *et al*. (2006)
Dioxins: Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/Fs)	*Comamonas* sp. strain KD7 and *Trifolium repens*	Aerobic	Wang and Oyaizu (2011)
	Mixed biofilm of *Burkholderia* sp. NK8 and *Pseudomonas aeruginosa* PAO1	Aerobic	Yoshida *et al*. (2009)
	Dehalococcoides mccartyi strain 195	Anaerobic	Liu *et al*. (2013)
Chlorinated ethenes:, tricholorethenes (TCE) and perchloroethenes (PCE)	*Dehalococcoides* genus	Anaerobic	Maymo-Gatell *et al*. (1999), Krajmalnik-Brown *et al*. (2007)
	Dehalobacter restrictus, *Dehalospirilum multivorans*, and *Geobacter lovleyi*	Anaerobic	Mattes *et al*. (2010)
TCE, PCE	Methanogens	Anaerobic co-metabolism	Maymo-Gatell *et al*. (1999)
	Acetogens		
	Sulfate-reducing bacteria		
Polycyclic aromatic hydrocarbons (PAHs)	*Pseudomonas*, *Sphingonomas*, *Mycobacterium*, *Nocardioides*, *Rhodococcus* and *Novosphingobium*	Aerobic	Lyu *et al*. (2014)
	Arthrobacter viscosus	Aerobic	Ferreira *et al*. (2013)
	Rhodococcus erythropolis and *Pseudomonas* spp.	Aerobic	Kurane (1997)
Brominated flame retardants: (1) polybrominated diphenyl ethers (PBDEs), (2) hexabromocyclododecanes, (3) tetrabromobisphenol A, (4) polybrominated biphenyls and (5) others	*Sulfurospirillum multivorans* and *Dehalococcoides* spp.	Anaerobic	He *et al*. (2006)
	Rhodococcus jostii	Aerobic	Robrock *et al*. (2009)
	Burkholderia xenovorans	Aerobic	Lu *et al*. (2008)
	Bacillus cereus		

provides an overview of microorganisms taking part in biodegradation of POPs. These chlorinated ethenes can also be co-metabolically dechlorinated via methanogens, acetogens, and sulfate-reducing bacteria that contain metal cofactors including haems, corrinoids, and cofactor F_{430} (Maymo-Gatell *et al.*, 1999). Degrading chloroethenes primarily convert to less chlorinated compounds and as the process goes further they will be converted to nontoxic ethane products (ETH) that are harmless (Maymo-Gatell *et al.*, 1999; Lohner *et al.*, 2011).

Polycyclic aromatic hydrocarbons (PAHs)

These are compounds with fused benzene rings that are ubiquitously produced in nature (Lyu *et al.*, 2014). PAHs are formed as a result of numerous human activities such as incomplete combustion of coal, gas, oil spills of petroleum, woods, municipal and industrial incinerators (Seo *et al.*, 2009). They have low water solubility and are thermodynamically stable giving rise to detrimental health effects (Johnsen and Karlson, 2004) (Lyu *et al.*, 2014). Naturally, they are biodegraded via multiple methods such as chemical and photo-oxidation, volatilization, and adsorption to sorptive surfaces (Rodriguez and Bishop, 2008; Lyu *et al.*, 2014). Additionally they are remediated through various bacterial communities' activities including *Pseudomonas*, *Sphingonomas*, *Mycobacterium*, *Nocardioides*, *Rhodococcus*, and *Novosphingobium* (Lyu *et al.*, 2014).

Brominated flame retardants

As civilization progresses, the discovery and use of new chemicals benefits society. Although some chemicals are produced and used with success for purposes that are in general considered good for the majority of the population, these chemicals can have an unexpected consequence after their primary use. This is the case of some flame retardants. Flame retardant chemicals are added to consumer products to reduce the heat release during a fire and ultimately to prevent or delay fires. They work as intended if used properly, however, they can be released from the consumer products and enter the environment. A particular class of flame retardants has become the subject of concern by the general public, environmental advocates and researchers: brominated flame retardants (BFRs).

BRFs are generally mixtures of flame retardant chemicals commonly used in plastics, textiles, electrical equipment, and construction materials (de Wit, 2002). There are five major classes of brominated flame retardants: (1) polybrominated diphenyl ethers (PBDEs), (2) hexabromocyclododecanes, (3) tetrabromobisphenol A, (4) polybrominated biphenyls, and (5) others. The first class, PBDEs, have been under intense scrutiny and, in the US, have gone through an industry voluntary phase-out of production (USEPA, 2009; USEPA, 2014). PBDEs have similar chemical structures as PCBs. Two of the PBDEs (BDE-47 and BDE-99) are now listed as persistent organic pollutants by the Stockholm Convention (UNEP, 2004). BDE-209 is the fully brominated congener and the main component of the deca-BDE commercial formulation and American manufacturers and importers of BDE-209 have announced a commitment to stop production and use by 2013 (USEPA, 2009).

PBDEs are hydrophobic molecules and have the tendency to accumulate in organic phases such as fatty tissue of humans and animals. In the external environment (not including mammalian tissues), PBDEs are ubiquitous and have been reported in house dust and indoor air (de Boer *et al.*, 2003; Harrad *et al.*, 2007; Voorspoels *et al.*, 2007), soils and biosolids (Andrade *et al.*, 2010), humans (Trudel *et al.*, 2011) and all other environmental compartments. The lowly brominated congeners are known to bioaccumulate, biomagnify, to easily adsorb, and to be more bioactive than the BDE-209 (McDonald, 2002; Stapleton *et al.*, 2005). PBDEs have also been shown to display endocrine disrupting potential in rats (Staskal *et al.*, 2005; Lilienthal *et al.*, 2006), but toxicity in humans is not well studied and lacks the availability of data. Most humans are exposed to PBDEs via food ingestion and breathing of air containing these chemicals and workers in the PBDE manufacturing industry are at a higher risk of exposure (Darnerud *et al.*, 2001).

Biodegradation of persistent organic pollutants using biofilms

Remediation of POPs from contaminated sites is a priority due to their ability to enter the food chain and their potent toxic and carcinogenic properties. Various approaches have been taken, but most POP remediation methods are costly and inefficient such as dredging and capping (USEPA, 2005). Dredging is not only expensive, but can also result in increased POP concentrations in the water phase due to re-suspension of contaminated particles into the water phase causing risk for bioaccumulation (Payne *et al.*, 2011; Martins *et al.*, 2012). Capping effectively controls POP equilibrium while keeping them out of the water phase by sequestration with activated carbon and some other substrates (Zimmerman *et al.*, 2004). But hazardous substances still remain in the environment and contaminated sediments can become recalcitrant to microbial degradation because of strong sequestering capacities of soil particles (Hatzinger and Alexander, 1995).

Research has shown that microbial communities can be established that can degrade organic pollutants aerobically and anaerobically (Kjellerup *et al.*, 2012). While *in situ* microbial degradation of POPs would represent a significant improvement in remediation efforts, previous attempts have failed due to POP stability, low bioavailability, and the low abundance and activity of naturally occurring POP-degrading microorganisms. To overcome these issues a solution would be to locate microbial communities onto sorptive surfaces such as activated carbon surfaces and apply these biofilm communities to contaminated sites.

PCBs

A number of organohalide respiring bacterial species have been identified for their ability to transform PCBs and some of these microbial communities have been established as potential agents for PCB bioremediation. Anaerobic bacteria can dechlorinate highly chlorinated PCBs as they use the chlorines as electron acceptor, while aerobic bacteria degrade PCBs with four or less chlorines by breaking the biphenyl ring structure open and give off water and carbon dioxide as their end products (Fagervold *et al.*, 2007; Payne *et al.*, 2011). Enhanced PCB dechlorination has been observed, when granular activated carbon (GAC) was used as a surface for biofilm formation (Edwards and Kjellerup, 2013; Kjellerup *et al.*, 2014; Mercier *et al.*, 2014). Co-localizing PCB-degrading microbes as biofilms and utilizing it as a microbial inoculum delivery system provides a number of benefits and have shown a high efficiency of GAC to quickly adsorb and sequester PCBs from aquatic sediments (Werner *et al.*, 2006; Edwards and Kjellerup, 2013). They also provide additional PCB-degrading microbes adjacent to sequestered PCBs that enhance the degradation capacity. As a result of this close spatial setting, microbes utilize PCB as an electron acceptor and enable subsequent degradation (Edwards and Kjellerup, 2013). Knowing both the aerobic and anaerobic degradation possibilities of PCBs, several studies tested both conditions and their efficiencies compared to one condition alone. A promising study by Payne *et al.* (2013) evaluated the simultaneous presence of DF1 and LB400 with GAC in bioaugmentation efficiency of contaminated sediment. After 3 months, the amount of PCBs had decreased by 80%, while a 25% decrease was observed in non-bioaugmented sediments after 12 months (Payne *et al.*, 2013).

Dioxins

In a similar fashion biodegradation of dioxins has been reported. Wang and Oyaizu (2011) reported that a biofilm of *Comamonas* sp. strain KD7 and *Trifolium repens* increased reduction rates of dioxins in soil samples by 22% after 3 months. Bioremediation efforts have demonstrated that presence of more than one bacterial species increased the degradation efficiency for dioxins. This strategy was evaluated, when a mixed biofilm of *Burkholderia* sp. NK8 and *Pseudomonas aeruginosa* PAO1 was applied that degraded chlorinated benzoates (Yoshida *et al.*, 2009). Studies have also shown that dioxins can be biodegraded by biofilms in unfavourable environments such as River Kymijoki Finland (Liu *et al.*, 2013). Here it was shown that the bioremediation of weathered dioxins present in the sediment biofilms

could be enhanced by stimulation with electron donors, co-substrate and/or bioaugmentation with *Dehalococcoides mccartyi* strain 195 (Liu *et al.*, 2013).

Chlorinated ethenes

In water, chlorinated ethenes can be degraded to harmless products. However, this process is not always completed, which results in formation of other toxic byproducts (Maymo-Gatell *et al.*, 1999; Mattes *et al.*, 2010). Chlorinated ethenes can be metabolized via several pathways: anaerobic reductive dechlorination, aerobic co-metabolism and anaerobic/anaerobic oxidation (Mattes *et al.*, 2010; Frascari *et al.*, 2013). Lower oxidized chlorinated ethenes can be degraded by anaerobic dechlorinating bacteria belonging to the *Dehalococcoides* genus, such as *Dehalobacter restrictus*, *Dehalospirilum multivorans*, and *Geobacter lovleyi* (Mattes *et al.*, 2010). A challenge for biodegradation in the environment is that these microbes are usually present in low numbers and their degradation reaction rates are low, unless biofilm based applications are utilized for instance in biobarriers or biowalls. Biowalls for remediation of TCE can be installed down gradient of the contaminated area and use the natural flow of groundwater to bring the contaminant through the matrix for degradation. The substrate for biofilm growth in biowalls can be plant mulch from pine, hardwood and cypress established as a reactive barrier are benefiting from the presence of indigenous TCE degrading organisms in the system (Lu *et al.*, 2008; Wei and Seo, 2010).

Polycyclic aromatic hydrocarbons (PAHs)

Given the high toxicity of PAHs, many studies have been conducted to evaluate the microbial interactions in PAHs remediation. Enhanced degradation is often reported in situations, where bacteria are associated with surfaces. Plosz *et al.* (2010) showed that PAHs removal occurred faster, when a biofilm delivery system was combined with ozonation (BIOZO system) and applied to landfill leachate. The study utilized a stage moving-bed biofilm reactor (SMBBR), while evaluating the efficiency of ozonation on removal of PAHs between a pre-anoxic zone and an aerobic zone. These results indicate that nitrate was reduced along with PAH degradation occurring in a reduced and anoxic environment. This dual removal of nitrate and PAH highlights the importance of co-metabolism in biofilm based remediation (Heidler and Halden, 2007; Lolas *et al.*, 2012).

Brominated flame retardants (PBDEs)

These substances are considered to be persistent in the environment, but research has shown the potential for biodegradation (Rayne *et al.*, 2003; He *et al.*, 2006; Vonderheide *et al.*, 2008). One anaerobic biodegradation study (Gerecke *et al.*, 2005) showed that BDE-209 can be degraded to nona- and octa-BDEs using biofilms from sewage sludge as the inoculum and the calculated half-life for BDE-209 was 700 days in the laboratory setting. (He *et al.*, 2006) identified two anaerobic bacterial species able to degrade deca-BDE and octa-BDE mixtures to lower brominated congeners: *Sulfurospirillum multivorans* and *Dehalococcoides* species. The anaerobic degradation of PBDEs has also been observed in sediments, where the sorption of PBDEs to sediment particles may play an important role in slowing down the rate of degradation (Tokarz *et al.*, 2008). In a study with BDE-contaminated sediment, the reductive debromination of BDE-209 was observed and formation of lower brominated congeners (from nona- to hexa-BDEs) occurred as a result. However, the half-life in sediment was estimated at well above a decade (Tokarz *et al.*, 2008). In soils, very little biodegradation was observed for a tri-BDE and BDE-209 in contaminated soils brought to the laboratory and incubated at aerobic and anaerobic conditions (Nyholm *et al.*, 2010). In the environment, BDE-209 was found in remote high altitude mountain lakes despite its low volatility (Bartrons *et al.*, 2011). Moreover, analysis of bottom rock and silt biofilms in the lakes revealed that the concentration of PBDEs in silt biofilms was much lower than the PBDEs concentration in rock biofilms, suggesting that PBDE biodegradation was favoured in silt biofilms due to longer anoxic periods (Bartrons *et al.*, 2011).

Aerobic degradation of PBDEs has been observed, though in laboratory

conditions. (Robrock *et al*., 2009) observed that the PCB-degrading bacteria *Rhodococcus jostii* and *Burkholderia xenovorans* were able to aerobically degrade PBDE congeners with 1–5 bromine atoms to lower brominated compounds. In another laboratory study, a metal-resistant bacterial strain, *Bacillus cereus*, was able to use BDE-209 as its carbon and energy source (Lu *et al*., 2008) and the authors suggested the possibility of using this bacterium for bioremediation of contaminated sites containing co-contaminants, such as other organic pollutants and metals. However, BDE-47 has shown some toxicity to marine bacteria, reducing the abundance of aerobic marine biofilms (Chiu *et al*., 2012). During laboratory experiments of aerated activated sludge treatment of wastewaters, biodegradation of PBDEs and the debromination of some PBDE congeners was observed. However, PBDE exposure to activated sludge bacteria caused a reduction in the community diversity for a short period of time (Langford *et al*., 2007). This suggests that creating the right conditions for anaerobic or aerobic degradation of PBDEs may be challenging, but one of the solutions might be to apply biofilms, where favourable conditions for debromination could be established by applying mixed cultures of other environmental species that can survive the toxic exposure to PBDEs. Although significant progress has been made in identifying bacterial species capable of biodegrading PBDEs, research is still needed for improved understanding of biodegradation pathways and to utilize biofilm based degradation as a potential remediation technology for PBDEs.

Applied biofilm based biodegradation methods

Despite the chemical stability and toxicity of POPs, many microorganisms and methods have been proposed that are able to transform these contaminants. Some of the more frequently applied solutions are discussed below.

Biofilm barriers

The lipophilicity and low water solubility of POPs make them persist in the environment and remain primarily in the solid phase. To prevent these contaminants from spreading into larger areas and simultaneously promote biodegradation, biobarriers consisting of biofilms established on support materials such as mulch, saw dust, and peat moss have been utilized (Seo and Bishop, 2008; Seo *et al*., 2009). In a lab scale mulch biofilm barrier (Seo *et al*., 2009) reported that after 150 days, a removal efficiency of 97–99% for phenanthrene and 99.9% for pyrene was observed. However, the mulch biobarrier itself could not entirely prevent migration of aqueous and solubilized PAHs that moved through the barrier without being degraded (Seo *et al*., 2009). In another study investigating PAH bioremediation (Ferreira *et al*., 2013) evaluated if the bacterium *Arthrobacter viscosus* would function as a permeable reactive biobarrier (PRBB) for treating PAH contaminated groundwater. It was observed that *A. viscosus* formed a functional and effective biobarrier, where benzo[a]anthracene and phenanthrene removals were observed after seven and three days, respectively. The study also showed that *A. viscosus* adhered readily to sepiolite thus providing a growth surface that can be applied as a solution for PAH remediation in groundwater environments (Ferreira *et al*., 2013).

Studies of biobarriers established for bioremediation of trichloroethylene (TCE) contaminated ground water has shown similar promising results as for PAHs. Instead of using solid surfaces as substratum for biofilm formation a polycolloid releasing substrate was established as a biobarrier system (Liang *et al*., 2013). The biobarrier consisted of biodegradable substrates such as vegetable oil (slow release), cane molasses (fast release), two types of surfactants to increase the solubility of TCE for microbial degradation (simple green and soya lecithin) that was formulated as an emulsion enhancing the reductive dechlorination taking place by indigenous dehalorespiring bacteria in the groundwater that was fed to the column experiment. The emulsion created anaerobic conditions and was shown to induce more complete removal of TCE due to increased sorption and subsequent microbial degradation. This concept was subsequently tested under field conditions to reduce TCE plume migration (Kuo *et al*., 2014). In the field study, the polycolloid biobarrier consisting of soybean oil, lactate and surfactants, which was employed

via injection wells (5 m intervals) into the TCE contaminated ground water. The monitoring lasted for approximately 100 days and significant reduction of TCE was observed after 35 days (87 to < 0.1 µg/l) simultaneously with the presence of anaerobic conditions (oxygen concentration decreased from 1.6 to < 0.1 mg/l) and reduced conditions due to the oxidation–reduction potential (ORP) going from 124 to −14 mV after 20 days of injection (Kuo *et al.*, 2014). During this time, TCE degradation products were detected in the ground water samples, which support the results showing reductive dechlorination of TCE taking place and reducing the importance of sorption.

A combination of the two above described approaches, solid surface for biofilm development and substrate application, was been taken for the bioremediation of the solvent tetrachloroethylene (PCE) that also is a groundwater contaminant (Kao *et al.*, 2001). The biobarrier was based on deploying a layer of peat, which was meant as the primary electron donor in order to continuously enhance the anaerobic reductive dechlorination of PCE. Experiments performed in continues-flow columns (laboratory scale) for 65 days were used to evaluate the viability of the system for microbial PCE degradation. The results showed up to 98% removal efficiency of PCE in this system due to the continuous supply of electron donor to the indigenous organohalide respiring bacteria from the peat layer, when synthetic PCE contaminated groundwater was tested (Kao *et al.*, 2001). A subsequent study that advanced the solution with peat for PCR bioremediation developed a two-layer biobarrier system that first consisted of organic-releasing layer (sludge cake from wastewater treatment) that was followed by an oxygen releasing layer (calcium peroxide) (Kao *et al.*, 2003). The sludge cake supplied primary substrates (electron donor) for reductive dechlorination of PCE, whereas the oxygen releasing layer enhanced aerobic degradation or co-metabolization of degradation products from the initial anaerobic processes. In this way complete mineralization of PCE and resulting degradation products that often stall the overall PCE remediation could be obtained *in situ*. Similar laboratory based column studies as described above with synthetic PCE contaminated groundwater were performed with this dual layer biobarrier system and showed up to 99% PCE removal efficiency (Kao *et al.*, 2003). If field tests of this system support these results, this dual-layer biobarrier solution will likely be a cost-effective PCE-remediation technology for contaminated groundwater aquifers.

Biobarriers are also deployed for bioremediation of nitrate, atrazine and other less common groundwater contaminants such as nitric acid, uranium and technetium (U(VI), Tc(VII)) (Michalsen *et al.*, 2009; Hunter and Shaner, 2010). Atrazine contaminated groundwater is often containing high levels as nitrate as well, which impedes the biological degradation of the recalcitrant pesticide. Therefore, a dual approach is needed to establish a bioremediation solution that can degrade both contaminants *in situ*. A model of *in situ* biobarriers was established by setting up two reactors in sequence that were inoculated with an atrazine-degrading consortium, where the first reactor was supplying a vegetable-oil-based solution creating denitrifying conditions to remove nitrate and the second reactor was kept aerobic to degrade atrazine. The experiment showed that the initial denitrifying barrier reduced the concentration of nitrate by 98%, making the subsequent aerobic degradation of atrazine possible. Overall, 99.9% of atrazine was removed after 30 weeks of operation (Hunter and Shaner, 2010), where, surprisingly, 30% of the atrazine was removed in the denitrifying reactor despite the oxygen-deprived conditions and the remaining 70% in the aerobic reactor. This dual-biobarrier set-up showed the potential for removal of recalcitrant contaminants such as atrazine that can take place *in situ* if air is injected into the groundwater system or other oxygenation processes such as the calcium peroxide process described above is introduced.

Groundwater contaminated with inorganic compounds such as metals and acids present a different challenge than recalcitrant organic contaminants such as TCE and PCE. The inorganic compounds cannot be biodegraded and instead the effort focuses on bioimmobilization, which is the case for Uranium and Technetium (Michalsen *et al.*, 2009). This was examined in a 21 month long study, where a biobarrier was established

in order to neutralize the nitric acid present in the groundwater (pH 4.7–6.9), remove nitrate and immobilize the present radionuclides in the groundwater. Ethanol was added to the groundwater system that also consisted of crushed limestone and sediment and the pH increased to 6.9, while nitrate was removed, 94% of total-U was immobilized and the sediment biomass in the form of biofilm was increased. Similar changes also occurred in the control system showing that the changes were not due to the addition of ethanol by itself. Instead the changes were shown to be caused by microbial activity, which was confirmed be the presence of dissimilatory nitrite reductase genes (nirS, nirK) supporting the increased denitrification. Analysis of the 16S rRNA from sediment samples showed that Beta-proteobacteria (incl. denitrifying bacteria) were dominant near the influent of acidic groundwater. This changed to Gamma- and Alpha-proteobacteria along the flow path where the pH increased and the concentration of nitrate decreased showing a shift in the microbial population (Michalsen *et al.*, 2009). Overall the results showed that this type of biobarrier can be applied for remediation of acidic radionuclide contaminated groundwater containing high concentrations of nitrate.

Activated sludge biofilms

One promising strategy to enhance POP bioremediation by biofilm is to exploit activated sludge's ability to co-metabolize. The ability of activated sludge to increase the rate of PAH bioremediation has been studied by (Kurane, 1997), where inoculation of *Rhodococcus erythropolis* and *Pseudomonas* sp. to activated sludge efficiently removed a group of PAHs. Additionally, Rodriguez and Bishop (2008) determined that a mixed activated sludge biofilm yielded higher biodegradation rates, when applied to a broad range of PAH substrates. This finding hinted that co-metabolism played an important role in degrading multiple PAHs simultaneously rather than the surfactant being the important factor as previously hypothesized (Rodriguez and Bishop, 2008). Furthermore, a concurrent biofilm delivery system with activated sludge demonstrated a successful approach for removal of micropollutants such as diclofenac (Falas *et al.*, 2013). This study emphasized that using biofilm or activated sludge methods separately did not yield significant results compared to the combined hybrid biofilm-activated sludge process.

Anaerobic biofilm processes in activated sludge wastewater treatment systems for instance digestion processes, which are used for biogas production in order to regain energy from the treatment processes, have shown also to be efficient for degradation of POPs such as PCBs (Bertin *et al.*, 2011). A membrane biological reactor (MBR) was fed with sludge that had been spiked with PCBs and run under both mesophilic (35°C) and thermophilic conditions (55°C). The results showed that more than 50% of the PCBs had been removed due to reductive dechlorination under methanogenic conditions in the digestion process. The microbial community consisted of both fermentative eubacteria as well as acetoclastic and hydrogenotrophic methanogens that all persisted exposure to the high PCB concentrations, while still producing methane. In a previous study of the anaerobic digestion process microbial degradation was also observed for PAHs thus showing that several organic contaminants have the potential to be metabolized during the digestion process (Bertin *et al.*, 2007). Based on this it was shown that biofilm based wastewater treatment systems can be multi-functional and produce energy while simultaneous degrading xenobiotic compounds, which afterwards can be used as fertilizer for agricultural purposes without risk from the presence of pathogenic organisms that also was significantly reduced during the process (Bertin *et al.*, 2007, 2011).

The application of a continuous stirred tank reactor system for treatment of naturally contaminated sewage sludge was also investigated for the biodegradation of six priority PCBs under aerobic and anaerobic conditions (Patureau and Trably, 2006). Less extensively chlorinated compounds experienced losses from aerobic and anaerobic processes that were higher than for the extensively chlorinated compounds (all abiotic losses were below 20%). The degradation was increased under methanogenic conditions, where more than 40% was removed independently of the PCB chlorination degree. The heaviest PCB congeners were more efficiently dechlorinated due to higher

anaerobic process rates (removal rate approximately 40%), whereas the degradation of lighter chlorinated PCBs was enhanced under aerobic conditions removal rate up to 100% (from 40% before). The results indicated that bioavailability of the PCBs in naturally contaminated sludge impacted the biodegradation significantly under both aerobic and anaerobic conditions despite the fact that the rates were enhanced in this continuous stirred tank reactor system (Patureau and Trably, 2006). In the system, the limiting PCB availability influenced the PCB removal negatively resulting in PCB concentrations that exceeded the current French/European regulations about the presence of PCBs in sewage sludge. Thus the biosolids cannot be applied onto farmland, since the reduction throughout the wastewater system did not reduce the concentration significantly (Patureau and Trably, 2006).

Not all xenobiotics can be degraded during the otherwise efficient activated sludge process such as PCE even though biodegradation was observed in biobarriers as described above. In one study it was shown that PCE (range 5–150 mg/l) and 2-CP (range 25–150 mg/l) decreased the microbial activity significantly measured by decrease in activity of the three key enzymes dehydrogenase, phosphatase and urease as well as a reduction of the microbial diversity (Li *et al.*, 2013). Short term exposures did not cause a shift in the microbial community, but longer term exposure induced a shift from Alpha- and Gamma-proteobacteria to firmicutes, bacteroidetes and synergistetes becoming dominant. *Actinobacteria* were eradiated during the long-term treatment.

Microbial fuel cells

One of the recent approaches is the application of microbial fuel cells (MFCs) that transform chemical energy from organic wastes into electrical energy with the organic waste being the electron donor (Yamamoto *et al.*, 2014). In MFCs, biofilm communities attach to the anode and assist in energy transfer. In a study conducted by (Ki *et al.*, 2008), it was concluded that as the diversity of microbial communities increased in a MFCs combined with activated sludge from a wastewater treatment plant, the electricity production was increased. MFCs can generate electricity from different sources and (Patil *et al.*, 2009) showed that wastewater from a chocolate factory generated higher currents compared to that of a membrane and salt bridge or glucose. The 16S rRNA results showed that while multiple bacterial groups participated in the MFC activity, Beta-proteobacteria was the predominant group, making up 51% of the community (Patil *et al.*, 2009). A symbiotic relationship was seen between the biofilm and the bacterial communities in the electrolyte that enhanced the current density to approximately 100–150 mA/m^2 (Yamamoto *et al.*, 2014; see also Chapter 11). 16S rRNA analysis of the MFC biofilms inoculated from activated sludge revealed the presence of *Dysgonomonas*, *Sporomusa*, and *Desulfovibrio* in anode and cathode biofilms, while *Geobacter* was only present in anode biofilm. Electricity production was in this study based on the organic substrate methanol as electron donor. However, other studies have shown that that POPs can be used in the same manner to be transformed into electrical energy. In a study by (Chun *et al.*, 2013) weathered PCBs were electrically stimulated by MFCs, thus enhancing the bioremediation rate and wastewater was treated simultaneously with electricity production, when a bio-electrochemical treatment system (similar to MFC) was applied (Velvizhi *et al.*, 2014).

For an affective production of electricity by MFCs, organic waste is fed to MFCs, while at the same time microbial communities are developed in the anode part. However, the challenge has been to harness a high density current that can be applied for external purposes and this aspect needs further research regarding identification of efficient current producing microbes, understanding of their ecology, and preparing optimal growth and nutrient conditions for the microbial communities.

Perspectives and conclusion

Recent efforts of bioremediation of POPs showed that biofilm microbial communities have significantly increased the removal rate from contaminated environments. This approach provides carbon and energy sources to the microbes as well as a support system to which they can adsorb, while transforming the contaminants (de Lipthay

et al., 2003; Petrie *et al.*, 2003). In return, the biofilm community converts the organic pollutants to harmless materials (de Lipthay *et al.*, 2003; Petrie *et al.*, 2003). Nevertheless, given the different situations and various microbial communities of POPs degraders, more research is necessary to elucidate the nature and biochemical pathways of these degraders.

As mentioned before, POPs are hydrophobic, toxic, and persistent compounds that make them less bioavailable for degradation (Choi *et al.*, 2009). Thus, many biofilm-based biotransformation reactions are added to organic surfaces such as GAC, enabling the POPs to adsorb to these surfaces thus enhancing the efficiency (Payne *et al.*, 2011; Kjellerup *et al.*, 2014). Another bioremediation challenge that the biofilm based approach would help to limit, is the low abundance and availability of indigenous microorganisms that often limits the rate of the bioremediation process (Fagervold *et al.*, 2007; Kjellerup *et al.*, 2008).

Although using microbial communities to enhance POPs bioremediation is a promising strategy, the lack of specific biodegradative pathways (lack of enzymes or cofactors) that are specific for degradation of different POPs might limit the complete mineralization of these compounds. In a study, it was found that *Geobacter* was useful for bioremediation of metals, but a lack of Fe(III) as the electron acceptor slowed the process (O'Neil *et al.*, 2008). Thus the availability of electron donors/acceptors and co-metabolism activities play a significant role in bioremediation (O'Neil *et al.*, 2008; Frascari *et al.*, 2013).

