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Review article

The use of molecular techniques to characterize the microbial communities in contaminated soil and water

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Abstract

Traditionally, the identification and characterization of microbial communities in contaminated soil and water has previously been limited to those microorganisms that are culturable. The application of molecular techniques to study microbial populations at contaminated sites without the need for culturing has led to the discovery of unique and previously unrecognized microorganisms as well as complex microbial diversity in contaminated soil and water which shows an exciting opportunity for bioremediation strategies. Nucleic acid extraction from contaminated sites and their subsequent amplification by polymerase chain reaction (PCR) has proved extremely useful in assessing the changes in microbial community structure by several microbial community profiling techniques. This review examines the current application of molecular techniques for the characterization of microbial communities in contaminated soil and water. Techniques that identify and quantify microbial population and catabolic genes involved in biodegradation are examined. In addition, methods that directly link microbial phylogeny to its ecological function at contaminated sites as well as high throughput methods for complex microbial community studies are discussed.
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Keywords: Molecular techniques; Microbial communities; Contaminated soil; Water; Microbial identification; Biodegradation

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1. Introduction

Traditionally, characterization of microbial community composition in contaminated soil and water has been limited to the ability to culture microorganisms from environmental samples. Unfortunately, only a fraction of the microorganisms involved in the biodegradation of contaminants in soil and water can currently be cultured in the laboratory. It has been estimated that the microbial community in one gram of soil may contain over one thousand different bacterial species (Rossello-Mora and Amann, 2001), but less than 1% of these may be culturable. It has been observed that fast growing organisms or strains best adapted to particular culture conditions grow preferentially than those which are not, and therefore do not accurately represent the actual microbial community composition of contaminated environments (Rappe and Giovannoni 2003; Gilbride et al., 2006). Hence culture-dependent characterization of microorganisms at contaminated sites may limit the scope of microbial biodiversity and the ecological importance of unculturable organisms at contaminated sites may go undetected (Brockman 1995; Van Hamme et al., 2003; Widada et al., 2002b).

Modern molecular techniques provide an exciting opportunity to overcome the requirement for culturing and have therefore greatly increased our understanding of microbial diversity and functionality in the environment. These methods rely on the characterization of cellular constituents such as nucleic acids, proteins, fatty acids and other taxa-specific compounds (Rossello-Mora and Amann, 2001). Such molecules can be extracted directly from environmental samples without the need for culturing and analysis of the molecular composition can be used to elucidate the composition of the microbial community (Amann et al., 1995; Greene and Voordouw, 2003). Another advantage of culture-independent molecular characterization includes the capacity to preserve *in situ* metabolic function and the microbial community composition by immediately preserving samples (Moller et al., 1998; Wilson et al., 1999a) or direct extraction of molecules of interest from environmental samples (Tsai and Olson, 1991).

This review is aimed at presenting and evaluating current molecular applications in the assessment of microbial community composition from contaminated soil and water environments in a bid to identify the dominant microbial communities or catabolic genes.

2. Biochemical methods

2.1. Phospholipids fatty acids (PLFA)

Phospholipids are important components of living cells membranes and constitute a significant proportion of organism biomass under natural conditions (Kozdroj and van Elsas, 2001). Microorganisms have the ability to change the lipid composition of their membranes in response to environmental conditions such as chemical stress (Frostegard et al., 1993) and temperatures fluctuations (Bartlett, 1999). PLFA rapidly degrade upon cell death thus making it good indicator of living organisms (Drenovsky et al., 2004) and changes in PLFA patterns under environmental stress conditions are a useful biomarker tool to describe the community structure and physiological state of certain microbial taxa (Vestal and White, 1989; Misko and Germida, 2002).

Changes in phospholipid profiles are generally related to the variation in the abundance of microbial groups and this can be interpreted by reference to a database of pure cultures and known biosynthetic pathways (Zelles, 1999). The extracted fatty acids are quantitatively analyzed by gas chromatography equipped with mass spectrometry (Zelles and Bai, 1993), while comparison of data with information on fatty acids database allows for the identification of extracted PLFAs (Widmer et al., 2001). Although direct extraction of PLFA from soil does not permit delineation down to species level, it is an efficient means by which gross changes in microbial community structure can be profiled (Nannipieri et al., 2003).

Several researchers have taken a variety of approaches to the interpretation of community fatty acids profiles (Haack et al., 1994). Methods such as tabulation of known or presumed unique fatty acids or comparisons of profiles on the basis of within-profile ratios of fatty acids have been used (Haack et al., 1994). Currently, PLFA analysis employs the use of multivariate statistics such as principal component analysis to discriminate between composite profiles (Haack et al., 1994; Langworthy et al., 1998).

