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ENVIRONMENTAL RESEARCH BRIEF

The Use of Molecular and Genomic Techniques Applied to Microbial Diversity, Community Structure, and Activities at DNAPL and Metal Contaminated Sites

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Abstract

The myriad of *in situ* subsurface remediation technologies currently in practice result in responses by indigenous or introduced microbial communities that can be measured with respect to alterations in biomass, structure, diversity, enzymatic activity, or consequent stress. Providing a highly developed understanding of subsurface ecologies has shown great promise, through the use of molecular and genomic techniques, in providing new approaches to soil and ground-water investigations by reducing the inherent parameter variability of more traditional approaches in bench and pilot scale investigations as well as full scale applications. In addition to providing a background on classic molecular and genomic sciences, the results and interpretation of their application to field-scale subsurface remediation activities is discussed.

Background

A wide variety of *in situ* subsurface remediation strategies have been developed to mitigate contamination by chlorinated solvent dense non-aqueous phase liquids (DNAPLS) and metals. Geochemical methods include: zerovalent iron emplacement, various electrolytic applications, electrosmotic mobilization, as well as the addition of various oxidants, or reductants, chelating agents, and surfactants. Physical methods (primarily for the chlorinated solvents) include *in situ* heating by various methods, steam injection/vacuum extraction, and gas sparging. Bioremediation methods applied to these classes of contaminants can be broadly categorized as active (enhanced) or passive (monitored natural attenuation; MNA) approaches. In the former, additions of carbon substrates including organic esters, acids, mulch, emulsified oils, or fats are intended to stimulate the growth of microorganisms capable of degrading chlorinated solvents or causing immobilization of metals, via reduction or removal as insoluble precipitates. Also, active approaches may be supplemented by the addition of cultured microorganisms capable of carrying out degradation or immobilization under selected conditions. This approach is called bioaugmentation.

Regardless of the basis for the remedial action, it is critical to recognize that subsurface microbial communities will respond to both the presence of the contaminants or the engineered manipulation of subsurface conditions. Responses of the microbiota may include stress or changes in diversity, community structure, biomass, and activity among others. Their response may have profound effects on remedial progress, effective time-frames to reach regulatory decision points, and consequences for the environment.

Therefore, it is quite important for subsurface scientists, engineers, regulatory officials, policy makers and the general public to recognize the roles that microorganisms play during *in situ* remedial efforts and the value of subsurface ecology to soil and water resources quality. It is also important that we employ and improve on modern methods to measure microbial responses to contamination and remediation strategies so that subsurface ecology may be better understood. The molecular and genomic techniques to identify and track the response of microbial ecosystems to human influences hold great promise in our efforts to protect the environment.

One should keep in mind that the inherent variability in subsurface hydrogeology, flow phenomena and geochemical parameters are of the order of ±20% (combined sampling and analytical error). Measured contaminant distributions can easily vary as much as ±140% over the time frame of initial site characterization or tracer release (Keeley and

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Barcelona, 2006). Therefore we should carefully consider how the measures of microbial responses to the presence of contaminants might be expected to vary considerably over time and space.

The purpose of this document is to provide an overview of the molecular and genetic techniques applicable in groundwater investigations and to present examples of the use of these techniques to draw site-specific inferences.

Overview of Molecular and Genomic Analysis Methods

Classical techniques to identify, measure, or estimate aspects of soil and subsurface microbial ecology (i.e., characterizing strains, active biomass, diversity, etc.) normally involved isolation followed by culturing the organisms. These methods are inadequate for complex soil or sediment environments, particularly when they are contaminated. Viable amounts of bacteria in environmental samples determined with classical culturing methods represent only a small fraction (0.1 to 10%) of the active microbial community (Amann et al., 1995; White et al., 1997). In the last twenty years, improvements in instrumentation, more selective and specific methods, as well as advanced statistical and mathematical interpretational tools have been developed. This progress has given subsurface scientists exciting opportunities to improve our understanding of indigenous microorganisms and their impact on the environment.

It should be clear that a major challenge in creating or utilizing an environmental diagnostic tool is to target and identify relevant biomarkers to provide the desired resolution (i.e., sensitivity and specificity). A biomarker however can be defined as any molecule that is specific to the microorganism(s) or the process of interest. A list of molecular and genomic tools is provided in Table 1. Most techniques target nucleic acids; however lipids and proteins are other targets with promising expansion in environmental settings (Table 1).

Table 1. Selected Molecular and Genomic Techniques Applied at Hazardous Waste Sites

Target	Technique	Observations	References
Molecular			
Lipids	PLFA	Sensitive and quantitative approach with available database support; knowledge of process-specific biomarkers and sophisticated and expensive equipment required. * (y), ** (y), ***(y)	Chang et al. (2001) *Appl. Environ. Microbiol.* 67:3149-3160.
Contaminants	CSIA	*In situ* monitoring tool, analyzes naturally occurring isotope ratios (C, N, H, and O) of dissolved contaminants, sophisticated and expensive equipment required. * (y), ** (y), ***(y)	Meckenstock et al. (2004) *J. Contam. Hydrol.* 75: 215-255.
Proteins	Dehydrogenase Enzyme Assay	Quantitative and inexpensive approach, time-consuming, useful for anaerobic soil and sediment metabolic monitoring. * (y), ** (y), ***(y)	Skujins & McLaren (1967) *Science* 22(158):1569-1570.
Genomic			
16S/fg	Direct PCR	Qualitative conventional approach, provide consistent information. * (n), ** (y), ***(y)	Major et al. (2002) *Environ. Sci. Technol.* 36:5106-5116.
16S/fg	Nested PCR	To enhance sensitivity, two successive PCR amplifications are made; an internal primer set is required. * (n), ** (y), ***(y)	Lendvay et al. (2003) *Environ. Sci. Technol.* 37:1422-1431.
16S/fg	DGGE	High resolution with group-specific primers, separates PCR amplicons due to their melting behavior in polyacrylamide gels, only useful for targeting a small region within a target gene. * (n), ** (y), ***(y)	Muyzer & Smalla (1998) *Antonie van Leeuwenhoek* 73:127-141.

Target	Technique	Observations	References
16S/fg	PCR/Cloning/ RFLP	Lengthy and time-consuming because of cloning (Transfer of a gene of interest into *E. coli* as a foreign host. * (n), ** (n), ***(y)	Macbeth et al. (2004) *Appl. Environ. Microbiol.* 70:7329-7341.
16S/fg	PCR/Cloning/ Sequencing	Most widely used technique, lengthy and time-consuming because of cloning (Transfer of a gene of interest into *E. coli* as a foreign host. * (n), ** (n), ***(y)	Chandler et al. (1997) *FEMS Microbiol. Rev.* 20:217-230.
16S	T-RFLP	Easy to perform, widely used, low resolution community fingerprints (16S rRNA gene) tool that only analyzes the terminal fragments generated in the restriction digests of PCR-amplified target gene(s). * (n), ** (n), ***(y)	Tiedje et al. (1999) *Appl. Soil Ecol.* 13(2):109-122.
16S/fg	RTmPCR	Quantitative technique for bioremediation monitoring in real time based on various detection chemistries including Taqman probes and SYBR Green. * (y), ** (y), ***(y)	Lendvay et al. (2003) *Environ. Sci. Technol.* 37:1422-1431.
16S	RTm T-RFLP	Quantitative fingerprinting of all microbial ribotypes in a community. * (y), ** (y), ***(n)	Yu et al. (2005) *Appl. Environ. Microbiol.* 71:1433-1444
fg mRNA	RT-RTm PCR	Provides sensitive detection of transcripts since RT-PCR quantifies the initial amount of starting template (specific mRNA) used in a PCR reaction. * (y), ** (y), ***(n)	Johnson et al. (2005) *Appl. Environ. Microbiol.* 71:3866-3871.
16S	FISH	Targets 16S rRNA gene(s); excellent tool but perhaps difficult to apply in bioremediation. Variations of this tool: microautoradiography (MAR-), substrate tracking autoradiography (STAR), Microautoradiography (MICRO)-FISH combine ^{14}C autoradiography to identify active cells. Catalyzed Reporter Deposition (CARD)-FISH can increase the sensitivity and allow the detection of functional genes and mRNA. * (y), ** (y), ***(n)	(1)DeLong et al. (1999) *Appl. Environ. Microbiol.* 65(12): 5554-5563. (2) Ouverney & Fuhrman (1999) *Appl. Environ. Microbiol.* 65:1746-1752. (3) Bakermans & Madsen (2002) *J. Microbiol. Meth.* 50:75-84.
16S/fg	*In situ* PCR	The target gene is PCR-amplified inside the cell followed by FISH detection; technique is technically challenging and must be optimized for different organisms. * (y), ** (y), ***(n)	Tani et al. (2002) *Appl. Environ. Microbiol.* 68:412-416.
16S/fg	Microarrays (PhyloChip Functional Gene Array)	This excellent research tool is a high throughput technology which allows the parallel screening of large numbers of genes. Results are non-quantitative and its use for environmental samples presents challenges in terms of specificity, sensitivity, and quantification. Sensitivity can be satisfactory if combined with PCR but results require cautious interpretation. * (y/n), ** (y/n), ***(n)	Wagner et al. (2006) *Microbial Ecol.* 53:498-506.

