

Chapter 2

Novel Molecular Tools to Assess Microbial Activity in Contaminated Environments

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2.1 Introduction

In order to understand the diversity and roles of microorganisms in global biogeochemical cycling, we must first recognize their distribution and several key factors controlling the environment. Bacterial cells arose ~3.5 billion years ago, based on fossils described by Schopf (1993) identifying “Cyanobacterium-like microorganisms.” It has been estimated that there are approximately $4\text{--}6 \times 10^{30}$ different prokaryotic cells placing prokaryotes as one of the dominant life forms on the planet (Santos Pontes et al. 2007). Given their long history it is not surprising that they have developed and adapted to a variety of—by human standards—hospitable and inhospitable environments. This explains their morphological, physiological, and in part their genetic diversity (Torsvik and Øvreås 2002). In a way bacteria are the “ultimate survivalists” because of their genetic variability allowing them to adapt relatively quickly. The ability of bacteria to adapt is a result of recombination events and induced mutations, which have allowed them to flourish during extreme environmental changes. Bacteria can often acquire or exchange genes via horizontal gene transfer (HGT) allowing them to adapt readily in any environment (Cohan 2001; Zeigler 2003; Santos Pontes et al. 2007). It is not surprising that bacteria have been recognized increasingly as a major catalyst in many biogeochemical reactions in both pristine and contaminated environments and play important roles in bioremediation, energy conversion, and biocatalysis in industrial and biomedical processes (Ferris 1993; Bull et al. 2000; Warren and Haak 2001; Crowe et al. 2007; Falkowski et al. 2008; Weisener et al. 2008; Schippers et al. 2010).

The biogeochemistry associated within environmental compartments is often complex. During the last 100 years pollution from varying sources such as mining, agriculture, burning of fossil fuels, and waste disposal activities into the hydrosphere

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and the terrestrial environment has culminated to such extent that many pristine environments no longer exist (Mielke et al. 2004). This has led to elevated concentrations of metals and metalloids within these locations. In general, the behavior of contaminants, particularly metals, depends on interactions between physicochemical parameters such as temperature and pH, solubility and formation of minerals, e.g., the rates and types of metabolisms and the specific metal/metalloids involved (such as Au, Cu, Ni, Pb, Zn, Se, As), and more importantly the influence of microbial activity (Schippers et al. 2010). Physicochemical conditions within the sediment compartment can often control and influence bacteria since certain variables such as pore size density, pH gradients, oxygen depletion, and nutrient matter can restrict or enhance microbial colonization. Prior to the 1990s, prokaryotic activity in natural and contaminated environments was often investigated using culture-based methodologies (Baldwin et al. 2005). In part due to their importance for public health, vast progress was made identifying specific protagonists in clinical microbiology during this period, while less so in the environmental counterparts of that discipline. The difficulties of cultivating bacteria from natural environments, such as soils and freshwater, have made the study of microbial diversity a difficult and not straightforward task (Fry 2000; Nee 2003; Kemp and Aller 2004). This is partly due to the complex interdependence of bacterial species and the complex chemistries for media enrichments.

During the 1990s, however, something akin to a revolution occurred with the advent of molecular-based approaches being directed toward environmental habitats. Giovannoni et al. (1990) were the first to phylogenetically analyze clone libraries created from eubacterial 16S rRNA extracted from picoplankton from the Sargasso Sea. Since then the use of molecular methods has resulted in a transformation in our understanding of microbial community structure in terms of function, phylogeny, and community composition within natural environments. This has led to a better understanding of the role microorganisms play in current and past biogeochemical systems. Continued advances and improvements to these approaches have provided data on their ecological roles and the evolution of bacterial species found in environmental samples. These new methods have led to the discovery of new bacterial species and function changing our concept of microbial diversity (Dunbar et al. 1999; Fry 2000; Kaeberlein et al. 2002; Jaspers and Overmann 2004; Santos Pontes et al. 2007). Although significant progress has been made, the complexities of environmental systems often require some degree of caution when evaluating the progression of events leading up to element and contaminant cycling. This holds especially true in contaminated environments since those are often unique and cannot necessarily be compared to naturally evolved geochemical systems. In part, the paucity in studies providing a greater understanding of such systems may be due to analytical challenges inherent in investigating such environmental systems in which many components are on a nano-scale and rely on a greater understanding of the bacterial community structure and function of the community as a whole. Some molecular methods used to investigate these consist of quantitative polymerase chain reaction (q-PCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction enzyme fragment length polymorphism (T-RFLP), and variations of fluorescent in situ hybridization