Frostegard et al. (1993) examined changes in microbial population profiles in soils artificially polluted with cadmium, copper, nickel, lead, or zinc using PLFA. They observed that certain fatty acid patterns characteristic of Gram-positive bacteria were reduced in both forest and arable soils spiked with metals and replaced by PLFA patterns indicative of a Gram-negative bacterial populations. Kamaludeen et al. (2003) investigated the ecotoxicity of long-term tannery waste contaminated soils by assessing the bacterial activity and community structure using PLFA. PLFA profiles of specific bacteria decreased significantly as the level of chromium contamination increased, indicating that the concentration of chromium in tannery waste contaminated soil had a significant effect on microbial community structure (Kamaludeen et al., 2003). In another study, a change in microbial community structure during bioremediation of explosives-contaminated soil in a molasses-fed bioslurry process was demonstrated using PLFA profiles (Fuller and Manning, 2004). PLFA analysis showed

that Gram-positive bacterial populations were more abundant after explosives compounds were reduced to non-inhibitory levels (Fuller and Manning, 2004). PLFA profiles have also been employed to characterize the microbial community in polycyclic aromatic hydrocarbon (PAH) contaminated freshwater sediments (Langworthy et al., 1998), characterization of sulphate-reducing bacteria in groundwater at a uranium mill (Chang et al., 2001) and the study of microbial community structure at uranium-contaminated ground water sources (Schryver et al., 2006).

Comparison of PLFA and 16S rRNA in a phylogenetic study of twenty five isolates of dissimilatory sulphate-reducing bacteria showed highly congruent clustering for twenty two isolates (Kohring et al., 1994) thus establishing the usefulness of PLFA in the determination of bacterial relationships. However, PLFA analyses are not without limitations as fatty acid composition can be influenced by temperature and nutrition (Graham et al., 1995). Furthermore, individual fatty acids cannot be used to represent specific species (a single microoorganism can have numerous fatty acids and the same fatty acids can occur in more than one species) (Kirk et al., 2004). PLFA as a microbial community profiling tool produces profiles of limited complexity thus PLFA is often used in conjunction with other molecular profiling methods to assess microbial diversity in contaminated soil and water (Ringelberg et al., 2001; Onstott et al., 2003; Pombo et al., 2005).

3. Nucleic acid based techniques

3.1. Polymerase chain reaction (PCR)

The polymerase chain reaction has the ability to produce millions of copies of a portion of a desired gene, entire gene or gene clusters with high fidelity within 3 to 4 h. It is the most widely used method for the amplification of 16S rRNA, or its gene, prior to fingerprinting studies. PCR-based methods have also used in the detection and quantification of microorganisms found in soil and water (Wilson et al., 1999b).

The technique can also be applied for the analysis of catabolic genes involved in the biodegradation of organic contaminants (Wilson et al., 1999a).

For the detection of organisms or genes from contaminated environments, two variants of the PCR technique are often used; simple PCR and multiplex PCR. Simple PCR uses a pair of primers in a single amplification reaction, while multiplex PCR uses multiple primer pairs simultaneously to amplify several genes in a single reaction (Markoulatos et al., 2002).

PCR amplification is dependent on the extraction and purification of nucleic acids of sufficient yields and quality from environmental samples. Insufficient lyses of cells could result in the preferential extraction of DNA from Gram-negative bacteria, while excessively harsh treatments may result in the shearing of DNA from readily lyzed cells (Wintzingerode et al., 1997). In addition, PCR amplification efficiency can severely be hampered by the presence of inhibitory substances which are co-extracted with nucleic acids which include humic acids, organic matter and clay particles (Kirk et al., 2004).

Methods employed for sample collection, transportation and storage prior to nucleic acid extraction are important in the way that bias may be introduced into subsequent microbial analysis of native communities (Schneegurt et al., 2003). Of particular concern is the temperature at which samples are stored and their exposure to oxygen (Jackson et al., 1998). With extended sample storage, both these factors may alter the microbial composition hence the need extract DNA/RNA after sample collection (Wintzingerode et al., 1997).

Knaebel and Crawford (1995) applied multiplex PCR to detect the petroleum-degrading microbial population in a petroleum contaminated soil. Baldwin et al. (2003) also employed a multiplex PCR technique for the detection of naphthalene dioxygenase, biphenyl dioxygenase, toluene dioxygenase, xylene monooxygenase, phenol monooxygenase and ring-hydroxylating toluene monooxygenase genes in a single PCR reaction.

Although multiplex PCR can save time and resources in the detection of microorganisms or genes involved in biodegradation, successful application depends on the combination of several primer pairs being able to perform reliably in a single reaction. Primer dimmer formation between the various primers is more likely to occur and this may lead to poor sensitivity and preferential amplification of certain targets (Gilbride et al., 2006).

