Chapter 2
From Microbial Ecology to Microbial Ecotoxicology

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Abstract Because of their ubiquity, abundance and metabolic activities, microorganisms play a crucial role in the biogeochemical cycling of elements in the environment. Any perturbations in the activity and diversity of the microbial community are likely to lead to significant impacts in terms not only of biogeochemical cycling but also ecosystem resilience. Human activities and industrialization have resulted in the release of millions of tonnes of chemicals and pollutants into the environments; some of these are toxic to living organisms. However there is a lack of information about the toxicity effects at the ecosystem level; where toxicity tests have been included in studies the basis has been the use of target species including plants (e.g. radish germination), worms (e.g. earthworm survival) and microbes (e.g. the Microtox bioassay test) to evaluate the effect of the pollutant on the target organisms. Microbial ecotoxicology represents an emerging discipline that encompasses microbial ecology, microbial toxicology, chemistry and physics and that offers great potential in the assessment of the fate and impact of environmental pollutants at the ecosystem level. In this introduction we discuss the importance of microbial ecology together with some of the advantages of the application of the recently established microbial ecotoxicology discipline in order to reliably assess the impact of contamination on the resilience and the functionality of the microbial community.

Keywords Biosensors · Bioreporters · Bioassays · Microorganisms · Ecotoxicology · Next generation sequencing · Environmental pollution

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2.1 Introduction

Microorganisms are the most abundant organisms on the planet, with recent estimates suggesting that the total numbers of microbes on the planet is between 9.2×10^{29} and 31.7×10^{29} (Kallmeyer et al. 2012). Bacteria represent the most diverse group of organisms; estimates suggest the number of bacterial species in the world range from 10^7 to 10^9 (Dykhuizen 1998; Curtis et al. 2002). Given these facts it is not surprising that microbes collectively exhibit the greatest metabolic diversity. Because of their ubiquity, abundance and metabolic activities, microorganisms play a crucial role in the biogeochemical cycling of elements in the environment. Understanding the role of microorganisms in the biogeochemical cycling is complex, requiring research into not only the structure and the function of microbial communities but also their synecological interactions. Historically this has taken place through a range of culture-dependent methods, which led to the recognition of the extraordinary diversity of microbial life. However, with the application of molecular microbial techniques, estimates of microbial diversity have increased dramatically. In particular, the advent of low cost, next generation sequencing technologies has led to an exponential increase in sequence-based microbial community studies investigating taxon diversity and community structure (e.g. via rRNA gene analysis) and/or microbial function via metagenomics of uncultivated microorganisms present within the environment. Such approaches have revealed a diverse wealth of hitherto unknown microbial taxa and provided new understanding of the ecological and biological functions and adaptations of environmental microbes. What is required now is to link this understanding of microbial diversity and complexity to ecosystem function. In natural environments, microorganisms interact with both biotic and abiotic components of their ecosystems; these interactions are essential for ecosystem function with key specific functions including biogeochemical cycling, biodegradation of pollutants and the impacts of microbes upon the activity and health of plants and animals, including humans. Defining the specific role of individual microorganisms in the environment is complex, due in part to the metabolic flexibility and diversity within individual species, and additionally by functional redundancy whereby diverse species can carry out the same biological activity.

In summary, microbial communities play a central role in ecosystem functioning. Any perturbations in the activity and diversity of the microbial community are likely to lead to significant impacts in terms of not only biogeochemical cycling but also ecosystem resilience. It is therefore not surprising that the ecological impacts of pollutants at the microbial scale and on the various functions that they carry out in the ecosystem together with the role of microbial communities in the ecodynamics of the pollutant-impacted ecosystem have formed the basis of microbial ecotoxicology (Ghiglione et al. 2016), a discipline that has emerged from microbial ecology. Here we discuss the importance of microbial ecology together with some of the advantages of the application of the recently established microbial ecotoxicology discipline in terms of assessing changes in the diversity and functionality of

the microbial community in pollutant-impacted environments. Specifically, we examine environmental pollution and the role of microorganisms in the removal of these pollutants. Following this we briefly review the techniques available for microbial ecology before looking at the development of microbial ecotoxicology and the use of biosensors before examining future prospects for this emerging area of research.

