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

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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

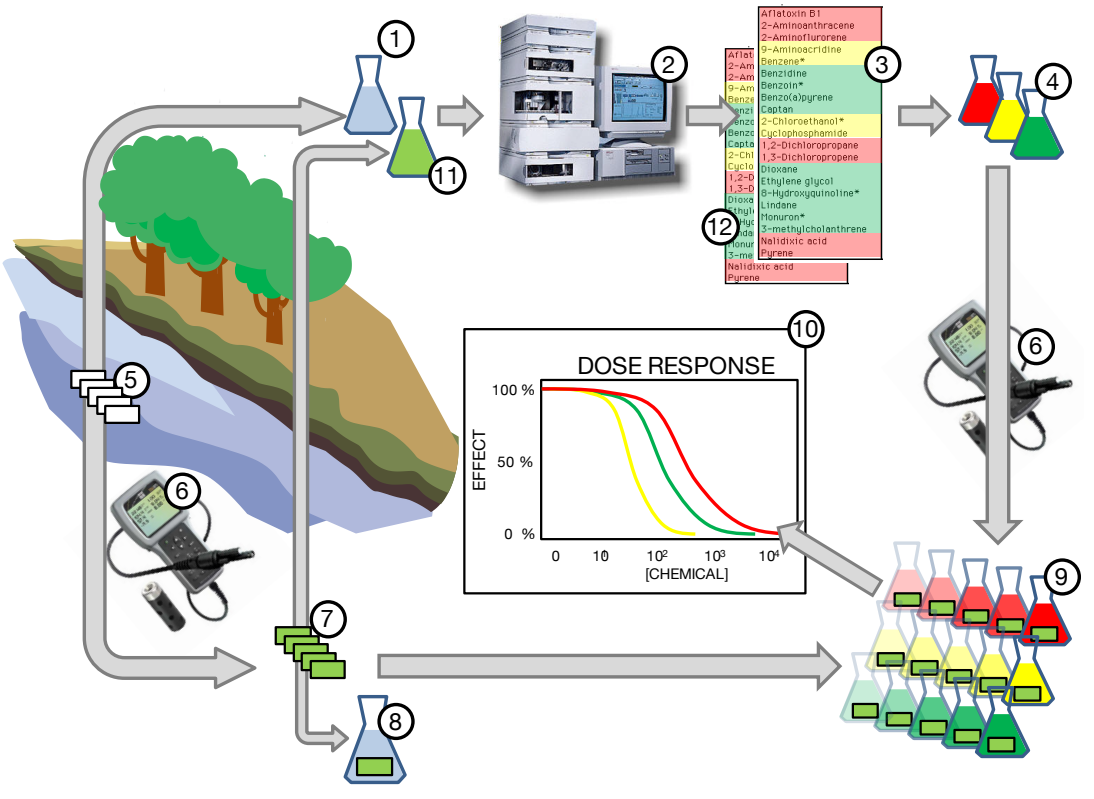


Figure 7.1 Conceptual framework for the use of freshwater biofilms in aquatic ecotoxicology. Water samples (1) from a river affected by chemical pollution are analysed (2). The chemicals present in the water (3) are classified according to their mode of action (different shaded colours), and a reduced 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,