The review of the current state-of-the-art for biodegradation of POPs and the application of biofilm based methods clearly show that most of this knowledge is based on laboratory experiments, where bacterial cultures are grown under controlled conditions. However, the natural conditions, lifestyle, and nutrient requirements of these microorganisms might vary depending on the POP in question and the environment such as co-contaminants and nutrient availability. Each new study adds to the knowledge of POP bioremediation by exploring the mechanism and interactions of these biofilm community members in transforming POPs. Effort should be made to imitate the natural environment, when performing experiments by setting up experiments under relevant environmental conditions for instance as mesocosms in the laboratory or in the environment to study the biodegradation POPs (Edwards and Kjellerup, 2013; Payne *et al.*, 2013). Experiments should account for factors such as system ecology (symbiosis with other organisms), physico-chemical properties of the environmental matrix, co-metabolism of existent microorganisms and sample heterogeneity by setting up a sampling grid and increase the sampling size. To overcome these challenges, applying robust and dense biofilm communities of specific microbes with the addition of electron donors/acceptors has been efficient to enhance the degradation of hazardous POPs contaminated sites. Nevertheless, given the complexity of metabolism pathways and the interaction of microorganisms and their various environments, more research in the field is necessary.

References

Accinelli, C., Sacca, M.L., Mencarelli, M., and Vicari, A. (2012). Application of bioplastic moving bed biofilm carriers for the removal of synthetic pollutants from wastewater. Bioresour. Technol. *120*, 180–186.

Andrade, N.A., McConnell, L.L., Torrents. A., and Ramirez, M.(2010). Persistence of polybrominated diphenyl ethers in agricultural soils after biosolids applications. J. Agric. Food. Chem. *58*, 3077–3084.

Baker, B.J., Tyson, G.W., Goosherst, L., and Banfield, J.F. (2009). Insights into the diversity of eukaryotes in acid mine drainage biofilm communities. Appl. Environ. Microbiol. *75*, 2192–2199.

Bartels, F., Backhaus, S., Moore, E.R.B., Timmis, K.N., and Hofer, B. (1999). Occurrence and expression of glutathiane-S-transferase-encoding bphK genes in *Burkholderia sp* strain LB400 and other biphenyl-utilizing bacteria. Microbiology UK *145*, 2821–2834.

Bartrons, M., Grimalt, J.O., and Catalan, J. (2011). Altitudinal distributions of BDE-209 and other polybromodiphenyl ethers in high mountain lakes. Environ. Pollut. *159*, 1816–1822.

Bertin, L., Capodicasa, S., Occulti, F., Girotti, S., Marchetti, L., and Fava, F. (2007). Microbial processes associated to the decontamination and detoxification of a polluted activated sludge during its anaerobic stabilization. Water Res. *41*, 2407–2416.

Bertin, L., Capodicasa, S., Fedi, S., Zannoni, D., Marchetti, L., and Fava, F. (2011). Biotransformation of a highly chlorinated PCB mixture in an activated sludge collected from a Membrane Biological Reactor (MBR) subjected to anaerobic digestion. J. Hazard. Mater. *186*, 2060–2067.

Buser, H.R., Rappe, C., and Bergqvist, P.A. (1985). Analysis of polychlorinated dibenzofurans, dioxins

and related compounds in environmental samples. Environ. Health Perspect. *60*, 293–302.

Chain, P.S., Denef, V.J., and Konstantinidis, K.T.(2006). *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. Proc. Natl. Acad. Sci. *103*, 15280–15287.

Chiu, J.M., Po, B.H., Chan, C.Y., Lam, M.H., Qian, P.Y., and Wu, R.S. (2012). Polybrominated diphenyl ethers (PBDEs) alter larval settlement of marine intertidal organisms across three phyla via reducing bacterial abundance on the biofilms. Environ. Sci. Technol. *46*, 7772–7781.

Cho, Y.M., Ghosh, U., and Kennedy, A.J.(2009). Field application of activated carbon amendment for in-situ stabilization of polychlorinated biphenyls in marine sediment. Environ. Sci. Technol. *43*, 3815–3823.

Choi, H., Agarwal, S., and Al-Abed, S.R. (2009). Adsorption and simultaneous dechlorination of PCBs on GAC/Fe/Pd: mechanistic aspects and reactive capping barrier concept. Environ. Sci. Technol. *43*, 488–493.

Chun, C.L., Payne, R.B., Sowers, K.R., and May, H.D. (2013). Electrical stimulation of microbial PCB degradation in sediment. Water Res. *47*, 141–152.

Cormier, S.A., Lomnicki, S., Backes, W., and Dellinger, B. (2006). Origin and health impacts of emissions of toxic by-products and fine particles from combustion and thermal treatment of hazardous wastes and materials. Environ. Health Perspect. *114*, 810–817.

Costerton, J.W. (1999). Introduction to biofilm. Int. J. Antimicrob. Agents. *11*, 217–221.

Darnerud, P.O., Eriksen, G.S., Johannesson, T., Larsen, P.B., and Viluksela, M. (2001).Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ. Health Perspect. *109* (Suppl. 1), 49–68.

de Boer, J., Wester, P.G., van der Horst, A., and Leonards, P.E. (2003). Polybrominated diphenyl ethers in influents, suspended particulate matter, sediments, sewage treatment plant and effluents and biota from the Netherlands. Environ. Pollut. *122*, 63–74.

de Lipthay, J.R., Tuxen, N., Johnsen, K., Hansen, L.H., Albrechtsen, H.J., Bjerg, P.L., and Aamand, J. (2003). In situ exposure to low herbicide concentrations affects microbial population composition and catabolic gene frequency in an aerobic shallow aquifer. Appl. Environ. Microbiol. *69*, 461–467.

de Wit, C.A. (2002). An overview of brominated flame retardants in the environment. Chemosphere *46*, 583–624.

Edwards, S.J., and Kjellerup, B.V. (2013). Application of biofilm-covered activated carbon particles as a microbial inoculum delivery system for enhanced bioaugmentation of PCBs in contaminated sediment. SERDP and ESTCP, Arlington, VA, USA.

Fagervold, S.K., May, H.D., and Sowers, K.R. (2007). Microbial reductive dechlorination of Aroclor 1260 in Baltimore Harbor sediment microcosms is catalyzed by three phylotypes within the *Chloroflexi*. Appl. Environ. Microbiol. *73*, 3009–3018.

Falas, P., Longree, P., la Cour Jansen, J., Siegrist, H., Hollender, J., and Joss, A. (2013). Micropollutant removal by attached and suspended growth in a hybrid biofilm-activated sludge process. Water Res. *47*, 4498–4506.

Ferreira, L., Cobas, M., Tavares, T., Sanroman, M.A., and Pazos, M. (2013). Assessment of *Arthrobacter viscosus* as reactive medium for forming permeable reactive biobarrier applied to PAHs remediation. Environ. Sci. Pollut. Res. Int. *20*, 7348–7354.

Ferrera, I., Massana, R., Casamayor, E.O., Balague, V., Sanchez, O., Pedros-Alio, C., and Mas, J. (2004). High-diversity biofilm for the oxidation of sulfide-containing effluents. Appl. Microbiol. Biotechnol. *64*, 726–734.

Frascari, D., Fraraccio, S., Nocentini, M., and Pinelli, D. (2013). Aerobic/anaerobic/aerobic sequenced biodegradation of a mixture of chlorinated ethenes, ethanes and methanes in batch bioreactors. Bioresour. Technol. *128*, 479–486.

Gerecke, A.C., Hartmann, P.C., and Heeb, N.V.(2005). Anaerobic degradation of decabromodiphenyl ether. Environ. Sci. Technol. *39*, 1078–1083.

Gross, R., Hauer, B., Otto, K., and Schmid, A. (2007). Microbial biofilms: new catalysts for maximizing productivity of long-term biotransformations. Biotechnol. Bioeng. *98*, 1123–1134.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. *2*, 95–108.

Harrad, S., Robson, M., Hazrati, S., Baxter-Plant, V.S., Deplanche, K., Redwood, M.D., and Macaskie, L.E. (2007). Dehalogenation of polychlorinated biphenyls and polybrominated diphenyl ethers using a hybrid bioinorganic catalyst. J. Environ. Monit. *9*, 314–318.

Hatzinger, P.B., and Alexander, M. (1995). Effect of aging of chemicals in their biodegradability and extractability. Environ. Sci. Tech. *29*, 537–545.

He, J., Robrock, K.R., and Alvarez-Cohen, L. (2006). Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs). Environ. Sci. Technol. *40*, 4429–4434.

Heidler, J., and Halden, R.U. (2007). Mass balance assessment of triclosan removal during conventional sewage treatment. Chemosphere *66*, 362–369.

Hu, J., Zheng, M., Liu, W., Nie, Z., Li, C., Liu, G., and Xiao, K. (2014). Characterization of polychlorinated dibenzo-*p*-dioxins and dibenzofurans, dioxin-like polychlorinated biphenyls, and polychlorinated naphthalenes in the environment surrounding secondary copper and aluminum metallurgical facilities in China. Environ. Pollut. *193*, 6–12.

Hunter, W.J., and Shaner, D.L. (2010), Biological remediation of groundwater containing both nitrate and atrazine. Curr. Microbiol. *60*, 42–46.

Johnsen, A.R., and Karlson, U. (2004). Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. Appl. Microbiol. Biotechnol. *63*, 452–459.

Kannan, K., Nakata, H., Stafford, R., Masson, G.R., Tanabe, S., and Giesy, J.P. (1998). Bioaccumulation and toxic potential of extremely hydrophobic polychlorinated biphenyl congeners in biota collected at a superfund site contaminated with Aroclor 1268. Environ. Sci. Technol. *32*, 1214–1221.

Kao, C.M., Chen, S.C., and Liu, J.K. (2001). Development of a biobarrier for the remediation of PCE-contaminated aquifer. Chemosphere *43*, 1071–1078.

Kao, C.M., Chen, S.C., Wang, J.Y., Chen, Y.L., and Lee, S.Z. (2003). Remediation of PCE-contaminated aquifer by an *in situ* two-layer biobarrier: laboratory batch and column studies. Water Res. *37*, 27–38.

Ki, D., Park, J., Lee, J., and Yoo, K. (2008). Microbial diversity and population dynamics of activated sludge microbial communities participating in electricity generation in microbial fuel cells. Water Sci. Technol. *58*, 2195–2201.

Kimbrough, R.D. (1995). Polychlorinated biphenyls (PCBs) and human health: an update. Crit. Rev. Toxicol. *25*, 133–163.

Kjellerup, B.V., Sun, X., Ghosh, U., May, H.D., and Sowers, K.R. (2008). Site specific microbial communities in three PCB-impacted sediments are associated with different *in situ* dechlorinating activities. Environ. Microbiol. *10*, 1296–1309.

Kjellerup, B.V., Paul, P., Ghosh, U., May, H.D., and Sowers, K.R. (2012). Spatial distribution of PCB dechlorinating bacteria and activities in contaminated soil. Appl. Env. Soil. Sci. 2012.

Kjellerup, B.V., Naff, C., Edwards, S.J., Ghosh, U., Baker, J.E., and Sowers, K.R. (2014). Effects of activated carbon on reductive dechlorination of PCBs by organohalide respiring bacteria indigenous to sediments. Water Res. *52*, 1–10.

Krajmalnik-Brown, R., Sung, Y., Ritalahti, K.M., Saunders, F.M., and Loffler, F.E. (2007), Environmental distribution of the trichloroethene reductive dehalogenase gene (tceA) suggests lateral gene transfer among *Dehalococcoides*. FEMS Microbiol. Ecol. *59*, 206–214.

Kumar, J., Lind, P.M., and Salihovic, S.(2014). Influence of persistent organic pollutants on the complement system in a population-based human sample. Environ. Int. *71*, 94–100.

Kuo, Y.C., Wang, S.Y., Chang, Y.M., Chen, S.H., and Kao, C.M. (2014). Control of trichloroethylene plume migration using a biobarrier system: a field-scale study. Water Sci. Technol. *69*, 2074–2078.

Kurane, R. (1997). Microbial degradation and treatment of polycyclic aromatic hydrocarbons and plasticizers. Ann. N.Y. Acad. Sci. *829*, 118–134.

Langford, K., Scrimshaw, M., and Lester, J. (2007). The impact of process variables on the removal of PBDEs and NPEOs during simulated activated sludge treatment. Arch. Environ. Contam. Toxicol. *53*, 1–7.

Li, H., Shen, T.T., and Wang, X.L.(2013). Biodegradation of perchloroethylene and chlorophenol co-contamination and toxic effect on activated sludge performance. Bioresour. Technol. *137*, 286–293.

Liang, S.H., Kuo, Y.C., Chen, S.H., Chen, C.Y., and Kao, C.M. (2013). Development of a slow polycolloid-releasing substrate (SPRS) biobarrier to remediate TCE-contaminated aquifers. J. Hazard. Mater. *254–255*, 107–115.

Lilienthal, H., Hack, A., Roth-Harer, A., Grande, S.W., and Talsness, C.E. (2006). Effects of developmental exposure to 2,2,4,4,5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. Environ. Health. Perspect. *114*, 194–201.

Liu, H., Park, J.W., Fennell, D.E., Rodenburg, L.A., Verta, M., and Haggblom, M.M. (2013). Microbially mediated reductive dechlorination of weathered polychlorinated dibenzofurans in Kymijoki sediment mesocosms. Chemosphere *91*, 212–221.

Lohman, K., and Seigneur, C. (2001). Atmospheric fate and transport of dioxins: local impacts. Chemosphere *45*, 161–171.

Lohner, S.T., Becker, D., Mangold, K.M., and Tiehm, A. (2011). Sequential reductive and oxidative biodegradation of chloroethenes stimulated in a coupled bioelectro-process. Environ. Sci. Technol. *45*, 6491–6497.

Lolas, I.B., Chen, X., Bester, K., and Nielsen, J.L. (2012). Identification of triclosan-degrading bacteria using stable isotope probing, fluorescence *in situ* hybridization and microautoradiography. Microbiology *158*, 2796–2804.

Lu, X., Wilson, J.T., Shen, H., Henry, B.M., and Kampbell, D.H. (2008). Remediation of TCE-contaminated groundwater by a permeable reactive barrier filled with plant mulch (Biowall). J. Environ. Sci. Health, Part A: Tox. Hazard. Subst. Environ. Eng. *43*, 24–35.

Lucas, W.D., and Hettige, H. (1993). Economic development, environmental regulation, and the international migration of toxic industrial pollution, 1960–88. Office of the Vice President, Development Economics, World Bank. Washington, DC, USA.

Lyu, Y., Zheng, W., Zheng, T., and Tian, Y. (2014). Biodegradation of polycyclic aromatic hydrocarbons by *Novosphingobium pentaromativorans* US6–1. PLoS One *9*, e101438.

Martinez, A., Wang, K., and Hornbuckle, K.C. (2010). Fate of PCB congeners in an industrial harbor of Lake Michigan. Environ. Sci. Technol. *44*, 2803–2808.

Martins, M., Costa, P.M., Raimundo, J., Vale, C., Ferreira, A.M., and Costa, M.H. (2012). Impact of remobilized contaminants in *Mytilus edulis* during dredging operations in a harbour area: bioaccumulation and biomarker responses. Ecotoxicol. Environ. Saf. *85*, 96–103.

Mattes, T.E., Alexander, A.K., and Coleman, N.V. (2010). Aerobic biodegradation of the chloroethenes: pathways, enzymes, ecology, and evolution. FEMS Microbiol. Rev. *34*, 445–475.

May, H.D., Miller, G.S., Kjellerup, B.V., and Sowers, K.R. (2008). Dehalorespiration with polychlorinated biphenyls by an anaerobic ultramicrobacterium. Appl. Environ. Microbiol. *74*, 2089–2094.

Maymo-Gatell, X., Anguish, T., and Zinder, S.H. (1999). Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by '*Dehalococcoides ethenogenes*' 195. Appl. Environ. Microbiol. *65*, 3108–3113.

McDonald, T.A. (2002). A perspective on the potential health risks of PBDEs. Chemosphere *46*, 745–755.

Mercier, A., Joulian, C., and Michel, C.(2014). Evaluation of three activated carbons for combined adsorption and biodegradation of PCBs in aquatic sediment. Water Res. *59*, 304–315.

Michalsen, M.M., Peacock, A.D., and Smithgal, A.N.(2009). Treatment of nitric acid-, U(VI)-, and Tc(VII)-contaminated groundwater in intermediate-scale physical models of an *in situ* biobarrier. Environ. Sci. Technol. *43*, 1952–1961.

Nyholm, J.R., Lundberg, C., and Andersson, P.L. (2010). Biodegradation kinetics of selected brominated flame retardants in aerobic and anaerobic soil. Environ. Pollut. *158*, 2235–2240.

O'Neil, R.A., Holmes, D.E., and Coppi, M.V.(2008). Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation. Environ. Microbiol. *10*, 1218–1230.

Patil, S.A., Surakasi, V.P., Koul, S., Ijmulwar, S., Vivek, A., Shouche, Y.S., and Kapadnis, B.P. (2009). Electricity generation using chocolate industry wastewater and its treatment in activated sludge based microbial fuel cell and analysis of developed microbial community in the anode chamber. Bioresour. Technol. *100*, 5132–5139.

Patureau, D., and Trably, E. (2006). Impact of anaerobic and aerobic processes on polychlorobiphenyl removal in contaminated sewage sludge. Biodegradation *17*, 9–17.

Payne, R.B., May, H.D., and Sowers, K.R. (2011). Enhanced reductive dechlorination of polychlorinated biphenyl impacted sediment by bioaugmentation with a dehalorespiring bacterium. Environ. Sci. Technol. *45*, 8772–8779.

Payne, R.B., Fagervold, S.K., May, H.D., and Sowers, K.R. (2013). Remediation of polychlorinated biphenyl impacted sediment by concurrent bioaugmentation with anaerobic halorespiring and aerobic degrading bacteria. Environ. Sci. Technol. *47*, 3807–3815.

Petrie, L., North, N.N., Dollhopf, S.L., Balkwill, D.L., and Kostka, J.E. (2003). Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl. Environ. Microbiol. *69*, 7467–7479.

Plosz, B.G., Vogelsang, C., Macrae, K., Heiaas, H.H., Lopez, A., Liltved, H., and Langford, K.H. (2010). The BIOZO process-a biofilm system combined with ozonation: occurrence of xenobiotic organic micro-pollutants in and removal of polycyclic aromatic hydrocarbons and nitrogen from landfill leachate. Water Sci. Technol. *61*, 3188–3197.

Rayne, S., Ikonomou, M.G., and Whale, M.D. (2003). Anaerobic microbial and photochemical degradation of 4,4'-dibromodiphenyl ether. Water Res. *37*, 551–560.

Robrock, K.R., Coelhan, M., Sedlak, D.L., and Alvarez-Cohen, L. (2009). Aerobic biotransformation of polybrominated diphenyl ethers (PBDEs) by bacterial isolates. Environ. Sci. Technol. *43*, 5705–5711.

Rodriguez, S., and Bishop, P. (2008). Enhancing the Biodegradation of Polycyclic Aromatic Hydrocarbons: Effects of Nonionic Surfactant Addition on Biofilm Function and Structure. J. Env. Eng. *134*, 505–512.

Safe, S., Connor, K., Ramamoorthy, K., Gaido, K., and Maness, S. (1997). Human exposure to endocrine-active chemicals: Hazard assessment problems. Regul. Toxicol. Pharmacol. *26*, 52–58.

Seo, Y., and Bishop, P.L. (2008). The monitoring of biofilm formation in a mulch biowall barrier and its effect on performance. Chemosphere *70*, 480–488.

Seo, Y., Lee, W.H., Sorial, G., and Bishop, P.L. (2009). The application of a mulch biofilm barrier for surfactant enhanced polycyclic aromatic hydrocarbon bioremediation. Environ. Pollut. *157*, 95–101.

Stapleton, H.M., Dodder, N.G., Offenberg, J.H., Schantz, M.M., and Wise, S.A. (2005). Polybrominated diphenyl ethers in house dust and clothes dryer lint. Environ. Sci. Technol. *39*, 925–931.

Staskal, D.F., Diliberto, J.J., Devito, M.J., and Birnbaum, L.S. (2005), Inhibition of human and rat CYP1A2 by TCDD and dioxin-like chemicals. Toxicol. Sci. *84*, 225–231.

Tobiszewski, M., and Namiesnik, J. (2012). Abiotic degradation of chlorinated ethanes and ethenes in water. Environ. Sci. Pollut. Res. Int. *19*, 1994–2006.

Tokarz, J.A., Ahn, M.Y., Leng, J., Filley, T.R., and Nies, L. (2008). Reductive debromination of polybrominated diphenyl ethers in anaerobic sediment and a biomimetic system. Environ. Sci. Technol. *42*, 1157–1164.

Trudel, D., Scheringer, M., von Goetz, N., and Hungerbuhler, K. (2011). Total consumer exposure to polybrominated diphenyl ethers in North America and Europe. Environ. Sci. Technol. *45*, 2391–2397.

UNEP (2004). The Stockholm Convention on Persistent Organic Pollutants. UNEP, Stockholm, Sweden.

USEPA (1992). US EPA 1992: Ground Water Issue. Vol. EPA/540/S-92/002. http://www.epa.gov/superfund/remedytech/tsp/download/tce.pdf USEPA.

USEPA (2005). Contaminated sediment remediation guidance for hazardous waste sites. US Environmental Protection Agency, Washington, DC, USA.

USEPA (2009). DecaBDE Phase-out Initiative. Washington, DC, USA.

USEPA (2014). Polybrominated Diphenyl Ethers (PBDEs) Action Plan Summary. Washington DC, USA.

Vater, S.T., Velazquez, S.F., and Cogliano, V.J. (1995). A case study of cancer data set combinations for PCBs. Regul. Toxicol. Pharmacol. *22*, 2–10.

Velvizhi, G., Goud, R.K., and Venkata Mohan, S. (2014). Anoxic bio-electrochemical system for treatment of complex chemical wastewater with simultaneous bioelectricity generation. Bioresour. Technol. *151*, 214–220.

Vonderheide, A.P., Mueller, K.E., Meija, J., and Welsh, G.L. (2008). Polybrominated diphenyl ethers: causes for concern and knowledge gaps regarding environmental distribution, fate and toxicity. Sci. Total. Environ. *400*, 425–436.

Voorspoels, S., Covaci, A., Jaspers, V.L., Neels, H., and Schepens, P. (2007). Biomagnification of PBDEs in three small terrestrial food chains. Environ. Sci. Technol. *41*, 411–416.

Wang, Y., and Oyaizu, H. (2011). Enhanced remediation of dioxins-spiked soil by a plant–microbe system using a dibenzofuran-degrading *Comamonas* sp. and *Trifolium repens L*. Chemosphere *85*, 1109–1114.

Wei, Z., and Seo, Y. (2010). Trichloroethylene (TCE) adsorption using sustainable organic mulch. J. Hazard. Mater. *181*, 147–153.

Werner, D., Ghosh, U., and Luthy, R.G. (2006). Modeling polychlorinated biphenyl mass transfer after amendment of contaminated sediment with activated carbon. Environ. Sci. Technol. *40*, 4211–4218.

Wu, K., Yau, Y.C., Matukas, L., and Waters, V. (2013). Biofilm compared to conventional antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. Antimicrob. Agents. Chemother. *57*, 1546–1548.

Yamamoto, S., Suzuki, K., and Araki, Y.(2014). Dynamics of different bacterial communities are capable of generating sustainable electricity from microbial fuel cells with organic waste. Microbes. Environ. *29*, 145–153.

Yoshida, S., Ogawa, N., Fujii, T., and Tsushima, S. (2009). Enhanced biofilm formation and 3-chlorobenzoate degrading activity by the bacterial consortium of *Burkholderia* sp. NK8 and *Pseudomonas aeruginosa* PAO1. J. Appl. Microbiol. *106*, 790–800.

Zimmerman, J.R., Ghosh, U., Millward, R.N., Bridges, T.S., and Luthy, R.G. (2004). Addition of carbon sorbents to reduce PCB and PAH bioavailability in marine sediments: physicochemical tests. Environ. Sci. Technol. *38*, 5458–5464.

Electroactive Biofilms in Water and Air Pollution Treatment

10

Anna Vilajeliu-Pons, Sebastià Puig, Alessandro Carmona-Martínez, Nicolas Bernet, Marta Coma, Federico Aulenta, Jesús Colprim and Maria Dolors Balaguer

Abstract

Biofilms are used in wastewater treatment and in the production of valuable compounds. Bioelectrochemical system (BES) technology represents one practical application of biofilms. In these systems, bioelectrogenic biofilms are a bacterial consortium capable of performing electron transfer to the conductive material on which they are grown. This capacity of these organisms has been used in environmental biotechnology to couple pollutant removal, mainly from water but also from air streams, for the production of energy or valuable products. The following chapter outlines the details of such a consortium, highlighting the mechanisms of extracellular electron transfer and their main applications.

Bioelectrochemical systems

Bioelectrochemical systems (BESs) were described for the first time approximately 100 years ago (Arends and Verstraete, 2012; Schröder, 2011). In a very general sense, BESs represent a group of technologies capable of (1) producing power simultaneously with air and liquid pollution treatment, and (2) recovering and/or producing chemical compounds. The concept of BESs started with Michael C. Potter, who described the electric activity of bacterial and yeast cultures in 1911 (Potter, 1911). After this discovery, it took 20 years until the additional development of the novel concept of BESs. BESs were possible due to Cohen who, like Potter, reported differences in the 'reduction potential' of a bacterial culture after a few days of incubation (Cohen, 1931). Interestingly, at that time, it was strongly believed that such power production was only possible due to the presence of exogenous molecules, such as electron mediators. Since then, BESs have come a long way in their research and development.

In the following decades, the idea of microbial electricity generation did not significantly progress, and the field received little attention until 1980–2000. Since then, BESs have been rapidly developed. Microbial fuel cells (MFCs) were the first BES used to produce electricity (Rabaey *et al.*, 2003). Thereafter, microbial electrolysis cells (MECs) were developed, which required applying a cell potential to overcome the energy barrier to remove pollutants, such as uranium (Gregory and Lovley, 2005) and chlorinated compounds (Aulenta *et al.*, 2010), or to produce high value products (Rabaey and Rozendal, 2010). Both bioelectrochemical cells consist of two electrodes placed in anode and a cathode compartments where oxidation and reduction reactions occur. An external electric circuit joins the anode and cathode electrodes. Unlike in the well-known electrochemical cells, at least one of the two reactions is catalysed by microorganisms in this system.

As a general overview of multiple processes occurring in BESs such as MFCs and MECs, bacteria in the anode compartment break down organic material under anaerobic conditions (Fig. 10.1) from which mainly electrons and protons are harvested. Some of the electrons and protons are used for biomass and CO_2 production (among other gases such as H_2 or CH_4). The electrons and protons migrate to the cathode compartment through an external circuit and an ionic membrane, respectively. In the cathode

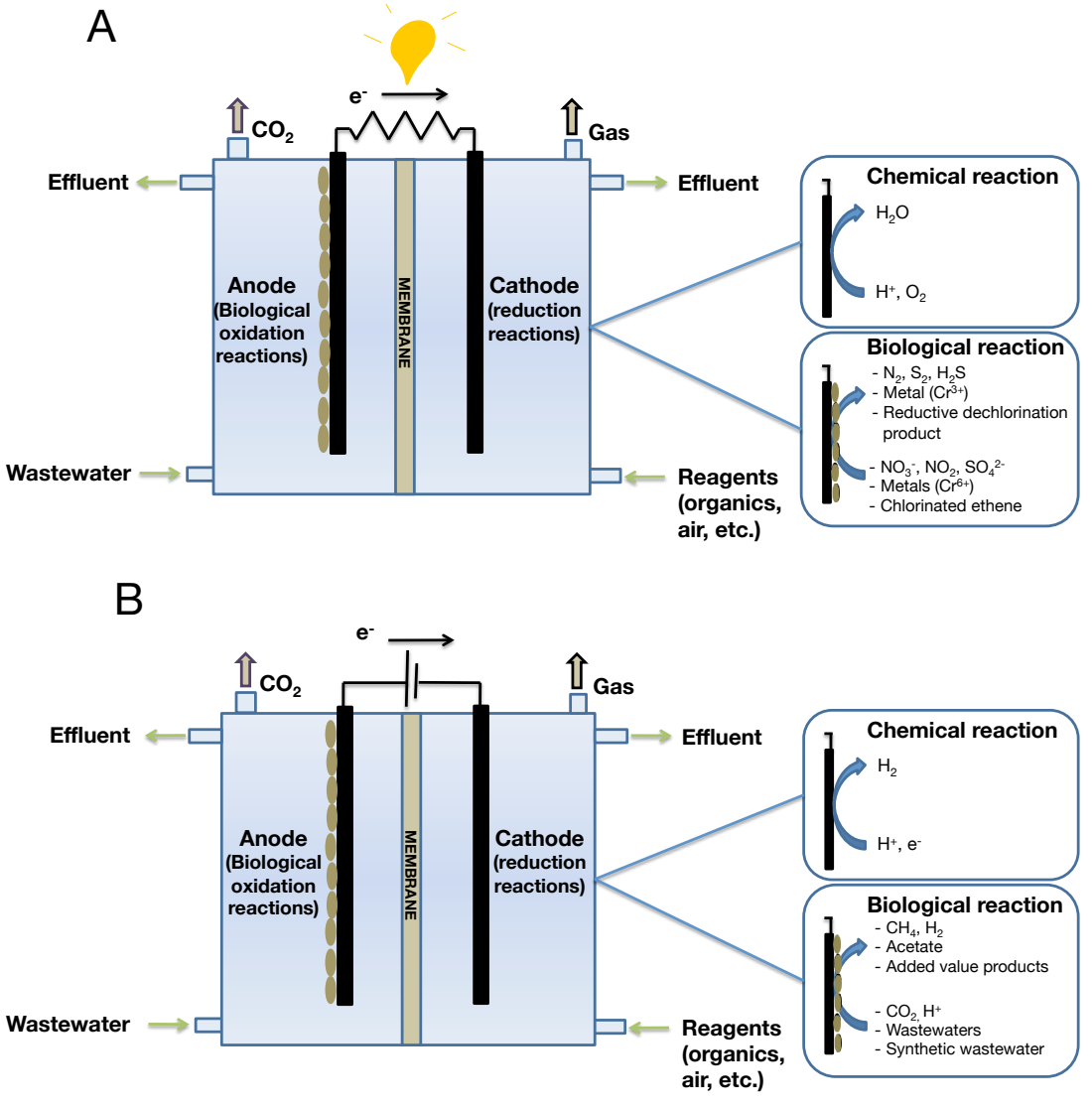


Figure 10.1 General overview of two configurations of bioelectrochemical systems (BESs) performing wastewater treatment. (A) Microbial fuel cell (MFC); (B) microbial electrolysis cell (MEC).

compartment, electrons and protons might be used to electrochemically reduce oxygen to water. They can be biologically captured to reduce different compounds in the so-called 'microbial electrosynthesis' process (Rabaey and Rozendal, 2010). A non-exhaustive list of the reduction reactions that occur at the cathode includes the following: the reduction of nitrate (Gregory *et al.*, 2004) and sulfate (Coma *et al.*, 2013) or the production of H_2 (Oh and Logan, 2005) or other chemical compounds, such as methane (Villano *et al.*, 2011) or acetate (Marshall *et al.*, 2012). The main difference between MFC and MEC is based on the production of electricity (MFC) or the use of electricity (MEC) to allow biological or chemical reactions at the cathode.