Note: *Quantitative: **
*Potential Application in Bioremediation: ***
*Applied in Bioremediation: ****
(y): Yes; (n): No
16S: 16S rRNA; fg: Functional Gene

Abbreviations: *CSIA, Compound-Specific Stable Isotope Analysis; DGGE, Denaturing Gradient Gel Electrophoresis; Direct PCR, Direct Polymerase Chain Reaction; FISH, Fluorescent In Situ Hybridization; MAR-FISH, Microautoradiography Fluorescent In Situ Hybridization; PhyloChip, DNA microarrays; PLFA, Phospholipids Fatty Acid; RFLP, Restriction Length Polymorphism; RTm PCR, Real-Time PCR; RTm T-RFLP, Real-Time PCR Terminal Restriction Fragment Length Polymorphism; T-RFLP, Terminal Restriction Fragment Length Polymorphism.*

Molecular Methods to Characterize Subsurface Microbial Communities

Lipids

Lipids make up ~ 5 to 9% (dry wt.) and the highest number of molecules in bacterial cells (Fritsche, 1999; Madigan et al., 2000). The phospholipids are the most abundant, providing structure to the lipid bilayer of microbial cell membranes (Ratledge and Wilkinson, 1988). The simplest intact phospholipid (IPP) is phosphatidic acid:

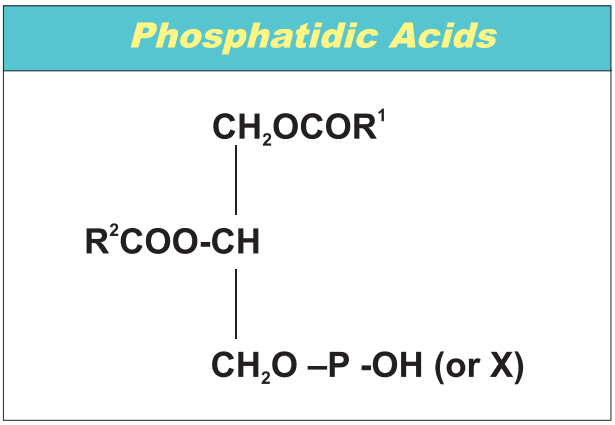

Figure 1. Phosphatidic Acids

Where R^1 and R^2 are often fatty acid esters, or ethers (i.e., in Archaea) on the three-carbon glycerol backbone. The X group can be a proton (as it is above), an alcohol, or various alkyl, alkylamine, amino acid, (e.g., serine), or other functional groups (Ratledge and Wilkinson, 1988). This part of the molecule is often referred to as the polar head group. Bacterial fatty acids are normally 12 to 24 carbons long. They are called phospholipid-linked fatty acids or PLFA. Often the phospholipids have one saturated fatty acid and one unsaturated acid. Though the degree of unsaturation may range up to six double bonds, polyunsaturated fatty acids are found in only a few bacterial groups (e.g., cyanobacteria) as well as eukaryotes, fungi, plants, and animals. The fatty acylchain may also contain branching as well as cyclopropane rings. A brief example of the nomenclature of PLFA compounds should prove helpful. Ex. 18:1w9c is a PLFA with eighteen carbons, 1 double bond between the 9 and 10 carbons from the methyl end of the molecule in the *cis* configuration. Branching and the incorporation of hydroxyl groups into the fatty acid chain call for a somewhat more complicated notation system.

It should also be noted that enzymatic hydrolysis of membrane phospholipids to diglycerides may not be immediate upon cell death particularly for those with alkyl ether side chains (Harvey et al., 1986). PLFA or IPP determinations require extraction of the lipid fractions from the bacteria (i.e., neutral lipids, glycolipids, and phospholipids) with organic solvents and further separation into three fractions including neutral or polar lipids and glycolipids.

For IPP, the fractions may be separated by liquid chromatography followed by electrospray ionization mass spectrometry LC/ESI/MS (Fang and Barcelona, 1998); or matrix assisted laser desorption/ionization time of flight mass spectrometry, MALDI-TOFMS (Ishida et al., 2002). There is significantly more useful information for microbial characterization in the intact phospholipids, but the tradeoffs are increased analysis time and more complex instrumentation.

In the case of PLFA, the lipid fractions are treated by saponification to separate the fatty acids and the backbone, methyl ester formation, extraction of the fatty acid methyl esters (FAMES), followed by analysis by gas chromatography (GC) or gas chromatography/mass spectrometry (GC-MS). Commercial kits are available to expedite FAME determinations (Instant FAME, www.midi-inc.com).

Interpretations of IPP or FAME data may vary as a number of variables may affect the total PLFA and the nature of the fatty acids. Among the variables are: culture conditions, carbon substrate, physiological or nutritional stress and growth phase.

With these potential complicating factors in mind, it is possible to estimate non-viable vs. viable biomass by determination of the diglyceride fatty acids and phospholipid fatty acids, respectively (Piotrowska-Seget and Mrozik, 2003). For viable biomass we can convert the phospholipid (µg/g sediment) to cells/µg PLFA using conversion factors 5.47×10^7 cells/µg PLFA from Balkwill et al. (1988), but see White et al. (1997) for an overview of some of the difficulties with making such conversions.

PLFA or IPP have been employed as biomarkers for different genera, species or microbial groups for over twenty years. For example, if ethanolamine and glycerol are predominant head groups and unsaturated this indicates that the IPP were from bacteria. Signature lipid biomarker analysis may be summarized (modified from Piotrowska Seget and Miozik (2003)) in Figure 2.

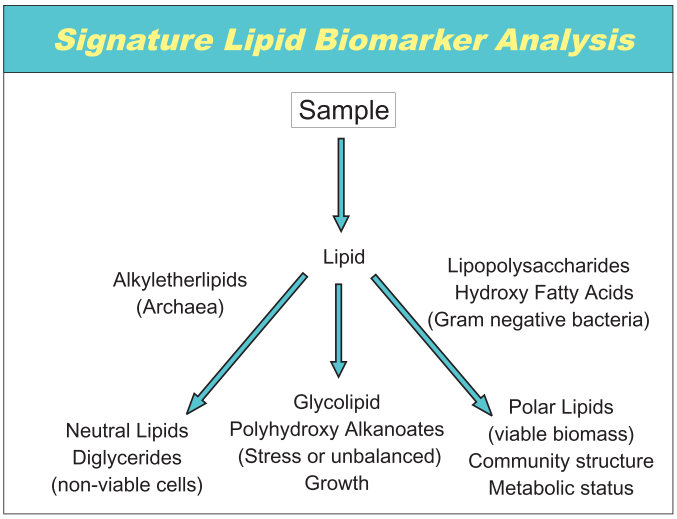

Figure 2. Signature Lipid Biomarker Analysis

Some characteristic biomarkers for microbial groups are shown in Table 2, which would enable one to examine community structure.