2.2 Tools Used in Microbial Ecology

A variety of methods have been developed to study microbial ecology and in particular to investigate microbial diversity and functions. Traditional methods involving the culturing of indigenous microflora to study microbial processes (e.g. plate counting) have been widely employed; however it is now well known that less than 1% of all environmental microorganisms can be cultured (Amann et al. 1995). Recent developments in molecular microbial ecology have led to new insights into microbial ecology and this has led to a far better understanding of the ecology of microbial communities in different matrices. Characterization of the activity and diversity of microorganisms in the environment has been an active area of research due to the crucial role of microbial taxonomy and functional diversity in the ecosystem. Many methods and approaches have been developed in order to allow microbiologists to better assess microbial diversity in natural ecosystems. Among these approaches are a number of classic culture-based techniques, including:

- Dilution plating and culturing methods developed using a variety of culturing techniques and culture media designed to increase the growth of certain microbial species (Boehm et al. 1997; Hill et al. 2000).
- Community-level physiological profiling, which can be performed by the BIOLOG® system. This has been used widely to analyse microbial communities based on the ability of microbes to utilize different carbon sources (Lehman et al. 1995; Hill et al. 2000).

Alternative, culture-independent techniques based either on the biochemical or nucleic acid composition of microorganisms or fluorescent microscopic approaches have also been developed and successfully applied in microbial ecology. These techniques include:-

- Phospholipid fatty acid (PLFA) analysis. Phospholipid fatty acids are essential components of the cell membrane of microorganisms which break down rapidly after cell death, serving as an indicator to distinguish between living and dead organisms. In addition, different PLFA profiles vary according to the composition of the microbial community, making this technique useful in distinguishing between microbial communities (Hill et al. 2000).
- Nucleic acid approaches which are the most widely used and arguably the most useful tools for studying microbial ecology as nucleic acids are present in all

forms of life (Woese et al. 1990) and contain unique and highly conserved regions. For example, fluorescent in situ hybridization (FISH), has been used to study microbial communities due to the ability of this technique to determine and quantify specific microbial groups (Watanabe 2001).

Nucleic acids approaches (mostly DNA, but also RNA) have often been used to underpin microbial community analysis. Some of the methods developed, such as FISH, microarray and whole metagenome sequence analysis (metagenomics) evaluate nucleic acids directly, while others, such as denaturing gradient gel electrophoresis (DGGE) terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA) or automated RISA (ARISA) and sequence analysis of 16 rDNA gene libraries require PCR to increase copies of a target gene for easier detection (Nakatsu 2007) (Fig. 2.1). Quantitative PCR (qPCR) is another useful, cost effective, sensitive and high throughput tool to study the abundance and expression of taxonomic or functional genes. Unlike other approaches, in this method the target genes can be fully quantified (Shahsavari et al. 2016).

Another technique used to establish the function of microorganisms in ecosystems is stable isotope probing (SIP); for example this technique has been used to identify the microorganisms responsible for the aerobic degradation of phenol (Manefield et al. 2002), the consumption of methanol by microorganisms (Radajewski et al. 2000) and the anaerobic degradation of benzene and toluene

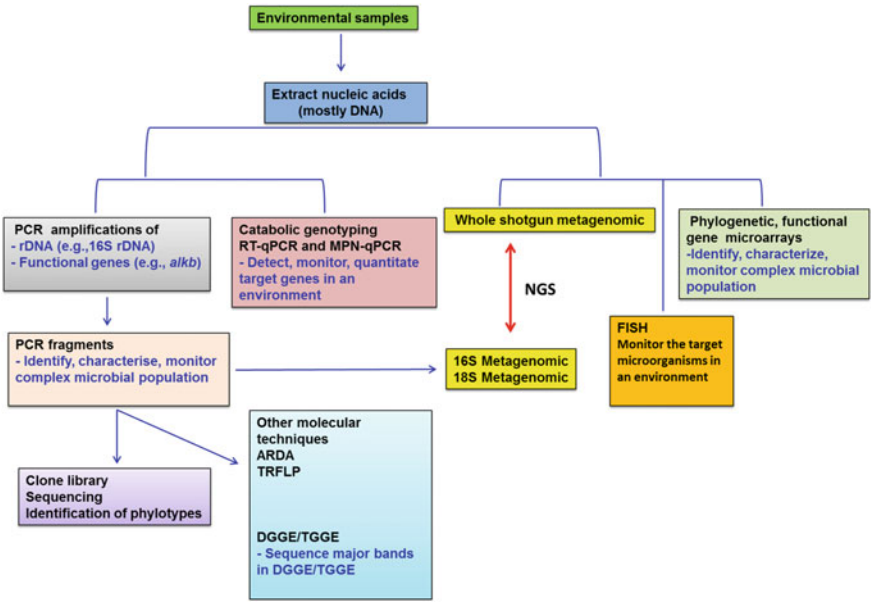

Fig. 2.1 Assessment of microbial communities using most common microbial molecular approaches [modified and updated from Whyte and Greer (2005)]