(FISH) and catalyzed reporter deposition-FISH (CARD-FISH). These molecular tools provide the unique opportunity to profile existing microbial ecophysiology and quantify individual groups of bacteria (Edwards et al. 2003; Huang et al. 2007; Fike et al. 2008; Norlund et al. 2009). Methods such as T-RLFP and DGGE have proven to be valuable preliminary assessment tools for fingerprinting more abundant microbial ribotypes within a community but often neglect the rarer types that may comprise the community. In this instance detailed metagenomic sequencing of relevant microbial species and communities may be required to shed light on the metabolic pathways and activities.

Recent advancements have led to a coupling of molecular-based approaches with physical characterization methods such as high-resolution microscopy, aiding in establishing the complexities of the interactions of bacteria with their environment and giving insights into mechanisms of contaminant cycling (Fike et al. 2008; Norlund et al. 2009). These investigations have contributed greatly to our understanding of complex bacteria/mineral relationships. FISH along with its variation CARD-FISH have proven to be very useful techniques for assessing bacteria mineral selectivity. Since they are based on the utilization of specific oligoprobes binding to their complementary DNA sequence and thereby labeling those organisms with that specific gene, certain parts of a community can be identified (Edwards et al. 2003; Peplies et al. 2006; Norlund et al. 2009). In fact, the combination of such molecular and spectroscopy-based techniques holds great promise for elucidating the complexities of bacterial communities and their influence on contaminants in sediment compartments (Neu et al. 2010). These methodologies will be discussed in a subsequent section of this chapter.

All of these methods can be used to assess microbial communities in terms of function, diversity, and population. With all molecular techniques there are inherent limitations, and caution should be used when interpreting results. For instance, fingerprinting techniques may only profile more abundant ribotypes within a community, while not detecting rarer ones. Metagenomic sequencing of microbial communities in impacted environments is a new perspective of metabolic activities and pathways for such systems (Hutchens 2009). The focus of this chapter will be on reviewing recent developments and advancements made to characterize microbial community composition and activity in contaminated environments with emphasis given to advantages and limitations. Future directions for elucidating microbial function in contaminated systems will be highlighted.

2.2 Using Culture-Based Techniques to Investigate Microbial Community Function and Development

Over the past 150 years microbiologists have relied on traditional approaches such as culture-based techniques, which rely on selective media, to identify microorganisms within a specific environment. This approach has resulted in a vast array of stored

pure culture collections from diverse habitats such as soils, marine estuaries, and freshwater ecosystems. Much of these investigations have derived from clinical studies as well as from studies investigating bacteria related to biogeochemical pathways in a quest to ascertain linkages to bacterial biodiversity. The classical approach to understanding diversity has relied on numerical taxonomy to compare isolates, identify them, and generally improve the taxonomy of the groups. The problem with this approach, however, is that it has led to confusion and uncertainties regarding the classification based on physiology and morphological comparisons. The development of molecular approaches described in this chapter has helped to shed light on these difficulties. These culture-independent approaches have illustrated that in most cases bacteria that grow as enrichment cultures are not the predominant species observed in natural systems (Fry 2004). The overall issue with using laboratory prepared media is that in some cases the numerically abundant species of bacteria will grow more slowly compared to less dominant species. An excellent review has been written by Fry (2004) who reviews some of the examples of the latest culture-based techniques used to successfully isolate dominant but uncultured bacteria using enrichment and micromanipulation techniques in a range of habitats.

2.3 Molecular Biological Analysis Techniques

While culture-based approaches, such as enrichments, are extremely useful for phylogenetic analyses of communities, they are limited to a few culturable organisms making up less than 1 % of the total microorganisms present in environmental samples (Ward et al. 1990; Amann et al. 1995). Additionally, culture-dependent methods are inherently biased through the choice of medium, selecting a specific portion of the present microbial community. The vast majority of microorganisms in the environment, though not having been cultured, have been characterized using culture-independent methods. Those methods are mainly based on the analysis of nucleic acids, lipids, and/or proteins from the sample matrix.