Table 2. Characteristic Biomarkers for Microbial Groups

Group	Fatty Acid Signature
Microeukaryotes	Polyunsaturated
Aerobic prokaryote	Monounsaturated
Gram-positive bacteria	Saturated and branched C_{16}-C_{19}
Anaerobic bacteria	Saturated and branched C_{16}-C_{19}
Sulfate-reducing bacteria	Saturated and branched C_{16}-C_{19}
Gram-negative bacteria	Cyclopropyl
Actinomycetes	Methyl branched (C_{10})
Cyanobacteria	Polyunsaturated
Fungi	C_{24}-C_{26} unsubstituted, linoleic acid

Microbial community response to metal (i.e., Cd, Cu, Ni, Pb, Zn) exposure in soils was tracked by Frostegard et al. (1996), who noted an increase in methyl branched fatty acids which reflected that increased actinomycetes were present. Fang et al. (2000a; 2000b) examined IPP in five strains of *Pseudomonas* noting significant changes in phospholipid content and composition as an adaptive response to toluene exposure. The results suggested that dissimilar bacteria utilized different mechanisms to adapt to the presence of the compound. They also showed that IPP was superior to FAME analysis to distinguish the individual strains using principal component analysis. However, the identification of all microbial species in a sediment sample with IPP or PLFA would not be possible due to the overlap of the lipids among species (White and Ringelberg, 1990).

Catalytic Proteins

Enzyme molecules are specialized proteins that catalyze nearly every biological reaction. These molecules are also highly specific in their catalytic ability in that each enzyme catalyzes only a single or a set of closely related reactions. Although enzymatic reactions are investigated for a variety of reasons, molecular microbiologists are often concerned in the detection of enzymatic activity as a function of various biological parameters. In a search which might be useful for comparison and prediction of soil microbiological activities, where they are associated with certain horizons and change significantly in their vertical distributions, the dehydrogenase activity emerges as a useful criterion for the characterization of soil biological state (Skujins and McLaren, 1967; Skujins, 1973). The effectiveness of the dehydrogenase measurement as a general index of metabolic rate(s) in subsurface microbial communities is due to their abundance among the sulfate-reducing bacteria (SRB), a consortium responsible for bioattenuation of contaminants under anaerobic conditions. The index is particularly useful in the augmented subsurface settings (i.e., PRBs) as shown by (Benner et al., 1999) and can be interpreted as endogenous respiration in the intrinsic environments (Ladd, 1978).

Nucleic Acid and Genomic Methods to Characterize Subsurface Microbial Communities

With the accelerated developments in sequencing capabilities, database expansion, bioinformatics, environmental genomics, transcriptomics, metabolomics, and proteomics; there exists a broad range of genomic tools to evaluate uncultured (i.e., not-yet-cultured) microorganisms. These tools, which have originally been developed for use in the medical fields, can now serve microbial ecologists with detection of specific microorganisms or characterization of an entire microbial community involving complex interactions of all community members. Environmental microbiologists are also equipped with an array of tools for detecting process-specific biomarkers and tracing genes responsible for degradation of contaminants (Löffler and Ritalahti, 2001). Probing the catabolic activity of the key microorganisms responsible for detoxification pathways is an ideal indicator for evaluating bioremediation progress and setting time frames for cleanup goals. In fact, the utility of the genomic biomarkers provides opportunities to advance bioremediation from a largely empirical practice into a predictable science (Lovley, 2003).

As may be observed in Table 1, the genomic tools include RNA/ribosomal DNA detection methods, quantitative PCR (i.e., polymerase chain reaction) amplification catalyzed reporter deposition, fluorescence *in situ* hybridization (FISH) (Cottrell and Kirchman, 2004) among other gene probing techniques.

It is generally believed that DNA provides evidence for the presence/absence of microorganisms and metabolic potential within the environment; mRNA provides specific activity and expression of metabolic processes or functions; and rRNA is an indicator of cell activity and viability (Olsen and Tsai, 1992; Stahl, 1997). Therefore, the genetic methods depend upon their ability to directly and representatively isolate DNA, mRNA, and rRNA, from contaminated sediment, soil, or water samples. At one time, this was accomplished by culturing organisms in the laboratory. However, as described earlier, the utility of cultural techniques in the community characterization should be considered as less than 10% of the microorganisms present in environmental samples which grow in culture media (White et al., 1997; Torsvik, et al., 1990) leading to a gross misrepresentation. A detailed work plan to define sampling and sample handling procedures should be in place prior to the selection of

genomic tools to ensure that the integrity of the biomarker(s) of interest is maintained. These strategies should address spatial and temporal sampling locations, sampling procedures, collection of solid versus liquid samples, sample transportation and storage, and sample processing.

As most environmental contaminants are typically located in saturated zones, more modern methods to recover nucleic acids are being developed to target solid matrices (i.e., soil, sediment, aquifer material). These techniques are normally a succession of isolation and purification steps. An example of such a scheme is shown in Figure 3 after the work of Ogram et al., 1987, who developed a whole sediment method. Previous methods utilized homogenization and fractional centrifugations. The purification scheme is self-explanatory.

In cases where little organic matter is available, dialysis may be employed to remove potential interference from inorganic material (Ogram et al., 1995). The less robust nucleic acid groups mRNA and RNA are accordingly isolated under less strenuous conditions (Stapleton et al., 1998).

More difficulty may be encountered in the preparation of sufficient amounts of genomic DNA from very small, low biomass samples as encountered in the subsurface, or where the efficiency of DNA isolation is limited. The recent robust tools to overcome some of these difficulties include commercially available kits to isolate microbial DNA from soil and other solid substrates. Once the nucleic acid fraction has been isolated and purified, amplification by PCR to develop gene probes and hybridization reactions to genetically match the probes may be performed (Steffen and Atlas, 1991; Innis and Gelfend, 1990).

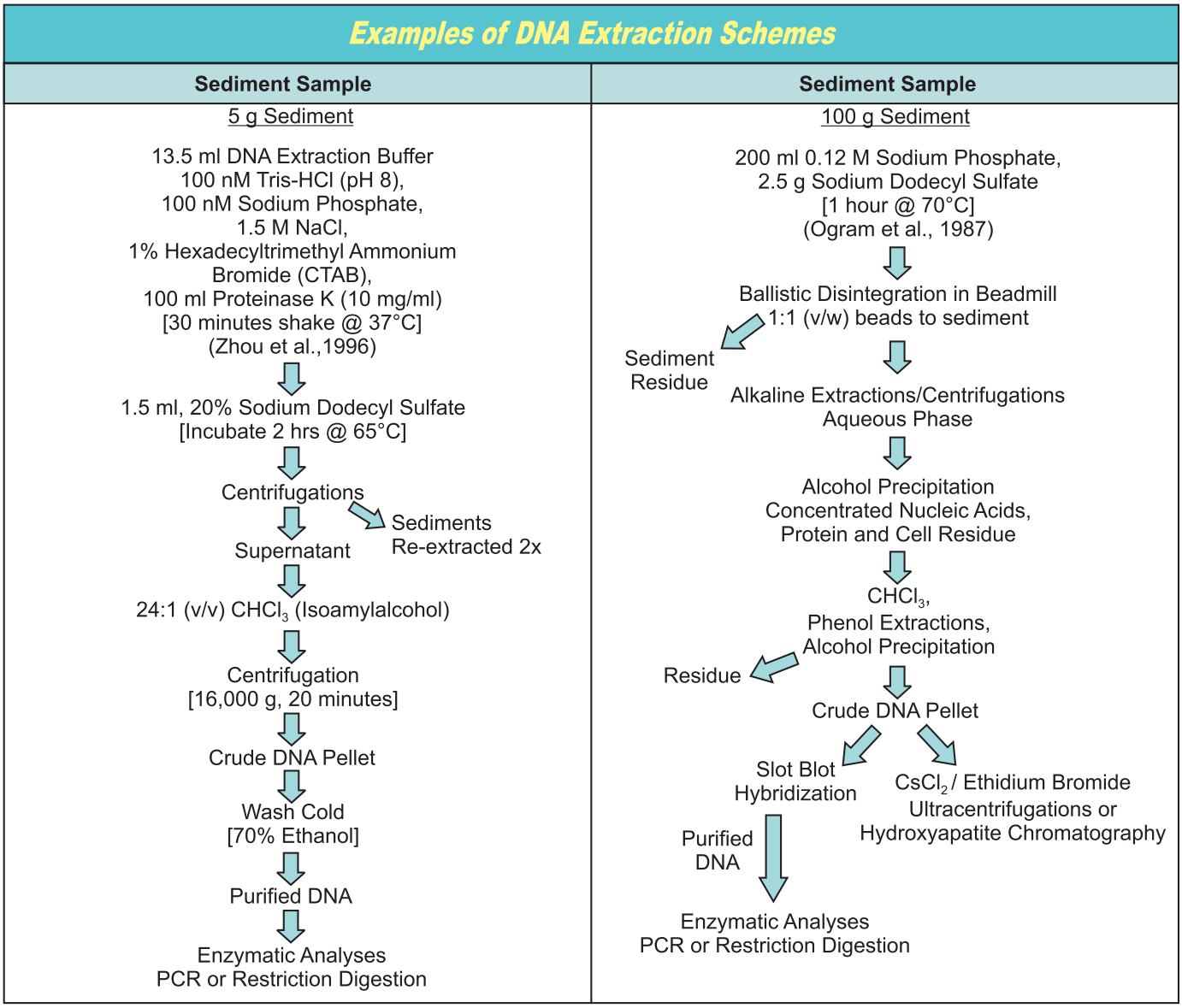

Figure 3. Example of DNA Extraction Scheme

PCR, employing a thermostable DNA polymerase, is capable of amplification of millions of copies of a specific DNA fragment that has been identified as the target sequence. Amplification is guided by a primer sequence, usually 10 to 25 bases in length; which generates a 3´ hydroxyl group facing the target sequence. Successive heating and cooling in the PCR reaction give the technique its amplification power but reagent concentrations, temperatures and cycle time are quite important (Innis and Gelfand, 1990).