Table 2.1 The main molecular tools used to assess microbial communities in polluted environments (Kasai 2011; Hirsch et al. 2010; Malik et al. 2008)

Method	Advantages	Disadvantages
RISA/ARISA	Rapid and simple rRNA fingerprinting Highly reproducible	Limited database for ribosomal intergenic spacer sequences
ARDRA	Simple Highly reproducible	Limited resolution Sequence information unavailable
Clone library and sequencing	Possible detection of species	Time consuming Expensive
DGGE/TGGE	Inexpensive Possible to sequencing the band to determine the related species High resolution (1 bp)	Overestimates diversity Several bands may come from one species Assessment of only 1–2% of the microbial population
TRFLP	Simple High reproducibility	Requires expensive equipment. Requires multiple restriction enzymes Sequence information unavailable Several peaks form from one species
FISH	DNA isolation and PCR bias independent	Limited number of probes available (about 3) Background fluorescence interferes with detection of organisms Probe permeability
Microarray technologies	High throughput PCR bias independent	Expensive Non-specific hybridisation Applicable to known sequences only
Metagenomic analysis	Assessment of whole microbial community Much more information provided	Expensive Generates huge amount of data Requires high-performance computing and automated software

(Aburto and Ball 2009; Cupples 2011; Herrmann et al. 2010; Kunapuli et al. 2007; Oka et al. 2008) among several other contaminants.

However, whether traditional, molecular approaches or NGS are to be employed, consideration must be given to the fact that each method has advantages and disadvantages (Table 2.1). Overall, due to the limitations associated with both traditional (culture-based methods) and molecular methods, a combination of both approaches is most often desirable in microbial ecological studies (Zhang et al. 2012).

Among the different molecular methods used, some have become widely used in microbial ecology; for example DGGE has been used over the past 20 years as a community fingerprinting technique, while over the past 5 years metagenomics has become the dominant analytical protocol. Table 2.1 provides details of these

various approaches. According to Whyte and Greer (2005), more than 1000 papers have been published by authors that have used DGGE for the analysis of various environmental microbial communities over a 10 year period. The author's estimation using Google Scholar and the search terms "environmental pollution" and "DGGE" resulted in 16,100 records by April 2016. DGGE has been used extensively since its introduction (Muyzer et al. 1993) to assess the effect of different chemical compounds such as petroleum hydrocarbons (Aburto-Medina et al. 2012; Adetutu et al. 2013; Shahsavari et al. 2013; Simons et al. 2013), tetrachloroethene (Patil et al. 2013) and metals on natural microbial communities (Reith et al. 2016).

DGGE and other gradient gel electrophoresis methods separate PCR-amplified DNA fragments from environmental samples (e.g. soil) based on differences in the GC content of the amplified gene (e.g. 16S rDNA). Sequences with different GC content show differential mobility through a DNA-denaturing gel (Whyte and Greer 2005). Denaturing conditions can be made with the use of urea and formamide in DGGE or by temperature in the case of TGGE. The main advantages of DGGE/TGGE are the ability to excise and directly sequence bands of interest which can then be compared with available sequences in online databases (GenBank or EMBL) to identify the putative microorganism. Also, DGGE is inexpensive and many samples can be run at the same time (for details about full DGGE's protocol see Green et al. 2010; Whyte and Greer 2005). Like other molecular microbial tools, DGGE has some disadvantages (Table 2.1) and the limitations of using this method should be considered when employing this technology for the evaluation of microbial communities.

The latest technique used by microbial ecologists is metagenomics. This is a preferred technique as it provides vast information that cannot be obtained with any other technique. The importance and ubiquity of this technique is such that it has been applied in many disciplines such as medicine, agriculture, energy production, bioforensics and bioremediation.