A wide variety of molecular techniques is available to analyze and characterize microbial community structure. Roughly these methods can be divided into those looking at certain fractions of the microbial community (Partial Community Analysis) and those looking at the whole microbial community (Whole Community Analysis).

2.3.1 *Fingerprinting Techniques*

One group of methods falling under the category of Partial Community Analysis is fingerprinting techniques. Those methods are techniques that characterize the structure of a microbial community based on the unique sequences of extracted nucleic acids (Muyzer 1999). In general fingerprinting techniques involve the

extraction of nucleic acids from the sample matrix, followed by amplification of specific molecular markers, such as the 16S rRNA gene, in a polymerase chain reaction (PCR). Results are restricted to differences within the sequences of these molecular markers. Pitfalls of PCR-based analyses from sample collection to analysis and cross checking of results have been reviewed by von Wintzingerode et al. (1997).

Fingerprinting methods are based on sequence variations between different organisms. Those differences result in different melting behaviors as well as differences in the locations of those parts of the sequences that represent restriction enzyme recognition sites. For different species, those sites are at different positions along the DNA strand. Two of the most commonly used techniques to analyze environmental samples of unknown microbial community composition are denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) and restriction fragment length polymorphism (RFLP), which both make use of sequence variations of PCR products amplified from environmental DNA.

Denaturing/temperature gradient gel electrophoresis (DGGE and TGGE) were introduced by Muyzer et al. (1993). Both techniques are based on electrophoresis of PCR amplified DNA fragments in a polyacrylamide gel, over either a gradient of increasing denaturants or temperature. Both methods rely on differences in the sequence-dependent melting behavior of double-stranded DNA. For this the extracted nucleic acids have to be amplified using primers that target specific molecular markers such as the 16S rRNA gene. For DGGE/TGGE the use of a 5'-GC clamped (30–50 nucleotides) forward primer is essential to avoid complete dissociation of the double strands. After loading the PCR product onto the gel, an electric current is applied, pulling the DNA fragments through the gel. Depending on the sequence of the amplicon, a higher or lower concentration of denaturant/temperature is needed for a complete dissociation of the double strands into single strands, causing the amplicons to stop moving through the gel at different positions. To determine the identities of bands separated on the gel, those bands can be excised from the gel and further analyzed via re-amplification, cloning and sequencing, or hybridization with molecular probes specific for particular taxonomic groups.

While these methods are valuable for the identification of changes in dominant species within a community, fingerprints resulting from DGGE/TGGE can be very complex, especially when using universal bacterial primers. This can lead to difficulties in separating bands of fragments with similar melting points. Additional care has to be taken, as rRNA operons of the same bacterium can show heterogeneity, leading to multiple bands and an overestimation of the microbial diversity. Also quantification of the extracted bands is not possible.

While DGGE and TGGE make use of the resulting different melting behaviors, various restriction fragment length polymorphism (RFLP) techniques make use of restriction enzyme recognition sites being present at different positions along the DNA strand. In amplified ribosomal DNA restriction analysis (ARDRA), the PCR product resulting from amplification of the extracted nucleic acids is digested with specific restriction enzymes. Depending on the location of the restriction enzyme

recognition sites, the DNA is cut into pieces of varying lengths. Subsequently the restricted fragments are separated via gel electrophoresis. This method has been used, e.g., by Smit et al. (1997) analyzing the effects of copper contamination on the soil microbial community. A challenge in the estimation of microbial diversity using this method is that the number of bands in the profile is larger than the number of different organisms in the sample, making the interpretation of the results difficult. This problem was resolved by Avaniiss-Aghajani et al. (1994) who developed terminal restriction fragment length polymorphism (T-RFLP) analysis. In T-RFLP analysis, extracted nucleic acids are amplified using a 5' fluorescently labeled primer. As with ARDRA, the resulting amplicons are digested with restriction enzymes. After digestion, the fragments are analyzed on an automatic DNA-sequencer, where only the terminal, fluorescently labeled fragments are detected.

Compared to DGGE/TGGE, T-RFLP analysis has the advantage that fragments can be quantified relatively by the intensity of the fluorescent signal, additionally to the method being quicker and less labor intensive. The inherent difficulty with T-RFLP, however, is that a collection or recovery of the fragments and thereby a subsequent analysis and identification of the microorganism via sequencing is not possible. To overcome this, fragments have been identified via comparison against databases of fragments produced by known gene sequences (Kent et al. 2003). Combining T-RFLP with clone library construction and sequencing Huang et al. (2011) were able to find close associations of the four most dominant operational taxonomic units detected in T-RFLP to phylum or genus level, when analyzing spatial and temporal variations of the microbial community in a tailings basin of a Pb–Zn mine.