Primers have been developed for a variety of genes including the universal 16S rDNA oligonucleotide (Stahl et al., 1988) that is useful in obtaining biomass estimates and normalizing specific genotypes as a percentage of the total population. Its use allows one to minimize variability in sample types or extractions and provides a basis of comparison for different sites, contaminant mixtures, or laboratories.

Catabolic gene probe values can be normalized using the 16S rDNA gene (total community value) to compare samples over space and time. The underlying assumptions are that there are seven copies of the 16S rRNA oligonucleotide per chromosome (Smith et al., 1987), however, the actual number can vary from one to fifteen, depending on the organism (Klappenbach et al., 2001). For example, in a study of accelerated anaerobic degradation in a mixed chlorinated and aromatic hydrocarbon plume at Dover Air Force Base, biomass estimates ranged from 8.78×10^6 organisms/g sediment (uncontaminated) to $2.11 \times 10^9 \pm 2.27 \times 10^7$organisms g/sediment (center of anaerobic arēa) (Stapleton et al., 1998). In the same study, catabolic gene probe data showed increased biomass of organisms possessing selected degradative enzymes.

The results developed with gene probes should be interpreted carefully as some DNA probes are susceptible to cross-hybridization with other genes and one may be misled as to the identity of the organisms responsible for degradation. Another caveat is that the "copy number" of 1 for the gene probes and 7 for 16S rDNA may vary substantially in different species of soil bacteria (Farelly et al., 1995).

The genomic tools that are applied after the completion of the isolation processes are broadly classified to present qualitative or quantitative approaches that can be used to assess the target biomarker(s). The 16S rRNA gene is the most extensively used target to qualitatively evaluate the presence of a specific group of organisms (Pace et al., 1986; Stahl, 1997). As a common practice, various techniques such as direct and nested PCR, T-RFLP, DGGE and others are routinely used to determine the presence of a specific 16S rRNA gene from phylogenically defined microbial groups. The utility of these biomarkers is particularly valuable if a link between 16S rRNA gene sequences and desired activity can be confidently established (Löffler and Ritalahti, 2001). Although there are indications that phylogeny and phenotype may not be representative of the cell's metabolic potentials (Amann and Ludwig, 2000), the application of 16S rRNA gene technologies has been proven to be successful in bioremediation in linking the presence of specific microbial groups with contaminant removal (Major et al., 2002; Gu et al., 2002; Lendvay et al., 2003; Anderson et al., 2003).

Another qualitative approach is to target the "functional genes" by tracking the process-specific biomarkers rather than the 16S rRNA genes. Only the functional genes for which a direct link with the process of interest has been recognized may be targeted by PCR amplification using primers for the functional gene of interest. For example, DGGE may be used to further separate DNA strands by differences in resistance to denaturation (e.g., more closely related to GC content) and to isolate a gene that codes for a specific catabolic enzyme, e.g., toluene dioxygenase to probe microbial activity at a fuel contamination site. In practice, to date, the utility of this approach has been very limited mainly due to the insufficient knowledge to identify the process-specific genes for many detoxification pathways (Kolker et al., 2005).

As described earlier, qualitative measurements are informative, in particular when site characterization is desired and in bioremediation when the process of interest is carried out by phylogenically defined bacterial groups. However, a promising tool for quantitative monitoring of 16S rRNA and functional gene copy numbers is quantitative real-time (RTm) PCR. During RTm PCR, an increase in fluorescent light emission during target gene amplification serves as a measure of the initial target concentrations. Therefore, a large number of the target nucleic acid in the original sample requires fewer cycles to accumulate amplification-associated fluorescence to a specific threshold level of detection (Ct value). The target molecules are quantified by extrapolation using linear standard curves for Ct values (Heid et al., 1996; Mackay, 2004). Both TaqMan and SYBR Green approaches are most commonly used in conjunction with RTm PCR (Walker, 2002; Mackay, 2004); however, various amplicon detection chemistries are also becoming available commercially. The advantage of RTm PCR over traditional end point PCR may include quantitation, enhanced speed and sensitivity, and lack of post-PCR steps (e.g., agarose gel). The utilities of RTm PCR for the quantitation of genes of interest in laboratory cultures (He et al., 2003a,b), or in environmental samples (Harms et al., 2003; Lendvay et al., 2003) have been shown. Appropriate database support is also currently available (i.e., GenBank, Ribosomal RNA Operon Copy Number Database). However, it should be noted that the accurate quantitation requires data on the target gene copy number per genome. As more bacterial genomes are increasingly sequenced and more data on gene copy numbers are becoming available in public databases, the use of RTm PCR will rise significantly.

The qualitative and quantitative genomic tools described thus far are intended to provide information on the presence of the target population or the gene of interest. These tools however cannot directly inform the user whether the target organisms are alive or metabolically active. Another approach to obtain livelihood or activity is by detecting mRNA transcripts. Various endpoint PCR or RTm PCR methods may be used for measuring the abundance of transcripts in samples, particularly those that may serve as biomarkers of contaminant degradation including *in situ* (Bagasra et al., 1994; Hodson et al., 1995; Tani et al., 1998, 2002) and *in situ* reverse transcriptase (Chen et al., 1997), and RNase protection assays (Sambrook and Russell, 2001). Among these

techniques, RT PCR is particularly sensitive and promising for measuring gene expression from the field samples (Freeman et al., 1999; Bustin, 2002). However, routine monitoring of mRNA in environmental samples may be challenging since mRNA molecules are less stable [short half-lives of transcripts, e.g., between 3-18 minutes in *E. coli* (Bernstein et al., 2002)] and prone to degradation in contrast to DNA. Other factors contributing to this challenge include the lack of protocols for extracting mRNA from soil and sediments, and difficulties in detecting low abundance transcripts. Many transcripts including those involved in contaminant degradation are present in 1 copy or fewer per cell (Velculescu et al., 1997). Therefore, in comparing field samples that are collected over spatial and temporal scales, it should be noted that different mRNAs have different half-lives and the sampling and analysis work plan should highlight the sample handling, extraction procedures, and standardization over time and space.

Proteomics is the most direct approach for monitoring microbial activity. It obtains information by detecting enzymes (catalytic proteins) that are involved in a specific process. The use of proteomic mass spectrometry for microbial detection offers distinct advantages over traditional approaches due to the speed of analysis. High throughput proteomic technologies are rapidly expanding (Fredrickson and Romine, 2005) and the potential for combined genome and proteome to monitor gene expression and activity from a biofilm community from an acid mine site have been demonstrated (Tyson et al., 2004; Ram et al., 2005). However, the application of proteomics technologies for the detection of a key catabolic enzyme by peptide mass fingerprinting (PMF) and peptide sequencing (PS) (Halden et al., 2005) is currently limited in bioremediation since a much more detailed understanding of protein function as well as database supports are required for routine monitoring practices.

Molecular and Genomic Case Studies

We have investigated the use of recent molecular and genomic techniques to characterize microbial communities and activities to assess their effectiveness at chlorinated and metals hazardous waste sites. Since currently there is no single technique that can rapidly, sensitively, specifically, and cost effectively detect and characterize the entire microbial communities in spatial and temporal scale, a multifaceted approach described here was selected across the contaminated sites to examine the utility of these tools.