Metagenomics is defined as the analysis of DNA from microbial communities in environmental samples without the requirement for microbial culturing (Oulas et al. 2015). Historically, it was categorized as PCR independent analysis; however metagenomic analysis can be carried out following PCR amplification of certain genes of interest (e.g. 16S rDNA or 18S rDNA). Recently whole shotgun metagenomics has been applied in different environments including those impacted by pollution. Perhaps the greatest advantage of metagenomics is that it also provides information on the functional gene composition of microbial communities rather than merely phylogenetic surveys. It therefore provides the opportunity to address two key microbial ecological questions: which microorganisms are present and what are their roles? Thus, the information relating to functional genes may potentially reveal novel enzymes and biocatalysts (Thomas et al. 2012).

Recent advances in high-throughput sequencing methods, also called next generation sequencing (as opposed to Sanger sequencing) have revolutionized metagenomic studies. The first NGS platform was developed by Roche (formerly Life Sciences), 454 pyrosequencing in 2005. Later, many sequencing platforms such as Illumina, Ion Torrent, PacBio and SOLiD were developed by different

vendors (van Dijk et al. 2014); however, the Illumina platforms (e.g. HiSeq or MiSeq) have become the most popular among researchers.

NGS technologies have been widely used recently to evaluate the effect of pollutants on microbial communities and their interactions, making them essential tools in microbial ecology (Adetutu et al. 2015; Costa et al. 2015; Shahi et al. 2016; Yergeau et al. 2012; Fang et al. 2013, 2014; Kao et al. 2016; Gołębiewski et al. 2014).

In summary, it can be seen that microbial ecology represents a key discipline in the field of contaminated environments. It is also apparent that the development of culture independent approaches has led to a much greater comprehension of microbial activity and diversity in the environment and the impact that pollutants have on both the activity and diversity of the microflora.

2.3 Environmental Pollution

Environmental pollution is a term which refers to the release, discharge or disposal of various pollutants and contaminants (solid, liquid or gases; physical, chemical and biological) as a consequence of pollution diffusion and industrial processes without appropriate treatment to meet the standard regulations required prior to discharge into the environment (water, air and soil), causing a threat to humans, animals, plants and to the entire environment (Wu et al. 2010). The current scale of environmental pollution is unprecedented; a recent Cornell University research survey concluded that about 40% of human deaths worldwide were caused by different types of pollution in the different environmental compartments (water, air and soil) (Pimentel et al. 2010). The situation is worse in developing countries. Unfortunately, approximately 90–95% of untreated urban sewage, toxic and carcinogenic chemicals generated are discharged directly into running waters in developing countries without appropriate treatment. Pollution is also a serious threat to water quality in developed countries, including the US, U.K. and Australia. In the UK it has been estimated that 110 tonnes of benzene and over one million tonnes of petroleum hydrocarbons are spilled into terrestrial ecosystems every year, mainly through vehicle emissions and from the chemical industry (Fahy et al. 2006). In Australia it is estimated that around 27 million litres of crude oil waste are illegally dumped annually without appropriate treatment (Aleer et al. 2011). Polluted water, air and soil all impact the biosphere, including humans due to the dependency on safe environmental conditions of the food-chain. Physical, chemical and biological contaminants that are directly or indirectly discharged into the environment will have an impact on living organisms.

The removal of these contaminants from the environment represents an essential step in restoring ecosystem functions and services that we depend on to maintain both ecosystem- and human-health. Biological systems and microorganisms in particular are playing an increasing role in the clean-up or remediation of contaminated sites (Arias et al. 2005). Bioremediation is already widely practiced as a

safe, environmentally friendly approach for the clean-up of various pollutants such as petroleum hydrocarbons (Lors et al. 2012) heavy metals (Mejáre and Bülow 2001), pesticides (Scott et al. 2008), and waste products from the dye (Senan and Abraham 2004), and pulp and paper industry (Paszczynski and Crawford 1995).

Microorganisms in particular are useful in the degradation of environmental contaminants in two general ways:

- Many microorganisms, including those indigenous to the particular environment can degrade the pollutants or transform them into less toxic compounds, reducing the concentration of pollutants and importantly reducing their toxic effects in the environment.
- Microorganisms themselves can serve as important indicators of environmental pollution since any environmental impact is translated in community changes that can be recorded. Moreover, since microorganisms are ubiquitous in the environment, a community response to environmental pollutants can be observed.