Different studies have used DGGE/TGGE and T-RFLP to assess microbial community composition in different contaminated environments. Spatial and temporal variations of microbial community composition were analyzed in different mining environments such as an acidic stream draining across a pyrite mine in China (Tan et al. 2009) or in a low-temperature, acidic, pyrite mine, where Kimura et al. (2011) were able to highlight the importance of bacteria species in iron transformation using T-RFLP and FISH. Kim et al. (2009) used DGGE to examine the effects of mine tailings and waste rocks on the hydrogeochemistry and microbiology of a stream and groundwater near an abandoned copper mine. The effects of metal pollution on microbial community structure and composition in a salt marsh were analyzed by Cordova-Kreylos et al. (2006) who used T-RFLP to aid them in the development of bioindicators of toxicant-induced stress and bio-availability of contaminants for wetland biota. In another study Gough and Stahl (2011) used T-RFLP to follow microbial community changes in lake sediments along a metal contamination gradient. In a recent study by Thavamani et al. (2012), the authors employed a holistic approach. To determine the soil microbial activity affected by a mix of polyaromatic hydrocarbons (PAHs) and heavy metals, they combined physicochemical, biological, and advanced molecular methods to analyze the activities of the soil microbial community in long-term mixed contaminated soils collected from a former manufactured gas plant (MGP) site. The study

highlighted the difficulties of implementing remediation strategies when dealing with mixed contaminations, as well as the importance of combining different analysis methods.

Other studies used those fingerprinting techniques when monitoring the effects of different remediation techniques such as amending mine tailings with a mixture of organic carbon sources to treat pore water and drainage (Lindsay et al. 2011), incorporating compost into a heavy metal-contaminated acidic soil (Farrell et al. 2010), or testing the effects of phytoremediation approaches (Martinez-Inigo et al. 2009; Tipayno et al. 2012) or landfarming on oil refinery sludge (Ros et al. 2010).

In laboratory-based studies, fingerprinting techniques have been used when testing the effect of different contaminants/elements on microbial communities. Jakobs-Schönwandt et al. (2010) investigated the shift of soil microbial communities when subjected to a biocide frequently found in wood preservatives. Brandt et al. (2010) compared a Cu-adapted and a corresponding nonadapted soil microbial community for their abilities to resist experimental Cu pollution. Other studies investigated the abilities of indigenous bacteria on arsenic mobilization (Corsini et al. 2011) or the ability of specialized mixed communities to selectively precipitate transition metals from acidic mine waters (Nancucheo and Johnson 2011) or the acid tolerance response of a bioremediation system based on sulfate reduction (Lu et al. 2011).

Fingerprinting techniques are extremely useful when starting to investigate an unknown microbial community; however, as mentioned previously, they have their limitations and can be time and labor extensive. On their own, both techniques can either quantify OR identify fragments, but not both.

2.3.2 *Quantitative-PCR*

In studies where changes in the amounts of specific groups or species of organisms or the expression of certain genes are of interest, quantitative or real-time PCR (qPCR) is a method of choice. Wherever a gene sequence can be utilized that uniquely identifies the organisms of interest, qPCR is a commonly used method to quantify these specific DNA sequences.

Quantitative PCR is an advancement of PCR in which the amount of the amplified targeted DNA is measured during each cycle of the PCR reaction using an intercalating dye such as SYBR[®] Green or fluorescently labeled probes (TaqMan[®]). The increase in fluorescence is measured during the reaction and software calculates the concentration from the intensity during the early exponential phase of the reaction, when concentrations are proportional to the starting template concentration. Quantitative PCR is especially useful observing gene expression, if the sequence of the functional gene of interest is known. For instance, in a study by Mouser et al. (2009), the authors used qPCR to follow the expression of the nitrogen fixation gene, *nifD*, and the ammonium transporter gene, *amtB*, in *Geobacter* species while evaluating in situ bioremediation of uranium-contaminated groundwater. In another study, Yergeau

et al. (2012) used reverse transcription qPCR assays to confirm the active expression of hydrocarbon degradation genes by *Pseudomonas* and *Rhodococcus* species in Arctic biopile soils.