Another variant of the PCR technique which can simultaneously detect and quantify the amplified product while the reaction is occurring is a real-time PCR. This approach enables the detection and quantification of PCR amplicons during the early exponential phase of the reaction (Higuchi et al., 1993; Heid et al., 1996). Real-time PCR has an increased capability for quantifying gene copy numbers present in a given sample (Lerat et al., 2005). It involves the use of fluorescent markers to quantify the product at the end of each amplification cycle and the amount of fluorescence is directly related to the amount of product at the end of each cycle in the PCR reaction (Saleh-Lakha et al., 2005).

Real-time PCR has been used in several environmental studies such as the monitoring of carbazole 1,9a-dioxygenase gene (*carAa*) numbers in soil slurry microcosms (Widada et al., 2002a), the measurement of the alpha-subunit of benzylsuccinate synthase gene (*bssA*) and atrazine catabolic gene (*atz*) (Beller et al., 2002; Devers et al., 2004) and the identification and quantification of the arsenate reductase gene (*arsC*) in soil and aromatic oxygenase genes (Baldwin et al., 2003; Sun et al., 2004). Recently, real-time PCR targeting the 16S rRNA genes and *Dehalococcoides* reductive dehalogenase (*RDase*) gene was used in the monitoring of *Dehalococcoides* strains (Ritalahti et al., 2006). It has also been used in quantifying the proportion of microorganisms containing alkane monooxygenase and the subsequent assessment of microbial community changes in hydrocarbon-contaminated Antarctic soil (Powell et al., 2006).

The advantages that real-time PCR offers include speed, sensitivity, accuracy and the possibility of robotic automation (Powell et al., 2006). Although real-time PCR can measure gene quantity, the results obtained do not link gene expression with a

specific measurable microbial activity or population. RNA extracted from soil and water samples are low in yield and often do not represent the soil microbial population (Saleh-Lakha et al., 2005). Moreover, RNA has a short half-life and rapidly degrades after extraction thus serving as a major challenge for the application of real-time PCR in environmental microbiology. Finally, specific probes used in the amplification reactions may fail to capture the sequence diversity that is present within environmental samples (Saleh-Lakha et al., 2005).

PCR molecular techniques have completely revolutionized the detection of DNA/RNA especially in microbial ecological studies. However, differential amplification of target genes such as 16S rRNA can bias PCR-based diversity studies (Wintzingerode et al., 1997). For example, sequences with lower guanine plus cytosine content are thought to separate more efficiently in the denaturing step of PCR and hence could be preferentially amplified (Wintzingerode et al., 1997). Also products seen on gels or in real-time may be as a result of artefacts or chimeric PCR product formation (Liesack et al., 1991). PCR is a very sensitive technique and in some cases may produce false positive or false-negative signals due to contamination (Spiegelman et al., 2005).

3.2. Microbial community profiling

In recent years, the application of molecular techniques have led to more rapid and accurate strategies for examining microbial diversity including the discovery and identification of novel organisms and their catabolic genes involved in the biodegradation of organic contaminants in soil and water (Amann et al., 1995; Wilson et al., 1999a,b; Murrell and Radajewski, 2000; Milcic-Terzic et al., 2001; Stokes et al., 2001; Greene and Voordouw, 2003; Mahmood et al., 2005; Gentry et al., 2006). Microbial identification and diversity characterization has been enhanced by utilising the highly conserved gene, 16S rRNA which is ubiquitous in all microorganisms (Olsen et al., 1986; Woese, 1987; Pace, 1997; Watanabe, 2001). 16S rRNA gene sequences are conserved enough to enable the design of PCR primers which target different taxonomic groups (from kingdom to genus), but have enough variability to provide phylogenetic comparisons of microbial communities (Woese, 1987).

Microbial community compositions can be analyzed based on profiles generated from the physical separation of rRNA or DNA sequences on a gel (Muyzer, 1999). In this regard, several techniques based on the amplification and comparisons of PCR-amplified DNA sequences have been developed and used to characterize microbial communities from contaminated environments. These methods detect differences between DNA/RNA sequences, which often include PCR-amplified 16S rRNA gene fragments.

The different genetic community profiling methods include amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE) and terminal-restriction length polymorphism (T-RFLP).

3.2.1. Amplified ribosomal DNA restriction analysis (ARDRA)

For amplified ribosomal DNA restriction analysis, PCR-amplified 16S rRNA fragments are digested or cut at specific sites with restriction enzymes and the resulting digest separated by gel electrophoresis. Different DNA sequences will be cut in different locations and will result in a profile unique to the community being analyzed. Divergence of the community rRNA restriction pattern on a gel is highly influenced by the type of restriction enzyme used (Gich et al., 2000). Banding patterns in ARDRA can be used to screen clones or be used to measure bacterial community structure (Kirk et al., 2004). ARDRA is simple, rapid and cost-effective, and as a result has been used in microbial identification (Vaneechoutte et al., 1992, 1995; Kita-Tsukamoto et al., 2006; Krizova et al., 2006) and microbial community studies (Weidner et al., 1996; Gich et al., 2000; Bai et al., 2006).