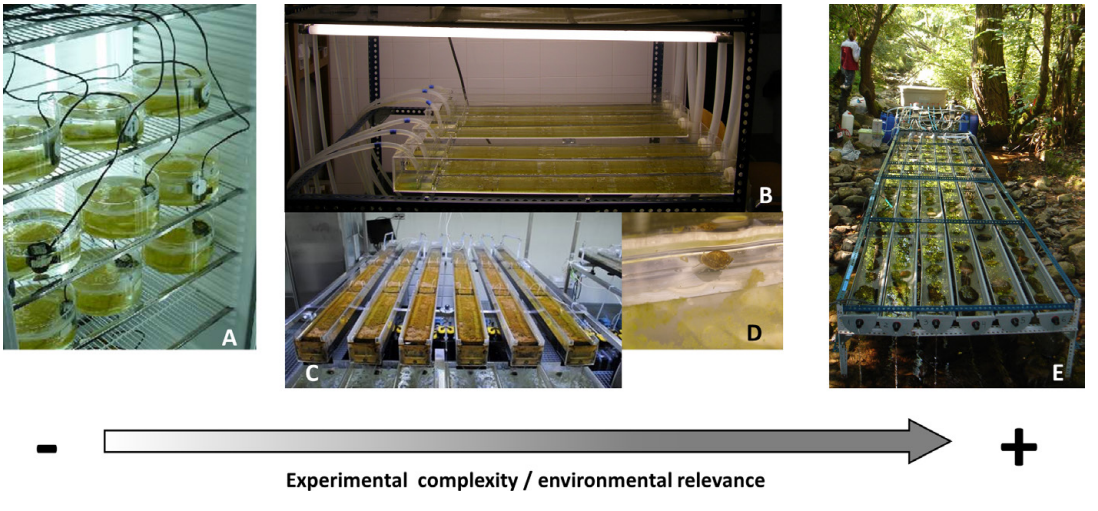


Figure 7.2 Experimental settings of biofilm ecotoxicology. Biofilm communities growing on artificial substrata (e.g. sandblasted glass substrata) are exposed to chemicals under controlled conditions. Exposure can be done in (A) crystallizing dishes (1.5 l volume) with recirculating water; (B) recirculating 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

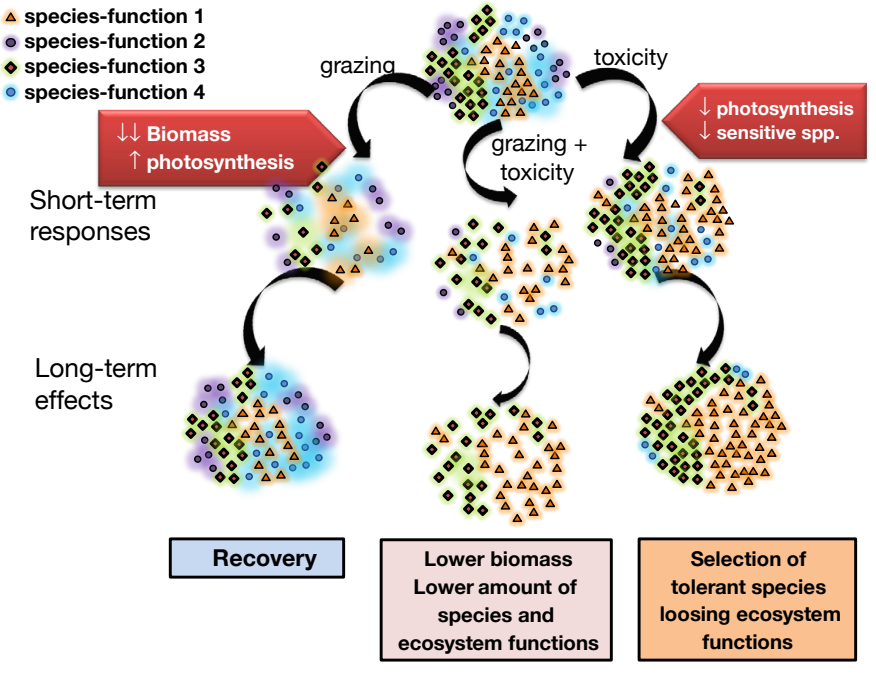


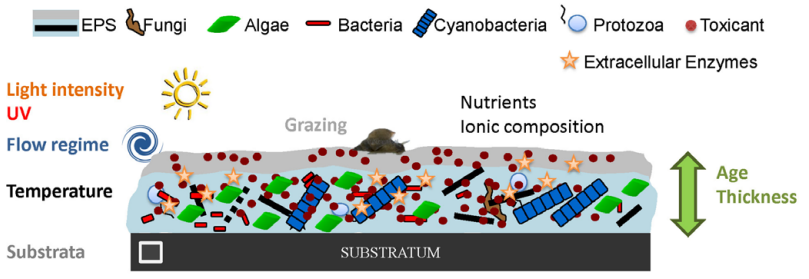
Figure 7.3 General model of the individual and combined effects of grazing and toxicity on biofilms. Based on a simple four-species biofilm model, it is expected that toxicity will constrain the ability of the community to recover from grazing pressure. In addition to the selection pressure exerted by 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.

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