2.4 Ecotoxicological Evaluation

Traditional chemical analyses are the usual way to evaluate the degradation of pollutants and monitor the contamination levels as well as to assess the impacts on various organisms. However, these techniques (gas chromatography-mass spectrometry, GCMS for hydrocarbons and inductively coupled plasma mass spectrometry, ICPMS for metals) are not effective in assessing the efficacy of the clean-up method in terms of toxicity reduction and bioavailability, or to evaluate the ecotoxicological outcomes of the process on the ecosystem at the treated sites (Molina-Barahona et al. 2005). A reduction in the concentration of contaminants does not necessarily mean a reduction in their toxicity due to many possible reasons as has been shown previously (Khudur et al. 2015). Therefore, integration between chemical analytical data, ecotoxicological assessments and the evaluation of the microbial community is required, though seldom carried out, to evaluate the efficiency of the approach used to treat the contaminated area and the resultant environmental outcomes in terms of its effects on ecosystems and humans.

The key indicators of the risk posed by chemicals to human health and other organisms in the environment is related to the bioavailability of the contaminant, which can be defined as the difference between the amount of the contaminant to which an organism is exposed and the actual dose of the substance the organism receives (Naidu 2011). Many factors determine the environmental bioavailability of a contaminant. These factors might include (a) the solubility of the contaminant in the soil solution (Lanno et al. 2004), (b) the chemical mass transfer rates, specifically the molecular weight of the desorbed fraction (Cornelissen et al. 1998), and (c) the type of organism exposed to the chemicals (Reid et al. 2000).

To examine the impact of chemicals on the biota, a variety of toxicity test methods and procedures have been proposed (Dutka and Kwan 1981). Inhibition of natural bacterial bioluminescence, which is known as the Microtox test, has been developed as a cost-effective, easy pre-screening test, which is based on measuring the inhibition in light emitted by a marine bacterial species, *Aliivibrio fischeri* (also known as *Vibrio fischeri* and formerly known as *Photobacterium phosphoreum*) (Kamlet et al. 1986). The bioluminescence results from a complex set of energy-producing reactions controlled by the expression of six genes with light output induced at high cell density. Therefore, inhibition of the enzymes by the pollutants alters the rate of gene expression and subsequently the amount of bioluminescence emitted.

The Microtox test is used to determine the toxicity of a contaminant and is commonly applied as a quick bioassay test, in an attempt to alert monitoring agencies of potential toxic conditions, as well as the rapid assessment of the changes in environmental quality. The endpoint of this test is the determination of the concentration of a contaminant which causes a reduction in the bioluminescence by 50% after a certain time, usually 5, 10 and 15 min. This is referred to as Effective Concentration 50 (EC_{50}) (Kamlet et al. 1986). On the other hand, a chronic toxicity test aims to evaluate the toxicity of a substance by detecting changes in physiological functions and usually performed on macroscopic organisms.

2.5 Microbial Ecotoxicology as a New Discipline

Microbial ecotoxicology is a new discipline that encompasses microbial ecology, microbial toxicology, and chemistry and physics. This new discipline may be distinguished from microbial toxicology since the latter has usually looked at the toxic effects of compounds on a certain microorganism and infer that toxicity to the whole community while microbial ecotoxicology aims to compile all the different approaches such as analytical methods, enzymatic measurements, toxicity measurements and culture-independent methods among others in order to have a more accurate assessment of the toxic compounds in the whole community. This is a significant development from simple microbial toxicology work, requiring the study of ecotoxicological approaches at the community level. However the outcome of microbial ecotoxicology is of ecological relevance. Consequently this discipline has recently been proposed and is currently being consolidated (Ghiglione et al. 2016, 2014; Gu and Wang 2014).

For some time microbial ecologists have been conducting and reporting successful studies on the bioremediation of toxic compounds (Aburto et al. 2009; Aburto-Medina et al. 2012; Shahsavari et al. 2013, 2015a, b). Most of these studies have relied on the use of analytical methods such as gas chromatography-mass spectrometry to confirm the degradation of the contaminants; however there is also a need to assess the toxicity of the remaining compounds following remediation with several toxicity methods such as the Microtox bioassay and the

Toxi-chromoPad test (Ahtiainen et al. 2002). These tests have been used for over twenty years (van Beelen and Doelman 1997) and can be classified as single species tests, carbon and nitrogen transformations, enzymatic tests (Margesin et al. 2000), biomass measurements (Margesin et al. 2000) and tests assessing changes in microbial diversity (Khudur et al. 2015).