Our comprehensive approach is further justifiable since it is well known that contaminant concentrations in the subsurface are often low (i.e., mg to µg per liter range). If the contaminant acts as an electron acceptor or donor, it is likely to support a non-substantial biomass of active populations. This is particularly true if it is assumed that no other substrate is available to support the growth of the target population and only 50% of the DNA could be recovered. Therefore, as reported by Gu et al. (2002), the biomass estimates based on DNA could generally be lower by several orders of magnitude than those based on the PLFA. In the present study, we have employed PLFA (FAMES) as indicators of biomass and community structure, enzyme assays for monitoring microbial activity, as well as more phylogenetically-specific DNA analyses for the presence of specific microbial groups.

FAMES and Intact Phospholipids

A number of investigators have utilized PLFA determinations to gauge microbial community responses to either the presence of contaminants or remedial action (Hansen et al., 2000; Pfiffner et al., 1997). Usually the fatty acid methylesters (FAMES) have been the focus rather than the intact phospholipids (IPP), to examine changes in viable biomass, community structure, and metabolic activity.

A - Chlorinated Solvents

Experimental Methods

The aquifer material for vertical profiling of PLFA key biomarkers at a TCE contaminated site in Florida was collected using a Cone-Penetrometer (CPT) equipped with a Mostap™ sampler (20-inch long with a 1.5-inch diameter). The sampler contained three sterile sleeves (brass or stainless steel) and one spacer. Each sleeve was 6 inches long and held approximately 250 grams of soil.

The coring was accomplished by driving the sample barrel to four different depths (7, 15, 30, and 40 ft) at each sampling location. Once the sample barrel was withdrawn, the three sleeves were extruded from the sample barrel. The sleeves were tightly capped on both ends with plastic end caps, sealed as quickly as possible, and labeled with the percentage of recovery recorded and marked to designate the top and bottom sections. The sleeves were immediately frozen in liquid nitrogen (-150°C) and shipped overnight (-70°C) to the laboratory for PLFA analysis.

At the laboratory, PLFA were analyzed by extraction of the total lipid (White et al., 1979) and then separation of the polar lipids by column chromatography. The polar lipid fatty acids were derivatized to fatty acid methyl esters, which were quantified using gas chromatography (Ringelberg et al., 1989). Fatty acid structures were verified by chromatography/mass spectrometry and equivalent chain length analysis.

In order to minimize sample contamination, a strict aseptic sampling procedure was adopted including sterilization of brass or stainless sleeves with isopropanol (70%) bath dipping (15 min), air drying at ambient temperature (~ 1h), and aluminum foil wrapping. Each three individually foil-wrapped sleeves were placed in an autoclave bag. The bag was placed in a heat-resistant plastic container, and was autoclaved (121°C, 30 min). The container was tightly capped, packed, and shipped to the field. Polyethylene sleeve caps were not autoclaved but were surface rinsed with isopropanol (70%) prior to use. Sterilization of drilling equipment involved steam cleaning between samples. After the samples were extruded, the sample barrel used to collect the soil sample was disassembled and decontaminated in Alconox® detergent mixed water. The sample barrel was then rinsed with tap water, de-ionized water, and isopropanol (70%) prior to complete air-drying (~ 1 h) and before it was

used again. Air exposure of the subsurface was minimized since core sampling was continued immediately after each extraction using two sets of the Mostap™ samplers. The sampler containing three cored sleeves was extracted, switched with another sterile one, and coring continued while the sampling crew conducted aseptic sample processing and decontamination procedures.

Results and Discussion

The microbial response, as measured by the fatty acid profile, to three remediation technologies applied at a TCE (trichloroethylene) contamination site at Cape Canaveral Air Station was investigated. The technologies included: *In situ* chemical oxidation (ISCO; $KMnO_4$); six-phase heating (SPH), and steam injection. Sediment cores were collected at four depth intervals before, during, and after remedial operation. The conversion factor of Balkwill et al. (1988) of 2 x10^6 cells/pmol of PLFA (FAMES) was used to determine total biomass. We observed relatively low levels of PLFA in control plot sediments ~ 9.7 to 18 picomoles/g dry wt. (1.9 x 10^5 to 3.6 x 10^5 $\pm$ 1 x 10^5 cells/g) and the lowest values were observed at the shallowest depth ~ 5m from land surface. This is counter to what most workers have noted where biomass (or its proxy, PLFA) is generally higher near the surface due to plant and microbial release of organic carbon in the root zone. It should be noted that the organic carbon level in these sandy sediments was quite low. The types of FAMES are shown in Table 3, along with their phylogenic microbial association, and response trend.

It is evident from the data in the table that ISCO had the greatest impact on biomass relative to the controls. The increase was from ~2.92 x 10^4 to 1.85 x 10^8 cells/g and was sharpest in the zone of initial $KMnO_4$ injection. Five months later in the treatment period, biomass generally decreased. One explanation for the increase in biomass may be that the permanganate may have reacted with native organic matter and released lower molecular weight compounds that served as a carbon source for microbial growth. The impact on community structure was evident for SPH and SI where biomarkers for thermophilic, Gram-negative anaerobes and Gram-positive bacteria clearly increased. ISCO resulted in the most significant influence on community structure evident in a number of phylogenic groups. It was most surprising that 12 months after the treatments were ended, the biomass and community structure in all three treatment plots returned to that of the control plot. Even though the biomass was low, the overall subsurface community structure reflected a mixed anaerobic and aerobic community similar to that reported by Ringelberg et al. (1997).

Table 3. Types of FAMES Observed, and Response Indicator Phylogenic Association

FAME	Phylogenic Association	ISCO	SPH	SI
Terminally Branched	Anaerobic Gram (-) and Gram (+) Thermophiles		+	+
C_{16} Monounsaturated	Proteobacteria	+++		
C_{18} Monounsaturated	Proteobacteria	+++		
Mid-Chain Branched	Sulfate-reducers Actinomycetes	++		
18:L w9c	Eukaryote (fungi)	++		
Polyunsaturated	Eukaryote (fungi)	++		
C_{13}-C_{18} saturated	Anaerobes			
C_{20}-C_{24} saturated	fungi			
Branched unsaturates	Metal-reducers			
Biomass		++		

B - Metals

We have used PLFA as indicators of biomass and community structure to investigate microbiology of ground water undergoing treatment with permeable reactive barriers (PRB) at three metal contaminated sites. The PRBs were selected on the basis of the reactive media that were used in their construction including a 100% zero valent iron (ZVI) barrier at North Carolina, a 50% ZVI:50% compost and pea gravel barrier at South Carolina, and a 0% ZVI:100% cow manure and limestone barrier at Louisiana.

Sediment core samples were collected from upgradient, upgradient/PRB interface, in the PRB, downgradient/PRB interface, and downgradient during quarterly or annual sampling. Each PRB was constructed in a trench stabilized during construction by a guar gum slurry. This is a common construction technique which ends up leaving a fair amount of organic matter in the subsurface despite application of the proprietary enzymatic degradation solution (Gu et al., 2002).

Experimental Methods

Core samples were collected to provide information in defining the horizontal and vertical distribution of microbes at the PRBs. The conventional coring techniques were adopted using a Geoprobe™ to obtain continuous coring for the entire depth of each aquifer. To minimize contamination, after a core was obtained using strict aseptic techniques, care was taken not to disturb or contaminate the sample. Processing was performed as quickly as possible under anaerobic and aseptic conditions while at the field. The entire core sleeve (2-feet long with a 1-inch diameter) was placed in an anaerobic field glove box. The glove box contained an atmosphere of argon and was shielded from direct sunlight. Each sleeve was labeled with the percentage of recovery recorded prior to opening and visual inspection for mineralogy. Surface layers of the core were scraped away using a sterile sampling device and discarded so that only the center of the core was placed in sterile thin-walled plastic sample bags (Whirl-Pak®). The portion used for DNA/PLFA analyses was rapidly frozen with liquid nitrogen and stored on dry ice at -70°C. The samples were transported to the laboratory at -70°C and stored at the same temperature prior to analysis.

Results and Discussion

One site at which a zero-valent iron permeable reactive barrier (ZVI-PRB) was used was at the U.S. Coast Guard Support Center at Elizabeth City, NC. This site had been a hard-chrome plating shop more than 30 years. According to Wilkin et al. (2002), chromate levels were in excess of 10 mg/L and TCE, c-DCE, and vinyl chloride were in the ppb ranges.