Microorganisms adhere to each other, creating a biofilm layer on the electrode surface with extracellular polymeric substances (EPS). This biofilm allows for successful electron transfer through the electrode surface and can comprise either a pure culture (Logan and Regan, 2006) or a mixed culture (complex microbial community) (Zhan *et al.*, 2014). The microorganism–electrode

interaction is not yet fully understood and is the subject of active research (Guo *et al.*, 2013). Different materials have been used as both electrode and carrier materials, including carbon, graphite, titanium and stainless steel (Wei *et al.*, 2011; Logan, 2008a). Now, granular graphite is one of the most common and inexpensive materials used because of its natural conductivity properties and affinity for microorganisms (Vilar-Sanz *et al.*, 2013; Arends *et al.*, 2012; Puig *et al.*, 2011a; Logan, 2008; Maignien *et al.*, 2006; Rabaey *et al.*, 2005). However, a different range of conductive materials has been used (Logan *et al.*, 2007), even though they have been modified in order to increase the level of microorganism surface adhesion and the conductivity of the material (Guo *et al.*, 2013).

In the anode compartment, the layer of biofilm contains microorganisms with the ability to transfer electrons extracellularly (Lovley, 2008a). These bacteria, which are commonly called exoelectrogens (Logan and Regan, 2006), have an important role in oxidizing and reducing metals in natural environments. In fact, the microbial electron transfer that some bacteria use in their role as insoluble metal electron acceptors is of special importance in several biogeochemical cycles. Moreover, this phenomenon has been applied to the bioremediation of contaminated sites since the early 1990s (Logan, 2008; Lovley *et al.*, 2004a; Nealson *et al.*, 1991). In the context of the direct conversion of organic wastes into electricity by exoelectrogen bacteria in BESs, it is worth noting that in order to improve bioenergy production, the research on BESs should explore the use of either robust mixed cultures or co-cultures with the ability to break down complex substrates. Such an approach should therefore continue because currently only a few attempts have successfully addressed this aspect (Miceli *et al.*, 2014; Speers *et al.*, 2014).

In the cathode compartment, microorganisms are able to reduce compounds by taking electrons from the electrode surface. These bacteria are called electrotrophs (Lovley, 2011). The role of these microorganisms in such specific systems has been hardly ever studied. Therefore, it is important to gain further fundamental knowledge about these microorganisms for the removal of pollutants from wastewater and for the production of high value products (Patil *et al.*, 2012).

Biofilms in bioelectrochemical systems

The study of MFC biofilms did not take off until 2005 (Rabaey and Verstraete, 2005). Since 2005, knowledge of the role of microorganisms was considered important in order to maximize the energy production and nutrient removal capacity or product production. Thereafter, the number of biofilm studies increased significantly. Some microorganisms responsible for current production, such as *Geobacter sulfurreducens* (Reguera *et al.*, 2005) and *Shewanella oneidensis* (Gorby *et al.*, 2006), were identified. Since then, the mechanisms of extracellular electron transfer between bacteria and electrode materials have been extensively studied by diverse research groups (Marsili and Zhang, 2009; Rosenbaum and Angenent, 2009). Currently, special attention is given to the syntrophic interactions within the biofilm and the obtained end-product because such types of interactions seem more relevant when working on the microbial electrosynthesis of added value products that do not necessarily depend on direct uptake electron transfer (Arends *et al.*, 2013). In the context of BESs, syntrophy is defined as the mutualistic interaction between microorganisms, where the main goal is maximizing the resource utilization (Lovley *et al.*, 2011). Synergy between communities helps degradation because some strains produce metabolites that can be used for other species to complete degradation. Single communities working individually are not capable of removing such substances. This concept is also related to the other communities present in extreme conditions, where microorganisms help each other to survive (McInerney *et al.*, 2008). In BESs, syntrophic interactions have been described on exoelectrogenic and non-exoelectrogenic bacteria in anode biofilms (Parameswaran *et al.*, 2009).

As stated previously, known biofilm structures consist of bacterial cells surrounded by self-produced EPS. Biofilms can be formed by single populations or by mixed communities. Microorganisms in the biofilm show heterogeneity due

to their interspersed distribution inside the EPS matrix (Davey and George, 2000). Usually, mixed bacterial cultures are used to inoculate BESs. Then, the biofilm is enriched by applying very specific operational conditions (such as a single substrate or a stable pH/temperature/redox potential) (Harnisch *et al.*, 2011). This enrichment is useful for increasing the biomass of the biofilm and its removal/production capacity. Once dominating bacteria are found in BES studies, isolated or purchased pure cultures can be used to study and acquire more fundamental and specific knowledge about the involved microorganisms and their metabolic pathways. On the other hand, mixed cultures have also been used to treat the complex organic matter that is usually contained in wastewaters due to their ability to adapt to changing conditions, such as those normally encountered in wastewater treatment plants. Consequently, a higher power output has been detected in BES studies based on mixed cultures, which seem to be more robust and resilient (Arends *et al.*, 2011). The maximum power output is not only related to the culture's source. In fact, there are many factors that can modify biofilm formation and behaviour, including the electrode surface, the nutrient availability, the pH (in pure cultures) and the hydrodynamic conditions (Franks *et al.*, 2010).

Exoelectrogenic microorganisms

Description

Lovley (2006) described a new form of microbial respiration in which microorganisms conserve energy for growth while transferring electrons extracellularly to the electrode. Lovley first called these bacteria *exoelectrogens*. Exoelectrogens are also known as anodophiles (Park and Zeikus, 2003), electrochemically active bacteria (EAB) (Chang *et al.*, 2006), anode-respiring bacteria (Torres *et al.*, 2007) and electrogenic microorganisms (Debabov, 2008).

Currently, the study of exoelectrogens has been prompted by their application in MFCs. In this alternative treatment, exoelectrogen microorganisms transform the acetate or complex organic matter from wastewater into electricity (Debabov, 2008; Lovley, 2008b). The most extensively studied microorganism able to produce high current densities and thick biofilms in MFCs is the Gram-negative *Geobacter sulfurreducens* (Bond and Lovley, 2003). Other bacteria with the ability to produce high current densities include *Rhodopseudomonas palustris* DX-1 (Xing *et al.*, 2008), *Thermincola ferriacetica* (Parameswaran *et al.*, 2013), *Geoalkalibacter ferrihydriticus* (Badalamenti *et al.*, 2013) and *Geoalkalibacter subterraneus* (Carmona-Martínez *et al.*, 2013). Such bacterial species have been well characterized, but they are not the only ones with a corroborated electrical activity (Gorby *et al.*, 2006). Different Gram-positive (*Pseudomonas* sp.) and Gram-negative (*Shewanella oneidensis*) bacteria have been identified in bioanodes. Therefore, their presence makes them likely to be responsible for the extracellular electron transfer process at the electrode surface (Arends *et al.*, 2011).

History and identification of exoelectrogens

Potter's discovery (1911) of microbial catalysed electrode reduction revolutionized the world of microbiology. He observed and reported electricity production with *Escherichia coli* and yeast cultures. It was not until the end of the twentieth century that Vargas *et al.* (1998) defined Fe(III) as the first external electron acceptor in MFC systems working with exoelectrogens.

The polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) techniques were used to identify bacterial populations in the biofilms (Davey and George, 2000). These techniques have since been used to identify exoelectrogens biofilms in BES systems.

Reimers *et al.* (2001) established microbes in seawater batteries, obtaining low power and voltage gradients. One year later, Bond *et al.* (2002) reproduced the work of Reimers by sequencing the microbial community responsible for electricity production. The 16S rRNA sequences obtained were analysed using the most probable number-polymerase chain reaction (MPN-PCR) technique, showing that the *Geobacteraceae* family of Delta-proteobacteria was the most abundant. Finding a bacterial species within the *Geobacteraceae* family (i.e. *Desulfuromonas acetoxidans*)

has been of great importance for the BESs field owing to the frequent appearance of these types of bacteria in electroactive biofilms (Yates *et al.*, 2012).

The interest on exoelectrogens has therefore rapidly increased with the identification of anode respiring bacteria in BESs, gradually achieving higher current and power outputs. The complete nucleotide sequences of the *Shewanella oneidensis* (Heidelberg *et al.*, 2002) and *Geobacter sulfurreducens* (Methé *et al.*, 2003) genomes have been described. The utilization of microarray technique (Nielsen *et al.*, 2003), polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) (Rabaey *et al.*, 2004) and pyrosequencing (Kiely *et al.*, 2011) have all proven their usefulness in the physiological identification and characterization of the microorganisms responsible for electricity generation. For instance, microarray and genetic analysis have successfully been used for the development of electron transfer mechanisms in *Geobacter sulfurreducens* (Holmes *et al.*, 2006). However, additional cloning, sequencing, and assembly techniques are needed to improve the microbial knowledge about exoelectrogens.

Once the first microorganisms were identified, scientists tried to unravel the mechanisms used to release electrons. The mechanisms used to transfer electrons can be direct (direct contact, conductive biofilm and nanowires) or indirect (mediators). Cylic voltammetry is a technique used to distinguish between the direct and indirect electron transfer processes in mixed or pure cultures (Harnisch and Freguia, 2012). Confocal Resonance Raman Microscopy (CRRM) has also been used to test variations in c-type cytochromes redox state (Virdis *et al.*, 2014).

Geobacter sulfurreducens has been widely studied as a model exoelectrogenic bacterial species that is frequently enriched in electroactive biofilms. Methé *et al.* (2003) interpreted the *G. sulfurreducens* genome, thus facilitating future studies about its electron transport mechanisms. The importance of conductive pili (also called nanowires) was realized when scientists began to understand their utility in electron transport processes (Malvankar *et al.*, 2011; Lovley *et al.*, 2011; Reguera *et al.*, 2005).

Nevin *et al.* (2008) compared pure (*G. sulfurreducens*) and mixed cultures in terms of the power production in different designs of MFCs. Different proportions between anode and cathode compartments have been tested, leading to the conclusion that the MFC design could have more important implications for power production than the use of pure or mixed biofilm cultures. However, other parameters have been evaluated, including biofilm conductivity. Malvankar *et al.* (2012) demonstrated that mixed cultures biofilms had higher electrical conductivity than pure cultures of *Geobacter sulfurreducens*. High conductivity allows for rapid electron transport between electrodes. Recent studies have demonstrated syntrophic interactions between exoelectrogen and non-exoelectrogen microorganisms (Parameswaran *et al.*, 2009) and the synergy in communities to remove complex compounds (Kiely *et al.*, 2011). Kim *et al.* (2011) showed that the proportion of exoelectrogens to non-exoelectrogens was lower at higher organic loading rates.

Finally, the most recent studies have focused on the effects of different parameters on the microbial community. Aelterman *et al.* (2008) studied the influence of anode potential. The microbial community was capable of self-regulating extracellular electron transfer (EET) pathways at different anode potentials and was not affected by this parameter. Moreover, the impact of media composition on the microbial community was studied by Puig *et al.* (2010) and Behera and Ghangrekar (2009). Different organic loading rates (OLRs) and pH values were tested by the authors mentioned above to achieve the maximum power production. It was shown that the microbial community activities improved at OLRs below 1 kg COD/kg VSS·d (where COD was the chemical oxygen demand and VSS was the volatile suspended solid in the anode compartment) with a basic pH of 8–9.5 producing the highest power production.

Applicability of BESs for waste treatment

The first exoelectrogen microorganism applications were studied using synthetic media. The most-used carbon source simulating wastewater was acetate, which is also used in genetic and

molecular studies (Lovley *et al.*, 2011; Kiely *et al.*, 2011; Logan and Regan, 2006; Logan *et al.*, 2006; Bond and Lovley, 2003; Bond *et al.*, 2002). Table 10.1 shows a representative but non-exhaustive set of studies regarding exoelectrogen wastewater treatment. In the study by Rabaey *et al.* (2005), microorganisms were able to oxidize 53% of the acetate with a Coulombic Efficiency (CE) of 36%. The term CE correlates with the electrons flowing through the BES, with the process occurring in one of the chambers (i.e. the anode or the cathode). It is the ratio between the electron actually transferred to the electrons potentially transferred to the electrical circuit considering the reaction occurring at the bioelectrode (equation 10.1).

$$CE = \frac{M_S I}{FnQ\ C} \cdot 100 \qquad (10.1)$$

Here, M_s is the molecular mass of the substrate (in g/mol), I is the current sourced from the MFC (in A) and F is Faraday's constant (96,485 C/mol). The term n identifies the number of electrons released for each mole of oxidized substrate, Q is the flow (l/day), and ΔC is the substrate concentration change (in mg/l) between the influent and effluent streams.

Thus, the CE from the anode is related to the percentage of organic matter removed by exoelectrogen bacteria in the BES. Several authors have studied the mechanisms involved in order to improve the efficiency of these systems. Recently, Villano *et al.* (2013) built a MEC with the ability to oxidize 94% of acetate in the influent with a CE of 91%.

In addition to acetate, a variety of other substrates have been used in the BES anode. For instance, glucose (Freguia *et al.*, 2008; Rabaey *et al.*, 2005, 2003) and fructose (Liamleam and

Table 10.1 Representative compilation of BESs studies for the treatment of synthetic media and raw wastewaters in terms of their percentage of removal capacity and coulombic efficiency (CE)

Substrate	Load (kg COD/ m^3·day)	Removal capacity (%)	CE (%)	Reference
Acetate	1.10	72	75 ± 7	Rabaey *et al*. (2005)
	2.10	53	36 ± 2	
Acetate	1.08	94	91 ± 2	Villano *et al*. (2013)
Glucose	1.10	84	59 ± 4	Rabaey *et al*. (2005)
Glucose	0.50	85	89 ± 4	Rabaey *et al*. (2003)
	5.00	41	10 ± 2	
Municipal wastewater	10–20	79	< 5	He *et al*. (2014)
Municipal wastewater	1.50	78–83	n.d.	Sevda and Sreekrishnan (2014)
Municipal wastewater	7.20	80	n.d.	Puig *et al*. (2011)
Municipal wastewater	9.77	43	n.d.	Puig *et al*. (2011a)
Municipal wastewater	0.10	75	20	Liu and Logan (2004)
Industrial wastewater: bakery	0.05	86	2 ± 1	Velasquez-Orta *et al*. (2011)
Industrial wastewater: brewery		85	2 ± 1	
Industrial wastewater: paper		78	26 ± 6	
Industrial wastewater: dairy		82	2 ± 1	
Industrial wastewater: brewery	6.70	44	7	Wen *et al*. (2010)
Landfill leachate	3.20	13	n.d.	Greenman *et al*. (2009)
Landfill leachate	2.20	10	< 2	Puig *et al*. (2011a)
Swine manure	4.50	27	8	Min *et al*. (2005)
Swine manure	2.00	62	n.d.	Lim *et al*. (2012)
Swine manure	1.20	84	< 1	Zhuang *et al*. (2012)
	4.90	77	< 1	

n.d., no data.

Annachhatre, 2007) have been tested. Interestingly, power outputs with both substances were lower than those of acetate due to previous fermentation processes, which produce hydrogen and acetate and methanogens that compete with the exoelectrogens for hydrogen. In contrast, non-synthetic media (i.e. raw wastewaters) have also been tested. For example, different wastes, including municipal wastewater (He *et al.*, 2014; Sevda and Sreekrishnan, 2014; Puig *et al.*, 2011; Liu and Logan, 2004) and industrial wastewaters from the bakery, brewery, paper, dairy and food industries (Jia *et al.*, 2013; Velasquez-Orta *et al.*, 2011; Wen *et al.*, 2010) have been used as the influent for BESs. Here, the organic loading rate has been generally higher than with the synthetic media based on easily degradable substrates. Despite the higher removal found when testing non-synthetic media, the exoelectrogen activity was lower due to the complex organic matter matrix in the influent, causing a more diversified range of side metabolic reactions that inevitably consume electrons.

Other substrates such as landfill leachate (Puig *et al.*, 2011a; Greenman *et al.*, 2009), swine manure (Lim *et al.*, 2012; Zhuang *et al.*, 2012; Min *et al.*, 2005) and urine (Ieropoulos *et al.*, 2012) have been analysed and found to have similar organic loading rates as wastewaters. The use of complex organic influents has resulted in low CEs and power production. However, such studies have proved the applicability of BESs.

Therefore, higher CEs are obtained when available biodegradable substrates such as acetate are used as the influent. However, other more complex substrates such as glucose or wastewaters have low CEs due to the requirements of earlier electron-consuming steps such as acid genesis (sugars to acetate) or hydrolysis.

Electrotrophic microorganisms

Description

An electrothoph has the microbial capacity to directly accept electrons from the electrode at the cathode compartment in the BES. These microorganisms have not been widely studied thus far. Early studies focused mainly on the anode compartment and on the exoelectrogen microorganisms. Currently, the interest in electrotrophs has increased, as researchers aim to develop biocathodes for the production of different added value molecules (Marshall *et al.*, 2012; Rosenbaum *et al.*, 2011).

Different reduction reactions have been reported in the literature. Some have been useful in the treatment of wastewaters with high nitrate content. For instance, Pous *et al.* (2013) demonstrated the successful conversion of nitrate and nitrite to dinitrogen gas. Recently, with the changing paradigm of converting wastes to new bioresources, other reactions producing high value products are being considered. For example, the conversion of carbon dioxide into methane has been recently demonstrated by Villano *et al.* (2013). Additionally, Marshall *et al.* (2013) showed that the carbon chain of certain organic acids commonly produced as the dead-end products of fermentation could be elongated. Rozendal *et al.* in 2008 provided a proof of concept of protons being reduced to hydrogen by means of a process that is microbiologically catalysed by an electroactive biofilm (Arends *et al.*, 2011). Hence, the variability of electron acceptors in cathode microbial reactions has demonstrated that the electrotroph capacity is a promising route to remove pollutants and/or to produce high-value products.

History and identification of electrotrophic metabolism

The study of these microorganisms was not developed until recently. Only a few studies have focused on the characterization of electrotrophic microorganisms. Wrighton *et al.* (2010) deeply analysed the denitrifying microbial communities of two different BESs and examined their phylogenetic affiliation and community structures. The study performed by Vilar-Sanz *et al.* (2013) about denitrifying communities focused on the functional genes of denitrification pathways.

Once the microorganisms were identified, efforts became more focused on the identification of the electron transfer mechanisms utilized by such electrotrophic microorganisms (Rosenbaum *et al.*, 2011; Lovley, 2011) and the relationship between members of the communities. Marshall *et al.* (2013) focused on the physical (scanning

electron microscope) and molecular (RNA, DNA) analysis of microbiomes (synergic communities) for electrosynthesis purposes. On the other hand, Ross *et al.* (2011) studied *Shewanella oneidensis* strain MR-1 to learn about the complex oxidation/reduction reactions occurring at the electrodes. This genus uses the Mtr respiratory pathway to catalyse electron flow from cytoplasmic oxidative reactions to electrodes. It has the ability to drive microbial reductive metabolism. This result suggested the possibility of obtaining valuable fuels and chemicals from that pathway.

Electrotrophic process applications

Electrotrophic microorganisms have been used in bioremediation and in the production/recovery of chemical compounds. A great variety of environmental pollutants have been treated by taking advantage of electrotrophic microorganisms in BES. For example, due to the intensive agriculture and livestock activities, the release of nitrate from water has become a serious problem, requiring large amounts of organic matter for its removal through conventional processes (denitrification). In this scenario, electrotrophs might provide a great benefit because they can reduce nitrates and/or nitrites from contaminated groundwater and wastewater to dinitrogen gas (Pous *et al.*, 2013; Kondaveeti and Min, 2013; Vilar-Sanz *et al.*, 2013; Puig *et al.*, 2012, 2011b; Wrighton *et al.*, 2010; Virdis *et al.*, 2008). Electron transport increases in highly conductive media but is limited in media with low conductivity (Pous *et al.*, 2013). Despite this, the possibility of treating groundwater without affecting the drinking water quality by BESs has been reported. Biocathodes can also reduce other types of inorganic contaminants, such as sulfates (Coma *et al.*, 2013), different metals such as uranium (Anderson *et al.*, 2003) and chromium (VI) (Tandukar *et al.*, 2009), and chlorinated compounds (Aulenta *et al.*, 2009). Table 10.2 provides a compilation of representative reducible pollutant studies using electrotrophic treatments.

Electrotrophic activity is not only linked to the removal of contaminants. Recent studies have suggested a promising future for these bacteria to produce (or recover) high value products from waste (Table 10.3). Different organic and inorganic products such as methane, hydrogen, acetate, ethanol, 1–3 propanediol and succinate have been obtained from the electrotroph activity (Logan and Rabaey, 2012). Hydrogen is the main product of water electrolysis and occurs easily in electrochemical cells. However, the presence of microorganisms in the cathode catalyses this reaction, such that production can be obtained at higher redox potentials.

Methane is another compound studied in BESs by different scientists and is of interest due to its high energetic value. Recently, the interest in this type of methane production route has gained increased attention. It has been demonstrated that methane production is feasible using synthetic and raw wastewaters and CO_2 carbon based substrates. For example, methane and acetate were

Table 10.2 Summary of literature studies about waste streams treated by electrotrophic bacteria and their percentage of removal capacity and Coulombic Efficiency (CE) with different substrates (S)

Substrate	Source (kg S/m^3·day)	Removal capacity (%)	CE (%)	Reference
Nitrate	0.06	64	60–80	Pous *et al*. (2013)
Nitrite	0.28	77	41±17	Vilar-Sanz *et al*. (2013)
Nitrate	0.37	15	85±11	
Nitrate	0.05	42	73±18	Puig *et al*. (2012)
Nitrate	0.50	35	48±11	Puig *et al*. (2011b)
Sulfates	0.21	<1	n.d.	Coma *et al*. (2013)
Chromium	0.01	99	n.d.	Tandukar *et al*. (2009)
Uranium	6.10^{-6}	70	n.d.	Anderson *et al*. (2003)

n.d., no data.

Table 10.3 Summary of literature studies about different high value ending products production of electrotrophic bacteria from different initial products and cathode potentials

Initial product (electron acceptor)	Final product	Potential (mV versus SHE)	Reference
H^+	Hydrogen	–1000	Batlle-Vilanova *et al*. (2014)
Synthetic wastewater	CH_4	<–1000	Xafenias and Mapelli (2014)
	Acetate		
Wastewater	CH_4	–590 to –900	Marshall *et al*. (2013)
CO_2	H_2		
H^+	Fatty acids		
CO_2	CH_4, H_2	–1047 to –1147	Jiang *et al*. (2013)
H^+	Acetate, CH_4, H_2	<–1147	
CO_2	CH_4, H_2	–590	Marshall *et al*. (2012)
H^+			
CO_2	Multicarbon organic compounds	–400	Nevin *et al*. (2011)
CO_2	Multicarbon organic compounds	–400	Nevin *et al*. (2010)
H^+	Hydrogen	–700	Rozendal *et al*. (2008)

n.d., no data.

the predominant products at a cathode potential of –590 mV versus SHE (Marshall *et al*., 2012). Another interesting example of the production of added-value molecules was recently described by Xafenias and Mapelli (2014), where the production of multicarbon organic compounds such as acetate was reported with significant efficiencies of approximately 60% when the cathode potential was poised below –1000 mV versus SHE (standard hydrogen electrode). This might be due to a synergistic reaction with the hydrogenotrophic methanogens, as this potential hydrogen could be produced.

Hydrogen production is one of the most well-studied reactions in biocathode compartments. The first attempts to use MECs to couple organic wastewater and hydrogen production were completed by Bruce Logan's group with small-scale prototypes. In 2009, Wagner *et al*. made a laboratory scale MEC for the treatment of swine wastewater in batch mode. They produced 0.9–1 m^3 H_2/m^3·day while removing up to 72% of the COD. The gas produced was up to 77% hydrogen but also contained up to 13% methane. Lalaurette *et al*. (2009) coupled a dark-fermentation reactor to a MEC to convert maize stover lignocellulose or cellobiose into hydrogen. Hydrogen yields approximately 11 H_2/g COD and 11 H_2/l·day were obtained. Cusick *et al*. (2010) evaluated the capacities for treatment and energy recovery of an MFC and MEC applied to winery and domestic wastewater. They concluded that energy recovery and organic removal from wastewater are more effective with MFCs than with MECs but that hydrogen production from wastewater-fed MECs can be cost-effective.

Cusick *et al*. (2011) published the first pilot-scale study carried out with a 1000 L-MEC treating winery wastewater in California. The reactor contained 144 electrode pairs in 24 modules. The development of an electroactive biofilm required approximately 60 days, which is longer than the period typically needed for laboratory-scale MECs. This was mainly due to the lack of volatile fatty acids (VFA) in the feeding, the low pH and the low and unstable temperature. Controlling these different parameters made it possible to enhance the performance in terms of current production, with a maximum current of 7.4 A/m^3 at the end of the test after 100 days of operation at a cathodic potential of 0.9 V. The maximum gas production was 0.19 ± 0.04 l/l·day. However, in the absence of a membrane, the hydrogen was directly converted to methane (86 ± 6% of product gas) by hydrogenotrophic methanogens.

In 2011, a 120 L-MEC was set up by Tom Curtis' group (Newcastle University, UK) in a municipal wastewater treatment plant in northern England. It was fed with raw wastewater at ambient temperatures. The organic loading rate was 0.14 kg COD/m^3·day, which is just below the typical loading rates for activated sludge. The energetic cost was 2.3 kJ/g DOD, which is below the values for activated sludge (2.5–7.2 kJ/g COD) (Heidrich *et al.*, 2013). The reactor maintained performance for 12 months (including over winter with influent temperatures as low as 6°C) achieving 70% electrical energy recovery and producing more than 1 litre of almost 100% pure hydrogen gas per day (Cotterill *et al.*, 2013). This work represents the first proof-of-concept demonstration of the feasibility of domestic wastewater treatment in a large-scale MEC at an ambient temperature.

Due to these results, a new prototype was developed and installed in another wastewater treatment plant in the north of England. The process was modified to reduce the hydrogen losses and to optimize the fluxes to prevent the bypass of the anode biofilm and sludge accumulation. The anode surface area was increased by 90% for the same volume compared to the previous prototype (Cotterill *et al.*, 2013). As in the previous pilot-reactor, the MEC was made of removable cassettes placed in a tank, following a design focused on improving the service ability of the reactor, minimizing hydrogen losses and improving flow distribution. Each cassette consisted of a carbon felt anode and a stainless steel wire wool cathode, with a battery separator instead of the expensive Nafion membrane generally used in laboratory-scale systems (Cotterill *et al.*, 2013).

Escapa *et al.* (2012) studied the potential inclusion of a MEC in an existing domestic wastewater treatment plant from an economic point of view. They estimated that for a full-scale MEC operating at a current density of 5 A/m^2 anode and an energy consumption of 0.9 kWh/kg COD, an anodic chamber cost of 1220 €/m^3 is the target purchase cost for which a break-even point can be reached after 7 years.

The main challenges for the development of MEC technology are enhancing the hydrogen-production rate and lowering the energy input (Liu *et al.*, 2010). This includes increasing the performance of the anodic biofilms in terms of current density, the development of efficient cathode electrode materials and novel MEC architecture to overcome the high cathode overpotential and the large internal resistance caused by the neutral pH conditions. In addition to the domestic wastewater, the development of cost-efficient pretreatment should favour the combined use of dark fermentation and MEC applied to high-strength wastewaters to significantly enhance the overall hydrogen-production rate and yield (Liu *et al.*, 2010).

Microbial extracellular electron transfer (EET) mechanisms

Certain microorganisms that are considered strict anaerobes and that are able to reduce metallic oxides such as iron are also able to transfer electrons via extracellular electron transfer (EET) to an electrode material (exoelectrogen). However, such metal oxide reduction ability does not necessarily confer EET ability (Richter *et al.*, 2007). Due to the original focus on the improvement of MFC performance, EET mechanisms have long remained unknown, although they represent a critical step in understanding the power generation phenomenon in BESs. Fortunately, advances in electrochemical techniques have clarified part of these EET mechanisms (Manohar *et al.*, 2008) through the size of the polarization curves (Logan, 2008a). Cyclic voltammetry (Harnisch and Freguia, 2012), electrochemical impedance spectroscopy (Dominguez-Benetton *et al.*, 2012) and surface-enhanced Resonance Raman Spectroscopy (Millo, 2012) are all techniques used to understand the EET mechanisms and to allow for BES characterization (Franks and Nevin, 2010).