Microbial community composition and succession in an aquifer exposed to phenol, toluene and chlorinated aliphatic hydrocarbons were assessed by ARDRA with the aim of identifying the dominant microbial community involved in the biodegradation of trichloroethene (TCE) following biostimulation (Fries et al., 1997). ARDRA revealed that the dominant microbial community members were stable and could be accounted for by the fingerprinting bands produced on the gel (Fries et al., 1997). In another study, Gich et al. (2000) used ARDRA to examine the microbial differences in activated sludge from treatment plants fed on domestic or industrial wastewater. It was observed that the bacterial communities in activated sludge were different from industrial and domestic waste water treatment plants. Hohnstock-Ashe et al. (2001), using ARDRA as a fingerprinting technique also observed that the microbial community composition in well waters contaminated with TCE had shifted toward a highly diverse community dominated by *Dehalococcoides ethenogenes*-like microorganisms.

ARDRA is useful for detecting structural changes in microbial communities but is unable to measure microbial diversity or detection of specific phylogenetic groups within a community fingerprinting profile (Liu et al., 1997). Optimization with restriction enzymes is required and is often difficult if sequences are unknown. As a result, further optimization may be required to produce fingerprinting patterns characteristics of the microbial community (Vaneechoutte et al., 1992; Spiegelman et al., 2005). In addition, banding patterns in diverse communities become too complex to analyze using ARDRA (Kirk et al., 2004). In recent studies, ARDRA has been combined with other molecular techniques such as T-RFLP and DGGE to characterize microbial communities from contaminated sources (Watts et al., 2001; Haack et al., 2004).

3.2.2. Ribosomal intergenic spacer analysis (RISA)

RISA is a PCR-based technique that amplifies the region between the 16S and 23S rRNA operons. The intergenic spacer region, depending on the species, has both sequence and length (50–1500 bp) variability (Ranjard et al., 2001) and this unique feature facilitates taxonomic identification of organisms (Spiegelman et al., 2005). RISA has been used to distinguish between different strains and closely related species of

Staphylococcus (Mendoza et al., 1998; Bes et al., 2002), *Bacillus* (Bourque et al., 1995; Daffonchio et al., 2003), *Vibrio* (Chun et al., 2002; Ghatak et al., 2005) and other medically important microorganisms.

In environmental studies, RISA has been used to detect microbial populations involved in the degradation of PAH at low temperature under aerobic and nitrate-reducing enriched soil conditions (Eriksson et al., 2003). RISA has also been used to define microbial diversity and community composition in freshwater environments (Fisher and Triplett, 1999).

RISA is a very rapid and simple rRNA fingerprinting method but its application in microbial community analysis from contaminated sources is limited partly due to the limited database for ribosomal intergenic spacer sequences is not as large or as comprehensive as the 16S sequence database (Spiegelman et al., 2005). As a result, community analysis using RISA could reduce its effectiveness in the identification of unknown or non-culturable microbial species from contaminated sources. Furthermore, RISA sequence variability may be too great for environmental applications. Its level of taxonomic resolution is greater than 16S rRNA and hence may lead to very complex community profiles (Spiegelman et al., 2005).

3.2.3. Denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (Fischer and Lerman, 1979, 1983) or temperature gradient gel electrophoresis (Rosenbaum and Riesner, 1987) separate amplified rDNA fragments of the same length but with different base pair compositions. The separation of bands in both DGGE and TGGE is dependent on the decreased electrophoretic mobility of partially melted double stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants or a linear temperature gradient (Muyzer and Smalla, 1998). The PCR-amplified DNA fragments are generally limited in size to 500 bp and are separated on the basis of sequence differences, not variation in length. The number of bands produced during DGGE or TGGE is proportional to the number of dominant species in the sample.

DGGE/TGGE is a method of choice when the desired information does not have to be as phylogenetically exhaustive as that provided by 16S rRNA gene clone libraries, but is still precise to determine the dominant members of microbial communities with medium phylogenetic resolution (Sanz and Kochling, 2007). For environmental or contaminated source samples where microbial diversity is largely unknown (Amann et al., 1995), DGGE/TGGE technique provides the opportunity for the identification of the microbial population through the excision and sequencing of bands (Forney et al., 2004).