The sediment cores collected at the Elizabeth City ZVI-PRB which has been in place since 1996 were analyzed to assess community structure and physiological status of the bacteria. The analyses focused on the samples collected in the midbarrier and in the zones of low and high corrosion particularly those at the upgradient interface of the wall from two consecutive sampling events 2 years apart.

In general, the abundance of biomass in sediment cores with high corrosion was one log factor higher than the samples with low corrosion. The lowest biomass was observed at an upgradient sandy location within the plume (1.03×10^5 cells/g) while the highest biomass was at a zone with the highest corrosion (5.09×10^6 cells/g). The increased microbial biomass at the upgradient interface of the PRB suggests a real potential for biofouling and reduced hydraulic performance of zero-valent iron PRBs.

The PLFA profiles of the collective samples were predominantly consistent with those of proteobacteria with the exception of the upgradient samples in which proteobacteria included 42% of their community structure; they had no biomarkers indicating metal reducers, or sulfate-reducing δ-proteobacteria. The fatty acids suggest that the community structure at the upgradient/PRB interface, within the PRB, and the downgradient/PRB interface consisted of sulfate and metal reducers ranging from 2.06% to 14.34% of the total community. Among these populations, metal reducers were present in every sample regardless of the detection of SRBs. However, zones of high corrosion were represented by the increased percentage SRBs than metal reducers. This could be attributed to the increase in pH which seemed to offset the positive influence of an abundance of hydrogen produced in the barrier.

The second permeable reactive barrier was constructed at a pilot scale to assess the efficacy of ZVI, leaf compost, and limestone and pea gravel as the reactive material to mitigate the principal contaminants that were arsenic (As), lead (Pb), and acidic pH conditions from an upgradient phosphate fertilizer manufacturing plant that had been decommissioned in 1972 in South Carolina. The combination of reactive materials was designed to promote microbiological sulfate reduction and to increase pH of the ground water from an acid producing to acid consuming condition.

The total microbial abundance based on the PLFA was several orders of magnitude higher in the barrier (10^{6-8} cells/gram) than in the background sediment samples (10^{4-5} cells/gram). The increase in the biomass was also positively correlated with depth. The highest biomass (6.63×10^8 cells/gram) was observed in a sample from 11 ft bgs suggesting that the microbial community adapted quickly to the strongly reducing conditions.

The increase in biomass continued over the next two consecutive sampling events which may reflect the adaptation of the population to degradation products of the compost. Mid-chain branched saturated PLFA structural groups increased significantly over time, especially compared to the Elizabeth City ZVI, suggesting that SRBs, and to a lesser extent metal reducers, were increasing at the expense of the other groups (i.e., Firmicutes, Proteobacteria, and non-specific populations). The increase in branched PLFA in the PRB, and the alteration of ground-water geochemistry, including the reduction of sulfate, resulted in Gram-positive anaerobic bacteria being a significant fraction of the overall community. In contrast, Polyenoic FAMES composed the most insignificant component of the total PLFA structural groups in the wall suggesting that eukaryotes were not supported by the reactive media. In general, the polyenegics are considered as a remnant of the guar gum (Gu et al., 2002).

The third field scale PRB comprised of pasteurized cow manure (50% by wt.) and limestone (50% by wt.) was constructed during 2003 in Louisiana to mitigate lead (Pb), cadmium (Cd), arsenic (As), and a low pH ground water. Analysis of the PLFA profiles at this site showed that the microbial community structures of the samples varied considerably among the samples. Estimated viable biomass, based on total PLFA concentrations ranged from $\sim 10^{5-7}$ cells/gram dry weight among the samples collected at 4 - 6 ft bgs with the majority of samples containing biomass of approximately 10^{7-9} cells/gram at the deeper horizons.

The majority of the samples had relatively diverse microbial communities in which the primary members were Firmicutes, which was $\geq$30% of the total PLFA. Firmicutes include Bacteroides and *Clostridia*-like fermenting bacteria. High

proportions of Firmicutes suggest that conditions were anaerobic at sampling locations within the barrier. Many of the samples also contained relatively large proportions of biomarkers for sulfate-reducing and metal-reducing bacteria, which further supports the presence of highly anaerobic conditions. In two-thirds of the samples the total "anaerobic" biomarkers accounted for ≥48% of the total PLFA. Methanogenesis is common in gut-dwelling archaea. Even though the biomarkers for methanogenic archaea may have some overlap with common actinobacteria, a strong correlation between these biomarkers and portions of the wall with significant methane production (as high as 50 mg/l) is suggestive of the presence of archaea PLFA.

The results from the organic based barrier in Louisiana suggested that there was a drastic difference in the most abundant population as compared to the Elizabeth City ZVI-PRB as Firmicutes were dominant in the manure wall but Proteobacteria were the most abundant group in the zero valent iron wall. Proteobacteria not only comprised a smaller proportion of the total population of the manure wall as compared to the ZVI wall, the physiological status of the Gram-negative Proteobacteria in the manure wall is suggestive of a slowed growth rate and/or of decreased permeability of their cell membrane. The second distinction between the community structures of the two barriers is that the PLFA from the manure wall contained a high percentage of normal saturated structural groups which are found in all organisms as compared to the ZVI wall.

Summary

The biomass estimates based on FAME were generally lower by several orders of magnitude from upgradient to the vicinity and within the three PRBs. FAME indicators of both the Gram-positive and Gram-negative-anaerobic microorganisms increased from core samples near and within the PRBs as evidenced by terminally branched and monoenoic fatty acids.

The FAME extracts from in or near the iron-containing PRBs showed a similar signal of increased representation of sulfate- and metal-reducing organisms extending well downgradient from the PRB. The presence of metal sulfides also supported the FAME analysis suggesting the microbial reduction of sulfates in the geochemical environment of the iron PRBs.

It is important to note that methanogens did not constitute a significant fraction of the total biomass of the South Carolina PRB. A similar result was also reported by Stapleton et al. (1998). Since there is generally a relative abundance of H_2 in the vicinity of ZVI-PRB produced by cathodic corrosion of the iron, it may be expected that the methanogens would predominate over the sulfate reducers. However, due to the high levels of $SO_4^=$ it is possible that the methanogens may have been at a competitive disadvantage (Lovley and Goodwin, 1988). Since the anaerobic microorganisms displayed a higher level of biomass, a reduction in the hydraulic performance of the ZVI-PRB may not result from biofouling which could be attributed to an accumulation of H_2. The role microorganisms play in the development of "green" rust, ferrous hydroxides, etc. in ZVI-PRBs remains unknown.

Catalytic Proteins

The activity of certain enzymes in soil and sediment samples reflects the metabolic rate of the microbial populations. One of the types of enzymes most studied under anaerobic conditions is dehydrogenases that are abundant in sulfate-reducing bacteria (SRB). This investigation examined over eighty samples derived from metal and chlorinated solvent contaminated sediments for dehydrogenases activities. The presence of SRBs among the tested samples was confirmed prior to the enzyme assay.

Experimental Methods

Triphenyl tetrazolium (TTC) chloride is a substrate for a number of non-specific dehydrogenases present in microbial communities and can be generally correlated with respiratory activity and used as an index of microbial activity (Ladd, 1978). In order to determine dehydrogenase activity (DH), 5 grams of core sample were placed in an anaerobic glove box. The samples then were buffered with $CaCO_3$ (to a pH >6), and 1.75 ml of 0.5% 2,3,5-triphenyl tetrazolium chloride and distilled water were added to each sediment sample. The samples were incubated for 24 hrs in an anaerobic glove box and were transferred to a chemical hood and extracted sequentially with two 10 ml aliquots of methanol and filtered through Whatman no. 42 paper. The aliquots were then combined in a volumetric flask and made up to 50 ml with methanol. Optical densities of the extract were measured at 485 µm. The enzyme activities were performed in triplicate and averaged. Controls were set up using samples with distilled water without TTC.

Since the results are interpreted in terms of enzyme activity rather than microbial number, a standard curve was prepared using TTF in methanol in the range of 0 to 1 mg/100 ml. The optical density was linear up to a concentration of 150 mM TTF/ml of methanol. Using this curve, the amount of TTF formed in each sample was converted into µl H and the results were expressed as µmoles H/g of core sample/hr (Johnson and Curl, 1972).