Recent biocathode studies have shown that electrochemically active bioanodes may be turned into biocathodes when the environmental and operation conditions change (Rozendal *et al.*, 2008). According to Rozendal *et al.* (2008), similar EET mechanisms as those occurring in bioanodes could occur in biocathodes. However, this hypothesis remains to be experimentally tested. One of the main differences between anodic and cathodic EET mechanisms is that the

redox active components could operate at higher redox potentials (Arends *et al.*, 2011).

Due to the knowledge generated by these techniques, scientists have been able to distinguish between different EET mechanisms, such as direct or indirect extracellular electron transfer. Interestingly, in mixed microbial biofilms, both mechanisms are believed to occur simultaneously in order to maximize the microbial benefits (Logan *et al.*, 2006).

Direct electron transfer mechanisms

Direct electron transfer (DET) is defined as the transport of electrons from the cofactor of a redox active enzyme (oxidoreductase) or a redox protein in the bacterial cell membrane to the electrode surface in the absence of redox mediators (Logan *et al.*, 2006). Thus, the main advantage of direct electron transport consists of the absence of diffusion limitations between microorganisms and the electrode (Rabaey and Rozendal, 2010).

Initial investigations of microbial DET were based on pure cultures. The microorganisms selected for this type of studies were *Geobacter sulfurreducens* (Gregory *et al.*, 2004; Bond and Lovley, 2003) and *Shewanella oneidensis* (Gorby *et al.*, 2006) because they are well known for being dissimilatory metal reducing organisms in nature (Lovley *et al.*, 2004; Nealson *et al.*, 1991). Studies on EET have focused on Gram-negative bacteria because they have shown more efficient EET capacities in terms of their achieved currents ($1\,A/m^2$) and greater EET diversity in both direct and mediated mechanisms (Arends *et al.*, 2011). However, *Thermincola ferriacetica* has recently been reported to produce current values of $8\,A/m^2$, indicating that Gram-positive bacteria can also efficiently conduct electrons via DET (Parameswaran *et al.*, 2013).

Microorganisms use three different direct electron transfer mechanisms, including the following: (1) direct contact between the membrane and the electrode, (2) conductive biofilms, and (3) conductive pili called nanowires.

Single-cell direct contact

Exoelectrogen microorganisms can attach to the electrode surface. *Geobacter sulfurreducens* is a clear example of this transfer mechanism. It has been demonstrated by spectroelectrochemical studies that electron transfer occur via c-type cytochromes displayed on the outer cell surface (Millo *et al.*, 2011; Busalmen *et al.*, 2008). Furthermore, *G. sulfurreducens* grows in layers, which allows for close contact between cells (Bond and Lovley, 2003) (Fig. 10.2).

On the other hand, *Shewanella oneidensis* is a well-known example of another anode respiring

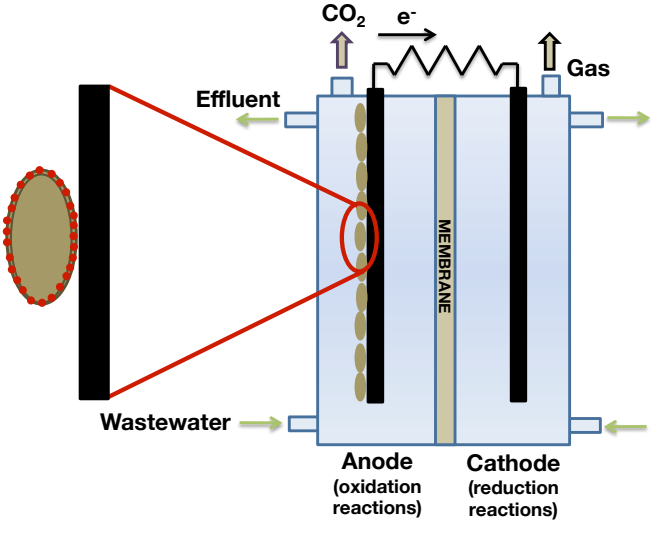


Figure 10.2 Scheme of direct electron transport mechanism. Small red circles represent c -type cytochromes. A colour characteristic of electroactive biofilms due to their high content of haems (Jensen *et al.*, 2010).

bacterium. Interestingly, it adheres to its substrate five times stronger in anaerobic conditions than in aerobic conditions, indicating that direct contact is a good fixation strategy for EET in adverse conditions. Therefore, based on such findings, EET studies between electrodes and microbes were initially accomplished under anaerobic conditions (Kim *et al.*, 1999).

Conductive biofilm

As mentioned above, exoelectrogen microorganisms within conductive biofilms attach to each other by EPS (Fig. 10.3). This strategy results in the formation of thick and conductive biofilm layers that allow for high current productions in BES (1A/m^2). For instance, it has been recently demonstrated that in pure-culture biofilms of *Geobacter sulfurreducens*, the microorganisms are able to release cytochromes to the matrix (Lovley *et al.*, 2011), thereby increasing the conductivity of the biofilm structure.

Conductive pili (Nanowires)

An additional mechanism of microbial DET in biofilms involves self-produced conductive hair-like filaments. These filaments are produced by some microorganisms, attach to the cell wall and are known as nanowires (Malvankar and Lovley, 2014, 2012). Such nanowires are involved in long-range EET due to the high content of c-type cytochromes in their structure. Additionally, the nanowires are shaped as thin single strands that connect the microorganism to the solid electrode (Fig. 10.4). Therefore, in studies where scanning

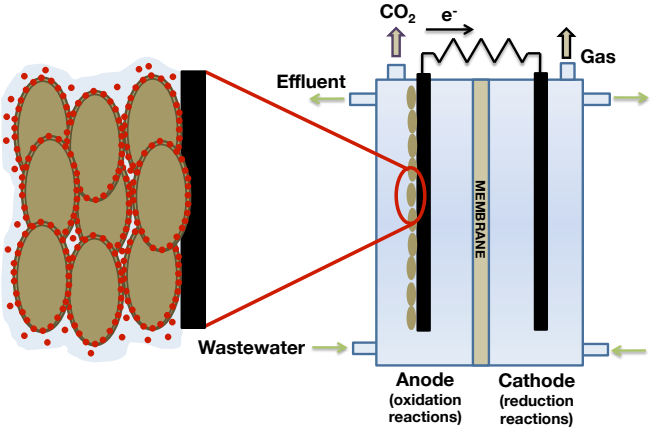


Figure 10.3 Scheme of direct electron transport mechanism of conductive biofilms. Blue matrix: EPS.

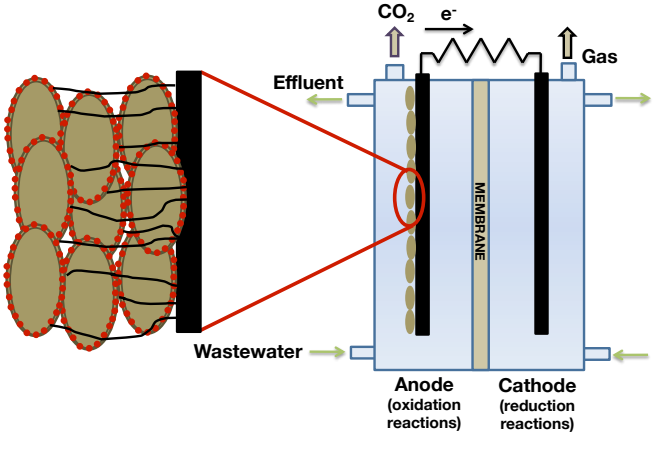


Figure 10.4 Scheme of direct electron transport mechanism by nanowires. Black lines: conductive pili.

electron microscopy is used for the inspection of electroactive biofilms, other web-like extracellular material resulting from the sample preparation could be wrongly identified as nanowires (Badalamenti *et al.*, 2013).

Nanowires have been proposed as a possible EET mechanism that is used by a variety of different microorganisms. For example, *Geobacteraceae* and *Shewanellaceae* species have been studied as nanowire producers by Reguera *et al.* (2006) and Gorby *et al.* (2006), respectively. Later, the work of Malvankar and Lovley (2014) has revealed that the function of the nanowire in these two organisms is different. While the nanowires in *Geobacter sulfurreducens* have metal conductivity and transfer electrons, the electrons seem to 'jump' between cytochromes located on the non-conductive pili in *Shewanella oneidensis*. This characteristic seems to confer *Geobacter sulfurreducens* a better ability to transfer electrons than *Shewanella oneidensis*. Moreover, *G. sulfurreducens* is able to produce a much thicker and uniform biofilm layer (> 50 mm) than *S. oneidensis*, which further supports experimental observations of *G. sulfurreducens* as a prominent electroactive bacteria, as both strains have been compared for current/power production in identical experimental set-ups (Call and Logan, 2011).

Indirect electron transfer mechanisms

Indirect electron transfer (IET) is defined as the transport of electrons from the bacterial cell to the electrode surface or from the cell to another bacterial cell through redox mediators (Rabaey and Rozendal, 2010) (Fig. 10.5).

These mediators could be chemically added or could be self-produced by the microorganisms as secondary metabolites (Arends *et al.*, 2011) (Table 10.4). Pyocyanin is one of the endogenous chemical mediators produced by *Pseudomonas aeruginosa* described in BES (Rabaey *et al.*, 2004).

A variety of chemicals have also been used to facilitate IET. These mediators include neutral red (Park *et al.*, 1999), anthraquinone-2–6, disulfonate (AQDS) (Holmes *et al.*, 2004), thionin, potassium ferricyanide (Bond *et al.*, 2002), methyl viologen, and others (Rabaey and Verstraete, 2005). The main problems of the chemical additions are related to the high cost of the mediators, their possible toxic effects and their wash-out effects.

Within the context of anodic biofilms, it is worth noting that the study of DET mechanisms has gained more attention over IET mechanisms. This has resulted in the partial abandon of IET by the scientific community. However, recent preliminary studies on microbial electrosynthesis of acetate at the cathode and the absence of evident biofilms suggest that an electron uptake based on IET mechanism might be responsible for the observed conversion of CO_2 into acetate (Arends *et al.*, 2013). Therefore, it is expected that the interest in IET will soon increase rapidly.

As in the case of anode EET mechanisms, cathode IET requires mediators to carry out the

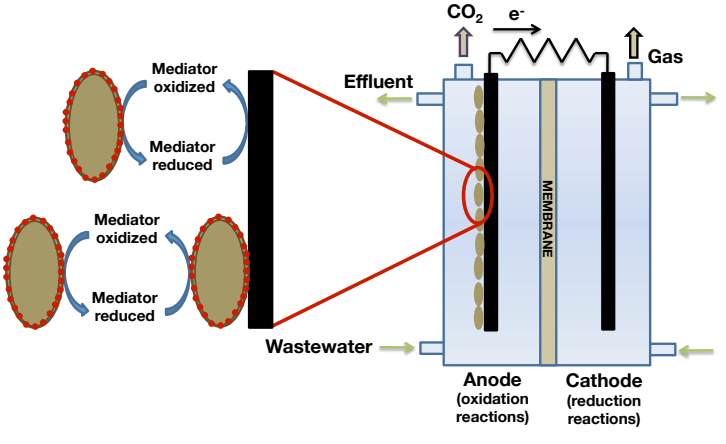


Figure 10.5 Scheme of indirect electron transport mechanism.

Table 10.4 Representative biogenic production of redox mediators

Microorganism	Mediator molecule	Reference
Sphingomonas xenophaga	4-Amino-1,2-naphthoquinone	Keck *et al*. (2002)
Pseudomonas aeruginosa	Phenazine-1-carboxylic acid	Price-Whelan *et al*. (2006)
Pseudomonas chlororaphis	Phenazine-1-carboxamide	van Rij *et al*. (2004)
Shewanella oneidensis	Flavin mononucleotide	von Canstein *et al*. (2008)
Shewanella algae	Melanin	Turick *et al*. (2002)
Bacillus pyocyaneus	Pyocyanine	Friedheim and Michaelis (1931)
Propionibacterium freundenreichii	2-Amino-3-carboxy-1,4-naphthoquinone	Hernandez and Newman (2001)
Shewanella alga	Cyanocobalamin	Workman *et al*. (1997)
Acetobacterium woodii	Hydroxycobalamin	Hashsham and Freedman (1999)
Pseudomonas stutzeri	Pyridine-2,6-bis	Lewis *et al*. (2001)
Methanosarcina thermophila	Porphorinogen-type molecules	Koons *et al*. (2001)
Shewanella oneidensis	1,4-Dihydroxy-2-naphthoate derivative	Ward *et al*. (2004)

More detailed information can be found in the following references: Hernandez and Newman, 2001; Li *et al*., 2009; Marsili and Zhang, 2009; Schröder, 2007; Watanabe *et al*., 2009.

reduction of certain final electron acceptors (see above). Although little information is available at present, it can be assumed that the mediators responsible for the cathode IET could be artificially added or biologically self-produced as has been described for the anode compartments. One recent study demonstrated the use of methyl viologen as a mediator for the electrochemically assisted microbial dechlorination of trichloroethene (TCE) and cis -dichloroethene (cis -DCE) (Aulenta *et al*., 2009). Other mediators have been applied to the cathodes, such as anthraquinone-2,6-disulfonate (Thrash *et al*., 2007) and neutral red (Park and Zeikus, 1999) for the reduction of perchlorate and fumarate, respectively. Taking into account all available information, it is expected that the cathodic IET mechanism will be very similar to the anodic IET (Thrash and Coates, 2008).

Microbial intracellular electron transport (ICET) mechanisms

The internal microbial mechanism to transport and release electrons inside the membranes of microorganisms is currently being researched. The study of these pathways is usually performed with pure cultures. One of the most studied species for ICET is *Geobacter sulfureducens*, which is studied due to its enrichment in multiple BES studies (Yates *et al*., 2012) and due to its demonstrated ability to produce high current densities (Chen *et al*., 2012). Despite these efforts, the mechanism of electron transfer remains unclear as unknown pathways exist in the process.

The mechanism that allows electron transfer from electron donor (reductant) to the electron acceptor (oxidant) occurs inside the lipid-membranes and involves the oxidoreductase enzyme (Hartshorne *et al*., 2009). First, electrons are generated by the NADH dehydrogenases located in the inner mitochondrial membrane (Fig. 10.6). This enzyme catalysis the conversion of NADH to NAD^+ and thereby releases protons to the periplasm zone. NAD^+ is reduced through a route that recycles it back to the active form via the Krebs cycle.

Electrons are transferred to the cytochrome b-c by the coenzyme ubiquinone. Coenzyme ubiquinone, also called Q10, has various functions related to its redox capacity, including electron transport between cytochromes. Once the electrons are in this cytochrome, more protons are released into the intermembrane space. Thereafter, electrons from the cytochrome b-c are transferred to the c-type cytochrome. The C-type cytochrome is mobile, connecting the inner membrane, the periplasm and the outer membrane.

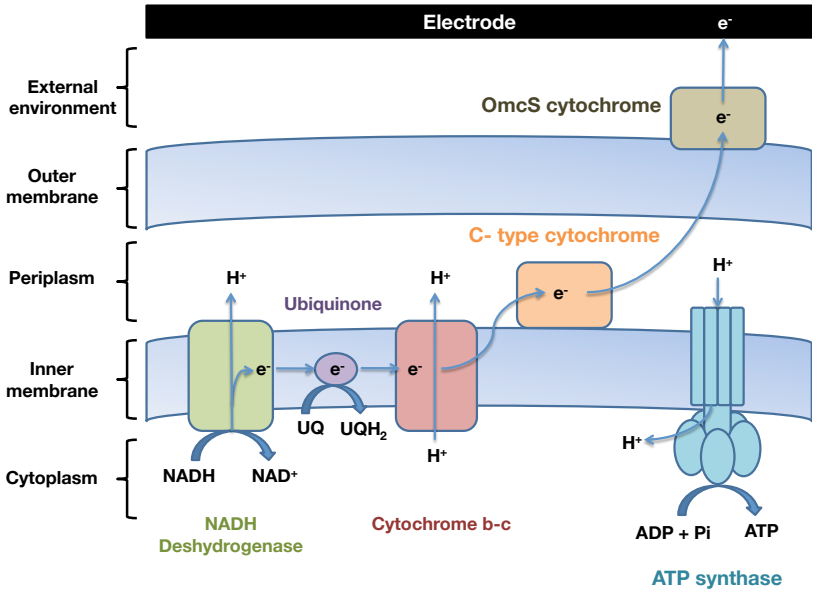


Figure 10.6 Electron transport chain to move electrons directly from the mitochondrial membrane to the external membrane until the electrode.

This cytochrome is responsible for the electron transfer between both membranes (Lovley *et al.*, 2004). Then, electrons are transferred through different cytochromes until they reach the OmcS cytochrome, which is in contact with the surface of the cell (Lovley, 2008b).

During electron transfer, the gradient of protons between the cytoplasm and the periplasm is used to generate energy in the form of adenosine triphosphate (ATP). The accumulation of protons is generated by proton pumping from the different cytochromes, increasing the pH of the periplasm. ATP synthase pumps these protons across the inner membrane and thereby produces ATP from adenosine diphosphate (ADP) and inorganic phosphate. This ability has been intensively studied with the aim of increasing the power production of MFCs. Different genetic engineering modifications have been applied to *Geobacter sulfurreducens* (Lovley *et al.*, 2011): cell abilities to produce more cytochromes, which increase the amount of electron transfer within the membrane and therefore increase the nanowire expression levels. These effects in turn enhance the contact cell–cell and/or cell–electrode contact, but not the current production. To improve the current production, Malvankar *et al.* (2011) increased the biofilm conductivity, observing a positive correlation. These findings highlight the necessity of a better understanding of the processes occurring inside the bacteria.

It is believed that the ability of *Geobacter sulfurreducens* to create very thick biofilms (> 50 mm) through the direct contact mechanism can be substituted by electron transport through conductive pili (Fig. 10.4). Such an additional DET mechanism is actively being discussed by the scientific community. However, recent studies on microbial nanowires suggest that these pili-like structures, which are located along the membranes on the external environment of the cell, are covered by OmcS cytochromes (Leang *et al.*, 2010; Lovley, 2006). Interestingly, this nanowire DET pathway allows the microorganisms to transfer electrons between them without intermediates, enabling biofilm communication (Logan and Regan, 2006).

The mechanisms of electron transfer through the membrane in electrotroph bacteria remain largely unknown. Scientists suspect that the process of moving electrons is similar to the exoelectrogenic process. Ross *et al.* (2011) studied *Shewanella oneidensis* strain MR-1 and demonstrated that the same pathway can be used for

oxidation and reduction reactions. The reduction of these compounds consumes protons in the cytoplasm, generating a proton gradient across the inner membrane (Lovley, 2011). Therefore, a proton gradient is necessary to generate energy by ATP synthase, as is the case for exoelectrogens.

Conclusions

This chapter provides a comprehensive description about the microbial aspects of bioelectrochemical systems. The potential of BES technology relies on its use as an alternative, cost-effective technology for the removal of contaminants (organic matter, carbon dioxide, nitrate, metals, chlorinated compounds, etc.) and the production of high value products (hydrogen, caustic soda, methane, volatile fatty acids, alcohols, etc.).

The rapid advances in electric and microbial techniques over the last decade have allowed for the improvement of such systems. Despite this, it remains necessary to gain fundamental and applied knowledge about the microbiological field to better understand the complex metabolic routes of the microorganisms involved. Several opportunities will be available once the wastewater treatment capacity and high-value production rate of BESs is improved.

Acknowledgement

This research was financially supported by the Spanish Government through the BEST ENERGY project (CTQ2011-23632).

References

Aelterman, P., Freguia, S., Keller, J., Verstraete, W., and Rabaey, K. (2008). The anode potential regulates bacterial activity in microbial fuel cells. Appl. Microbiol. Biotechnol. *78*, 409–418.

Aelterman, P., Rabaey, K., Clauwaert, P., and Verstraete, W. (2006). Microbial fuel cells for wastewater treatment. Water Sci. Technol. *54*, 1–9.

Anderson, R.T., Vrionis, H.A., Ortiz-bernad, I., Resch, C.T., Long, P.E., Dayvault, R., Marutzky, S., Metzler, D.R., Peacock, A., White, D.C., Lowe, M., Lovley, D.R., and Karp, K. (2003). Stimulating the In Situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl. Environ. Microbiol. *69*, 5884–5891.

Arends, J.B.A., Blondeel, E., Tennison, S.R., Boon, N., and Verstraete, W. (2012). Suitability of granular carbon as an anode material for sediment microbial fuel cells. J. Soils Sediments *12*, 1197–1206.

Arends, J.B.A., Boon, N., and Rabaey, K. (2013). Microbial Electrosynthesis on Stainless Steel Electrodes by a Homoacetogenic Community: Are Carbon Electrodes Needed?, in: Microbial Fuel Cells, 4th International Conference, Abstracts. pp. 34–35.

Arends, J.B.A., Desloover, J., Puig, S., and Verstraete, W. (2011). Principles and technology of Microbial Fuel Cells (Wiley-VCH Books, West Sussex, UK).

Arends, J.B.A., and Verstraete, W. (2012). 100 years of microbial electricity production: three concepts for the future. Microb. Biotechnol. *5*, 333–346.

Aulenta, F., Canosa, A., De Roma, L., Reale, P., Panero, S., Rossetti, S., and Majone, M. (2009). Influence of mediator immobilization on the electrochemically assisted microbial dechlorination of trichloroethene (TCE) and cis -dichloroethene (cis -DCE). J. Chem. Technol. Biotechnol. *84*, 864–870.

Aulenta, F., Reale, P., Canosa, A., Rossetti, S., Panero, S., and Majone, M. (2010). Characterization of an electro-active biocathode capable of dechlorinating trichloroethene and cis-dichloroethene to ethene. Biosens. Bioelectron. *25*, 1796–1802.

Badalamenti, J.P., Krajmalnik-brown, R., and Torres, C.I. (2013). Generation of high current densities by pure cultures of Anode- Respiring *Geoalkalibacter* spp. under alkaline and saline conditions in Microbial Electrochemical Cells. Am. Soc. Microbiol. *4*, e00144–e00213.

Batlle-Vilanova, P., Puig, S., Gonzalez-Olmos, R., Vilajeliu-Pons, A., Bañeras, L., Balaguer, M.D., and Colprim, J. (2014). Assessment of biotic and abiotic graphite cathodes for hydrogen production in microbial electrolysis cells. Int. J. Hydrogen Energy *39*, 1297–1305.

Behera, M., and Ghangrekar, M.M. (2009). Performance of microbial fuel cell in response to change in sludge loading rate at different anodic feed pH. Bioresour. Technol. *100*, 5114–5121.

Bond, D.R., and Lovley, D.R. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl. Environ. Microbiol. *69*, 1548–1555.

Bond, D.R., Holmes, D.E., Tender, L.M., and Lovley, D.R. (2002). Electrode-reducing microorganisms that harvest energy from marine sediments. Science *295*, 483–485.

Busalmen, J.P., Esteve-Núñez, A., Berná, A., and Feliu, J.M. (2008). C-type cytochromes wire electricity-producing bacteria to electrodes. Angew. Chem. *47*, 4874–4877.

Call, D.F., and Logan, B.E. (2011). Lactate oxidation coupled to iron or electrode reduction by *Geobacter sulfurreducens* PCA. Appl. Environ. Microbiol. *77*, 8791–8794.

Carmona-Martínez, A.A., Pierra, M., Trably, E., and Bernet, N. (2013). High current density via direct electron transfer by the halophilic anode respiring bacterium *Geoalkalibacter subterraneus*. Phys. Chem. Chem. Phys. *15*, 19699–19707.

Chang, I.S., Moon, H., Bretschger, O., Jang, J.K., Park, H. Il, Nealson, K.H., and Kim, B.H. (2006). Electrochemically active bacteria (EAB) and

mediator-less microbial fuel cells. J. Microbiol. Biotechnol. *16*, 163–177.

Chen, S., He, G., Liu, Q., Harnisch, F., Zhou, Y., Chen, Y., Hanif, M., Wang, S., Peng, X., Hou, H., and Schröder, U. (2012). Layered corrugated electrode macrostructures boost microbial bioelectrocatalysis. Energy Environ. Sci. *5*, 9769–9774.

Cohen, B. (1931). J. Bacteriol. *21*, 18–19.

Coma, M., Puig, S., Pous, N., Balaguer, M.D., and Colprim, J. (2013). Biocatalysed sulphate removal in a BES cathode. Bioresour. Technol. *130*, 218–223.

Cotterill, S., Dolfing, J., Jones, C., and Curtis, T.P. (2013). Plant-scale development of a Microbial Electrolysis Cell, in: Microbial Fuel Cells, 4th International Conference, Abstracts. pp. 218–223.

Cusick, R.D., Bryan, B., Parker, D.S., Merrill, M.D., Mehanna, M., Kiely, P.D., Liu, G.,and Logan, B.E. (2011). Performance of a pilot-scale continuous flow microbial electrolysis cell fed winery wastewater. Appl. Microbiol. Biotechnol. *89*, 2053–2063.

Cusick, R.D., Kiely, P.D., and Logan, B.E. (2010). A monetary comparison of energy recovered from microbial fuel cells and microbial electrolysis cells fed winery or domestic wastewaters. Int. J. Hydrogen Energy *35*, 8855–8861.

Davey, M.E., and George, A.O. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. Microbiol. Mol. Biol. Rev. *64*, 847–867.

Debabov, V.G. (2008). Electricity from microorganisms. Microbiology *77*, 123–131.

Dominguez-Benetton, X., Sevda, S., Vanbroekhoven, K., and Pant, D. (2012). The accurate use of impedance analysis for the study of microbial electrochemical systems. Chem. Soc. Rev. *41*, 7228–7246.

Escapa, A., Gómez, X., Tartakovsky, B., and Morán, A. (2012). Estimating microbial electrolysis cell (MEC) investment costs in wastewater treatment plants: Case study. Int. J. Hydrogen Energy *37*, 18641–18653.

Franks, A.E., Malvankar, N., and Nevin, K.P. (2010). Bacterial biofilms: the powerhouse of a microbial fuel cell. Biofuels *1*, 589–604.

Franks, A.E., and Nevin, K.P. (2010). Microbial Fuel Cells, A Current Review. Energies *3*, 899–919.

Freguia, S., Rabaey, K., Yuan, Z., and Keller, J. (2008). Syntrophic processes drive the conversion of glucose in Microbial Fuel Cell anodes. Environ. Sci. Technol. *42*, 7937–7943.

Friedheim, E., and Michaelis, L. (1931). Potentiometric study of piocyanine. J. Biol. Chem. *91*, 355–368.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., *et al.* (2006). Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc. Natl. Acad. Sci. U.S.A. *103*, 11358–11363.

Greenman, J., Gálvez, A., Giusti, L., and Ieropoulos, I. (2009). Electricity from landfill leachate using microbial fuel cells: Comparison with a biological aerated filter. Enzyme Microb. Technol. *44*, 112–119.

Gregory, K.B., Bond, D.R., and Lovley, D.R. (2004). Graphite electrodes as electron donors for anaerobic respiration. Environ. Microbiol. *6*, 596–604.

Gregory, K.B., and Lovley, D.R. (2005). Remediation and recovery of uranium from contaminated subsurface environments with electrodes. Environ. Sci. Technol. *39*, 8943–8947.

Guo, K., Freguia, S., Dennis, P.G., Chen, X., Donose, B.C., Keller, J., Gooding, J.J., and Rabaey, K. (2013). Effects of surface charge and hydrophobicity on anodic biofilm formation, community composition, and current generation in Bioelectrochemical Systems. Environ. Sci. Technol. *47*, 7563–7570.

Harnisch, F., and Freguia, S. (2012). A basic tutorial on cyclic voltammetry for the investigation of electroactive microbial biofilms. Chem. Asian J. *7*, 466–475.

Harnisch, F., Koch, C., Patil, S.A., Hübschmann, T., Müller, S., and Schröder, U. (2011). Revealing the electrochemically driven selection in natural community derived microbial biofilms using flow-cytometry. Energy Environ. Sci. *4*, 1265–1272.

Hartshorne, R.S., Reardon, C.L., Ross, D., Nuester, J., Clarke, T.A., Gates, A.J., Mills, P.C., Fredrickson, J.K., Zachara, J.M., Shi, L., *et al.* (2009). Characterization of an electron conduit between bacteria and the extracellular environment. Proc. Natl. Acad. Sci. U.S.A. *106*, 22169–22174.

Hashsham, S.A., and Freedman, D.L. (1999). Enhanced biotransformation of carbon tetrachloride by *Acetobacterium woodii* upon addition of hydroxocobalamin and fructose. Appl. Environ. Microbiol. *65*, 4537–4542.

He, W., Feng, Y., Liu, J., Wang, X., Qu, Y., and Ren, N. (2014). A horizontal plug flow and stackable pilot microbial fuel cell for municipal wastewater treatment. Bioresour. Technol. *156*, 132–138.

Heidrich, E.S., Dolfing, J., Scott, K., Edwards, S.R., Jones, C., and Curtis, T.P. (2013). Production of hydrogen from domestic wastewater in a pilot-scale microbial electrolysis cell. Appl. Microbiol. Biotechnol. *97*, 6979–6989.

Hernandez, M.E., and Newman, D.K. (2001). Cellular and Molecular Life Sciences Extracellular electron transfer. Cell. Mol. Life Sci. *58*, 1562–1571.

Holmes, D.E., Bond, D.R., and Lovley, D.R. (2004). Electron Transfer by *Desulfobulbus propionicus* to Fe (III) and Graphite Electrodes. Appl. Environ. Microbiol. *70*, 1234–1238.