Furthermore, the tests have been used to assess the toxicity of different contaminants such as herbicides (Bonnet et al. 2007, 2008), hydrocarbons (Płaza et al. 2009), dyes (Ogawa et al. 1988), wastewater (Sazykin et al. 2016) and heavy metals (Preston et al. 2000) among others. Some of the advantages of microbial toxicology tests are:

- They are generally simple to carry out.
- Some assays are almost as accurate as the chemical methods.
- Importantly they provide information on the biological effects of contaminants.
- This approach allows the establishment of toxicologically safe endpoints.

In parallel, other tests that have been carried out may provide a better representation of the status of the whole community and they include the measurement of growth, basal respiration and enzymatic potential in order to evaluate the effect of contaminants on the microorganisms in the ecosystem. Specifically, previous studies have reported the use of microbial biomass (Ingham et al. 1986; Muñoz-Leoz et al. 2011), photosynthesis (Sabater et al. 2007) basal respiration (Gong et al. 2001; Kumpiene et al. 2009; Muñoz-Leoz et al. 2011), enzyme activities (Gong et al. 2001; Kumpiene et al. 2009; Liu et al. 2009; Mora et al. 2005; Muñoz-Leoz et al. 2011; Renella et al. 2008; Tscherko and Kandeler 1997) and nitrification (Muñoz-Leoz et al. 2011; Sverdrup et al. 2002) as indicators of the effects of contaminants such as hydrocarbons, metals, pesticides and explosives on the microbial community. Some of the enzymes that have been used as indicators of the microbial community status include dehydrogenases, ureases, arylsulfatases, phosphatases and β-glucosidases.

The measurement of enzyme activities is a good parameter to determine the toxicity of the matrix. Arylsulfatase, β-glucosidase and dehydrogenase were used to evaluate the toxicity of a heavy metal contaminated soil treated with organic and inorganic amendments (Mora et al. 2005). Similarly, Kumpiene and colleagues used enzyme activities as indicators in the phytostabilisaton of a Pb and Cu contaminated soil. Phosphatase, glycosidase, sulfatase and urease were measured and their increased activity indicated sustainable management of the treated soils since these enzymes are involved in organic matter decomposition and the biogeochemical cycle of macronutrients (Kumpiene et al. 2009). Another study also confirmed that the ratio of arylsulphatase to microbial biomass is a sensitive index to evaluate contamination (Tscherko and Kandeler 1997).

Soil basal respiration and substrate-induced respiration indicate the actual respiratory microbial activity and the maximum potential respiratory microbial activity respectively. These are also good indicators of soil microbial activity and have been measured to assess the effect of the fungicide tebuconazole (Muñoz-Leoz et al. 2011) and the explosive compound RDX (Gong et al. 2001) on the microbial community.

The Microtox, Microtox solid Phase test assay, the P450 reporter gene system and the Toxi-chromo Pad have also been very useful in evaluating the toxicity of hydrocarbon contaminated sediments (Mueller et al. 2003) and creosote contaminated soil (Ahtiainen et al. 2002). Although the Microtox result is based on a single microorganism, it has been extrapolated to the whole community, in order to overcome this drawback, biofilms have also recently been used as indicators of the effects of chemicals on the microbial community (Sabater et al. 2007) or to assess the quality of riverine systems (Burns and Ryder 2001).

Thus, the evaluation of the Microtox, the biogeochemical cycle parameters and biofilms is a good indication of the toxic effects of contaminants on the microbial community and confirms there is a strong link between microbial ecotoxicological studies and the microbial ecology of the system.

In summary, assessment of the activity of key microbial enzymes or processes within a biogeochemical cycle provides a good indication of the toxic effects of contaminants on the microbial community. For example nitrification has been used for microbial toxicological assessment of the impact of a contaminant. Smolders et al. (2001) used potential nitrification rate tests (PNR) to evaluate metal toxicity in metal salt–spiked soils, uncontaminated soils and field soil contaminated with metals from previous smelting activities. The authors concluded that nitrification was sensitive to metal, although they stated that overall it was not a useful soil assay. In another study Broos et al. (2007) measured substrate-induced nitrification (SIN) and substrate-induced respiration (SIR) in the top soils of 12 Australian soils amended with $ZnSO_4$ or $CuSO_4$. The median effect concentration (EC_{50}) values for Zn and Cu based on total metal concentrations varied between 107 and 8298 mg kg^{-1} for Zn and 108 and 2155 mg kg^{-1} respectively among soils. The results of this study showed significant relationships between the EC50 values for SIR and background Zn concentrations and the cation exchange capacity (CEC0 for Zn, and the presence of clay and log CEC for Cu.