Results and Discussion

We have used dehydrogenase measurement as an index of metabolic activity at a metal contaminated site in Louisiana and at a chlorinated solvents site in Florida. While the ground-water remedy at the Louisiana site included a permeable reactive barrier constructed from cow manure and limestone, as discussed earlier, the Cape Canaveral project in Florida was designed to field-test and compare three technologies for the remediation of ground water contaminated with TCE. The technologies included Six-Phase Heating (SPH), Steam Injection (SI), and In Situ Chemical Oxidation (ISCO). A critical aspect of the project was to determine the effects of the remediation activities on the indigenous microbial populations which are relied upon for the final polishing phase of the treatment train.

The results indicated that dehydrogenase activity in the sediment samples was not always correlated to the microbial number. However, in amended environmental samples such as the PRB barrier samples, the enzyme activity increased

with increased microbial populations. It was also determined that air drying the core samples resulted in a 30 to 50 percent loss of dehydrogenase activity while storage of wet sediment at –80°C was found to be most satisfactory for retaining dehydrogenase activity. Therefore, the assays were performed on fresh core sediments that were frozen immediately upon extraction in the field.

The Louisiana PRB was constructed in two phases; the first portion was emplaced in the aquifer for the treatability study which is now termed "the matured wall" with an extension to the wall completed one year later in May 2003, at the full-scale which is now termed "the new wall". Data show that the enzymatic activities were significantly higher among the bacterial populations of the newly installed permeable reactive barrier as compared to that of the more mature wall. The results indicated that the vertical average enzyme activity of core samples in the old wall rose from 0.547 µmoles H/g/hr in December 2004, to 0.677 µmoles H/g/hr in April 2006 (16 months). In the newer extension, the average activity rose from 0.788 µmoles H/g/hr in December 2004 to 2.075 µmoles H/g/h in February 2005 (3 months). These differences in enzyme activity are also reflected by the appearance of the cores upon extraction. While in the older wall the more active depths rendered brown cores, those from the more active depths in the newer wall were black indicating more reduced conditions. In either event, these results are indicative of the ample carbon compounds or H_2 that are made bioavailable from the reactive material in the more recently installed wall.

Although all of the core samples suggest that the highest enzyme activity occurred in the deeper zones, it is clear that enzyme activity in the new construction exceeded that of the older wall. For example, representative cores (April, 2006) show that activity from 5-9 feet in the old wall was 0.329 µmoles H/g/hr and was 1.024 µmoles H/g/hr in the 10-12 foot core. In the newer wall, however, the activity was 0.659 µmoles H/g/hr in the 5-9 foot core and 6.240 µmoles H/g/hr in the 11-12 foot core.

When analyzing cores from the Cape Canaveral project from the control site as well as the ISCO treatment plot before initiation of the remediation, it was determined that the enzyme activity increased linearly with depth, as in the other treatment plots. The cores above 17 feet in depth varied between 0.556 and 0.767 µmoles H/g/hr while the activity increased to 4.682 µmoles H/g/hr at 31 feet and 5.796 µmoles H/g/hr at 41 feet.

Cores from the first post treatment sampling event (0 month after ISCO termination) showed that in the ISCO plot the activity had actually increased over the background (3.178 versus 0.556 µmoles H/g/hr) at 17 feet bgs, but was below background (3.465 versus 4.682 µmoles H/g/hr) at 31 feet bgs. In the SPH plot cores from the first post treatment sampling event demonstrated that the enzyme activity still increased with depth (0.138 at 7 ft, 1.984 at 27 ft, and 4.230 at 41 ft) but varied between 60-70 percent of background. One second post treatment sampling (6 months after ISCO termination) from the SPH plot shows the enzyme activity at 8 feet bgs was only 0.003 µmoles H/g/hr.

Summary

The dehydrogenase assay used in this research for the measurement of enzyme activity under anaerobic conditions is based on the principle that when metabolizing cells come in contact with an aqueous solution of TTC, it is converted into triphenlyformazon (TTF) which can then be measured colorimetrically. Regardless of the remedial options used, ISCO, SPH, or permeable reactive barriers, the results indicated that there was an increased bacterial activity as measured by the elevated enzyme levels particularly in response to enhanced electron acceptors/donors present in the aquifers. Therefore, it can be concluded that this assay can be confidently used as a sensitive index for bacterial activity at various contaminated sites.

Genomic

Experimental Methods

DNA Extraction

Various DNA extraction protocols were evaluated on samples from two permeable reactive sites: the North Carolina ZVI-PRB, and the Louisiana cow manure-PRB. All protocols utilized a bead-beating method to lyse bacterial cells and release DNA (Mini Bead-Beater, BioSpec Products, Bartlesville, OK, Borneman et al., 1996; Duncan et al., 2003). Phenol-chloroform extraction, followed by ethanol precipitation (Rios-Hernandez et al., 2003), and four commercially available kits were compared, following the manufacturers' protocols: FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, Duncan et al., 2003), Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc, Carlsbad, CA), UltraClean Soil DNA Kit (Mo Bio Laboratories), and UltraClean Mega Soil DNA Kit (Mo Bio Laboratories). Sample weights of 0.25, 0.5, 2, and 5 g were compared. Bead-beating rates of 25,000 and 36,000 rpm and beating times of 60 seconds and 90 seconds were compared for the FastDNA Spin Kit for Soil. DNA fragment size was examined and the concentration verified by agarose gel electrophoresis of an aliquot of the DNA sample and viewing the Sybr®Safe (Invitrogen Inc., Carlsbad, CA)-stained gel under UV transillumination.

PCR, Cloning, and DGGE

Primers were synthesized by Invitrogen Corp. (Carlsbad, CA). Other reagents and enzymes were purchased from Fisher Scientific or Sigma-Aldrich.

Eubacteria

1500 bp fragment: Nearly full length eubacterial 16S rDNA (*Escherichia coli* positions 8-1492) for cloning was obtained from DNA purified from cells by amplification with "universal" eubacterial primers targeting conserved regions and the cycling conditions described in Herrick et al. (1993). The PCR products were cloned into the TOPO-TA vector (Invitrogen Inc., Carlsbad, CA) following the manufacturer's recommendations to increase the number of transformants when cloning from a pool of sequences. The inserted fragment was amplified from randomly selected single white colonies using primers complementary to the M13

sequences present in the TOPO-TA vector, and then purified for sequencing as described below (DNA sequencing).

550 bp fragment (nested): Primers GM5F and D907R (Muyzer et al., 1993) were used to amplify a 500-bp region of the 1500 bp PCR template above for denaturing gradient gel electrophoresis (DGGE) analysis of differences in sequence diversity among the samples. DGGE was performed using a D-Code 16/16-cm gel apparatus (BioRad, Hercules, CA), at a constant voltage of 65 V for 16 hours and a constant temperature of 60°C (Muyzer et al., 1993). The gradient was formed of 6% polyacrylamide in TAE buffer with between 40 and 60% denaturant (7M urea and 40% formamide is defined as 100% denaturant). After electrophoresis, the gel was stained in a solution of Sybr®Safe and a permanent image was captured by the NucleoCam Digital Image Documentation System (NucleoTech Corp., San Mateo, CA). Selected bands were cut from the gel and incubated in PCR-grade water, then used as template DNA in subsequent PCR reactions, as previously described (Rios-Hernandez et al., 2003). The PCR product was prepared for sequencing as described below (DNA sequencing).

Archaea

600 bp fragment: 16S rRNA PCR products of approximately 600 bp in size were obtained for cloning by amplification with the archaeal primers ARC333GC and 958R (Reysenbach and Pace, 1995; Watanabe et al., 2002). Amplification followed the touch-down procedure described in Muyzer et al. (1993). Cloning and subsequent preparation for DNA sequencing followed the procedure described for the eubacterial 1500 bp fragment.

220 bp fragment (nested): A "nested" PCR reaction was performed using archaeal 16S primers ARC20 and 958R for the first amplification, and primers ARC333 (Reysenbach and Pace, 1995) and P2 (Muyzer et al., 1993) for the second PCR reaction. DGGE was performed to separate the resulting 220 bp fragments (8% polyacrylamide, 0-100% denaturant), and bands were cut from the gel for purification and subsequent sequencing, as described above for the eubacterial 500 bp fragment.