DGGE in particular has been widely used for the assessment of microbial community structure in contaminated soil and water in a number of studies (Macnaughton et al., 1999; Chang et al., 2000; Ralebitso et al., 2000; Watanabe et al., 2000; Kleikemper et al., 2002; Cummings et al., 2003; El-Latif Hesham et al., 2006). Apart from microbial community profiling, the DGGE technique has also been used to examine gene clusters such as dissimilatory sulphite reductase beta-subunit (*dsrB*) genes in sulphate-reducing bacterial communities (Geets et al., 2006) and benzene, toluene, ethylbenzene and xylene (BTEX) monooxygenase genes from bacterial strains obtained from hydrocarbon-polluted aquifers (Hendrickx et al., 2006).

The main advantages of DGGE/TGGE are that; it enables the monitoring of the spatial/temporal changes in microbial community structure and provides a simple view of the dominant microbial species within a sample. The limitations of DGGE/TGGE in microbial community studies include; sequence information derived from microbial populations is limited to 500 bp fragments of 16S rRNA sequences which may lack the specificity required for the phylogenetic identification of some organisms (Gilbride et al., 2006); due to the existence of multiple copies of rRNA in an organism, multiple bands for a single species may occur (Nubel et al., 1997); and different 16S rRNA sequences may have identical mobilities. Band intensity may not truly reflect the abundance of microbial population (strong band may just mean more copies) and perceived community diversity may be underestimated. Also, DGGE/TGGE analysis of microbial communities produces a complex profile which can be quite sensitive to spatial and temporal sampling variation (Murray et al., 1998).

3.2.4. Terminal-restriction fragment length polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism is a modification of ARDRA. The PCR primers used in T-RFLP analysis are fluorescently labelled and the resultant PCR products are visualised and quantified (Liu et al., 1997). T-RFLP relies on variations in the positions of restriction sites among sequences and the determination of the length of fluorescently labelled terminal restriction fragments by high-resolution gel electrophoresis on an automated DNA sequencer. The use of fluorescently tagged primers limits the analysis to only the terminal fragments of the digestion (Marsh, 1999). This simplifies the banding pattern, hence enabling the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Tiedje et al., 1999).

The microbial community composition of lake sediments contaminated with copper as a consequence of mine milling disposal over a 100-year period was studied using T-RFLP (Konstantinidis et al., 2003). T-RFLP has also been used to characterize microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer (Reardon et al., 2004) and dechlorinating bacteria from a basalt aquifer (Macbeth et al., 2004).

Recently, Fahy et al. (2005) using T-RFLP observed that the long-term presence of benzene in groundwater reduced bacterial diversity and community structure compared with that of clean groundwater sources. In addition, the reliability of T-RFLP for monitoring microbial populations characterized by low diversity and high relative abundances of a few dominant groups was assessed in a hydrocarbon-polluted marine environment (Denaro et al., 2005).

In contaminated soils, T-RFLP has also been used successfully in describing bacterial communities of polychlorinated biphenyl (PCB) contaminated soils (Fedi et al., 2005) and

microbial communities that reductively dechlorinate TCE to ethene (Richardson et al., 2002).

The use of automated detection systems and capillary electrophoresis in T-RFLP analysis allows high throughput and more accurate quantitative analysis of microbial community samples than any of the genetic fingerprinting method discussed in this paper.

Despite the high resolution and sensitivity, T-RFLP is highly dependent on PCR amplification of 16S rRNA which is affected by DNA extraction method, PCR biases and the choice of universal primers (Kirk et al., 2004). Different enzymes will produce different community fingerprints and incomplete digestion by the restriction enzymes may lead to an overestimation of diversity (Dunbar et al., 2000; Osborn et al., 2000). It is therefore important to use at least two to four restriction enzymes (Tiedje et al., 1999) as T-RFLP profiles generated by a single restriction enzyme in a complex microbial community may lead to erroneous conclusions about the abundance of a particular strain or species (Osborne et al., 2006).

4. Fluorescent *in situ* hybridization (FISH)

FISH is a method used to quantify the presence and relative abundance of microbial populations in a community sample. Microbial cells are treated with fixative, hybridized with specific probes (usually 15–25 bp oligonucleotide-fluorescently labelled probes) on a glass slide then visualised with either epiflourescence or confocal laser microscopy (Sanz and Kochling, 2007).

Hybridization with rRNA-targeted probes enhances the characterization of uncultured microorganisms and also facilitates the description of complex microbial communities (Edgcomb et al., 1999). FISH is a taxonomic method that is mostly used for the examination of whether members of a specific phylogenetic affiliation are present and provides direct visualisation of uncultured microorganisms and also facilitates the quantification of specific microbial groups (Sanz and Kochling, 2007). FISH use alone does not provide any insight to metabolic function of microorganisms. However, it can be coupled with other techniques such as microautoradiography to describe the functional properties of microorganisms in their natural environment (Wagner et al., 2006).