It is also important to point out that molecular tools can detect changes in the microbial community that can be missed by the measurement of only community level end points (Widenfalk et al. 2008). Therefore, an ideal ecotoxicology study should include the assessment of the biogeochemical parameters and the use of molecular tools, especially with the advent of the next generation sequencing, which provides the largest amount of data (up to one billion short reads per run) with a relatively low cost (Metzker 2010; Schuster 2007).

Biological tools such as biosensors and biomarkers are extremely helpful to provide signals for potential damage in the environment and have been widely used in microbial toxicology (Hansen 2008; Hansen and Usedom 1997). Biosensors act as sensing systems and biomarkers, providing biochemical responses that indicate the initial level of damage and provide information in order to take precautionary action. Thus, the recognition of an early sign of environmental damage will prevent eventual larger damage to the environment.

Genetic engineering has provided the tools to introduce genes encoding enzymes and luminescent proteins into bacterial species that act as reporter genes (van der Meer and Belkin 2010). Some of these genes include the *lux* genes of

bioluminescent bacteria such as *Aliivibrio fischeri*, *Vibrio harveyi* and *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*). Other reporter genes are the lucFF from the firefly luciferase and the *gfp* genes encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* among others. An excellent review on the use of reporter and sensor proteins used in ecotoxicology studies has been prepared by Van der Meer and Belkin (van der Meer and Belkin 2010).

These recombinant microorganisms are usually termed bioreporters and they allow the quantification of bioavailability which is a direct indicator of the contaminants effect on living organisms (Farré and Barceló 2009). *Escherichia coli* has been extensively used as a bioreporter (Robbens et al. 2010), used to detect bioavailable iron (Bachmann 2003) ionic mercury (Xu et al. 2015), arsenic (Yoon et al. 2015) and zinc (Maderova et al. 2011) among other contaminants. Other engineered microorganisms include *Pseudomonas fluorescens* and *Pseudomonas putida* which have been used for the detection of halogenated compounds and heavy metals (Preston et al. 2000; Sütterlin et al. 2008; Weitz et al. 2001). A recent review also indicates the use of cyanobacteria as bioreporters (Mateo et al. 2015). Microalgae such as *Chlorella vulgaris, Chlorella fusca*, (formerly *Selenastrum capricornotum*) and *Dunaliella salina* are sensitive to pesticides and have also been used as toxicity indicators. Several reviews have compiled the advances of biosensors at different time points (D'Souza 2001; Rodriguez-Mozaz et al. 2003; Su et al. 2011; van der Meer and Belkin 2010). Furthermore biosensors have been recently used to assess the eco and genotoxicity of airborne emissions (Kováts and Horváth 2016) and to compare the ecotoxicological contamination of wastewaters in Russia and Germany (Sazykin et al. 2016). Some of the most common microorganisms used as biosensors to different contaminants are listed in Table 2.2.

Thus, another challenge of microbial ecotoxicology as an emerging discipline will be to merge and perform in parallel all the methods mentioned above (targeting single species and the whole community). This will provide a better assessment of the site toxicity and in turn provide crucial information about the success of any remediation intervention.

2.6 Future Aspects

So far, microbial ecotoxicology approaches include the measurement of biogeochemical parameters (photosynthesis, respiration, denitrification, decomposition, and enzymatic potential), the Microtox, biosensors and bioreporters in order to evaluate the effects of contaminants on the microbial community.

However, there is a requirement for a better toxicity indicator for the entire microbial community in the system in order to relate specific functions (e.g. enzyme activities) to certain groups of organisms and their abundance. With the advent of next generation sequencing, we are able to detect differences in functional genes at the community level *alkB* genes involved in degradation alkane and if we couple