Holmes, D.E., Chaudhuri, S.K., Nevin, K.P., Mehta, T., Methé, B.A., Liu, A., Ward, J.E., Woodard, T.L., Webster, J., and Lovley, D.R. (2006). Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. Environ. Microbiol. *8*, 1805–1815.

Ieropoulos, I., Greenman, J., and Melhuish, C. (2012). Urine utilisation by microbial fuel cells; energy fuel for the future. Phys. Chem. Chem. Phys. *14*, 94–98.

Jia, J., Tang, Y., Liu, B., Wu, D., Ren, N., and Xing, D. (2013). Electricity generation from food wastes and microbial community structure in microbial fuel cells. Bioresour. Technol. *144*, 94–99.

Jiang, Y., Su, M., Zhang, Y., Zhan, G., Tao, Y., and Li, D. (2013). Bioelectrochemical systems for simultaneously production of methane and acetate from carbon

dioxide at relatively high rate. Int. J. Hydrogen Energy *38*, 3497–3502.

Keck, A., Rau, J., Reemtsma, T., Mattes, R., Stolz, A., and Klein, J. (2002). Identification of quinoide redox mediators that are formed during the degradation of Naphthalene-2-Sulfonate by *Sphingomonas xenophaga* BN6. Appl. Environ. Microbiol. *68*, 4341–4349.

Kiely, P.D., Regan, J.M., and Logan, B.E. (2011). The electric picnic: synergistic requirements for exoelectrogenic microbial communities. Curr. Opin. Biotechnol. *22*, 378–385.

Kim, B.H., Ikeda, T., Park, H.S., Kim, H.J., Hyun, M.S., Kano, K., Takagi, K., and Tatsumi, H. (1999). Electrochemical activity of an Fe (III)-reducing bacterium, *Shewanella putrefaciens* IR-1, in the presence of alternative electron acceptors *200*, 475–478.

Kim, J.R., Rodríguez, J., Hawkes, F.R., Dinsdale, R.M., Guwy, A.J., and Premier, G.C. (2011). Increasing power recovery and organic removal efficiency using extended longitudinal tubular microbial fuel cell (MFC) reactors. Energy Environ. Sci. *4*, 459–464.

Koons, B.W., Baeseman, J.L., and Novak, P.J. (2001). Investigation of cell exudates active in carbon tetrachloride and chloroform degradation. Biotechnol. Bioeng. *74*, 12–17.

Lalaurette, E., Thammannagowda, S., Mohagheghi, A., Maness, P.-C., and Logan, B.E. (2009). Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis. Int. J. Hydrogen Energy *34*, 6201–6210.

Leang, C., Qian, X., Mester, T., and Lovley, D.R. (2010). Alignment of the c-type cytochrome OmcS along pili of *Geobacter sulfurreducens*. Appl. Environ. Microbiol. *76*, 4080–4084.

Lewis, T.A., Paszczynski, A., Gordon-Wylie, S.W., Jeedigunta, S., Lee, C.H., and Crawford, R.L. (2001). Carbon tetrachloride dechlorination by the bacterial transition metal chelator pyridine-2,6-bis(thiocarboxylic acid). Environ. Sci. Technol. *35*, 552–559.

Li, X.M., Zhou, S.G., Li, F.B., Wu, C.Y., Zhuang, L., Xu, W., and Liu, L. (2009). Fe(III) oxide reduction and carbon tetrachloride dechlorination by a newly isolated *Klebsiella pneumoniae* strain L17. J. Appl. Microbiol. *106*, 130–139.

Liamleam, W., and Annachhatre, A.P. (2007). Electron donors for biological sulfate reduction. Biotechnol. Adv. *25*, 452–463.

Lim, S.J., Park, W., Kim, T.-H., and Shin, I.H. (2012). Swine wastewater treatment using a unique sequence of ion exchange membranes and bioelectrochemical system. Bioresour. Technol. *118*, 163–169.

Liu, H., Hu, H., Chignell, J., and Fan, Y. (2010). Microbial electrolysis: novel technology for hydrogen production from biomass. Biofuels *1*, 129–142.

Liu, H., and Logan, B.E. (2004). Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. Environ. Sci. Technol. *38*, 4040–4046.

Logan, B.E. (2008a). Power generation, in: Microbial Fuel Cells, 4th International Conference, Abstracts. pp. 44–61.

Logan, B.E. (2008b). Microbial Fuel Cells (New Jesey, USA: John Wiley and Sons, Inc.).

Logan, B.E., and Rabaey, K. (2012). Conversion of wastes into bioelectricity and chemicals by using microbial electrochemical technologies. Science *337*, 686–690.

Logan, B.E., and Regan, J.M. (2006). Electricity-producing bacterial communities in microbial fuel cells. Trends Microbiol. *14*, 512–518.

Logan, B.E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W., and Rabaey, K. (2006). Microbial fuel cells: methodology and technology. Environ. Sci. Technol. *40*, 5181–5192.

Logan, B., Cheng, S., Watson, V., and Estadt, G. (2007). Graphite fiber brush anodes for increased power production in air-cathode microbial fuel cells. Environ. Sci. Technol. *41*, 3341–3346.

Lovley, D.R. (2006). Bug juice: harvesting electricity with microorganisms. Nat. Rev. Microbiol. *4*, 497–508.

Lovley, D.R. (2008a). Extracellular electron transfer: wires, capacitors, iron lungs, and more. Geobiology *6*, 225–231.

Lovley, D.R. (2008b). The microbe electric: conversion of organic matter to electricity. Curr. Opin. Biotechnol. *19*, 564–571.

Lovley, D.R. (2011). Powering microbes with electricity: direct electron transfer from electrodes to microbes. Environ. Microbiol. Rep. *3*, 27–35.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004). Dissimilatory Fe (III) and Mn (IV) reduction. Adv. Microb. Physiol. *49*, 219–286.

Lovley, D.R., Ueki, T., Zhang, T., Malvankar, N.S., Shrestha, P.M., Flanagan, K.A., Aklujkar, M., Butler, J.E., Giloteaux, L., Rotaru, A.-E., *et al.* (2011). *Geobacter*: the microbe electric's physiology, ecology, and practical applications. Adv. Microb. Physiol. *59*, 1–100.

Malvankar, N.S., and Lovley, D.R. (2012). Microbial nanowires: a new paradigm for biological electron transfer and bioelectronics. ChemSusChem *5*, 1039–1046.

Malvankar, N.S., and Lovley, D.R. (2014). Microbial nanowires for bioenergy applications. Curr. Opin. Biotechnol. *27C*, 88–95.

Malvankar, N.S., Vargas, M., Nevin, K.P., Franks, A.E., Leang, C., Kim, B.-C., Inoue, K., Mester, T., Covalla, S.F., Johnson, J.P., Rotello, V.M., Tuominen, M.T., and Lovley, D.R. (2011). Tunable metallic-like conductivity in microbial nanowire networks. Nat. Nanotechnol. *6*, 573–579.

Malvankar, N.S., Lau, J., Nevin, K.P., Franks, A.E., Tuominen, M.T., and Lovley, D.R. (2012). Electrical conductivity in a mixed-species biofilm. Appl. Environ. Microbiol. *78*, 5967–5971.

Manohar, A.K., Bretschger, O., Nealson, K.H., and Mansfeld, F. (2008). The use of electrochemical impedance spectroscopy (EIS) for the evaluation of the electrochemical properties of bioelectrochemical systems. Bioelectrochemistry *72*, 149–154.

Marshall, C.W., LaBelle, E.V., and May, H.D. (2013). Production of fuels and chemicals from waste by microbiomes. Curr. Opin. Biotechnol. *24*, 391–397.

Marshall, C.W., Ross, D.E., Fichot, E.B., Norman, R.S., and May, H.D. (2012). Electrosynthesis of commodity chemicals by an autotrophic microbial community. Appl. Environ. Microbiol. *78*, 8412–8420.

Marsili, E., and Zhang, X. (2009). Shuttling via soluble compounds. In Bioelectrochemical Systems: From Extracellular Electron Transfer to Biotechnological Application, Rabaey, K., Angenent, L., Schroder, U., and Keller, K., eds. (IWA Publishing, London, UK), pp. 59–75.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J.M., Schink, B., Rohlin, L., and Gunsalus, R.P. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N.Y. Acad. Sci. *1125*, 58–72.

Methé, B.A., Nelson, K.E., Eisen, J.A., Paulsen, I.T., Nelson, W., Heidelberg, J.F., Wu, D., Wu, M., Ward, N., Beanan, M.J., *et al.* (2003). Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. Science *302*, 1967–1969.

Miceli, J.F., Garcia-Peña, I., Parameswaran, P., Torres, C.I., and Krajmalnik-Brown, R. (2014). Combining microbial cultures for efficient production of electricity from butyrate in a microbial electrochemical cell. Bioresour. Technol. *169C*, 169–174.

Millo, D. (2012). Spectroelectrochemical analyses of electroactive microbial biofilms. Biochem. Soc. Trans. *40*, 1284–1290.

Millo, D., Harnisch, F., Patil, S.A., Ly, H.K., Schröder, U., and Hildebrandt, P. (2011). In situ spectroelectrochemical investigation of electrocatalytic microbial biofilms by surface-enhanced resonance Raman spectroscopy. Angew. Chem. Int. Ed. Engl. *50*, 2625–2627.

Min, B., Kim, J., Oh, S., Regan, J.M., and Logan, B.E. (2005). Electricity generation from swine wastewater using microbial fuel cells. Water Res. *39*, 4961–4968.

Nealson, K.H., Myers, C.R., and Wimpee, B.B. (1991). Isolation and identification of manganese-reducing bacteria and estimates of microbial Mn (IV)-reducing potential in the Black Sea. Deep Sea Res. Part A. Oceanogr. Res. Pap. *38*, S907–S920.

Nevin, K.P., Hensley, S. a, Franks, A.E., Summers, Z.M., Ou, J., Woodard, T.L., Snoeyenbos-West, O.L., and Lovley, D.R. (2011). Electrosynthesis of organic compounds from carbon dioxide is catalyzed by a diversity of acetogenic microorganisms. Appl. Environ. Microbiol. *77*, 2882–2886.

Nevin, K.P., Richter, H., Covalla, S.F., Johnson, J.P., Woodard, T.L., Orloff, A.L., Jia, H., Zhang, M., and Lovley, D.R. (2008). Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells. Environ. Microbiol. *10*, 2505–2514.

Nevin, K.P., Woodard, T.L., and Franks, A.E. (2010). Microbial Electrosynthesis: Feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. Am. Soc. Microbiol. *1*, e00103–e00110.

Nielsen, J.L., Wagner, M., and Nielsen, P.H. (2003). Use of microautoradiography to study *in situ* physiology of bacteria in biofilms. Environ. Sci. Biotechnol. *2*, 261–268.

Oh, S.E., and Logan, B.E. (2005). Hydrogen and electricity production from a food processing wastewater using fermentation and microbial fuel cell technologies. Water Res. *39*, 4673–4682.

Parameswaran, P., Bry, T., Popat, S.C., Lusk, B.G., Rittmann, B.E., and Torres, C.I. (2013). Kinetic, electrochemical, and microscopic characterization of the thermophilic, anode-respiring bacterium *Thermincola ferriacetica*. Environ. Sci. Technol. *47*, 4934–4940.

Parameswaran, P., Torres, C.I., Lee, H.-S., Krajmalnik-Brown, R., and Rittmann, B.E. (2009). Syntrophic interactions among anode respiring bacteria (ARB) and Non-ARB in a biofilm anode: electron balances. Biotechnol. Bioeng. *103*, 513–523.

Park, D.H., Laivenieks, M., Guettler, M.V., Jain, M.K., and Zeikus, J.G. (1999). Microbial utilization of electrically reduced neutral red as the sole electron donor for growth and metabolite production. Appl. Environ. Microbiol. *65*, 2912–2917.

Park, D.H., and Zeikus, J.G. (1999). Utilization of electrically reduced neutral red by *Actinobacillus succinogenes*: Physiological function of neutral red in membrane-driven fumarate reduction and energy conservation. J. Bacteriol. *181*, 2403–2411.

Park, D.H., and Zeikus, J.G. (2003). Improved fuel cell and electrode designs for producing electricity from microbial degradation. Biotechnol. Bioeng. *81*, 348–355.

Patil, S.A., Hägerhäll, C., and Gorton, L. (2012). Electron transfer mechanisms between microorganisms and electrodes in bioelectrochemical systems. Bioanal. Rev. *4*, 159–192.

Potter, M.C. (1911). Electrical effects accompanying the decomposition of organic compounds. Proc. R. Soc. London. Ser. B. *84*, 260–276.

Pous, N., Puig, S., Coma, M., Balaguer, M.D., and Colprim, J. (2013). Bioremediation of nitrate-polluted groundwater in a microbial fuel cell. J. Chem. Technol. Biotechnol. *88*, 1690–1696.

Price-Whelan, A., Dietrich, L., and Newman, D. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nat. Chem. Biol. *2*, 221–225.

Puig, S., Coma, M., Desloover, J., Boon, N., Colprim, J., and Balaguer, M.D. (2012). Autotrophic denitrification in microbial fuel cells treating low ionic strength waters. Environ. Sci. Technol. *46*, 2309–2315.

Puig, S., Serra, M., Coma, M., Balaguer, M.D., and Colprim, J. (2011). Simultaneous domestic wastewater treatment and renewable energy production using microbial fuel cells (MFCs). Water Sci. Technol. *64*, 904–910.

Puig, S., Serra, M., Coma, M., Cabré, M., Balaguer, M.D., and Colprim, J. (2010). Effect of pH on nutrient dynamics and electricity production using microbial fuel cells. Bioresour. Technol. *101*, 9594–9599.

Puig, S., Serra, M., Coma, M., Cabré, M., Balaguer, M.D., and Colprim, J. (2011a). Microbial fuel cell application in landfill leachate treatment. J. Hazard. Mater. *185*, 763–767.

Puig, S., Serra, M., Vilar-Sanz, A., Cabré, M., Bañeras, L., Colprim, J., and Balaguer, M.D. (2011b). Autotrophic

nitrite removal in the cathode of microbial fuel cells. Bioresour. Technol. *102*, 4462–4467.

Rabaey, K., Boon, N., Siciliano, S.D., Verstraete, W., and Verhaege, M. (2004). Biofuel cells select for microbial consortia that self-mediate electron transfer. Appl. Environ. Microbiol. *70*, 5373–5383.

Rabaey, K., Clauwaert, P., Aelterman, P., and Verstraete, W. (2005). Tubular microbial fuel cells for efficient electricity generation. Environ. Sci. Technol. *39*, 8077–8082.

Rabaey, K., Lissens, G., Siciliano, S.D., and Verstraete, W. (2003). A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency. Biotechnol. Lett. *25*, 1531–1535.

Rabaey, K., and Rozendal, R.A. (2010). Microbial electrosynthesis – revisiting the electrical route for microbial production. Nat. Rev. Microbiol. *8*, 706–716.

Rabaey, K., Van der Sompel, K., Maignien, L., Boon, N., Aelterman, P., Clauwaert, P., De Schamphelaire, L., Pham, H.T., Vermeulen, J., Verhaege, M., Lens, P., and Verstraete, W. (2006). Microbial Fuel Cells for sulfide removal. Environ. Sci. Technol. *40*, 5218–5224.

Rabaey, K., and Verstraete, W. (2005). Microbial fuel cells: novel biotechnology for energy generation. Trends Biotechnol. *23*, 291–298.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005). Extracellular electron transfer via microbial nanowires. Nature *435*, 1098–1101.

Reguera, G., Nevin, K.P., Nicoll, J.S., Covalla, S.F., Woodard, T.L., and Lovley, D.R. (2006). Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. Appl. Environ. Microbiol. *72*, 7345–7348.

Reimers, C.E., Tender, L.M., Fertig, S., and Wang, W. (2001). Harvesting energy from the marine sediment–water interface. Environ. Sci. Technol. *35*, 192–195.

Richter, H., Lanthier, M., Nevin, K.P., and Lovley, D.R. (2007). Lack of electricity production by *Pelobacter carbinolicus* indicates that the capacity for Fe(III) oxide reduction does not necessarily confer electron transfer ability to fuel cell anodes. Appl. Environ. Microbiol. *73*, 5347–5353.

Rosenbaum, M., and Angenent, L.T. (2009). Genetically modified microorganisms for bioelectrochemical systems. In Bioelectrochemical Systems: From Extracellular Electron Transfer to Biotechnological Application, Rabaey, K., Angenent, L., Schroder, U., and Keller, K., eds. (IWA Publishing, London, UK), pp. 101–113.

Rosenbaum, M., Aulenta, F., Villano, M., and Angenent, L.T. (2011). Cathodes as electron donors for microbial metabolism: which extracellular electron transfer mechanisms are involved? Bioresour. Technol. *102*, 324–333.

Ross, D.E., Flynn, J.M., Baron, D.B., Gralnick, J.A., and Bond, D.R. (2011). Towards electrosynthesis in *shewanella*: energetics of reversing the mtr pathway for reductive metabolism. PLoS One *6*, e16649–e16658.

Rozendal, R.A., Jeremiasse, A.W., Hamelers, H.V.M., and Buisman, C.J.N. (2008). Hydrogen production with a Microbial Biocathode. Environ. Sci. Technol. *42*, 629–634.

Schröder, U. (2007). Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. Phys. Chem. Chem. Phys. *9*, 2619–2629.

Schröder, U. (2011). Discover the possibilities: microbial bioelectrochemical systems and the revival of a 100-year old discovery. J. Solid State Electrochem. *15*, 1481–1486.

Sevda, S., and Sreekrishnan, T.R. (2014). Removal of organic matters and nitrogenous pollutants simultaneously from two different wastewaters using biocathode microbial fuel cell. J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. *49*, 1265–1275.

Speers, A.M., Young, J.M., and Reguera, G. (2014). Fermentation of glycerol into ethanol in a microbial electrolysis cell driven by a customized consortium. Environ. Sci. Technol. *48*, 6350–6358.

Tandukar, M., Huber, S.J., Onodera, T., and Pavlostathis, S.G. (2009). Biological chromium (VI) reduction in the cathode of a microbial fuel cell. Environ. Sci. Technol. *43*, 8159–8165.

Thrash, J.C., and Coates, J.D. (2008). Review: direct and indirect electrical stimulation of microbial metabolism. Environ. Sci. Technol. *42*, 3921–3931.

Thrash, J.C., Van Trump, J.I., Weber, K. a, Miller, E., Achenbach, L. a, Coates, J.D. (2007). Electrochemical stimulation of microbial perchlorate reduction. Environ. Sci. Technol. *41*, 1740–1746.

Torres, C.I., Marcus, A.K., and Rittmann, B.E. (2007). Kinetics of consumption of fermentation products by anode-respiring bacteria. Appl. Microbiol. Biotechnol. *77*, 689–697.

Turick, C.E., Tisa, L.S., and Caccavo, F. (2002). Melanin production and use as a soluble electron shuttle for Fe (III) oxide reduction and as a terminal electron acceptor by *Shewanella algae* BrY. Appl. Environ. Microbiol. *68*, 2436–2444.

Van Rij, E.T., Wesselink, M., Chin-A-Woeng, T.F.C., Bloemberg, G.V., and Lugtenberg, B.J.J. (2004). Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol. Plant. Microbe. Interact. *17*, 557–566.

Vargas, M., Kashefi, K., Blunt-Harris, E.L., and Lovley, D.R. (1998). Fe (III) reduction on early Earth. Lett. to Nat. *395*, 65–68.

Velasquez-Orta, S.B., Head, I.M., Curtis, T.P., and Scott, K. (2011). Factors affecting current production in microbial fuel cells using different industrial wastewaters. Bioresour. Technol. *102*, 5105–5112.

Vilar-Sanz, A., Puig, S., García-Lledó, A., Trias, R., Balaguer, M.D., Colprim, J., and Bañeras, L. (2013). Denitrifying bacterial communities affect current production and nitrous oxide accumulation in a microbial fuel cell. PLoS One *8*, e63460–63471.

Villano, M., Monaco, G., Aulenta, F., and Majone, M. (2011). Electrochemically assisted methane production in a biofilm reactor. J. Power Sources *196*, 9467–9472.

Villano, M., Scardala, S., Aulenta, F., and Majone, M. (2013). Carbon and nitrogen removal and enhanced

methane production in a microbial electrolysis cell. Bioresour. Technol. *130*, 366–371.

Virdis, B., Millo, D., Donose, B.C., and Batstone, D.J. (2014). Real-time measurements of the redox states of c-type cytochromes in electroactive biofilms: a confocal resonance Raman Microscopy study. PLoS One *9*, e89918–89928.

Virdis, B., Rabaey, K., Yuan, Z., and Keller, J. (2008). Microbial fuel cells for simultaneous carbon and nitrogen removal. Water Res. *42*, 3013–3024.

Von Canstein, H., Ogawa, J., Shimizu, S., and Lloyd, J.R. (2008). Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Appl. Environ. Microbiol. *74*, 615–623.

Wagner, R.C., Regan, J.M., Oh, S.-E., Zuo, Y., and Logan, B.E. (2009). Hydrogen and methane production from swine wastewater using microbial electrolysis cells. Water Res. *43*, 1480–1488.

Ward, M.J., Fu, Q.S., Rhoads, K.R., Yeung, C.H.J., Spormann, A.M., and Criddle, C.S. (2004). A derivative of the menaquinone precursor 1,4-dihydroxy-2-naphthoate is involved in the reductive transformation of carbon tetrachloride by aerobically grown Shewanella oneidensis MR-1. Appl. Microbiol. Biotechnol. *63*, 571–577.

Watanabe, K., Manefield, M., Lee, M., and Kouzuma, A. (2009). Electron shuttles in biotechnology. Curr. Opin. Biotechnol. *20*, 633–641.

Wei, J., Liang, P., and Huang, X. (2011). Recent progress in electrodes for microbial fuel cells. Bioresour. Technol. *102*, 9335–9344.

Wen, Q., Wu, Y., Zhao, L., and Sun, Q. (2010). Production of electricity from the treatment of continuous brewery wastewater using a microbial fuel cell. Fuel *89*, 1381–1385.

Workman, D.J., Woods, S.L., Gorby, Y.A., Fredrickson, J.K., and Truex, M.J. (1997). Microbial reduction of vitamin B 12 by *Shewanella alga* Strain BRY with subsequent transformation of carbon tetrachloride. Environ. Sci. Technol. *31*, 2292–2297.

Wrighton, K.C., Virdis, B., Clauwaert, P., Read, S.T., Daly, R.A., Boon, N., Piceno, Y., Andersen, G.L., Coates, J.D., and Rabaey, K. (2010). Bacterial community structure corresponds to performance during cathodic nitrate reduction. ISME J. *4*, 1443–1455.

Xafenias, N., and Mapelli, V. (2014). Performance and bacterial enrichment of bioelectrochemical systems during methane and acetate production. Int. J. Hydrogen Energy *3*, 1–12.

Xing, D., Zuo, Y., Cheng, S., Regan, J.M., and Logan, B.E. (2008). Electricity generation by Rhodopseudomonas palustris DX-1. Environ. Sci. Technol. *42*, 4146–4151.

Yates, M.D., Kiely, P.D., Call, D.F., Rismani-Yazdi, H., Bibby, K., Peccia, J., Regan, J.M., and Logan, B.E. (2012). Convergent development of anodic bacterial communities in microbial fuel cells. ISME J. *6*, 2002–2013.

Zhan, G., Zhang, L., Tao, Y., Wang, Y., Zhu, X., and Li, D. (2014). Anodic ammonia oxidation to nitrogen gas catalyzed by mixed biofilms in bioelectrochemical systems. Electrochim. Acta *135*, 345–350.

Zhuang, L., Zheng, Y., Zhou, S., Yuan, Y., Yuan, H., and Chen, Y. (2012). Scalable microbial fuel cell (MFC) stack for continuous real wastewater treatment. Bioresour. Technol. *106*, 82–88.

Biofilms for One-stage Autotrophic Nitrogen Removal

11

José M. Carvajal-Arroyo, Tiago Rogeiro Vitor Akaboci, Maël Ruscalleda, Jesús Colprim, Emilie Courtens and Siegfried E. Vlaeminck

Abstract

About 20 years after the discovery of microbial anoxic ammonium oxidation (anammox), the autotrophic nitrogen removal through partial nitritation–anammox (PNA) for ammoniacal wastewater treatment has become a mature technology. The application of these slow growing anoxic ammonium-oxidizing bacteria (AnAOB) requires engineered systems with efficient biomass retention. In the last decade, several one-stage PNA technologies have been developed that promote the growth of AnAOB in biofilms along with aerobic ammonium-oxidizing bacteria (AerAOB). Such biofilms grow on the surface of a carrier material or in mm-scale bio-aggregates (granules). Thanks to the easy retention of biofilm carriers or good settleability of granules, long sludge retention times can be maintained. Additionally, diffusional oxygen transfer limitation within the biofilm allows for the creation of aerobic and anoxic microniches where AerAOB and AnAOB, respectively, can thrive. This chapter describes and discusses the engineering and ecological characteristics of the different technologies developed so far, including rotating biological contactors (RBC), moving bed biofilm reactors (MBBR), membrane-aerated biofilm reactors (MABR) and granular systems. Moreover, the recent literature on operation parameters that influence the greenhouse gas emissions (i.e. N_2O) during PNA are described. Finally the future trends in the biofilm-PNA applications to new effluents, with special attention to mainstream sewage treatment, are discussed.

Introduction

The discovery of the anoxic ammonium-oxidizing (anammox) bacteria in the early 1990s enabled for the resource-efficient treatment of ammonium rich wastewaters with low biodegradable organic matter content, through completely autotrophic microbial nitrogen transformations. Autotrophic nitrogen removal occurs through two sequential conversion steps. First, about half of the ammonium in the wastewater is converted to nitrite (partial nitritation). This reaction is performed by aerobic ammonium-oxidizing bacteria (AerAOB). Subsequently, the residual ammonium is oxidized by anoxic ammonium-oxidizing bacteria (AnAOB) using the nitrite formed in the first step as a terminal electron acceptor, and yielding nitrogen gas as the main product as well as some nitrate. Although nitrite oxidizing bacteria (NOB) naturally occur in one-stage PNA biofilms, nitratation is minimized to avoid nitrate production which would turn into N removal inefficiency. The combination of these two microbial conversions for completely autotrophic nitrogen removal, in a one-stage process, accepts different terminologies, like oxygen limited autotrophic nitrification-denitrification (OLAND) (Kuai and Verstraete, 1998), completely autotrophic nitrogen removal over nitrite (CANON) (Third *et al.*, 2001), or single-stage nitrogen removal using anammox and partial nitritation (SNAP) (Furukawa *et al.*, 2005). The industry has adopted the name of deammonification. The complete process of PNA, can be carried out in two separate reactors (aerobic and anoxic), or in a single stage, with nitritation and anammox reactions separated in time or in space (within the biofilm).

AerAOB are chemolithoautotrophic bacteria, falling predominantly within the taxonomic group of Beta-proteobacteria. *Nitrosomonas* is the dominant genus of AerAOB in one-stage PNA reactors (Vlaeminck *et al.*, 2012), among which *N. europaea* is the best known species. In the AerAOB metabolic pathway, NH_3 oxidation is catalysed by an ammonia monooxygenase (AMO), yielding hydroxylamine (NH_2OH) as a reaction intermediate. NH_2OH is further oxidized to nitrite by hydroxylamine oxidoreductase (HAO). The oxidation of NH_2OH yields four electrons. Two electrons are returned to AMO to allow for NH_3 oxidation, and the remaining two electrons are used in cell metabolism, including fixation of inorganic carbon (Arp and Stein, 2003) (Fig. 11.1A).

AnAOB are chemolithoautotrophic bacteria belonging to the phylum Planctomycetes. So far, five different genera (*Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua*) have been described (Kartal *et al.*, 2007; Kuypers *et al.*, 2003; Quan *et al.*, 2008; Schmid *et al.*, 2000; Strous *et al.*, 1999a). The first four genera, have been found in freshwater natural ecosystems, and bioreactors (except *Anammoxoglobus*), while *Scalindua* spp. thrive in saline water such as sea water, or highly saline anammox bioreactors (Kartal *et al.*, 2006). In the anammox reaction, nitrite is initially reduced to nitric oxide (NO), by the enzyme nitrite oxidase (NirS). Hydrazine synthase (HZS) combines NO and ammonium to generate hydrazine, which is finally oxidized to dinitrogen gas by hydrazine dehydrogenase (HDH). Carbon fixation in AnAOB is supported by nitrite oxidation to nitrate, which is catalysed by a nitrate reductase (NAR) (Fig. 11.1B) (Kartal *et al.*, 2011).

The stoichiometry of the one-stage PNA (Vlaeminck *et al.*, 2012) can be obtained by combination of the two independent reactions of nitritation (Barnes and Bliss, 1983) and anammox (Strous *et al.*, 1998) were AerAOB cells are accounted as $CH_{1.4}O_{0.4}N_{0.2}$ and AnAOB are accounted for as $CH_2O_{0.5}N_{0.15}$ (equation 11.1).

$$NH_4^+ + 0.792O_2 + 0.080HCO_3^- \rightarrow 0.435N_2 + 0.111NO_3^- + 1.029H^+ + 0.052CH_{1.4}O_{0.4}N_{0.2} + 0.028CH_2O_{0.5}N_{0.15} + 1.460H_2O \quad (11.1)$$

When compared with the conventional nitrification/denitrification, one-stage PNA provides significant advantages. Firstly, only about half of the ammonium needs to be oxidized to nitrite, which translates in up to 57% savings in aeration requirements. Secondly, the lithoautotrophicity of PNA eliminates the need for addition of organic carbon for 100%. Finally, PNA generates ca. 80% less biomass, which significantly reduces the sludge management costs.