Two types of FISH probes based on conserved or unique regions of 16S rRNA genes can be developed; domain-or-group-specific probe and strain-specific probes. Domain-or-group-specific probes discriminate or identify members of larger phylogenetic group, while strain-specific probes quantify or assess the abundance of a specific species or strain within a microbial community (Dubey et al., 2006). Wagner et al. (1993, 1994) used both group- and species-specific rRNA-targeted oligonucleotide probes to define probacteria and *Acinetobacter* from activated sludge respectively. Richardson et al. (2002) combined group-specific FISH and T-RFLP in the characterization of microbial communities engaged in TCE biodegradation. From the FISH analysis, the authors observed that the number of organisms such as *Cytophaga*, *Flavobacterium* and *Bacteroides* were abundant than the TCE degrader *D. ethenogenes* in the microbial consortium. However, the lack of functional gene analysis in the study meant that the relative abundance of these organisms and their ecological importance for TCE biodegradation could not be established.

FISH techniques are often used in conjunction with other genetic fingerprinting methods such as DGGE (Straub and Buchholz-Cleven, 1998; Onda et al., 2002; Ebie et al., 2002; Collins et al., 2006) and T-RFLP (Richardson et al., 2002; Kotsyurbenko et al., 2004; Jardillier et al., 2005; Collins et al., 2006) for the enumeration and characterization of microbial population from contaminated sources.

The draw back of FISH is that a limited number of probes can be used in a single hybridization experiment and background fluorescence can be problematic in some samples (Dubey et al., 2006; Sanz and Kochling, 2007). A prior knowledge of the sample and the microorganisms most likely to be detected is necessary (*i.e.* rRNA sequence) for the design of specific probes. Finally, the design, validation, and optimization of hybridization conditions for a new probe and quantification of specific microbial groups can be time consuming and complex (Sanz and Kochling, 2007).

5. Techniques linking microbial identity to ecological function

5.1. Stable isotope probing (SIP)

Stable isotope probing (SIP) enables the characterization or identification of microbial populations actively involved in specific metabolic processes in the environment with the aim of linking the microbial phylogeny with function (Radajewski et al., 2000). SIP involves the incorporation of stable isotope-labelled substrates into cellular biomarkers that can be used to identify organisms assimilating the substrate (Boschker and Middelburg, 2002). Labelled biomarkers such as PLFA (Bull et al., 2000), rRNA (Manefield et al., 2002) and DNA (Radajewski et al., 2000) have been developed and used in microbial ecological studies.

SIP techniques have successfully been applied for the characterization and identification of active methanotroph microbial populations in soda lakes (Lin et al., 2004), microbial communities in activated sludge (Manefield et al., 2005), isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater (Kasai et al., 2006), identification of uncultured bacteria from PAH contaminated soil (Singleton et al., 2006) and molecular analysis of arsenic reducing bacteria in arsenic rich groundwater sources (Lear et al., 2007).

Apart from microbial identification, DNA-SIP techniques can also be used in the isolation of large DNA fragments from uncultured microbial communities for metagenomic analysis (Dumont et al., 2006).

SIP like any other techniques used for microbial characterization has limitations in relation to the required sensitivity, methods used for extracting and purifying DNA/RNA and down stream analysis of the labelled material. Insufficient substrate incorporation and incubation with DNA/RNA-SIP can lead to enrichment bias that does not represent the natural substrate metabolism in the environment and there is a potential for enhanced cross-feeding of substrate (Neufeld et al., 2007).

Finally, PLFA-SIP lacks the taxonomic identification associated with microorganisms as previously discussed under Section 2.1.

5.2. Reverse sample genome probing (RSGP)

This approach involves the isolation of chromosomal DNA in pure culture from standard microbial species followed by cross hybridization (Voordouw et al., 1991). It requires pure bacterial cultures to develop an array, which can then be used to analyze the extracted DNA. Genomes that exhibit more than 70% cross hybridization are often regarded as the same species (Greene and Voordouw, 2003). A genome array or master filter is then prepared from the genome with less cross hybridization. Community DNA probes from an area of interest are prepared and are then used to hybridize with the genome array (Voordouw et al., 1991; Greene and Voordouw, 2003). Since its introduction, RSGP has been used in the characterization of sulphate-reducing bacteria (SRB) from an oil field (Voordouw et al., 1991, 1992) and in the identification of hydrocarbon-degrading bacteria in soil (Shen et al., 1998).

RSGP has also been used in the characterization of microbial communities enriched with a mixture of aromatic hydrocarbons (Greene et al., 2000). RSGP analysis from contaminated and uncontaminated soils revealed no differences in the microbial population while the succession of microbial populations as detected by RSGP showed a reduction in *Pseudomonas* and *Rhodococcus* spp. and eventual domination by *Alcaligenes* spp. (Greene et al., 2000). Similar observations of a decrease in microbial succession as a result of contamination have been reported using other fingerprinting techniques (Frostegard et al., 1993; Fahy et al., 2005). In a more recent RSGP study investigating TCE contaminated soils, it was observed that TCE affected the microbial community composition when TCE was actively metabolised in soils (Hubert et al., 2005).