2.4 The Use of Gene Arrays for Community Structure Determinations

In environmental sciences, microarrays have mainly been used to provide a high-throughput and comprehensive view of the microbial community (Gentry et al. 2006). Similar to hybridization techniques, microarrays exploit complementary base pairing. Microarrays consist of multiple spots containing thousands to hundreds of thousands of probes of sequences of interest which will be used to bind complementary nucleic acids extracted from the sample. Based on the information they provide, microarrays can be divided into two major groups: those that investigate (1) phylogeny and those investigating (2) function (Rastogi and Sani 2011). Based on the target genes, Gentry et al. (2006) divided those arrays further into five major groups (a–e).

(1) Phylogenetic information can be gained from phylogenetic arrays (PGA) and community genome arrays (CGA). (a) PGAs are used to compare communities in different environments or follow changes in their composition over time and are based on conserved marker genes such as the 16S rRNA gene. (b) Community genome arrays on the other hand are made up of the whole genomic DNA extracted from cultured organisms and can be used to describe a community based on its relationship to the cultured organisms. (2) Functional information can be gained from functional gene arrays (FGA) and metagenomic arrays (MGA). (c) FGAs are used to gain information about the presence of known genes encoding proteins catalyzing biogeochemical processes of interest, such as sulfur, carbon, or nitrogen cycles, and can provide information about the microbial populations involved. (d) The probes used on metagenomic arrays on the other hand are made from environmental DNA and can be used without any prior knowledge about the sequences of the community. (e) A fifth type of array, the whole-genome open-reading frame array (WGA), can provide information on both phylogeny as well as function. Those arrays hold probes for all open-reading frames—the part of a gene that encodes a protein—and can be used to compare genomes as well as to analyze the interactions of different organisms at the transcriptional level. Specific applications of these five major types of arrays are discussed in a review by Gentry et al. (2006).

Microarrays have originally been developed for gene expression analysis of individual organisms or pure cultures and their use with environmental samples bears special challenges (Zhou and Thompson 2002; Gentry et al. 2006). Some difficulties are associated with the sample matrix, such as the presence of substances like humic acids, metals, and organic contaminants which can interfere with the hybridization step, or the fact that some environments contain only low levels of

biomass creating the need to amplify nucleic acids, which as mentioned before may introduce bias. The possibly biggest challenge is the vast number of unknown organisms in the sample and therefore unknown DNA sequences. Organisms without a corresponding probe on the microarray will be overlooked, even if they may be important and dominant in the system. Additionally, probes can cross-hybridize to similar unknown sequences resulting in a false signal due to binding of a different gene or to an underestimation of a signal due to a weaker binding of a slightly divergent sequence preventing a binding of the target sequence (Gentry et al. 2006). Nonetheless, microarrays have been successfully applied in microbial ecological studies.

Neufeld et al. (2006) designed a habitat-specific array to investigate the microbial community structures of hexachlorocyclohexane (HCH) contaminated and uncontaminated soils. The authors found strong correlations between HCH contamination and probe signals for organisms belonging to the genus *Sphingomonas* and to different organisms with acid-tolerant phenotypes. Using a functional gene array (GeoChip), Liang et al. (2009) examined the diversity of microbial communities along a contamination gradient along an oil field in China. With increasing contamination level, the authors found a decrease in the diversity and number of functional genes, as well as in archaea. A study by Xiong et al. (2010) investigated the effects of arsenic contamination on microbial communities. Comparing soil samples with different arsenic contamination levels from the rhizosphere of the arsenic accumulating plant *Pteris vittata* and non-rhizosphere areas the authors not only found that, in comparison to arsenic contaminated soil, the uncontaminated soil showed a higher heterogeneity and more unique genes, but they also detected distinct differences in arsenic resistance, sulfur reduction, phosphorus utilization, and denitrification genes between rhizosphere and non-rhizosphere samples. Their results suggest that bacteria associated with the rhizosphere of *P. vittata* play an important role in the plants soil arsenic uptake and accumulation.