Efficient retention of biomass is a major requirement for autotrophic nitrogen removal. AnAOB are slow growing bacteria (Strous *et al.*, 1998). For example, the cellular doubling times

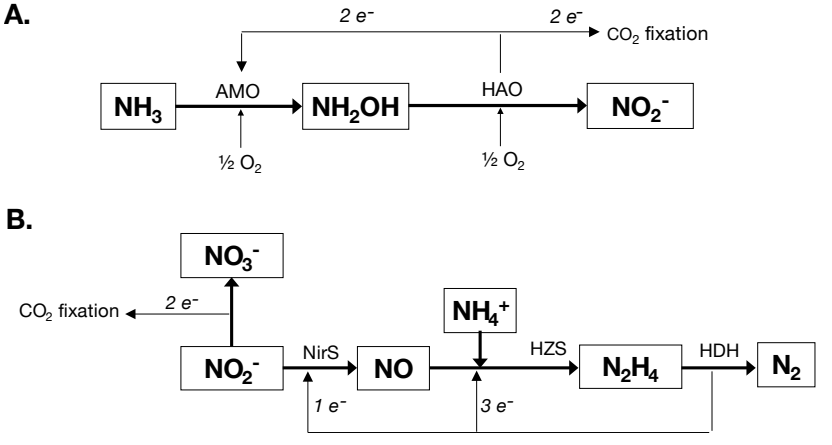


Figure 11.1 A: Metabolic pathway of nitritation by AerAOB. B: Metabolic pathway of anammox by AnAOB.

measured in full scale installations range from 11 to 27 days (Joss *et al.*, 2009; van der Star *et al.*, 2007), although the shortest observed doubling time for AnAOB is 3.3 days, in a laboratory-scale membrane anammox reactor (Lotti *et al.*, 2014). Therefore, the retention time of the biomass in the reactor must exceed those numbers. In general, 30–45 days of sludge retention time (SRT) is recommended in reactors operated at 30–35°C (Vlaeminck *et al.*, 2012). This can be achieved by promoting bacterial growth in biofilms, either on synthetic carriers that are easily retained, or in dense bio-aggregates or granules with fast settling properties. In fact, AnAOB tend to grow in aggregates (flocs or biofilm), and only under strictly anoxic conditions AnAOB are able to grow as free cells (Lotti *et al.*, 2014). The cell density seems to play an important role in AnAOB growth, since AnAOB activity can only be detected above a certain threshold (Strous *et al.*, 1999a). Moreover, De Clippeleir and colleagues (2011) observed higher specific AnAOB activity at high cell densities than in low density cultures, and proved the stimulating role of AerAOB-produced quorum sensing molecules in a PNA biofilm.

Growth of PNA biomass in biofilms offers several advantages aside from improved retention of the microorganisms. Because biofilms have higher cell densities, compared to suspended growth systems, a higher concentration of biocatalyst (microorganisms) can be achieved in the reactor, which translates in a significantly smaller reactor size, and consequently lower capital costs. As a result, full scale plants based on biofilms accept higher volumetric nitrogen loading rates than suspended growth installations (Lackner *et al.*, 2014).

Biofilms for one-stage PNA

The diffusive transport and conversion of the substrates (molecular oxygen and nitrogen species) in one-stage PNA biofilms allows for the formation of microniches with optimal conditions for the growth of both groups of bacteria (Fig. 11.2). In the aerobic part of the biofilm, AerAOB consume the oxygen and produce nitrite. The oxygen penetration depth in PNA depends on ammonium concentration, bulk dissolved oxygen (DO) level, and size of the biofilm, in the range of 0.06–0.02 mm for granules sized 0.79–2.55 mm diameter (Volcke *et al.*, 2012). The depletion of oxygen by AerAOB allows for the growth of AnAOB in the anoxic part of the biofilm, -which would otherwise be inhibited by oxygen (Strous *et al.*, 1999b) – where they benefit from the *in situ* production of nitrite, their terminal electron acceptor. DO inhibition of AnAOB has been further described in the literature (Egli *et al.*, 2001) and a 50% inhibitory concentration (IC_{50}) of 2.2 mg O_2 l^{-1} was calculated from batch tests (Carvajal-Arroyo *et al.*, 2013). However, AnAOB inhibition by DO is reversible, which allows the separation of nitritation and anammox reaction not only in space, but also in time, by alternating imposition of aerobic and anoxic conditions in the reactor.

The immediate uptake of nitrite by AnAOB in PNA biofilms represents an additional advantage over two-stage systems. Maintaining low nitrite concentrations is crucial for a stable and well performing process, since nitrite is a potential inhibitor for both groups of bacteria. While for AerAOB, the undissociated form HNO_2 (free nitrous acid, FNA) is responsible for the bactericidal effect in the range 0.2–2.8 mg FNA-N/l (Anthonisen *et al.*, 1976), ionized nitrite causes toxicity to AnAOB (Puyol *et al.*, 2014). The inhibitory effect of nitrite on AnAOB is strongly exacerbated when the electron donating substrate ammonium is not available (Carvajal-Arroyo *et al.*, 2014). This situation may occur if nitritation and anammox rates are not well balanced.

Aerobic nitrate generation by NOB has to be avoided as it lowers the N removal efficiency, in case this is not compensated by denitrifying activity (Fig. 11.2). Additionally, NOB compete for oxygen with AerAOB. Several strategies are used to reduce NOB growth in PNA reactors. Firstly, the prevention of nitrite accumulation helps maintaining low NOB activity as AnAOB have higher affinity for nitrite than NOB (Lackner *et al.*, 2008). Besides, the temperature plays another important role in allowing AerAOB to outcompete of NOB. AerAOB grow faster than NOB at high temperatures. Although some authors fix the breakthrough temperature in the range 15–18°C (Hellinga *et al.*, 1998; Wyffels *et al.*,

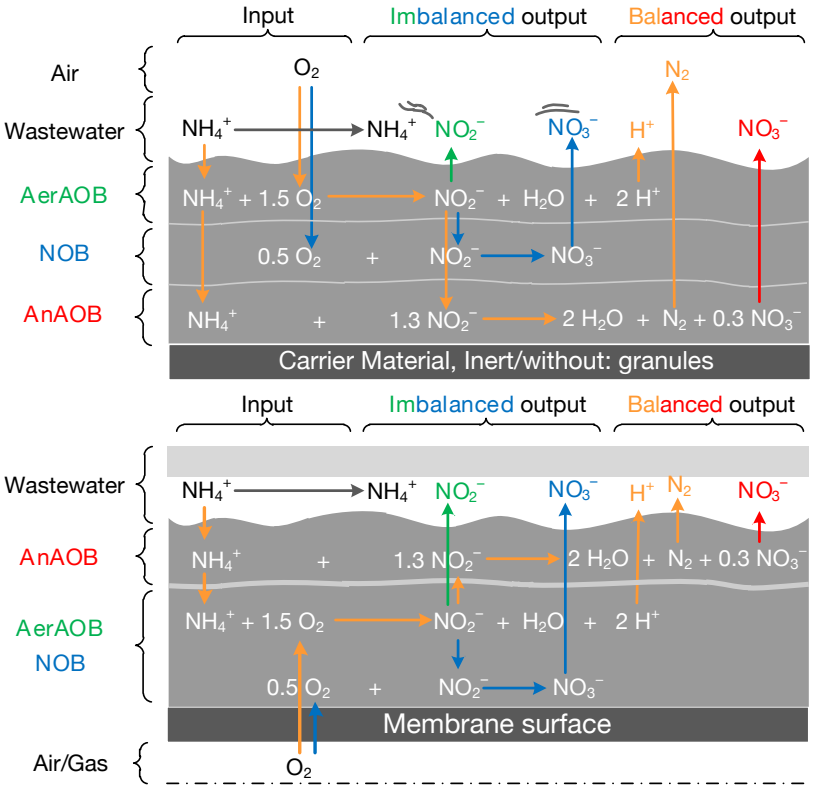


Figure 11.2 Scheme of PNA biofilms with co-diffusion (upper diagram) and counter-diffusion (lower diagram) of ammonium and oxygen. Yellow arrows: microbial transformations leading to balanced performance of PNA biofilms (maximum theoretical N removal, according to stoichiometry in equation 1). Red arrows: NO_3^- production inherent to a balanced PNA biofilm, limiting the N removal efficiency. Green arrows: Excessive nitritation leading to imbalanced PNA outcome. Blue arrows: nitratation (by NOB) leading to imbalanced PNA system (N removal efficiency lower than maximum theoretical). In counter-diffusive systems (MABR) AerAOB and NOB share space in the aerobic zone of the biofilm (Pellicer-Nacher *et al.*, 2014).

2003), other reports show NOB growth greater than AerAOB growth up to 28°C (Knowles *et al.*, 1965). Although adjusting the sludge age would allow for selective washout of NOB, biofilm reactors do not allow for such strategy, since the high SRT maintained in PNA-biofilm reactors enables growth of bacteria with very different growth rates (Fux *et al.*, 2004). Finally, in systems treating high strength effluents, depending on the feeding strategy, the concentrations of free ammonia (FA) can be high enough (>5 mg N/l) to cause inhibition of NOB, giving advantage to AerAOB growth. However, in more diluted effluents FA inhibition of NOB is not likely and other methods need to be applied (see 'Future trends', below).

The first full-scale installations were two-stage systems, in which PNA occurred in separate compartments. Currently however, the combination of both processes in a single reactor is largely preferred. By 2014, around 100 full scale plants were in operation, among which only four facilities operated with two stage systems (Lackner *et al.*, 2014). Biofilm based reactors for one-stage PNA, have been applied in full scale for the treatment of a wide variety of high strength ammoniacal wastewaters. The most popular application of one-stage PNA reactors is the treatment of reject water from sludge digesters. Although, other ammonium rich effluents are currently treated through one-stage PNA, including landfill leachate, food industry effluents (winery, distillery, starch production, sweetener production, potato and meat processing), monosodium glutamate production, or digested black water (Lackner *et al.*, 2014).

Several biofilm reactor configurations that are currently in use at full-scale are rotating biological contactors (RBC) (Hippen *et al.*, 1997), moving bed biofilm reactors (MBBR) (Rosenwinkel and Cornelius, 2005) and granular systems (Abma *et al.*, 2010) (Table 11.1). Additionally membrane-aerated biofilm reactors (MABR) (Gong *et al.*, 2007) have been evaluated at laboratory scale. A detailed description of these technologies can be found in the following sections.

Reactor configurations

Rotating biological contactor (RBC)

A reactor configuration suitable for the application of one-stage autotrophic nitrogen removal is the rotating biological contactor (RBC). First observations of nitrogen losses in the absence of biodegradable organic carbon for denitrification, occurred in RBC treating landfill leachate in Germany (Hippen *et al.*, 1997), Switzerland (Siegrist *et al.*, 1998), and UK (Schmid *et al.*, 2003). AnAOB were later identified as responsible for the N losses.

RBCs have been widely used for wastewater treatment, and their application to autotrophic nitrogen removal has an extensive track record (Patwardhan, 2003; Pynaert *et al.*, 2002). A RBC consists of a series parallel discs mounted on a horizontal shaft that is partially submerged in the wastewater (Fig. 11.3). The biofilm grows on the surface of the discs, and oxygenation is facilitated by continuous rotation of the shaft, alternately exposing the biomass to the air and to the wastewater. Another type of RBC consists of rotating cages, instead of discs, that enclose biofilm carriers, similar to those used in MBBR reactors (Mathure *et al.*, 2005). Although the RBC technology has proved robust and its operation is cost-effective, the nature of the design leaves little flexibility in terms of control strategies.

In RBC, aeration occurs through three different mechanisms (Kim and Molof, 1982): (i) oxygen transfer to the liquid film on the surface of the biofilm during the air exposure period; (ii) direct oxygen uptake by the bacteria exposed to the air; and (iii) oxygen transfer from the air to the bulk liquid in the reactor, which is enhanced by the turbulence caused by the rotation of the discs. The final oxygen transfer coefficient ($K_L a$) is influenced by the rotational speed and the immersion level of the discs. Several works have reported an enhancement of the oxygen transfer rates due to the presence of oxygen consumming bacteria (Kim and Molof, 1982; Paolini, 1986; Courtens *et al.*, 2013), and introduce an 'enhancement factor' in the oxygenation mass balance (equation 11.2):

$$O_{UR} = E \times \alpha K_L a \times (C_{sat} - C_{bulk}) \qquad (11.2)$$

Table 11.1 Qualitative comparison of one-stage PNA reactor configurations based on biofilms

	Reactor configuration			
	RBC	MBBR	MABR	Granules
Investment costs	High	Medium	High	Low
Operational costs	Low	Medium/high	Medium/high	Medium
Area requirement	High	Low	Low	Low
Aeration	Passive	Active	Active*	Active
Ease of DO control	Medium	Medium/high	Low	High
Inoculation feasibility	Low/medium	Medium/high	Low/medium	High
Risk for mechanical failure	High	Low	High	Low
Operational flexibility	Low	Medium	Low	Medium/high
Full-scale installations§	6	9	0	18

*Aeration in MABR occurs by oxygen diffusion through the pores of the membrane without generating bubbles, and it is controlled through the gas pressure in the lumen of the membrane fibres.
§Data from Lackner *et al*. (2014).
Adapted from Vlaeminck *et al*. (2012).

Figure 11.3 Lab-Scale OLAND-rotating biological contactor at the Laboratory of Microbial Ecology and Technology (Ghent University), started up in 2000 (Pynaert *et al*., 2002). Picture from 2014.

where O_{UR} is the oxygen uptake rate, E is the enhancement factor, $\alpha K_L a$ is the oxygen transfer coefficient in wastewater, C_{sat} is the saturation DO and C_{bulk} is the DO in the bulk liquid.

Overall, lower immersion level and faster rotational speed lead to better oxygenation, although excessive rotation velocity can cause biomass sloughing (Cortez *et al*., 2008). As in other technologies for autotrophic nitrogen removal, the management of the oxygen budget in RBC affects the balance between nitritation and anammox rates, and plays a role in the suppression of nitratation activity. Courtens *et al*. (2013) showed that high immersion level (80%) effectively suppressed NOB activity. Two mechanisms were identified to contribute to the minimization of aerobic nitrate generation. Firstly, lower oxygen availability caused accumulation of higher FA concentration, inducing NOB inhibition. Secondly, the exposure to anoxic conditions during longer periods, probably caused lag phases in NOB activity.

As in other biofilm technologies, AnAOB grow in the anoxic zones of the biofilm. In a study carried out in a RBC treating landfill leachate in Kölliken, Switzerland, *K. stuttgartiensis* dominated the AnAOB community in the biofilm (Egli *et al*., 2003). In another RBC treating synthetic wastewater, *K. stuttgartiensis* shared the anoxic areas of the biofilm with *B. anammoxidans* (Pynaert *et al*., 2003). In the work of Egli and colleagues, AerAOB affiliated to *N. europaea* and *N. eutropha* could be found in a dense layer in the outer part of the biofilm, whereas in a laboratory scale RBC, AerAOB were evenly distributed in the biofilm, including putatively anoxic areas (Pynaert *et al*., 2003). Along with AerAOB and AnAOB, a great diversity of heterotrophic Planctomycetes (*Pirellula*, *Gemmata*, *Isosphaera*, and *Planctomyces*) were found to coexist. The presence of heterotrophic bacteria in a reactor fed with a medium devoid of organic carbon, was explained by the decay of biomass in the biofilm (Pynaert *et al*., 2003). Indeed, *Pirellula* has been demonstrated capable of denitrification (Fuerst, 1995), which would further improve total N removal. Unlike the genera *Kuenenia* and *Brocadia*, *Scalindua* is commonly associated with samples from sediments and oxygen minimum zones in the ocean (Jetten *et al*., 2003).

Although the application of RBC for one-stage autotrophic nitrogen removal has been widely explored at the laboratory scale, there are only two references of RBC intentionally designed to perform PNA at full scale. In Sneek, The Netherlands, a 6-m^3 PNA RBC (cage type) is operated to treat digested black water, serving 464 population equivalents. In this facility, the rotation speed is controlled (1–4 rpm) to achieve the DO set point (0.60–0.65 mg O_2/l), and the pH is maintained in the range 7.0–7.5 by NaOH dosage. Another RBC was built by Advanced Wastewater Solutions (AWWS) in Hulst, The Netherlands, to treat the effluent from a fertilizer production industry. In this case, the DO is controlled by variation of the disc immersion level and rotational velocity. The feeding control is performed on the basis of online measurement of ammonium concentration in the effluent, and the pH is adjusted by acid/base dosage.

Moving-bed biofilm reactor (MBBR)

MBBR have been used for municipal and industrial wastewater treatment, such as pharmaceutical, paper industry, refinery and poultry processing, among others (Barwal and Chaudhary, 2014). MBBR technology utilizes mobile carrier materials to support biomass growth in biofilms. The carriers are kept in suspension inside the reactor, occupying the whole working reactor volume, by mechanical stirring or internal recirculation, together with active aeration in aerobic reactors. In contrast with other biofilm processes, the MBBR dispensed the need for sludge recirculation and backwashing, as well as problems related to clogging, common in fixed biofilm reactors (Ødegaard, 2006).

After the discovery of the anammox process, the application of MBBR configuration to one-stage PNA gained attention due to its high biomass retention efficiency. Since both nitritation and anammox pathways can occur simultaneously in the biofilm, there is no limitation for the anoxic/aerobic reaction times. In MBBR the biofilm is based on a co-diffusion scheme for oxygen and ammonium. AerAOB preferably settle in the outer layer (aerobic layer) while AnAOB grow in the inner layer (anoxic layer), as showed in Fig. 11.2 (Helmer *et al.*, 1999). According to microbial community analysis in a MBBR performing PNA, the biofilm developed is dominated by *Nitrosomonas europaea* and *N. eutropha*, for AerAOB, and Candidatus *Brocadia fulgida*, Candidatus *Anammoxoglobus propionius* and Candidatus *Kuenenia stuttgartiensis* for AnAOB (Almstrand *et al.*, 2014; Gilbert *et al.*, 2014b; Helmer *et al.*, 2002).

Most MBBR performing PNA have been started-up utilizing biofilm carriers developed previously (Christensson *et al.*, 2013), corresponding mainly for commercial application. The development of biofilm carrier from conventional activated sludge can be used as initial start-up strategy, but generally it takes long time for the biofilm to develop (Mehrdad *et al.*, 2014). A successful start-up strategy consists of an initial inoculation with nitrifying biomass, which is subjected to oxygen limitation in order to promote accumulation of nitrite. Subsequently, anammox biomass is seeded enabling for N removal (Daverey *et al.*, 2013).

Biofilm thickness and density are dependent on the hydrodynamics and biochemical reactions, and it influences the nitrogen removal efficiency in MBBR. A biofilm with thickness less than 0.2 mm might not support AnAOB growth due to the difficulty to obtain an anoxic layer under aerobic conditions (Hao *et al.*, 2002). However, no changes in nitrogen removal rate were obtained when the biofilm thickness increased from 0.27 mm to 0.77 mm during operation of a PNA- MBBR (Cema *et al.*, 2011). It took about 30 days for AerAOB enrichment and attachment on carrier, under low turbulence, and about 150 days for AnAOB growth in the biofilm (Mehrdad *et al.*, 2014). The biofilm in the carrier is protected from shearing forces and, therefore, the biofilm thickness is only limited by the carrier itself. The structure and activity of the microbial community in the biofilm developed in carriers, and consequently, the nitrogen removal efficiency are influenced by the operational conditions. Real-time DO control is the most common strategy, since it allows for the management of the nitrite level in the reactor. Ammonium, nitrite and nitrate are monitored in the influent and effluent with the help of online sensors. The DO set point can be increased or decreased, if the ratio $NO_{3\ produced}^{-}/NH_{4\ removed}^{+}$ is lower or higher than 11%, respectively. This strategy minimizes the growth of NOB in the reactor (Christensson *et al.*, 2013). Conductivity and pH can also be used for process performance assessment (Szatkowska *et al.*, 2007). The pH values decrease during partial nitritation due to consumption of alkalinity and to a lower extent, increase due to anammox reaction. In turn, conductivity is depleted by removal of the main ions (ammonium and hydrogen carbonate).

A significant number of publications show the applications for MBBR for nitrogen removal in one-stage reactor and several full-scale MBBR are currently in operation with several commercial applications in the market (Lackner *et al.*, 2014).

Granular systems

Compared to other biofilm reactors, granular biomass requires neither a carrier nor chemicals for microbial attachment. Granular biomass was first applied for anaerobic digestion of low strength wastewaters (Lettinga *et al.*, 1980) although later

on, its main application shifted to highly loaded wastewaters, including agroindustrial wastewaters. Years later, granulation was shown feasible also in aerobic reactors for sewage treatment (Mishima and Nakamura, 1991).

In the last years, some studies demonstrated the feasibility of enriching aerobic nitrifying biomass in granular systems (Campos *et al.*, 2000). As AnAOB also grow in granules (Abma *et al.*, 2007), autotrophic nitrogen removal is carried out by combination of partial nitritation and anammox processes in granules. Similar to other biofilm systems, the microbial community is stratified in an outer aerobic layer – dominated by AerAOB – surrounding the anoxic zone – with AnAOB – in the core of the granule (Fig. 11.4).

Granular nitritation-anammox processes have been mainly carried out in sequencing batch reactor (SBR), but also in airlift and up-flow column reactors (Vlaeminck *et al.*, 2009; Winkler *et al.*, 2012; Zhang *et al.*, 2012;Wang *et al.*, 2014).

Different strategies are applied to start up autotrophic nitrogen removal in reactors with granular biomass, depending on the source of inoculum. One of them consists in inoculating the reactor with AnAOB sludge and enriching the reactor by feeding ammonium and nitrite. Once stable nitrogen removal is obtained, nitrifying biomass can be added in the reactor, changing influent to contain only ammonium as nitrogen source. Simultaneously, oxygen must be provided for ammonium oxidation into nitrite, necessary for anammox reaction (Zhang *et al.*, 2012). Other strategies consist in obtaining first stable partial nitritation from nitrifying biomass, under oxygen limitation, and then proceed with AnAOB inoculation (Vázquez-Padín *et al.*, 2009). According to Vázquez-Padín (2009), the second start-up strategy seems to be more suitable for two reasons: (i) AnAOB activity in the first strategy can decrease after nitrifying biomass inoculation, and (ii) less AnAOB sludge is necessary to start up the process. In case of neither nitrifying nor anammox biomass are available, cultivating AerAOB and AnAOB from activated sludge can be also utilized (Hu *et al.*, 2013), requiring a long start-up period due to slow microbial growth rate.

Microbial balance between AerAOB and AnAOB is also relevant for autotrophic nitrogen removal in granular systems. This balance is determined by granule size and substrate concentration in the bulk liquid, which will influence substrate mass transfer into the granule, and consequently the activities in each granule layer. Under DO limitation (~5 μm O_2), Nielsen and colleagues (2005) verified that large type aggregates (>500 μm), accounted for 68% of anammox potential, with AerAOB located at outer granule

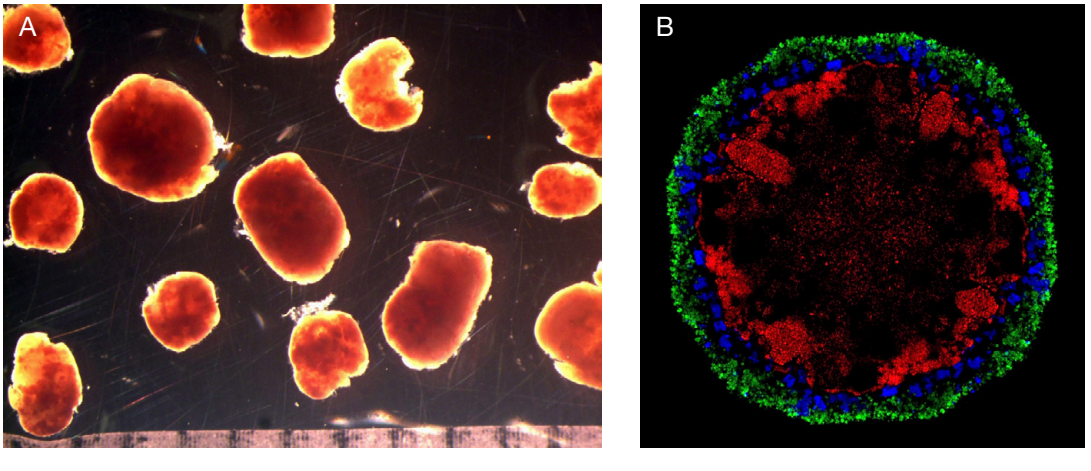


Figure 11.4 (A) Micrograph of a granular biofilm. (B) Fluorescence *in situ* hybridization (FISH) micrograph of a PNA granule, showing stratification of AerAOB (green, probes Nso1225 and Nso190), NOB (*Nitrospira* spp.) (blue, probe Ntspa662) and AnAOB (red, probe Amx820). Image extrapolated from Figure 1 in Vlaeminck *et al*. (2010).

layer and AnAOB at inner granule, whereas 65% of the nitrification potential was found in the smaller aggregates (<500 μm), primarily composed by AerAOB. Therefore, hydrodynamic conditions and sludge age, both of which affect the size distribution of the aggregates (or the biofilm depth), are key to obtain efficient nitrogen removal, minimizing both ammonium and nitrite in the effluent of the reactors. Accumulation of nitrite provides and advantage for NOB growth, and can result in excessive accumulation of nitrate.

Although granulation mechanisms are still not so clear, some factors have been pointed out to trigger biomass aggregation into anaerobic and aerobic granules. Hydrodynamics can be considered one of the most relevant among these factors. Granulation pathways in one-stage partial nitritation and anammox process were investigated by Vlaeminck and colleagues (2010), who hypothesized that granules replicate by division and budding, driven by bacterial growth and/or decay based on species-specific physiology and by hydrodynamic shear and mixing. Internal decay and subsequent shear on collisions of a weakened granule, can lead to granule division at a certain size. These new, small aggregates can grow into a new granule.

Short hydraulic retention time (HRT) allows the formation of larger and denser granules (Jin *et al.*, 2008), by selecting bigger granules that have good settling velocity. This parameter can be used in reactors in continuous operation, such as airlift and up-flow column reactor, and in batch mode operation, e.g. SBR. On the other hand, ammonium conversion can be optimized by adjusting the HRT (Vázquez-Padín *et al.*, 2010). For SBR reactor, settling time also could be a parameter to wash-out the particles with smaller diameter (De Clippeleir *et al.*, 2009). Applying this conditions, flocs and smaller aggregates composed majorly by AerAOB leave the system, avoiding an excess of nitritation rate.

The ecology of granular biomass can be influenced by organic carbon, due to competition of heterotrophic denitrifying bacteria with AerAOB and AnAOB for oxygen and nitrite, respectively. Based on this, PNA has been extensively applied as an alternative to denitrify wastewater with low chemical oxygen demand (COD):N ratio content (around 0.5). Nonetheless, growth of heterotrophic microorganisms in presence of organic matter can cause an increase in sludge production, which translates in a shorter SRT of the autotrophic biomass, impacting the stability of the autotrophic process. Moreover, heterotrophic growth negatively affects the settleability of the biomass, contributing further to biomass loss (Jenni *et al.*, 2014).

Membrane-aerated biofilm reactors

The application of membrane-aerated biofilm reactors (MABR) to one-stage autotrophic nitrogen removal has been explored at laboratory scale. In MABR, a hydrophobic membrane is used to provide oxygenation, and it also serves as carrier material for biofilm growth, while ammonium is present in the bulk liquid. Therefore, the electron donor (ammonium) and the terminal electron acceptor (oxygen) flow in counter-diffusion through the biofilm (Fig 11.2). In MABR, the oxygenation occurs by diffusion of the oxygen across the pores of the membrane, without producing bubbles. This avoids biofilm detachment from the carrier (the surface of the membrane), and additionally, it minimizes stripping of NH_3, NO and N_2O. As a consequence of the counter-diffusive feeding of ammonium and oxygen, the stratification pattern in MABR is different from other biofilms formed in systems where both ammonium and oxygen are present in the bulk liquid. AerAOB grow in the interface biofilm-membrane, were DO concentrations are highest. Then, a DO gradient is created as oxygen is consumed for ammonium oxidation, creating anoxic conditions near the biofilm–liquid interface, where AnAOB grow (Fig. 11.5)

The feasibility of one-stage autotrophic nitrogen removal in a MABR was first proved by (Gong *et al.*, 2007). In this study, the surface of the membrane was covered by a non-woven fabric as carrier material and a maximum N removal efficiency of 89% was achieved. This is the maximum achievable N removal efficiency according to PNA stoichiometry, therefore some endogenous heterotrophic denitrification may have occurred, which is expectable in a thick biofilm supported in such a carrier. In a modelling study, Terada and colleagues (2007) revealed the importance of the

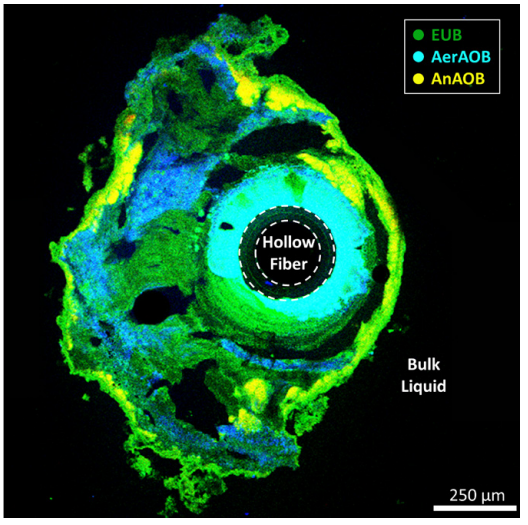


Figure 11.5 Fluorescence *in situ* hybridization (FISH) micrograph of cross-sectional stratification of bacteria in the biofilm of a MBBR for PNA reported in Pellicer-Nacher *et al*., 2014. Eubacteria (EUB mix) stained with FLUO (green), AerAOB (Nso190-Nmo218-Cluster 6a192) stained with Cy3 (blue) and AnAOB (AMX820) stained with Cy5 (red).

biofilm depth for high N removal efficiency in MABR. While thick biofilms (450–1400 µm) provided superior performance of counter-diffusive systems, greater thicknesses caused the removal rates to decrease, likely due to ammonium mass transfer limitation. Additionally, denitrification fuelled by biomass decay may improve total N removal. However, excessive COD in the influent can compromise the stability of the reactor, and influents with COD/N greater than 2 may not be treatable in a one-stage autotrophic MABR (Lackner *et al*., 2008).