Table 2.2 Microorganisms used as biosensors for a wide range of contaminants

Microorganism	Contaminant	Reference
Achromobacter	Surfactant detection	Taranova et al. (2002)
Acinetobacter	Phenol	Abd-El-Haleem et al. (2002)
Aliivibrio fischeri (formerly *Vibrio fischeri*)	Landfill leachate Oil polluted soil Insecticides: Ametryn	Thomas et al. (2009), Bundy et al. (2004), Farré et al. (2014)
Bacillus subtilis	Dyes Zinc, Pb and Cd	Ogawa et al. (1988), Kahru et al. (2005)
Chlorella vulgaris	Heavy metals Atrazine, simazine and diuron Herbicides	Durrieu and Tran-Minh (2002), Naessens et al. (2000), González-Barreiro et al. (2006)
Escherichia coli	Heavy metals Pollutants Zinc, Cu and Cd Aluminium Dioxins Wastewater Zinc bioavailability in soils PAHs Dibenzo-p-dioxins Arsenic Mercury	Vollmer et al. (1997), Bechor et al. (2002), Preston et al. (2000, Guzzo et al. (1992), Min et al. (2003), Sazykin et al. (2016), Maderova and Paton (2013), Gu and Chang (2001), Yoon et al. (2015), Xu et al. (2015)
Janthinobacterium lividum	Organic compounds and heavy metals	Cho et al. (2004)
Photobacterium leiognathi	Metals, pesticides, PAHs	Ulitzur et al. (2002)
Pseudomonas fluorescens	Zinc, Cu and Cd Olive mill wastewater Cu, Zn and 3,5-DCP Cu in soils Naphthalene	Preston et al. (2000), Mekki et al. (2008), Weitz et al. (2001), Maderova et al. (2011), King et al. (1990)
Pseudomonas putida	Benzalkonium chloride Cu, Zn and 3,5-DCP	Sütterlin et al. (2008), Weitz et al. (2001)
Raphidocelis subcapitata (formerly *Selenastrum capricornotum*)	Mercury Zinc, Pb and Cd	Juneau and Popovic (1999), Kahru et al. (2005)
Synechococus (cyanobacteria)	Marine oil spills	Brussaard et al. (2016)
Synechocystis	Herbicides	Shao et al. (2002)
Sphingomonas yanoikuyae	Fluorene	Bastiaens et al. (2001)
Spirostomum ambiguum (ciliate)	Heavy metals & hydrocarbons	Płaza et al. (2009)
Spirulina subsalsa	Heavy metals, triazinic herbicides, carbamate insecticides	Campanella et al. (2001)

(continued)

Table 2.2 (continued)

Microorganism	Contaminant	Reference
Symbiodinium	Herbicides: diuron and atrazine	Jones et al. (2003)
Tetrahymena pyriformis (ciliate)	Herbicides	Bonnet et al. (2007), (2008)
Tetrahymena termofila (ciliate)	Micotoxins	Benitez et al. (1994)
Tetrahymena pyriformis (ciliate)	Heavy metals	Gutiérrez et al. (2003)
Trichosporum cutaneum (fungi)	Wastewater BOD sensor Alkylbenzene sulfonate	Marty et al. (1997), Nomura et al. (1998)
Vibrio harveyi	Aluminium	Guzzo et al. (1992)
Vibrio aquamarinus	Wastewater	Sazykin et al. (2016)

this technique with microbial ecotoxicity tests, it will be easier to assess the toxicity level for the whole community. Therefore, one of the challenges in microbial ecotoxicology may be to incorporate and establish metagenomics as an aid in the collection of reliable toxicity levels.

Moreover, there is a need for bioremediation studies to conduct post-treatment toxicity tests in order to confirm the lack of toxicity at the site. Some bioremediation studies aim to reduce the contaminant below the local Environmental Protection Agency maximum limit; however, even if the contaminant concentration (or that of the intermediates) is within the desired limits it may still be toxic to several organisms.

Furthermore, apart from the classical toxicity indicators such as EC_{50}, LC_{50} and LD_{50}, it is important for microbial toxicological tests such as bioreporters to be accepted internationally in order to have a consensus on maximum toxicity levels. The establishment of microbial ecotoxicology as a new discipline should aid in such a task.

2.7 Conclusions

Molecular techniques such as clone libraries, DGGE, TRFLP and next generation sequencing combined with analytical methods (HPLC, GC-MS, ICPMS, etc.) have been extremely useful for microbial ecologists to identify the microorganisms responsible for a specific function such as the degradation of the contaminants or the production of a specific enzyme, while the analytical methods have helped to reliably measure chemical compounds of interest within a process.

Although microbial toxicology studies have also been performed previously, the results have generally been reported elsewhere in specialized toxicology journals.

Thus, one of the challenges of the recently established microbial ecotoxicology will be to bring together the results from the analytical methods, microbial toxicology and microbial ecology.

There is no doubt that the development of microbial ecotoxicology as a new discipline was both timely and crucial and its establishment will be fundamental to scientists assessing not only the impact of environmental contamination but also that of natural changes in the environment as well as the success of any remediation intervention.

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