2.5 Microbial Diversity Investigations Via Sequencing

Throughout the last five years the use of high-throughput sequencing (HTS) techniques has revolutionized the way microbial communities are analyzed (Logares et al. 2012). Instead of sequencing individual DNA clones, these methods sequence hundreds of thousands to tens of millions of DNA molecules in parallel. Since December 2008 three HTS platforms are commercially available, 454 (Roche/454 Life Sciences), Solexa (Illumina), and SOLiD (ABI), which can generate gigabases of sequences in a single experiment (Jones 2010). Compared to traditional sequencing, where obtained sequences are typically over 800 nucleotides in length, sequences obtained with these next-generation techniques are much shorter with 25–50 nucleotides (Illumina and ABI) and 200–400 nucleotides (454) in length (Graveley 2008). Those short sequences, however, make it difficult to match the sequences to the reference genome and gain sufficient information to relate DNA segments to species or functional groups. A comparison of the three different

platforms has been provided by Mardis (2008). Due to the larger fragment length obtained, the Roche/454 Life Science Sequencer is the most widely used HTS platform and its HTS process, including considerations and drawbacks such as the large amount of DNA needed as well as the required computing power for data analysis, has been reviewed by Jones (2010). New technologies are being developed at an incredible speed and existing techniques have constantly been improved in throughput as well as read length. Since 2010 further next-generation sequencing methods are available, such as the Ion Torrent (Life Technologies) and the SMRT (Pacific Biosciences). A more recent review of next-generation sequencing technologies with an emphasis on their application in environmental DNA analysis has been published by Shokralla et al. (2012). Figure 2.1, originally published in that review paper, displays the development and advances of next-generation sequencing methods.

In 2006, a study by Edwards et al. was the first one describing an application of HTS to environmental samples. The authors used 454-sequencing to compare microbial communities of two sites in an iron mine. Sequence analysis revealed distinct differences between the communities and their metabolic pathways. While the authors could explain much of the correlation between occurring microbial metabolism and the geochemical conditions, they highlighted the fact that many pathways in the environment are still unexplained. Bowman et al. (2012) advanced our knowledge of the effects and possible implications of climate change, using 454-sequencing to analyze microbial communities in the Arctic ice identifying microbial diversity to improve our understanding of the consequences of the potential disappearance of this environment.

In contaminated environments, metagenomic analyses are especially useful when studying the underlying pathways and investigating metabolic activities and bioremediation potential. Bioremediation is a potentially effective and inexpensive way to restore contaminated environments; however, its implementation is often limited by a lack of information on the microbial communities present and the factors influencing their growth and metabolism (Lovley 2003). Several studies have used cloning-based sequencing methods looking at genes, functions, and pathways to look at bioremediation of environments affected by different contaminants such as jet fuel (Brennerova et al. 2009), hydrocarbons and chlorinated solvents (Dojka et al. 1998), oil (Röling et al. 2002), acid mine drainage (Martins et al. 2009), or uranium (Seifert et al. 2008). Generally those studies have used sequencing in combination with other molecular techniques such as fingerprinting. While they give valuable information on specific genes and function, they were inherently biased and restricted to the genes used for analysis. HTS, however, is a method of particular interest as it can possibly lead to the discovery of novel organisms or genes that might be missed by traditional methods. Yergeau et al. (2012) were the first ones to use metagenomic sequencing analysis in a soil bioremediation experiment. The authors reported the presence of *Caulobacter* who “could be involved in alkane degradation in Arctic soils, a role that has not been previously reported.” Additionally they found sequences related to uncultured or not classified microorganisms and genes, which potentially represent novel hydrocarbon degradation genes.

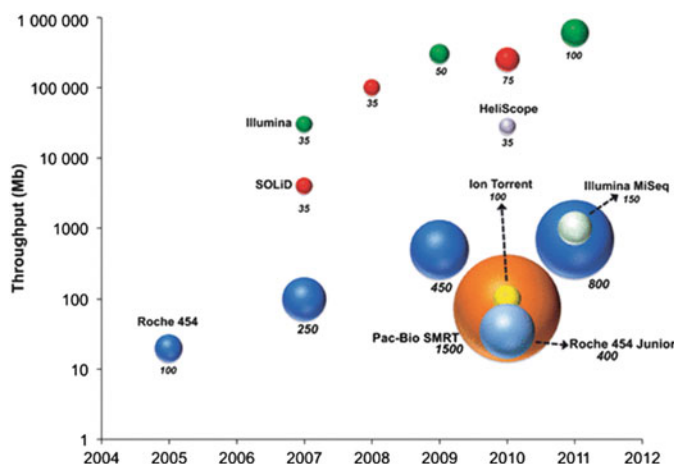


Fig. 2.1 Historical development of next-generation sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform [in base pairs (bp)]. Colors correspond to individual platforms (Shokralla et al. 2012)