A major drawback of counter-diffusion of the main substrates in MABR is the difficulty to control NOB growth. The exact DO level that NOB experience is hard to control. Furthermore, DO concentration is highest at the surface of the membrane. Here, the concentration of ammonium is the lowest, and therefore inhibition of NOB by free ammonia is unlikely. Different strategies have been evaluated to suppress NOB activity in MABR. For example, the application of intermittent aeration proved successful in limiting nitrite uptake by NOB. The exposure to transient anoxic conditions caused a lag phase in NOB activity, giving AerAOB an advantage to compete for oxygen, and lowering nitratation from 85%, during continuous aeration, to 9% of total nitrite consumption, during sequential aeration (Pellicer-Nàcher *et al*., 2010). Another viable strategy to outcompete NOB in MABR is the control of the O_2:NH_4^+ surface loading ratio (Terada *et al*., 2007). Gilmore and colleagues (2013) were able to maintain relatively low nitratation rates (14–19% of nitrite consumption) by applying a low O_2:NH_4^+ loading ratio (2.18 g O_2/g NH_4^+-N) in a continuously aerated MABR performing PNA.

Molecular characterization of MABR biofilms by fluorescence *in situ* hybridization, has shown the stratification of the different functional groups, with AerAOB growing in the vicinity of the membrane (aerobic region), and AnAOB growing in the outer region of the biofilm (anoxic) (Gong *et al*., 2008). However, the aeration strategy further affects the spatial disposition of AnAOB. When intermittent aeration is provided, AnAOB grow closer to AerAOB (Pellicer-Nàcher *et al*., 2010), than in a continuously aerated reactor, where the stratification is more evident (Gilmore *et al*., 2013).

MABR provide an option for environments where the off-gas production is restricted, or where gas bubbles are to be avoided in the liquid (e.g. zero-gravity, space flight conditions).

Nitrous oxide emissions during autotrophic nitrogen removal

N_2O is an important greenhouse gas with a global warming potential 300 greater than CO_2. Anthropogenic N_2O emissions after the industrial revolution have triggered a 20% increase in the N_2O atmospheric concentration. About 40% of the total N_2O dumped into the atmosphere (17 Tg/year) originates from human activities (chemical: combustion, incineration, industrial production processes and atmospheric deposition; and biological: agriculture, livestock wastes) (IPCC 2007, Physical scientific basis). Greenhouse gas (GHG) emissions from wastewater treatment occur during biological nitrogen removal. Global N_2O emissions during sewage treatment have been estimated at 0.22 Tg N/year, which accounts for 3.2% of the total anthropogenic

emissions (Mosier *et al.*, 1999), and up to 10.2% if nitrogen removal during manure treatment is included in the estimate (Scheehle *et al.*, 2006). Nonetheless, those figures may greatly underestimate the total N_2O emissions from wastewater treatment, since no data are available about N_2O emissions derived from nitrogen removal from industrial wastewaters or from landfill leachate, both of which may significantly contribute to the total GHGs emissions (Desloover *et al.*, 2012). Measurements made in full scale plants have revealed that N_2O can be produced in substantial quantities during wastewater treatment. The N_2O emissions in full-scale WWTPs range 0–14.6% of the total N-load of the plant (Kampschreur *et al.*, 2009).

N_2O is generated by both chemical and biological reactions (Fig. 11.6) (Desloover *et al.*, 2012). Chemical N_2O generation occurs by NH_2OH oxidation or nitrite and NO reduction by Fe^{2+}. The biological sources of N_2O are nitritation carried out by AerAOB, and by heterotrophic denitrifiers. During nitritation, two possible mechanisms lead to N_2O production. Firstly, in aerobic conditions, N_2O is generated from the HAO-catalysed oxidation of hydroxylamine, via the unstable intermediate HNO (Hooper and Terry, 1979; Poughon *et al.*, 2001). Secondly, under anoxic conditions, AerAOB are able to perform dissimilatory nitrite reduction to N_2O (using ammonium or H_2 as electron donor) (Bock *et al.*, 1995), with NIR and NOR reductases catalysing the reduction of nitrite and NO, respectively (nitrifier denitrification). Heterotrophic denitrification pathway includes N_2O and NO as intermediates during nitrite reduction to N_2. N_2O emissions arise when limiting electron donor does not allow for complete denitrification (Zumft, 1997). Finally, N_2O production has been detected from full scale anammox bioreactors (Kampschreur *et al.*, 2008). Although AnAOB do not have the genetic potential for N_2O production, one of the intermediates of anammox reaction, NO, is an important precursor of N_2O. Indeed, NO emission has been identified as one of the consequences of nitrite inhibition of AnAOB (Carvajal-Arroyo *et al.*, 2014). A N_2O production pathway in anammox reactors, has been recently hypothesized, consisting in the N_2O generation from chemical NO reduction, with Fe^{2+} as electron donor, and with anammox catalysing Fe^{2+} regeneration (Kampschreur *et al.*, 2011).

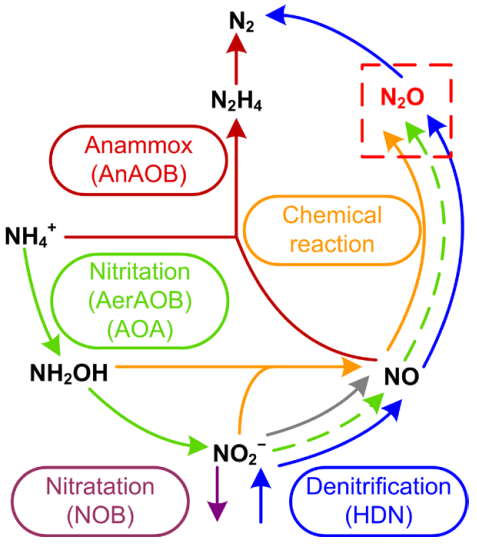


Figure 11.6 Routes of N_2O generation in partial nitritation–anammox reactors. Redrafted after Desloover *et al.* (2012). Green dashed line shows nitrifier-denitrification pathway for N_2O generation.

Different operational parameters have been found to play a major role in the production (and emission) of N_2O during autotrophic nitrogen removal. The DO concentration is one of the main factors controlling N_2O production. As NIR and NOR encoding genes are up-regulated during anoxia, oxygen limitation leads to increase N_2O production due to nitrifier denitrification (Tallec *et al.*, 2006). On the other hand, excess aeration may also enhance N_2O emissions (Kampschreur *et al.*, 2008). Therefore, aeration must be minimized to avoid aerobic N_2O production while maintaining enough DO to elude AerAOB denitrification. A bubbleless approach, in a MABR performing one-stage PNA, has been evaluated at laboratory scale, proving N_2O emissions to be 100-fold lower than in conventional systems, although no full scale confirmation exists (Pellicer-Nàcher *et al.*, 2010).

Other factors that affect N_2O emissions are nitrite and ammonia concentrations. Nitrite, as the main precursor of N_2O, is known to influence GHGs emissions during nitritation. Overall, higher nitrite concentrations (or sudden pulses in nitrite concentration) lead to greater N_2O

emissions (Colliver and Stephenson, 2000; Tallec *et al.*, 2006). In addition, ammonium concentration also influences N_2O emissions, since hydroxylamine production by HAO is regulated by ammonium. Accumulation of ammonium during the anoxic phase has been shown to trigger proportional N_2O peaks at the beginning of the following aerobic phase in a partial nitritation reactor (Yu *et al.*, 2010).

Although the reports on N_2O emissions in full-scale wastewater treatment plants are scarce, the available literature indicates that plants performing PNA emit a higher percentage of the nitrogen load as N_2O than plants designed for traditional nitrification-denitrification (by one to two orders of magnitude) (Desloover *et al.*, 2012). This can be partially explained by the fact that PNA plants treat more concentrated effluents (e.g. sludge centrate, industrial wastewaters), which results in highly variable conditions in the reactors, i.e. ammonium and nitrite concentrations, leading to higher N_2O emissions.

Overall, one-stage systems for autotrophic nitrogen removal have lower N_2O emissions than two-stage configurations. Measurements from one-stage full scale plants show N_2O emissions in the range of 1.2–1.3% of the total N load (Kampschreur *et al.*, 2009; Weissenbacher *et al.*, 2010), and maximum N_2O losses of 0.0015% were quantified in a lab-scale MABR (Pellicer-Nàcher *et al.*, 2010). N_2O emissions of 6.6% of the total nitrogen load were observed in a two-stage PNA system treating effluent of potato industry (Desloover *et al.*, 2011). Indeed, in one-stage biofilm systems, nitrite accumulation is generally 10–50 times lower than in two-stage systems (Desloover *et al.*, 2012), as it is immediately consumed by AnAOB. The preventative effect of AnAOB in the PNA biofilm can also be extended to the consumption of NO, which can be used by AnAOB when ammonium is available (Carvajal-Arroyo *et al.*, 2014; Kartal *et al.*, 2010b).

Application of PNA to the mainstream of sewage treatment

PNA technologies are a well-established alternative for the treatment of ammonium rich wastewaters, with influent concentrations higher than 500 mg NH_4^+-N l^{-1} (Lackner *et al.*, 2014). The fast implementation of the PNA process to the full scale has been driven by the significant savings brought about in terms of aeration, addition of chemicals and sludge management. Siegrist *et al.* (2008) estimated that about 40–50% of the total operation costs in a wastewater treatment plant could be saved by applying PNA to the treatment of sludge digester supernatant. For example, the implementation of this process allowed for net energy generation in a municipal wastewater treatment plant in Austria (Wett *et al.*, 2007). Nonetheless, the N load in sidestream is only about 15–25% of the total nitrogen load of the treatment plant. Therefore, even higher energy efficiency could be expected for future wastewater treatment plants, where the enhanced energy recovery from organic carbon is complemented with a minimum cost of nitrogen removal by application of PNA in the mainstream of sewage treatment (Kartal *et al.*, 2010a; Verstraete and Vlaeminck, 2011).

When compared to sludge liquor, sewage has lower N concentrations (20–60 mg N/l), lower temperatures (5–25°C) that are subjected to seasonal variations, and higher COD/N ratios. These differences pose additional challenges to the application of one-stage PNA to N removal in the mainstream. Low temperatures and low ammonium concentrations reduce both AnAOB and AerAOB rates. Dosta *et al.* (2008) showed almost complete loss of AnAOB activity when decreasing the temperature from 18 to 15°C in an anammox airlift reactor. Although AnAOB retrieved from low temperature (–1.7 to 4°C) natural environments have shown temperature optima at 15°C or even 12°C (Dalsgaard *et al.*, 2005; Rysgaard *et al.*, 2004), cultures obtained by adaptation of mesophilic reactors to low temperatures show higher optimum temperatures. A AnAOB rate of 39 mg N/g volatile suspended solids (VSS) d was observed in a laboratory scale anammox airlift at 10°C. Despite the difficulties, successful operation of one-stage PNA treating low strength synthetic wastewater, has been shown in MBBR at 13°C and 10°C (Gilbert *et al.*, 2014b; Persson *et al.*, 2014b), in a SBR at 12°C (Hu *et al.*, 2013) and in RBC at 15°C (De Clippeleir *et al.*, 2013).

Low N concentrations in the mainstream can further reduce AerAOB and AnAOB rates. AerAOB saturation constant for ammonium was estimated in 2.4 mg NH_4^+ -N/l (Wiesmann, 1994), which is already in the order of the ammonium concentrations expected in the reactor. While in suspended growth systems, AnAOB saturation constants for ammonium and nitrite are much lower than mainstream N concentrations, e.g.: $K_{s,}$ NO_2^- = 0.035 mg NO_2^- -N/l (Lotti *et al.*, 2014), $K_{s,}$ NH_4^+ = 0.07 mg NH_4^+ -N/l (Strous *et al.*, 1998), mass transfer limitation within biofilms generate high apparent saturation constants in the order of N concentrations in the water line, i.e.: high apparent saturation constants were calculated for AnAOB granules (2.4 ± 0.6 mm diameter) at 30°C, $K_{s,}$ NO_2^- = 4.90 mg NO_2^- -N/l, $K_{s,\ NH4+}$ = 8.96 mg NH_4^+ – N/l(Puyol *et al.*, 2013). Indeed, a decrease in N conversion rates due to low substrate availability was observed in a MBBR operated at 20°C, when treating sewage-like N concentrations (Gilbert *et al.*, 2014b).

The suppression of NOB activity is the main challenge brought by low temperature and low ammonium influent concentrations. High FA concentrations, usually present in reactors treating high strength ammoniacal wastewaters, pose inhibition to NOB, giving an advantage to AerAOB and AnAOB growth. Since NH_3 inhibition of NOB is unlikely during treatment of sewage, other strategies need to be followed to suppress aerobic nitrite oxidation. In suspended or hybrid growth systems and at moderate temperatures (25–30°C), maintaining low aerobic SRT favours NOB washout. This strategy is not possible in biofilm reactors, due to the impossibility to uncouple aerobic SRT from anoxic SRT. The application of low DO levels, a common strategy at moderate temperatures, cannot ensure NOB suppression at low temperatures, as *Nitrospira* spp. are K-strategists, with high affinity for oxygen, which facilitates NOB acclimation to low DO (Laanbroek and Gerards, 1993; Sliekers *et al.*, 2005). Some authors have put their efforts in the exploration of another strategy to suppress NOB activity, which is based on the application of alternating anoxic and aerobic conditions in the reactor. A delay or lag time in nitrate production by NOB, with respect to AerAOB oxidation of ammonia, has been observed when changing from anoxic to aerobic conditions (Alleman, 1984; Turk and Mavinic, 1986; Villaverde *et al.*, 2000). This phenomenon has been further studied with *Nitrospira* spp. at temperatures as low as 10°C, where short anoxic periods (5–20 min) were able to shut down NOB metabolism causing lag phases in nitrate production after restoration of aerobic conditions (Gilbert *et al.*, 2014a). This strategy has been successfully applied in full-scale hybrid growth PNA systems treating high strength wastewater at 30°C (Jardin and Hennerkes, 2011).

While application of one-stage PNA for mainstream N removal is a promising technology, important challenges still need to be solved. Stringent treatment standards cannot be met without effective suppression of NOB. Additionally, low removal rates, accompanied by the low growth rates of AnAOB at low temperature, require high catalyst concentrations, as well as very long SRT. This can only be achieved by efficient separation of the biomass, which is very much facilitated in biofilm systems.

Future trends

Existing applications of one stage PNA process are restricted to nitrogenous wastewaters from sludge digestion, landfill leachates and industry. Nonetheless, current research efforts aim to expand the field of application of PNA to other abundant nitrogen-containing wastewaters with low COD:N ratio in high volumetric loading rate processes. For example, the treatment of diluted source-separated urine has been demonstrated at the laboratory scale (Udert *et al.*, 2008) and technical scale trials have been done to treat digested black water (Meulman *et al.*, 2010; Vlaeminck *et al.*, 2009). The livestock industry generates waste manure with high N concentration which may be treated through PNA. Given the high concentration of N in manure (3–5 g N/l), treatment with PNA would bring considerable savings. The treatment of manure digestate with anammox based technologies has been explored at lab scale (Bernet and Béline, 2009; Hwang *et al.*, 2005; Molinuevo *et al.*, 2009; Scaglione *et al.*, 2013; Yamamoto *et al.*, 2011). However in these studies, the N removal is performed in two-stage systems,

and the influent is diluted to mitigate the toxicity caused by the complex organic matrix. To date, the application of one stage systems for PNA in manure remains to be demonstrated.

References

Abma, W.R., Driessen, W., Haarhuis, R., and van Loosdrecht, M.C.M. (2010). Upgrading of sewage treatment plant by sustainable and cost-effective separate treatment of industrial wastewater. Water Sci. Technol. *61*, 1715–1722.

Abma, W.R., Schultz, C.E., Mulder, J.W., van der Star, W.R.L., Strous, M., Tokutomi, T., and van Loosdrecht, M.C.M. (2007). Full-scale granular sludge anammox process. Water Sci. Technol. *55*, 27–33.

Alleman, J.E. (1984). Elevated occurrence of nitrite in biological wastewater treatment sustems. Water Sci. Technol. *17*, 409–419.

Almstrand, R., Persson, F., Daims, H., Ekenberg, M., Christensson, M., Wilén, B.-M., Sörensson, F., and Hermansson, M. (2014). Three-dimensional stratification of bacterial biofilm populations in a moving bed biofilm reactor for nitritation-anammox. Int. J. Mol. Sci. *15*, 2191–2206.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., and Srinath, E.G. (1976). Inhibition of nitrification by ammonia and nitrous-acid. J. Water Pollut. Con. F. *48*, 835–852.

Arp, D.J., and Stein, L.Y. (2003). Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. Crit. Rev. Biochem. Mol. *38*, 471–495.

Aspegren, H., Nyberg, U., Andersson, B., Gotthardsson, S., and Jansen, J.l.C. (1998). Post denitrification in a moving bed biofilm reactor process. Water Sci. Technol. *38*, 31–38.

Barnes, D., and Bliss, P.J. (1983). Biological control of nitrogen in wastewater treatment (E., and FN. Spon., London, UK).

Barwal, A., and Chaudhary, R. (2014). To study the performance of biocarriers in moving bed biofilm reactor (MBBR) technology and kinetics of biofilm for retrofitting the existing aerobic treatment systems: a review. Rev. Environ. Sci. Biotechnol. *13*, 285–299.

Bernet, N., and Béline, F. (2009). Challenges and innovations on biological treatment of livestock effluents. Bioresource Technol. *100*, 5431–5436.

Bock, E., Schmidt, I., Stuven, R., and Zart, D. (1995). Nitrogen loss caused by denitrifying Nitrosomonas cells using ammonium or hydrogen as electron-donors and nitrite as electron-acceptor. Arch. Microbiol. *163*, 16–20.

Campos, J.L., Mendez, R., and Lema, J.M. (2000). Operation of a nitrifying activated sludge airlift (NASA) reactor without biomass carrier. Water Sci. Technol. *41*, 113–120.

Carvajal-Arroyo, J.M., Puyol, D., Li, G.B., Lucero-Acuna, A., Sierra-Alvarez, R., and Field, J.A. (2014). Pre-exposure to nitrite in the absence of ammonium strongly inhibits anammox. Water Res. *48*, 52–60.

Carvajal-Arroyo, J.M., Sun, W.J., Sierra-Alvarez, R., and Field, J.A. (2013). Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents. Chemosphere *91*, 22–27.

Cema, G., Płaza, E., Trela, J., and Surmacz-Górska, J. (2011). Dissolved oxygen as a factor influencing nitrogen removal rates in a one-stage system with partial nitritation and Anammox process. Water Sci. Technol. *64*, 1009–1015.

Christensson, M., Ekström, S., Andersson Chan, A., Le Vaillant, E., and Lemaire, R. (2013). Experience from start-ups of the first ANITA Mox Plants. Water Sci. Technol. *67*, 2677–2684.

Colliver, B.B., and Stephenson, T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. Biotechnol. Adv. *18*, 219–232.

Cortez, S., Teixeira, P., Oliveira, R., and Mota, M. (2008). Rotating biological contactors: a review on main factors affecting performance. Rev. Environ. Sci. Biotechnol. *7*, 155–172.

Courtens, E.N.P., Boon, N., De Clippeleir, H., Berckmoes, K., Mosquera, M., Seuntjens, D., and Vlaeminck, S.E. (2014). Control of nitratation in an oxygen-limited autotrophic nitrification/denitrification rotating biological contactor through disc immersion level variation. Bioresource Technol. *155*, 182–188.

Dalsgaard, T., Thamdrup, B., and Canfield, D.E. (2005). Anaerobic ammonium oxidation (anammox) in the marine environment. Res. Microbiol. *156*, 457–464.

Daverey, A., Su, S.-H., Huang, Y.-T., Chen, S.-S., Sung, S., and Lin, J.-G. (2013). Partial nitrification and anammox process: A method for high strength optoelectronic industrial wastewater treatment. Water Res. *47*, 2929–2937.

De Clippeleir, H., Defoirdt, T., Vanhaecke, L., Vlaeminck, S.E., Carballa, M., Verstraete, W., and Boon, N. (2011). Long-chain acylhomoserine lactones increase the anoxic ammonium oxidation rate in an OLAND biofilm. Appl. Microbiol. Biot. *90*, 1511–1519.

De Clippeleir, H., Vlaeminck, S.E., Carballa, M., and Verstraete, W. (2009). A low volumetric exchange ratio allows high autotrophic nitrogen removal in a sequencing batch reactor. Bioresource Technol. *100*, 5010–5015.

De Clippeleir, H., Vlaeminck, S.E., De Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., and Boon, N. (2013). One-stage partial nitritation/anammox at 15A degrees C on pretreated sewage: feasibility demonstration at lab-scale. Appl. Microbiol. Biot. *97*, 10199–10210.

Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W., and Vlaeminck, S.E. (2011). Floc-based sequential partial nitritation and anammox at full scale with contrasting N2O emissions. Water Res. *45*, 2811–2821.

Desloover, J., Vlaeminck, S.E., Clauwaert, P., Verstraete, W., and Boon, N. (2012). Strategies to mitigate N2O emissions from biological nitrogen removal systems. Curr. Opin. Biotechnol. *23*, 474–482.

Di Trapani, D., Christensson, M., Torregrossa, M., Viviani, G., and Ødegaard, H. (2013). Performance of a hybrid

activated sludge/biofilm process for wastewater treatment in a cold climate region: Influence of operating conditions. Biochem. Eng. J. *77*, 214–219.

Dosta, J., Fernandez, I., Vazquez-Padin, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J., and Mendez, R. (2008). Short- and long-term effects of temperature on the Anammox process. J. Hazard. Mater. *154*, 688–693.

Egli, K., Bosshard, F., Werlen, C., Lais, P., Siegrist, H., Zehnder, A.J.B., and van der Meer, J.R. (2003). Microbial composition and structure of a rotating biological contactor biofilm treating ammonium-rich wastewater without organic carbon. Microb. Ecol. *45*, 419–432.

Egli, K., Fanger, U., Alvarez, P.J.J., Siegrist, H., van der Meer, J.R., and Zehnder, A.J.B. (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch. Microbiol. *175*, 198–207.

Fuerst, J.A. (1995). The Planctomycetes – Emerging models for microbial ecology, evolution and cell biology. Microbiology UK *141*, 1493–1506.

Furukawa, K., Lieu, P.K., Tokitoh, H., and Fujii, T. (2006). Single-stage nitrogen removal using ANAMMOX and partial nitritation (SNAP). Water Sci. Technol. *53*, 83–90.

Fux, C., Huang, D., Monti, A., and Siegrist, H. (2004). Difficulties in maintaining long-term partial nitritation of ammonium-rich sludge digester liquids in a moving-bed biofilm reactor (MBBR). Water Sci. Technol. *49*, 53–60.

Gilbert, E.M., Agrawal, S., Brunner, F., Schwartz, T., Horn, H., and Lackner, S. (2014a). Response of different Nitrospira species to anoxic periods depends on operational DO. Environ. Sci. Technol. *48*, 2934–2941.

Gilbert, E.M., Agrawal, S., Karst, S.M., Horn, H., Nielsen, P.H., and Lackner, S. (2014b). Low temperature partial nitritation/anammox in a moving bed biofilm reactor treating low strength wastewater. Environ. Sci. Technol. *48*, 8784–8792.

Gilmore, K.R., Terada, A., Smets, B.F., Love, N.G., and Garland, J.L. (2013). Autotrophic nitrogen removal in a membrane-aerated biofilm reactor under continuous aeration: a demonstration. Environ. Eng. Sci. *30*, 38–45.

Gong, Z., Liu, S.T., Yang, F.L., Bao, H., and Furukawa, K. (2008). Characterization of functional microbial community in a membrane-aerated biofilm reactor operated for completely autotrophic nitrogen removal. Bioresource Technol. *99*, 2749–2756.

Gong, Z., Yang, F., Liu, S., Bao, H., Hu, S., and Furukawa, K. (2007). Feasibility of a membrane-aerated biofilm reactor to achieve single-stage autotrophic nitrogen removal based on Anammox. Chemosphere *69*, 776–784.

Hao, X., Heijnen, J.J., and van Loosdrecht, M.C.M. (2002). Sensitivity analysis of a biofilm model describing a one-stage completely autotrophic nitrogen removal (CANON) process. Biotechnol. Bioeng. *77*, 266–277.

Hellinga, C., Schellen, A., Mulder, J.W., van Loosdrecht, M.C.M., and Heijnen, J.J. (1998). The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. Water Sci. Technol. *37*, 135–142.

Helmer, C., Kunst, S., Juretschko, S., Schmid, M.C., Schleifer, K.H., and Wagner, M. (1999). Nitrogen loss in a nitrifying biofilm system. Water Sci. Technol. *39*, 13–21.

Helmer, C., Schmid, M., Filipov, E., Gaul, T., Hippen, A., Rosenwinkel, K.-H., Seyfried, C.F., Wagner, M., and Kunst, S. (2002). Deammonification in biofilm systems: population structure and function. Water Sci. Technol. *46*, 223–231.

Hippen, A., Rosenwinkel, K.-H., Baumgarten, G., and Seyfried, C.F. (1997). Aerobic deammonification: A new experience in the treatment of waste waters. Water Sci. Technol. *35*, 111–120.

Hooper, A.B., and Terry, K.R. (1979). Hydroxylamine oxidoreductase of Nitrosomonas. Production of nitric-oxide from hydroxylamine. Biochim. Biophys. Acta *571*, 12–20.

Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M., Kruit, J., Jetten, M.S.M., and Kartal, B. (2013). Nitrogen removal by a nitritation-anammox bioreactor at low temperature. Applied and Environ. Microbiol. *79*, 2807–2812.

Hwang, I.S., Min, K.S., Choi, E., and Yun, Z. (2005). Nitrogen removal from piggery waste using the combined SHARON and ANAMMOX process. Water Sci. Technol. *52*, 487–494.

Jardin, N., and Hennerkes, J. (2011). Full scale experience with the deammonification process to treat high strength sludge water – A case study. Paper presented at: 11th IWA Specialised Conference on Design, Operation and Economics of Large Wastewater Treatment Plants (Budapest).

Jenni, S., Vlaeminck, S.E., Morgenroth, E., and Udert, K.M. (2014). Successful application of nitritation/anammox to wastewater with elevated organic carbon to ammonia ratios. Water Res. *49*, 316–326.

Jetten, M.S.M., Sliekers, O., Kuypers, M., Dalsgaard, T., van Niftrik, L., Cirpus, I., van de Pas-Schoonen, K., Lavik, G., Thamdrup, B., Le Paslier, D., *et al.* (2003). Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. Appl. Microbiol. Biot. *63*, 107–114.

Jin, R.-C., Zheng, P., Mahmood, Q., and Zhang, L. (2008). Performance of a nitrifying airlift reactor using granular sludge. Sep. Purif. Technol. *63*, 670–675.

Joss, A., Salzgeber, D., Eugster, J., König, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., and Siegrist, H. (2009). Full-scale nitrogen removal from digester liquid with partial nitritation and anammox in one SBR. Environ. Sci. Technol. *43*, 5301–5306.

Kampschreur, M.J., Kleerebezem, R., de Vet, W.W.J.M., and van Loosdrecht, M.C.M. (2011). Reduced iron induced nitric oxide and nitrous oxide emission. Water Res. *45*, 5945–5952.

Kampschreur, M.J., Poldermans, R., Kleerebezem, R., van der Star, W.R.L., Haarhuis, R., Abma, W.R., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2009). Emission of nitrous oxide and nitric oxide from a full-scale single-stage nitritation-anammox reactor. Water Sci. Technol. *60*, 3211–3217.

Kampschreur, M.J., van der Star, W.R.L., Wielders, H.A., Mulder, J.W., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2008). Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. Water Res. *42*, 812–826.

Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M., and Strous, M. (2006). Adaptation of a freshwater anammox population to high salinity wastewater. J. Biotechnol. *126*, 546–553.

Kartal, B., Kuenen, J.G., and van Loosdrecht, M.C.M. (2010a). Sewage treatment with anammox. Science *328*, 702–703.

Kartal, B., Maalcke, W.J., de Almeida, N.M., Cirpus, I., Gloerich, J., Geerts, W., den Camp, H., Harhangi, H.R., Janssen-Megens, E.M., Francoijs, K.J., *et al.* (2011). Molecular mechanism of anaerobic ammonium oxidation. Nature *479*, 127-U159.

Kartal, B., Rattray, J., van Niftrik, L.A., van de Vossenberg, J., Schmid, M.C., Webb, R.I., Schouten, S., Fuerst, J.A., Damste, J.S.S., Jetten, M.S.M., *et al.* (2007). Candidatus 'Anammoxoglobus propionicus' a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. *30*, 39–49.

Kartal, B., Tan, N.C.G., Van de Biezen, E., Kampschreur, M.J., Van Loosdrecht, M.C.M., and Jetten, M.S.M. (2010b). Effect of nitric oxide on anammox bacteria. Applied and Environ. Microbiol. *76*, 6304–6306.

Kim, B.J., and Molof, A.H. (1982). The scale-up and limitation of physical oxygen-transfer in rotating biological contactors. Water Sci. Technol. *14*, 569–579.