The application of RSGP analysis to environmental samples is limited to the development of suitable master filters or genome arrays representative of the microbial community (Voordouw et al., 1991). The major draw back of this technique is the choice of organism to be used as the standard in the preparation of the genome arrays. In contaminated soil and water, where the identity, physiology and biochemistry of the degrading microbial populations are unknown or non-culturable, the RSGP application in such an environment is very limited.

6. Microarray technologies

DNA microarray technology is a very powerful taxonomic and functional tool that is widely used to study biological processes, including mixed microbial communities. This technique is similar to FISH, but provides a means for simultaneous analysis of many genes (Cho and Tiedje, 2002). DNA microarray is a miniaturized array of complementary DNA probes (~ 500–5000 nucleotides in length) or oligonucleotides (15–70 bp) attached directly to a solid support, which permits simultaneous hybridization of a large set of probes complementary to their corresponding DNA/RNA targets in a sample (Fodor et al., 1993).

Microarray technology has been used successfully in the analysis of global gene expression in pure culture studies (Schena et al., 1995; DeRisi et al., 1997), but is complicated for environmental samples due to numerous challenges such as specificity, sensitivity and quantification (Zhou and Thompson, 2002). Despite these challenges, three major forms of environmental microarray formats namely, functional gene arrays (FGA) (Wu et al., 2001; Rhee et al., 2004; Tiquia et al., 2004), community genome arrays (CGA) (Wu et al., 2004, 2006) and phylogenetic oligonucleotide arrays (Loy et al., 2002; El Fantroussi et al., 2003; Chandler et al., 2006) have been developed for microbial community analyses of environmental samples.

Functional gene arrays (FGA) identify or measure genes encoding key enzymes in a metabolic process (Zhou, 2003; Rhee et al., 2004). Such an approach provides vital information about the presence of important genes as well as the expression of the genes in the environment by measuring the mRNA (Gentry et al., 2006). Many studies have used the FGA approach to investigate microbial involvement in environmental processes such as nitrogen fixation and nitrification (Taroncher-Oldenburg et al., 2003; Steward et al., 2004; Gentry et al., 2006). For biodegradation of contaminants, FGA techniques have been developed for the detection of specific aromatic oxygenase genes in a soil community degrading PCB (Denef et al., 2003) and the presence and expression of naphthalene-degrading genes from soil contaminated with PAH (Rhee et al., 2004).

Community genome array (CGA) is similar in concept to reverse sample genome probing (RSGP) (Voordouw et al., 1991) except that CGA uses nonporous hybridization surfaces and fluorescence based detection systems for high throughput analysis but decreased sensitivity (Wu et al., 2004). Wu et al. (2004) pioneered the development and testing of CGA as a tool to detect specific microorganisms within a natural microbial community. CGA has been shown to achieve species-to-strain-level differentiation depending on hybridization temperature and has an added potential for the determination of genomic relatedness of isolated bacteria (Gentry et al., 2006). The major disadvantage of CGA is similar to RSGP in that, culturable organisms are needed in the array preparation thus making the CGA application on the field apart from laboratory studies almost impossible.

Phylogenetic oligonucleotide arrays (POA) rely on the use of 16S rRNA as already discussed in Section 3.2 for the identification of microorganisms in the environment. Due to a high throughput capacity of microarrays and the availability of extensive rRNA sequence databases, POA provides a very convenient means of simultaneously identifying many microorganisms from a sample. Several studies have employed POA in environmental investigations of microbial populations in water (Rudi et al., 2000; Castiglioni et al., 2004), soil (Small et al., 2001; Loy et al., 2004) and activated sludge (Adamczyk et al., 2003).

The application of microarrays in environmental microbiology, specifically in the examination of microbial populations engaged in biodegradation has the potential for organism identification as well as defining their ecological role (Wu et al., 2001; Rhee et al., 2004).

However, more rigorous and systematic assessment and development are needed to realize the full potential of microarrays for microbial detection and community analysis (Zhou, 2003). Microarrays currently detect only the dominant populations in many environments (Rhee et al., 2004). In addition, probes designed to be specific to known sequences can cross-hybridize to similar or unknown sequences and may produce misleading signals (Gentry et al., 2006). Moreover, soil, water and sediments often contain humic acids and other organic materials which may inhibit DNA hybridization on microarrays (Saleh-Lakha et al., 2005). Finally, limitations in quality RNA extraction from many environmental samples imply that advances in RNA extraction and purification and amplification methods are needed to make microarray gene expression analysis possible for a broader range of samples (Gentry et al., 2006).