2.6 Coupling Molecular Based with Microscopic and Isotopic Approaches

Advances made in the development of new molecular tools have contributed greatly to the advancement of our understanding of complex bacteria/mineral relationships in some environmental systems. As mentioned earlier, fluorescent in situ hybridization (FISH), similar to other hybridization methods, uses specific fluorescently labeled oligoprobes binding to their complementary DNA sequence. FISH along with its different variations, such as catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH) or MAR-FISH, the combination of FISH with microautoradiography (MAR), have proven invaluable for the detection and enumeration of specific bacterial taxa and the investigation of microbial activities in complex communities, as well as for imaging their spatial association with mineral phases (Edwards et al. 2003; Okabe et al. 2004; Peplies et al. 2006; Norlund et al. 2009; Tischer et al. 2012).

Like other molecular biological methods, the standard FISH technique has its limitations. One of those is that, when working with intact cells, not all bacterial and archaeal cells can undergo permeabilization by the oligonucleotide probes. Additionally, using rRNA probes hampers the identification of cells with low ribosome contents and thereby restricts the sensitivity of the method (Wagner et al. 2003). Many limitations have been overcome by advances made over the last 10 years improving the method itself as well as combining it with other methods. Some of those have been reviewed by Wagner et al. (2003) along with new trends in the use of FISH techniques to identify and analyze functional groups of microorganisms (Wagner and Haider 2012).

CARD-FISH has proven valuable for assessing complexities of microbial communities associated with hydrocarbon contaminated aquifer environments (Tischer et al. 2012). To investigate both bacteria and archaea simultaneously, the authors used a dual hybridization approach followed by two consecutive stainings. Additionally, the authors were able to improve the CARD-FISH detection for their materials by applying microwave irradiation during the permeabilization and hybridization steps. These improvements to the standard approach will allow more detailed investigations at higher spatial resolution to elucidate the ecology of possible hydrocarbon contaminant degraders (Tischer et al. 2012).

MAR-FISH can be used to obtain detailed information pertaining to the activity of defined groups of prokaryotic cells within natural environments. In this approach radioactively labeled substrates are used to determine the physiological activity of bacteria within a given environment system. The technique not only determines general physiological conditions but can also be used to investigate the ecophysiology of organisms defined by oligonucleotide probes (Ito et al. 2002; Wagner and Loy 2002). Nielsen et al. (2002) successfully quantified acetate consuming iron-reducing bacteria in activated sludge by incubating the biomass with radio-labeled acetate while inhibiting sulfate reducing and methanogenic prokaryotes. The authors were able to show that besides being useful for enumeration, MAR-FISH is also suitable to classify functional groups of bacteria phylogenetically.

Microscopy and imaging techniques are invaluable and irreplaceable methods in the analysis of complex environmental systems. Neu et al. (2010) reviewed some applications of laser scanning microscopy (LSM), magnetic resonance imaging (MRI), and scanning transmission X-ray microscopy (STXM). By combining different techniques, such as STXM using synchrotron radiation, laser scanning microscopy (LSM), and FISH-based oligonucleotide probes (e.g., immunogold), it is possible to examine phylogenetic identity and metabolic activity from individual cells thus differentiating processes at the species level similar to MAR-FISH. These microscopic techniques present a powerful way to visualize and examine mixed environmental microbial communities, especially as microbial communities often appear in films or as aggregates (Neu et al. 2010).

Other combinations of methods that have been used to differentiate and identify community structure and metabolic activity in natural sediments include the combination of CARD-FISH with Raman microspectroscopy (Huang et al. 2007; Wagner 2009) stable isotope labeling, and high resolution secondary ion mass spectrometry (SIMS) (Fike et al. 2008; Orphan and House 2009).