Knowles, G., Downing, A.L., and Barrett, M.J. (1965). Determination of kinetic constants for nitrifying bacteria in mixed culture with aid of an electronic computer. J. Gen. Microbiol. *38*, 263–278.

Kuai, L.P., and Verstraete, W. (1998). Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system. Applied and Environ. Microbiol. *64*, 4500–4506.

Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jorgensen, B.B., Kuenen, J.G., Damste, J.S.S., Strous, M., and Jetten, M.S.M. (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature *422*, 608–611.

Laanbroek, H.J., and Gerards, S. (1993). Competition for limiting amounts of oxygen between Nitrosomonas europaea and Nitrobacter winogradskyi grown in mixed continous cultures. Arch. Microbiol. *159*, 453–459.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., and van Loosdrecht, M.C.M. (2014). Full-scale partial nitritation/anammox experiences – An application survey. Water Res. *55*, 292–303.

Lackner, S., Terada, A., and Smets, B.F. (2008). Heterotrophic activity compromises autotrophic nitrogen removal in membrane-aerated biofilms: Results of a modeling study. Water Res. *42*, 1102–1112.

Lemaire, R., Webb, R.I., and Yuan, Z.G. (2008). Micro-scale observations of the structure of aerobic microbial granules used for the treatment of nutrient-rich industrial wastewater. ISME J. *2*, 528–541.

Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw, W., and Klapwijk, A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. *22*, 699–734.

Lotti, T., Kleerebezem, R., Lubello, C., and van Loosdrecht, M.C.M. (2014). Physiological and kinetic characterization of a suspended cell anammox culture. Water Res. *60*, 1–14.

Mathure, P., and Patwardhan, A. (2005). Comparison of mass transfer efficiency in horizontal rotating packed beds and rotating biological contactors. J. Chem. Technol. Biot. *80*, 413–419.

Mehrdad, M., Park, H., Ramalingam, K., Fillos, J., Beckmann, K., Deur, A., and Chandran, K. (2014). Anammox moving bed biofilm reactor pilot at the 26th Ward wastewater treatment plants in Brooklyn, New York: Start-up, biofilm population diversity and performance optimization. Water Sci. Technol. *Uncorrected Proof.*

Meulman, B., Elzinga, N., Gorter, K., Zeeman, G., Buisman, C.J.N., Vlaeminck, S.E., and Verstraete, W. (2010). Pilotscale demonstration of sustainable C and N removal from concentrated black water. In WA World Water Congress and Exhibition (Montréal, QC, Canada).

Mishima, K., and Nakamura, M. (1991). Self-immobilization of aerobic activated sludge—A pilot study of the aerobic upflow sludge blanket process in municipal sewage treatment. Water Sci. Technol. *23*, 981–990.

Molinuevo, B., Garcia, M.C., Karakashev, D., and Angelidaki, I. (2009). Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance. Bioresource Technol. *100*, 2171–2175.

Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Sybil, S., and van Cleemput, O. (1999). An overview of the revised 1996 IPCC guidelines for national greenhouse gas inventory methodology for nitrous oxide from agriculture. Environ. Sci. Policy *2*, 325–333.

Nielsen, M., Bollmann, A., Sliekers, O., Jetten, M., Schmid, M., Strous, M., Schmidt, I., Larsen, L.H., Nielsen, L.P., and Revsbech, N.P. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. FEMS Microbiol. Ecol. *51*, 247–256.

Ødegaard, H. (2006). Innovations in wastewater treatment: -the moving bed biofilm process. Water Sci. Technol. *53*, 17–33.

Paolini, A.E. (1986). Effect of biomass on oxygen-transfer in rbc systems. J. Water Pollut. Con. F. *58*, 306–311.

Pastorelli, G., Canziani, R., Pedrazzi, L., and Rozzi, A. (1999). Phosphorus and nitrogen removal in moving-bed sequencing batch biofilm reactors. Water Sci. Technol. *40*, 169–176.

Patwardhan, A.W. (2003). Rotating biological contactors: a review. Industrial and Engineering Chemistry Research *42*, 2035–2051.

Pellicer-Nacher, C., Franck, S., Gulay, A., Ruscalleda, M., Terada, A., Al-Soud, W.A., Hansen, M.A., Sorensen,

S.J., and Smets, B.F. (2014). Sequentially aerated membrane biofilm reactors for autotrophic nitrogen removal: microbial community composition and dynamics. Microb. Biotechnol. *7*, 32–43.

Pellicer-Nàcher, C., Sun, S., Lackner, S., Terada, A., Schreiber, F., Zhou, Q., and Smets, B.F. (2010). Sequential aeration of membrane-aerated biofilm reactors for high-rate autotrophic nitrogen removal: experimental demonstration. Environ. Sci. Technol. *44*, 7628–7634.

Persson, F., Sultana, R., Suarez, M., Hermansson, M., Plaza, E., and Wilén, B.-M. (2014a). Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation–anammox at low temperatures. Bioresource Technol. *154*, 267–273.

Persson, F., Sultana, R., Suarez, M., Hermansson, M., Plaza, E., and Wilen, B.M. (2014b). Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation-anammox at low temperatures. Bioresource Technol. *154*, 267–273.

Poughon, L., Dussap, C.-G., and Gros, J.-B. (2001). Energy model and metabolic flux analysis for autotrophic nitrifiers. Biotechnol. Bioeng. *72*, 416–433.

Puyol, D., Carvajal-Arroyo, J.M., Garcia, B., Sierra-Alvarez, R., and Field, J.A. (2013). Kinetic characterization of Brocadia spp.-dominated anammox cultures. Bioresource Technol. *139*, 94–100.

Puyol, D., Carvajal-Arroyo, J.M., Sierra-Alvarez, R., and Field, J.A. (2014). Nitrite (not free nitrous acid) is the main inhibitor of the anammox process at common pH conditions. Biotechnol. Lett. *36*, 547–551.

Pynaert, K., Smets, B.F., Wyffels, S., Beheydt, D., Siciliano, S.D., and Verstraete, W. (2003). Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. Applied and Environ. Microbiol. *69*, 3626–3635.

Pynaert, K., Sprengers, R., Laenen, J., and Verstraete, W. (2002). Oxygen-limited nitrification and denitrification in a lab-scale rotating biological contactor. Environ. Technol. *23*, 353–362.

Quan, Z.X., Rhee, S.K., Zuo, J.E., Yang, Y., Bae, J.W., Park, J.R., Lee, S.T., and Park, Y.H. (2008). Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. Environ. Microbiol. *10*, 3130–3139.

Rosenwinkel, K.H., and Cornelius, A. (2005). Deammonification in the moving-bed process for the treatment of wastewater with high ammonia content. Chem. Eng. Technol. *28*, 49–52.

Rusten, B., Ødegaard, H., and Lundar, A. (1992). Treatment of dairy wastewater in a novel moving bed biofilm reactor. Water Sci. Technol. *26*, 703–711.

Rysgaard, S., Glud, R.N., Risgaard-Petersen, N., and Dalsgaard, T. (2004). Denitrification and anammox activity in Arctic marine sediments. Limnol. Oceanogr. *49*, 1493–1502.

Scaglione, D., Tornotti, G., Teli, A., Ficara, E., R., C., and F., M. (2013). Advanced bioprocesses for N removal from the liquid fraction of co-digestated piggery/poultry manure and agro-wastes. In 13th World congress on anaerobic digestion (Santiago de Compostela, Spain).

Scheehle, E., Godwin, D., and Ottinger, D. (2006). Global anthropogenic non-CO2 greenhouse gas emissions: 1999–2020 (Washington, DC, U.S. Environmental Protection Agency).

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.H., and Wagner, M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. *23*, 93–106.

Schmid, M., Walsh, K., Webb, R., Rijpstra, W.I.C., van de Pas-Schoonen, K., Verbruggen, M.J., Hill, T., Moffett, B., Fuerst, J., Schouten, S., *et al.* (2003). Candidatus 'Scalindua brodae', sp nov., Candidatus 'Scalindua wagneri', sp nov., two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. *26*, 529–538.

Siegrist, H., Reithaar, S., Koch, G., and Lais, P. (1998). Nitrogen loss in a nitrifying rotating contactor treating ammonium-rich wastewater without organic carbon. Water Sci. Technol. *38*, 241–248.

Siegrist, H., Salzgeber, D., Eugster, J., and Joss, A. (2008). Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal. Water Sci. Technol. *57*, 383–388.

Sliekers, A.O., Haaijer, S.M., Stafsnes, M., Kuenen, J.G., and Jetten, M.M. (2005). Competition and coexistence of aerobic ammonium- and nitrite-oxidizing bacteria at low oxygen concentrations. Appl. Microbiol. Biot. *68*, 808–817.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., and Miller, H.L. (2007). In Climate Change 2007: The Physical Science Basis, S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, A.B. Averyt, M. Tignor, and H.L. Miller, eds. (Cambridge University Press, Cambridge, UK), pp. 21–92

van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.W., Tokutomi, T., Strous, M., Picioreanu, C., and Van Loosdrecht, M.C.M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Res. *41*, 4149–4163.

Strous, M., Heijnen, J.J., Kuenen, J.G., and Jetten, M.S.M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biot. *50*, 589–596.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., and Jetten, M.S.M. (1999a). Missing lithotroph identified as new planctomycete. Nature *400*, 446–449.

Strous, M., Kuenen, J.G., and Jetten, M.S.M. (1999b). Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microb. *65*, 3248–3250.

Szatkowska, B., Cema, G., Plaza, E., Trela, J., and Hultman, B. (2007). A one-stage system with partial nitritation and Anammox processes in the moving-bed biofilm reactor. Water Sci. Technol. *55*, 19–26.

Tallec, G., Garnier, J., Billen, G., and Gousailles, M. (2006). Nitrous oxide emissions from secondary activated sludge in nitrifying conditions of urban wastewater treatment plants: Effect of oxygenation level. Water Res. *40*, 2972–2980.

Terada, A., Lackner, S., Tsuneda, S., and Smets, B.F. (2007). Redox-stratification controlled biofilm (ReSCoBi) for completely autotrophic nitrogen removal: The effect of co- versus counter-diffusion on reactor performance. Biotechnol. Bioeng. *97*, 40–51.

Third, K.A., Sliekers, A.O., Kuenen, J.G., and Jetten, M.S.M. (2001). The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria. Syst. Appl. Microbiol. *24*, 588–596.

Turk, O., and Mavinic, D.S. (1986). Preliminary assessment of a shortcut in nitrogen removal from wastewater. Can. J. Civil Eng. *13*, 600–605.

Udert, K.M., Kind, E., Teunissen, M., Jenni, S., and Larsen, T.A. (2008). Effect of heterotrophic growth on nitritation/anammox in a single sequencing batch reactor. Water Sci. Technol. *58*, 277–284.

Vázquez-Padín, J., Mosquera-Corral, A., Campos, J.L., Méndez, R., and Revsbech, N.P. (2010). Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. Water Res. *44*, 4359–4370.

Vázquez-Padín, J.R., Figueroa, M., Fernández, I., Mosquera-Corral, A., Campos, J.L., and Méndez, R. (2009). Post-treatment of effluents from anaerobic digesters by the Anammox process. Water Sci. Technol. *60*, 1135–1143.

Verstraete, W., and Vlaeminck, S.E. (2011). ZeroWasteWater: short-cycling of wastewater resources for sustainable cities of the future. Int. J. Sust. Dev. World *18*, 253–264.

Villaverde, S., Encina, P.A.G., Lacalle, M.L., and Fdz-Polanco, F. (2000). New operational strategy for SBR technology for total nitrogen removal from industrial wastewaters highly loaded with nitrogen. Water Sci. Technol. *41*, 85–93.

Vlaeminck, S.E., Cloetens, L.F., Carballa, M., Boon, N., and Verstraete, W. (2009). Granular biomass capable of partial nitritation and anammox. Water Sci. Technol. *58*, 1113–1120.

Vlaeminck, S.E., De Clippeleir, H., and Verstraete, W. (2012). Microbial resource management of one-stage partial nitritation/anammox. Microb. Biotechnol. *5*, 433–448.

Vlaeminck, S.E., Terada, A., Smets, B.F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N., Carballa, M., *et al.* (2010). Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. Applied and Environ. Microbiol. *76*, 900–909.

Vlaeminck, S.E., Terada, A., Smets, B.F., Van der Linden, D., Boon, N., Verstraete, W., and Carballa, M. (2009). Nitrogen removal from digested black water by one-stage partial nitritation and anammox. Environ. Sci. Technol. *43*, 5035–5041.

Volcke, E.I.P., Picioreanu, C., De Baets, B., and van Loosdrecht, M.C.M. (2012). The granule size distribution in an anammox-based granular sludge reactor affects the conversion-Implications for modeling. Biotechnol. Bioeng. *109*, 1629–1636.

Wang, L., Zheng, P., Xing, Y., Li, W., Yang, J., Abbas, G., Liu, S., He, Z., Zhang, J., Zhang, H., *et al.* (2014). Effect of particle size on the performance of autotrophic nitrogen removal in the granular sludge bed reactor and microbiological mechanisms. Bioresour. Technol. *157*, 240–246.

Weissenbacher, N., Takacs, I., Murthy, S., Fuerhacker, M., and Wett, B. (2010). Gaseous nitrogen and carbon emissions from a full-scale deammonification plant. Water Environ. Res. *82*, 169–175.

Wett, B., Buchauer, K., and Fimml, C. (2007). Energy self sufficiency as a feasible concept for wastewater treatment systems. Paper presented at: IWA Leading Edge Technology Conference (Singapore).

Wiesmann, U. (1994). Biological nitrogen removal from wastewater. In Biotechnics/Wastewater, Fiechter, A., ed. (Springer, Berlin/Heidelberg, Germany), pp. 113–154.

Winkler, M.K., Kleerebezem, R., and van Loosdrecht, M.C. (2012). Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. Water Res. *46*, 136–144.

Wyffels, S., Boeckx, P., Pynaert, K., Verstraete, W., and Van Cleemput, O. (2003). Sustained nitrite accumulation in a membrane-assisted bioreactor (MBR) for the treatment of ammonium-rich wastewater. J. Chem. Technol. Biot. *78*, 412–419.

Yamamoto, T., Wakamatsu, S., Qiao, S., Hira, D., Fujii, T., and Furukawa, K. (2011). Partial nitritation and anammox of a livestock manure digester liquor and analysis of its microbial community. Bioresource Technol. *102*, 2342–2347.

Yu, R., Kampschreur, M.J., van Loosdrecht, M.C.M., and Chandran, K. (2010). Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. Environ. Sci. Technol. *44*, 1313–1319.

Zhang, L., Jiang, J., Yang, J., Hira, D., and Furukawa, K. (2012). High rate nitrogen removal by the CANON process at ambient temperature. Water Sci. Technol. *65*, 1826–1833.

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews *61*, 533–616.

Glossary

The following glossary includes the definition of key concepts used among the different chapters throughout the entire book.

Alpha-diversity: the local diversity of a community contained in a habitat patch.

Aquatic biofilms: complex microbial communities attached and growing on living and non-living surfaces found in marine or freshwater or man-made environment. In biofilms microbes are attached to each other and embedded in the matrix of self-produced extra polymeric substances.

Autotroph: organism that is capable of producing nutritive organic molecules from inorganic sources, using different energy sources (light, chemical reactions).

Benthos: community of organisms living in close relationship with their substrate, in general permanently attached.

Beta-diversity: the variability of species identities among communities across space and time. Beta-diversity can occur as directional turnover along a gradient or as non-directional variation.

Bioaccumulation: general term referring to the accumulation of chemical substances, such as metals or organic pollutants, in the biota. Bioaccumulation generally provides a good proxy of the bioavailability of compounds. The term *accumulation* is often used for biofilms when the methodology does not discern between the portion of bioaccumulated chemical from the portion remaining outside of the cells, mainly adsorbed to EPS and/or inorganic particles.

Biofilm biobarriers: are physical structures where biofilm develops that are constructed to enhance bioremediation of pollutants in aqueous environments such as groundwater and surface water.

Biodiversity/Diversity: the total variety and variability among living organisms, the communities and ecosystems they are part of. Diversity measures may consider the number of species (or some other biological units), i.e. richness, their relative abundances, and the varying dissimilarities between species.

Bioelectrochemical system (BES): biological technology capable to produce electrical power or chemical compounds by the action of a biocatalyst (exoelectrogenic microorganims) generally using wastewater as anodic fuel.

Biofouling: attachment and growth of microorganisms, algae, and invertebrates on submerged surfaces. The term is specially used for man-made surfaces, such as in water distribution systems. It is also called *microbial fouling*. Such accumulation is also referred to as *epibiosis* when the host surface is another organism and the relationship is not parasitic.

Community: a group of interacting species that overlap in time and space.

Community ecotoxicology: is the study of the effects of toxicants on ecological systems focusing on the effects at community level.

Community level physiological profile (CLPP): is a rapid community-level culture approach which is used to characterize the metabolic profile of microbial communities by measuring the utilization of a range of different carbon sources.

Confocal laser scanning microscopy (CLSM): microscopy technique which allows the visualization of images at different depths of the observed sample (such as a biofilm) to compile a three-dimension final image. Confocal laser scanning microscopy may be used to detect reflection, autofluorescence signals (such as pigments of phototrophs) or the emission signals of specific fluorochromes most commonly targeting nucleic acids, lipids, carbohydrates, or proteins.

Cooperation: beneficial interactions between organisms.

Coulombic efficiency (CE): parameter used to evaluate the electrical efficiency in Bioelectrochemical Systems (BES). CE is the ratio between electrons actually transferred and the electrons potentially transferred to the electrical circuit considering the oxidation-reduction reaction occurring at the bioelectrode.

Co-diffusion and Counter-diffusion: related to the direction of diffusion of substances in biofilms. Transport of substances occurs by diffusion within the biofilm layer and is driven by a concentration gradient. In co-diffusive systems, the gradients of the electron donor and electron acceptor are parallel. On the other

hand, in counter-diffusive systems, both gradients are opposed and thus electron donor and acceptor diffuse in opposite directions.

Dispersal: the movement of individuals from one habitat patch to another. Microorganisms are assumed to have higher dispersal rates due to their high population sizes, high transportability and short generation times, which should increase their chance to reach new habitats and establish populations therein.

Dissolved organic matter (DOM): the organic fraction of the dissolved material in water (defined as those passing a 0.5 μm filter). DOM contains a mix of organic compounds (including humic substances, polysaccharides, peptides, lipids). DOM is usually quantified in carbon units and then referred as DOC (dissolved organic carbon).

Electron transfer mechanisms: the microbial mechanisms to transport and release electrons inside the membranes (intracellular electron transfer (ICET)) and to/from an electrode material (extracellular electron transfer (EET)). Different EET mechanisms are defined, such as direct (direct contact, conductive biofilm and nanowire) or indirect (mediators) extracellular electron transfer. Both mechanisms can take place simultaneously in order to maximize the microbial benefits.

Electrotrophic microorganism: group of microorganisms capable to directly receive electrons from an electrode to grow. These microorganisms are able to reduce compounds taking electrons from the electrode surface.

Epilithic biofilm: biofilm attached on rocks or cobbles. It is also named *epilithon*.

Epiphytic biofilm: biofilm growing on living plants such as that developing on macrophytes. Significant interactions with plant (which might be both synergic and antagonistic) typically occur in this biofilm type.

Epipelic biofilm: biofilm attached to the particles of cohesive sediments (clay and silt). Typical epipelic biofilms occur in intertidal areas where biofilms are mainly colonized by diatoms. It is also named *epipelon*.

Epipsammic biofilm: Biofilm attached to the particles of sandy sediments (sand and gravel). It is also called *epipsammon*.

Epixylic biofilm: Biofilm growing on dead plant material such as wood and leaves. Epixylic biofilm is mainly formed by fungi due to their ability to degrade lignocellulose compounds which are the main constituent of plant tissues. It is also called *epixylon*.

Evenness: a measure for the variation in species abundances in a community and reaches its maximum value when all species are equally abundant.

Exoelectrogenic microorganism: group of microorganisms that have the ability to transfer electrons extracellularly to the electrode material. In practical applications, exoelectrogenic microorganisms are able to oxidize organic matter from wastewater transferring electrons to the electrode material and generating electricity.

Extracellular enzymes: enzymes bound to the cell surface of microorganisms or in the periplasmatic space in gram-negative bacteria, acting outside the cell. In biofilms, free enzymes are also found within the EPS matrix. Extracellular enzymes play a key role in the decomposition of dissolved organic matter in aquatic environments since they degrade polymeric and macromolecular organic matter into low molecular weight molecules which can cross the bacterial cell membranes.

Extracellular polymeric substances (EPS): mainly composed by polysaccharides (but also by proteins, lipids, particulate material and detritus), EPS provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells. This matrix provides a protection from predation, toxic substances and physical perturbations.

Functional diversity: diversity of physiological or ecological units in a community. It is also a diversity measure (see Biodiversity/Diversity definition) which includes a functional notion of dissimilarity between species.

Gamma-diversity: is the diversity of a region holding multiple habitat patches with contained communities.

Granular biomass: compact granules and dense aggregates with an approximately spherical external appearance that do not coagulate under decreased hydrodynamic shear conditions and settle faster than flocs, allowing for better biomass retention and high volumetric conversion in a reactor.

Heterotroph: organism that obtains carbon from organic compounds.

Macrophytes: aquatic plants that grow in or near water. They can be floating, submersed or emerged. Aquatic macrophytes provide a substrate for algae and epiphytic biofilms.

Mass effects perspective: a meta-community perspective which emphasizes that spatial dynamics, such as source-sink effects or rescue effects, affect local community structures.

Meta-community: a set of local communities which are linked by dispersal of potentially interacting species. Several perspectives within the framework of meta-community ecology emphasize different mechanisms as potential drivers of local community composition (see the mass effect, the neutral, and the species sorting perspective definitions).

Methanogenesis: biogenic formation of methane (CH_4) as a form of anaerobic respiration in which the terminal electron acceptors are carbon compounds of low molecular weight. Methanogenesis occurring in sewers is carried out by Methanogenic Archaea (MA).

Microbially induced concrete corrosion (MICC): biological process occurring in biofilms growing on the crown of sewer pipes, surface exposed to the gas phase, that leads to the corrosion of sewers, cracking of the pipes and ultimately, structural collapse. MICC is caused by the biological production of sulfuric acid (H_2SO_4) from oxidation of hydrogen sulfide (H_2S) with oxygen (O_2) in sewers atmosphere.

Microphytobentos: synonym of the term "biofilm" but usually referred to populations of photoautotrophic microorganisms such as diatoms, euglenids, crysophyceans, dinoflagelates that colonize benthic substrata in marine systems, especially in intertidal and lower supra-tidal sediments where the light arrives.

Multichannel imaging: application of up to five separate excitation–detection combinations either simultaneously or sequentially in conventional CLSM.

Neutral perspective: a meta-community perspective, in which all species are similar in their competitive abilities. Dynamics are derived from dispersal and stochastic demographic processes.

Nestedness: degree of order/organization of a community, in which the number (due to gain and loss) of species is related to site-specific factors.

Next-generation sequencing (NGS): also known as high-throughput sequencing, is the catch-all term used to describe recent technologies (including, among others, Illumina (Solexa) sequencing and Roche 454 sequencing), which allow us to sequence relatively short DNA and RNA sequences along the entire genome much more quickly and cheaply than the previously used Sanger sequencing.

Nutrient uptake length: is the physical distance a molecule of a nutrient is transported in the flowing water until it is uptaken by microorganisms. In streams, it can be measured by adding a known content of nutrient (usually performed with phosphate, ammonia or nitrate) and follow its disappearance downstream. Nutrient uptake length is an important parameter for quantifying nutrient cycling in streams.

Nutrient stoichiometry: is the molar ratio between major nutrients (i.e. C, N and P) in organisms and in their food. The first author defining stoichiometry of aquatic organisms was Redfield who defined the molar C:N:P ratio of 106:16:1 for marine plankton.

Periphyton: synonym of the term "biofilm" and specially used in studies focusing on photosynthetic organisms.

Persistent organic pollutants (POPs): are chemical compounds that are carbon based and due to their structure possess chemical and physical characteristics that make them persistent to biodegradation in the environment for many years. They are distributed as a result of natural processes involving soil/sediment, water and air. The consequence is accumulation in fatty tissues and biomagnification in the food web, where they can cause diverse harmful effects due to their toxicity.

Phylogenetic diversity: a concept of diversity, which includes the phylogenetic dissimilarity between species, additionally to their presence/absence or abundance.

Pollusensitivity: sensitivity value of species towards levels of pollution (of various natures). This value (or the sensitivity profile of a species) is usually calculated from large datasets and describes species probability of presence in relationship with water quality.

Polychlorinated biphenyls (PCBs): are organic compounds that consist of two biphenyl rings onto which 1–10 chlorines are attached. All congeners are hydrophobic with a high biomagnification potential in the food chain, exhibit toxicity to a varying degree and a significant proportion display dioxin-like toxicity.

Quorum sensing: a form of bacterial population density depended chemical cell-to-cell communication and gene regulation.

Recovery: ability of a community under perturbation (e.g. a chemical) to restore its functional and structural attributes to initial values (measured before the perturbation) after the perturbation has ended.

Resistance: ability of a community to remain unchanged under perturbation (e.g. by the presence of a contaminant).

Sensitivity: in ecotoxicological studies it refers to the degree of modification in the function and structure of a community in response to a perturbation, such as the presence of a contaminant.

Sewers: underground network of physical structure-installations composed of pipelines, pump stations, manholes and channels that convey the wastewater from its source to the point where it is treated and discharged.

Solid retention time (SRT): is defined as the ratio between total biomass contained in a bioreactor and the rate at which biomass is washed out in the effluent. If the solid retention time is too short, slow growing microorganisms are not able to maintain their populations in the reactor and will eventually be completely washed out of the reactor.

Species sorting perspective: a meta-community perspective, which emphasizes spatial niche separation and local interactions between species and their abiotic environment.

Stable isotope probing (SIP): is a technique that is used to identify the microorganisms in environmental samples that use a particular growth substrate (i. e. organic pesticides). SIP is based on the incorporation of ^{13}C-labelled substrate into cellular biomarkers such as nucleic acids (DNA and rRNA), the separation of labelled from unlabeled nucleic acids by density gradient centrifugation, and molecular identification of active populations carrying labelled nucleic acid.

Stromatolites: ancient microbial mats which were abundant and diverse in the shallow zone of the oceans in the Proterozoic. Usually they are formed by precipitation and microbial carbonate sedimentation, resulting in a layered structure where typically cyanobacteria are present. Stromatolites played a crucial role for the early establishment of life since they consumed CO_2 and produced free O_2 and H_2.

Sulfate-reducing bacteria (SRB): group of bacteria predominant in anaerobic sewers that uses sulfate respiration to obtain energy while oxidizing organic compounds and reducing sulfate (SO_4^{-2}) to hydrogen sulfide (H_2S).

Suspended aggregates: highly fragile structures suspended in fresh and seawater made of microorganisms, organic and inorganic particles. Suspended aggregates typically occur during bloom periods after an increased input of nutrients. They are also called *lake snow*, *river snow* or *marine snow*.

Synergy: cooperation, not obligatory between microorganisms, in different processes that result in a greater benefit or production than if microorganisms were individual. In communities, cooperation helps in degradation because some strains produce the metabolites that are used for other species to complete degradation. In BESs, syntrophic interactions have been described on exoelectrogenic and non-exoelectrogenic bacteria in anode biofilms.

Syntrophic interactions: the use of one organism's metabolic intermediates and by-products by another organism as a metabolic substrate. It serves to increase internal nutrient cycling, reduce energy expenditures on resource acquisition, and can create favourable environmental conditions for novel niche development.

Taxonomic diversity: diversity based on the presence/absence or the abundance of taxonomic units, such as species, and is insensitive towards phylogenetic or functional differences among taxa.

Tolerance: the capacity of a community to support an alteration of its environment (e.g. exposure to toxicants), with no significant modifications in its structure and function.

Toxicity endpoint: is the measurement of a biological effect caused by exposure to a toxicant, usually measured as the 50% lost of the specific endpoint (i.e. metabolic activity such as organism growth, survival or reproduction, or biomass).

Xenobiotics: group of chemicals including for instance pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and antibiotics which is foreign to biological systems, but is commonly detected in the environment.

Index

Aquatic Biofilms

Ecology, Water Quality and Wastewater Treatment

Biofilms in aquatic ecosystems colonize various surfaces (sand, rocks, leaves) and play a key role in the environment. Aquatic biofilms supply energy and organic matter to the food chain, they are important in recycling organic matter and contribute to water quality.

This book is a concise review of the current knowledge on aquatic biofilms with an emphasis on the characteristics and ecology of biofilms in natural ecosystems and a focus on biofilm applications linked to water pollution problems. The volume is divided into three sections: Biofilm Mode of Life; Biofilms and Pollution; and New Technologies Using Biofilms. In the first section the aquatic biofilm mode of life is described and reviewed. Key aspects covered include the three-dimensional structure and cell-to-cell communication of biofilms, their dynamic prokaryotic diversity and their vital role in biogeochemical cycles. In the second part of the book the use of biofilms in water quality is comprehensively covered. Chapters discuss biofilms in water quality, environmental risk assessment, monitoring and ecotoxicological approaches. Further topics include biofilm development in sewage pipes and the potential for microbial transformations in these systems. The final section focuses on important examples of novel technologies based on biofilms for water treatment, including the biodegradation of pollutants, the application of bioelectrogenic biofilms and the biofilm capacity for nitrogen removal.

With contributions from ecologists, engineers and microbiologists this book presents scientists and technicians with up-to-date knowledge and a clear understanding of aquatic biofilms from different and complementary points of view. An essential reference book for anyone working with biofilms.

ISBN 978-1-910190-17-3

9 781910 190173

www.caister.com