DNA sequencing

Amplified DNA was purified from primers and unincorporated nucleotides and concentrated with Millipore Ultrafree-MC 30,000 NMWL Filter Devices to 20-100 ng/µL. Sequencing of the purified PCR products was performed on an ABI Model 377 automated sequencer, using Ampli-TaqFS DNA polymerase and fluorescent-labeled dNTPs in a cycle-sequencing kit (ABI Prizm Dye Terminator Kit, PE Applied Biosystems, Inc., Foster City, CA). The M13 primers and two internal primers (704f f, 907r, Johnson, 1994) were employed to sequence 1500 bp eubacterial 16S rDNA. The M13 primers were used to sequence the 600 bp archaeal 16S rDNA. The amplification primers were used to sequence the nested eubacterial (550 bp) and archaeal (220 bp) fragments. Sequencher® (Gene Codes Corp., Ann Arbor, MI) was used to assemble the fragments. The assembled sequences were compared to those in GenBank using BLASTN (Altschul et al., 1997, National Center for Biotechnology Information). Sequences from a BLASTN search, that most closely matched the sequences from the clones and sequences of selected outgroup strains, were aligned using CLUSTAL X (ver. 1.81) (Thompson et al., 1997). A dendrogram was constructed from the distance matrix using the neighbor-joining method in CLUSTALX and 1000 bootstrap replicates were performed to estimate the support for each branch (Felsenstein, 1985).

Results and Discussion

DNA extraction

All methods gave qualitatively the same results: DNA was obtained from five sites of sufficient quantity and quality to be amplified using universal eubacterial primers. We were not initially able to amplify DNA from two of the Elizabeth City samples. Tests confirmed that PCR-inhibiting substances were not responsible for the lack of amplification, therefore, based on the high percentage sand composition of these samples, we assumed that biomass might be lower in these two samples. PLFA values confirmed that the number of microorganisms was very low in samples taken from these as well as similar sites. Our procedures were accordingly modified to increase the amount of sample processed and to concentrate the extracted DNA. In brief, four 0.5 g subsamples were processed separately until an intermediate stage in the protocol of the MoBio Power Soil DNA Isolation Kit, and then combined prior to filtration and final elution of the DNA. These modifications, together with decreasing the final elution volume, allowed us to increase the final DNA concentration by a factor of approximately 16 (standard protocols call for single 0.25 g samples and elution in a volume of 50 µL rather than 100 µL). Using the modified protocol, eubacterial and archaeal16S rRNA was successfully amplified from two Elizabeth City samples (eubacterial only from two additional samples) and six samples from the permeable reactive barrier in Louisiana.

Molecular identification of eubacteria and archaea

Although DGGE profiles suggested that 16S rDNA sequence diversity was fairly low in the Elizabeth City sites, five distinct groups of eubacterial sequences were found. A total of 9 eubacterial and 5 archaeal sequences were obtained from cloning. The majority of the eubacterial sequences (all from Elizabeth City, NC) showed highest affinities to those from Firmicutes, especially to members of the high G+C groups (Figure 4). Two sequences had very high similarity (>99%) to *Rhodococcus* strains, including one with 1 base difference from *Rhodococcus* sp. UFZ-B528 (AF235012, sequence of an isolate from a chlorobenzene-contaminated aquifer), three were most closely affiliated with *Desulfotomaculum* (genus of Gram-positive sulfate-reducing bacteria), and two were most similar to uncultured *Clostridium* sequences obtained from a bioreactor producing methane from landfill leachate. Two sequences were almost identical (>99%) to that of *Pseudomonas libaniensis*, isolated from spring water. Short sequences (122-161 bp) were obtained by cutting bands from the DGGE gels and reamplifying--to the extent the sequences from cut bands and clones overlapped, the

former confirmed the presence of the eubacterial groups obtained by cloning. The cloned sequences were of much higher quality as well as longer, both factors are beneficial for phylogenetic inference.

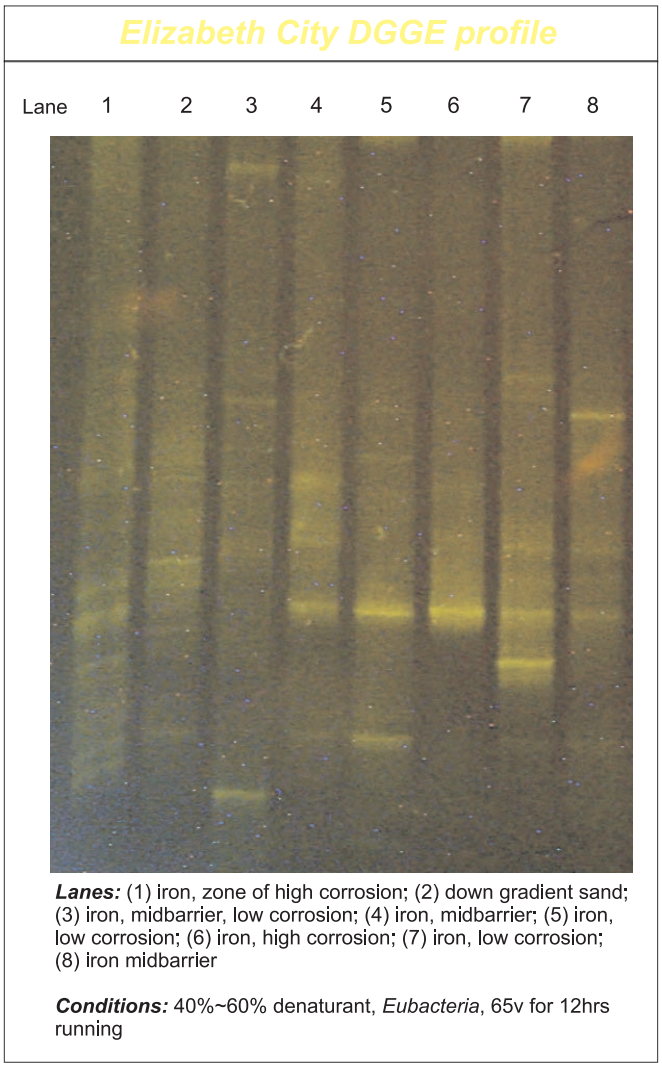

Figure 4. Elizabeth City DGGE profile

As discussed earlier, methanogenic archaea PLFA were found within the Louisiana wall, especially at the regions of high methane production. Further genomic analysis of this PRB has also shown sequences most closely associated with methanogens. For example, the archaeal sequences from cloned 16S sequences (all from the Louisiana wall) were primarily affiliated with *Methanosarcinacea* (4/5); the fifth was similar (98%) to *Methanobacterium.*

Summary

It is clear that typical protocols used for soil analysis would certainly fail to adequately interrogate ground-water treatment systems unless they were substantially modified. The modifications found necessary to compensate for the low biomass in the Elizabeth City samples were two-fold: to increase the size of the sample processed, and to use nested PCR for visualization of diversity patterns by DGGE. However, cloning longer fragments is recommended for sequence analysis.

It should be noted that the presence of the iron or sorption of DNA macromolecules on the minerals or Fe surfaces was not encountered during our research. Eubacterial diversity was limited in the Elizabeth City site, but nevertheless contained members from four different groups of eubacteria. Some of the sequences had highest affinity to those obtained from sites contaminated with chlorobenzene.

Conclusions

It should be clear to the reader that the PLFA indicators of microbial biomass and community structure complement the use of DNA for community structure, diversity and specific microbial species in the subsurface. The presence of contaminants superimposed on both site geochemistry and physical/chemical interventions to remediate chlorinated solvent and metal contamination evoke a profound change in the abundance, diversity and activity of microbiota. Increases in biomass alone show that microorganisms can adapt, and in some cases thrive under otherwise adverse dynamic conditions.

In these disturbed environments, there may be a need for more effective DNA extraction methods to magnify the sample size which would enable improved understanding of the microbial ecology of the subsurface. As more specific gene probes are developed, improved DNA extraction techniques could provide a much more in-depth picture of microbial function, diversity, activity, and interdependence.

It must be kept in mind that nearly exponential changes in microbial biomarkers can occur due to contamination and remediation while the geochemical parameters remain far more stable in time and space. There is a definite need for the application of more accurate, reliable and statistically responsive indicators of microbial reactions to geochemical stressors.

Notice

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