The combination of stable isotope characterization methods with genomic analysis provides direct insight into the expression of key functional genes and community structure. Stable isotope methods are extremely powerful since they allow the determination of chemical bonding environments associated with the microbial cells. For example, the incorporation of ^{13}C isotopic tracers into the cell membrane causes significant changes in the observed resonance spectra compared to its parent ^{12}C spectra (Huang et al. 2004). The observed changes result from modifications to the bond vibrational state brought on by the heavier isotope. The observed shift in vibrational spectra is termed a “red shift” due to the production of

longer wavelengths within the Raman Spectra associated with ^{13}C -labeled cells (Huang et al. 2004). While this approach maintains the resolution of the MAR-FISH technique, it does provide a link between highly resolved single cell studies and population-based sequence strategies, which rely on stable isotopes. Studies investigating naphthalene-degrading consortia in contaminated aquifers have proven the validity of the method (Huang et al. 2007). Here the authors incubated labeled ^{13}C contaminated ground water with a single-specific FISH probe to observe the in situ activity of pseudomonads as naphthalene degraders. By using this approach, the authors noted that the bacteria's direct metabolism could be quantified using the labeled compound. Interestingly, the authors noted differences in ^{13}C concentrations for the different ^{13}C -labeled pseudomonad cells they attributed those to differences in the activities of individuals, within different strains or species targeted by the oligonucleotide probe.

The application of nano-SIMS imaging, a higher resolution version of the SIMS technique, by itself or in combination with specific isotope labeling experiments has proven very successful for assessing ecophysiology of microbes in various environments (Fayek et al. 2005; Wagner 2009) and could be effective for elucidating bacterial effects on metals in mine waste spoils. For example, the analyses of stable isotopes of oxygen, iron, copper, and sulfur as well as low weight carboxylic acids using SIMS can yield valuable information on biogeochemical element cycling in mine dumps and has the potential of becoming a tool for the prediction and control of metals released in acid mine drainage (AMD) generation. What is still required is further development of other molecular-based approaches for assessing mine waste spoils.

2.7 Conclusions

Like culture-based methods, molecular techniques have their advantages and disadvantages and can be biased. Molecular analyses methods generally depend on the extraction of DNA or RNA from the sample matrix. Preferential or incomplete lysis of microbial cells can produce a distorted view of the community structure. Additionally, DNA can persist in the environment for prolonged periods, making the differentiation between dead, alive, and active cells difficult if not impossible (Romanowski et al. 1992; Nielsen et al. 2007). To overcome the problem of detecting DNA from dead as well as live cells, recently selective intercalating dyes ethidium monoazide (EMA) and propidium monoazide (PMA) have been used to exclude DNA from dead cells (Bae and Wuertz 2009; Nocker and Camper 2009; Taskin et al. 2011). DNA analyses are useful for studying the functional potential of the microbial community; it, however, does not provide any information on the activity of the microbial community or parts of it.

Information of bacterial activity can be gained from rRNA or mRNA extracted from environmental samples. The number of ribosomes in a cell (rRNA) is known to correlate with growth rate, with some exceptions having been found for slow growing bacteria (Quiros et al. 1989), while the detection of mRNA is a definitive

indicator of activity (Wellington et al. 2003). However, due to the possibility of the modification of proteins following mRNA transcription, its detection might not necessarily equate with the phenotypic expression of the targeted gene (Wellington et al. 2003). Those uncertainties, as well as possible biases during DNA/RNA amplification need to be taken into consideration, especially when analyzing environmental samples. T-RLFP and real-time PCR have proven to be valuable preliminary assessment tools for fingerprinting important functional members of the bacterial community. To shed light on the metabolic pathways and activities contributing to, e.g., metal mobility and biomineral-induced metal mobilization, further analyses are required. Sequencing methods have enabled researchers to detect slight changes in the composition of microbial communities in samples that may occur following alterations of the environment which can happen naturally or as a consequence of anthropogenic activities (Leininger et al. 2006; Fierer et al. 2007; Shokralla et al. 2012). However, even those methods are not foolproof and especially in natural systems it is not always clear if a change in community composition is directly related to the change of a specific environmental factor.

Overall, limitations of methods must be considered and a combination of methods is recommended to minimize misinterpretation as well as oversight of less abundant, but important members of the microbial community. Studies using a combination of methods, such as the study by Kao et al. (2010) who used DGGE and qPCR plus culturing (enrichments) in a bioremediation study at a petroleum–hydrocarbon-contaminated site, give a more comprehensive view of environmental occurrences such as success or failure of remediation strategies.

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