7. Conclusion

Culture independent molecular tools applied for the analyses of mixed microbial communities from contaminated soil and water have undoubtedly advanced our knowledge and understanding about microbial diversity and biochemistry of contaminants catabolism. Molecular techniques have contributed significantly to the detection and identification of microorganisms and catabolic genes especially in non-culturable organisms as well as the quantification or enumeration of the relative abundance of organisms from contaminated sources.

Currently, there is no single molecular technique that can adequately describe the entire microbial diversity and the associated catabolic genes at contaminated sites (Table 1). Each molecular technique has its own limitations with respect to the introduction of biases for the investigation of microbial diversity and catabolic gene analysis. Several factors controlling the growth, microbial interaction and metabolism of microorganisms in contaminated environment are poorly understood (Lovley, 2003).

Techniques such as microarray and SIP provide much needed information about microbial phylogeny in relation to ecological function. However, several key issues will need to be addressed with these techniques in order to improve the accuracy and sensitivity in relation to microbial studies from contaminated sources.

Currently, gene expression analysis relies on extracted RNA from soil or water samples. It is worthy of note that, RNA is an indirect measurement of activity as oppose to the translated

Table 1
Summary of the various molecular techniques used in the study of soil and water microbial population at contaminated sites

Techniques	Application	Limitations	References
Phospholipids fatty acids (PLFA)	• Elucidate the structure and physiological state of microbial populations	• Many fatty acids are common to different microorganisms	Vestal and White (1989)
Simple, multiplex and real-time PCR	• Target amplification and quantification of gene of interest	• Presence of inhibitors of PCR reaction • Non-specific primer annealing • Primer artifacts and DNA contamination	Liesack et al. (1991)
Amplified ribosomal DNA restriction analysis (ARDRA)	• Simple method for microbial identification and ecological studies	• Microbial community fingerprint is highly influenced by the restriction enzyme • Unable to identify specific phylogenetic group within a community fingerprint	Gich et al. (2000); Vaneechoutte et al. (1992); Spiegelman et al. (2005)
Ribosomal intergenic spacer analysis (RISA)	• Phylogenetic identification of organisms	• It has relatively small database for comprehensive identification of non-culturable organisms	Spiegelman et al. (2005)
Denaturing gradient/temperature gradient gel electrophoresis (DGGE/TGGE)	• Profile microbial communities and identify populations through excision and sequencing of bands	• Sequence information from microbial population is limited to 500 bp fragment of 16S rRNA	Muyzer et al. (1993)
		• Heterogeneity in 16S rRNA exist in some organism and may produce multiple bands on gels	Nubel et al. (1997); Gilbride et al. (2006)
Terminal-restriction fragment length polymorphism (T-RFLP)	• Possibility of high throughput and quantification of microbial community	• Multiple restriction enzymes are needed to describe a microbial population	Osborne et al. (2006)
Fluorescent *in situ* hybridization (FISH)	• Quantification and identification of specific organism *in situ*	• Limited number of probes (about 3) can be used in an experiment • Background fluorescence can interfere with detection of organism • Probe permeability	Dubey et al. (2006)
Stable isotope probing (SIP)	• Directly link microbial phylogeny with function	• Lack sensitivity • Enrichment bias may not reflect substrate metabolism in the environment	Radajewski et al. (2000); Neufeld et al. (2007)
Reverse sample genome probing (RSGP)	• It identifies and characterises bacteria	• Not suitable for environment where prior pure culture isolation has not taken place	Voordouw et al. (1991)
Microarray technologies	• Identify organism and define its ecological role	• Non-specific hybridization may produce misleading signals • Lack of specificity, sensitivity and quantification	Zhou and Thompson (2002); Gentry et al. (2006)

proteins which measures the active biological function. By directly analysing the proteome (proteomics) of the environmental sample rather than looking at DNA/RNA, the active pool of microorganisms involved in the biological breakdown of contaminant as well as the functional contribution of certain proteins in the environmental process can be identified (Aebersold and Mann, 2003; Tyers and Mann, 2003). Although the proteomics approach in environmental studies has received little attention (Lopez-Barea and Gomez-Ariza, 2006), it has an enormous potential to become a very powerful tool in describing the biology and ecological functions of many environments in the future.

Finally, recent advances in metagenomics in which collective microbial genomes are sequenced and screened for functional genes and phylogenetic markers (Eyers et al., 2004; Handelsman; 2004; Garcia-Martin et al., 2006), provide an opportunity for linking microbial diversity with function. In this regard, the development of metagenomic arrays for uncultured microorganisms from contaminated soil and water can greatly improve our understanding of microbial interaction and metabolism to facilitate the development of suitable bioremediation strategies for environment clean up.

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