

# Biodegradation in Contaminated Aquifers: Incorporating Microbial/Molecular Methods

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## Abstract

In order to evaluate natural attenuation in contaminated aquifers, there has been a recent recognition that a multidisciplinary approach, incorporating microbial and molecular methods, is required. Observed decreases in contaminant mass and identified footprints of biogeochemical reactions are often used as evidence of intrinsic bioremediation, but characterizing the structure and function of the microbial populations at contaminated sites is needed. In this paper, we review the experimental approaches and microbial methods that are available as tools to evaluate the controls on microbially mediated degradation processes in contaminated aquifers. We discuss the emerging technologies used in biogeochemical studies and present a synthesis of recent studies that serve as models of integrating microbiological approaches with more traditional geochemical and hydrogeologic approaches in order to address important biogeochemical questions about contaminant fate.

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The fate of biologically reactive compounds in ground water has been an area of intense study in recent decades. Much of the focus has been on investigating the role of intrinsic bioremediation (a subset of natural attenuation) in the treatment of ground water contamination, particularly for hydrocarbons (for reviews, see Christensen et al. 2000b; Chapelle 2000; Rogers et al. 2002). In order to document natural attenuation, observed decreases in contaminant concentrations must be tied to the responsible underlying mechanisms (National Research Council 2000). Because intrinsic bioremediation is mediated by the indigenous microbial community, a thorough evaluation of contaminant degradation in aquifers requires an approach that integrates qualitative and quantitative geochemical measurements of contaminant transformations with measurements of the identity, abundance, and function of the key microorganisms involved in the degradation process. In addition, understanding the mechanisms

responsible for biodegradation allows for the enhancement of these processes through an engineering approach.

Many ground water scientists have used geochemical methods, targeting inorganic and organic species in the aqueous, gaseous, and solid phases in the subsurface, to assess microbial activity (see review by Cozzarelli and Weiss 2007). For example, mass loss of contaminants (Barker et al. 1986; Eganhouse et al. 2001), production of metabolites (Beller 2000; Cozzarelli et al. 1990), changes in redox-sensitive species (Christensen et al. 2000a), and isotopic fractionation of reactants or products (Kelley et al. 1997; Peter et al. 2004) have all been used to document and quantify microbial transformation processes. Although geochemical data can help researchers understand environmental conditions and the fate and transformation rates of contaminants, few investigators combine these observations with direct knowledge of members of the indigenous microbial community and what factors may limit their *in situ* activity. Because the structure and function of microbial communities exhibit significant spatial and temporal variability, collecting microbial data is also a necessary complement to routine geochemical analyses. This combination of microbial and geochemical data is essential in evaluating and implementing remediation strategies which are inherently driven by the composition and function of the microbial community.

In this review paper, we present an overview of the experimental approaches and microbial/molecular methods

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for investigating the controls on microbially mediated degradation processes in contaminated aquifers (Figure 1; Table 1). Because there are several excellent reviews of many of these techniques (e.g., Goldscheider et al. 2006; Green and Scow 2000; Madsen 2006; Spiegelman et al. 2005), the focus of this paper will be on emerging technologies and recent state-of-the-art studies that serve as models for integrating microbiological approaches with more traditional geochemical and hydrogeologic approaches to better understand the controls on contaminant fate. We conclude with a general discussion of the future directions of research in this field and the recommendation for a balanced methodological approach that combines geochemical techniques with both culture-based and molecular approaches.

## Experimental Design

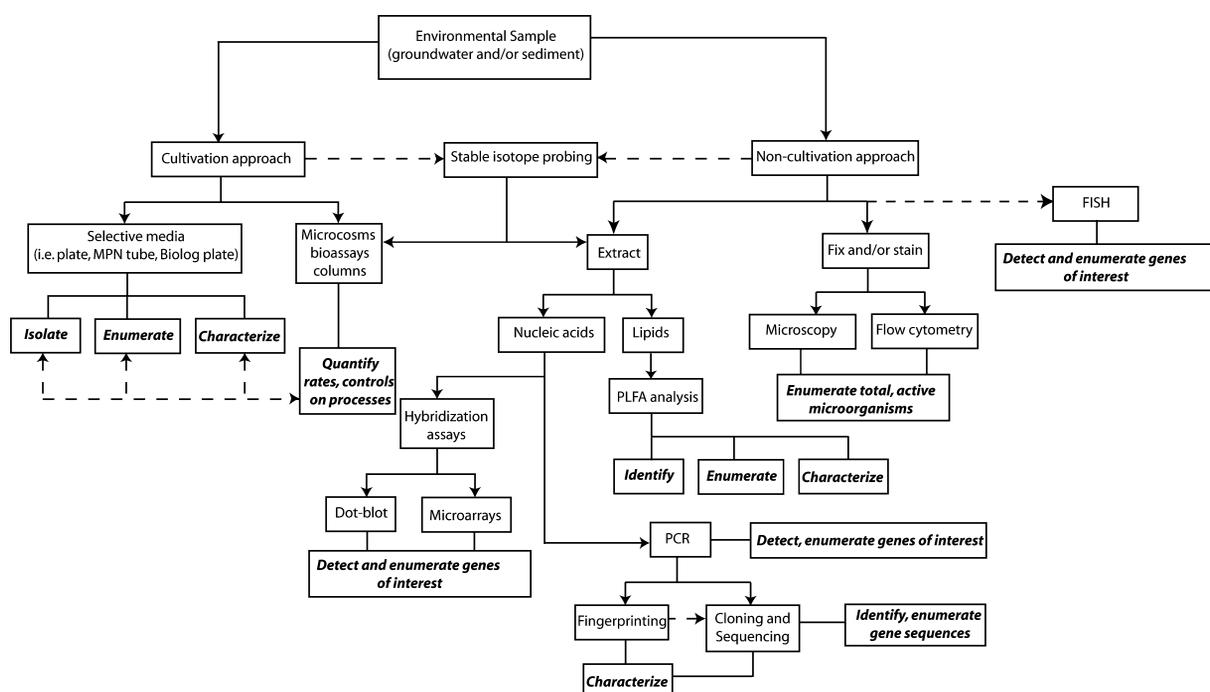
Many traditional geochemical approaches serve as the foundation for the collection of microbial data (Cozzarelli and Weiss 2007; Scow and Hicks 2005; Smets and Pritchard 2003). The following section outlines different approaches that can be expanded to incorporate microbiological assays and discusses how the inclusion of such methods may affect the experimental design.

## Methodology

The laboratory microcosm approach (often referred to as a bioassay or batch experiment) can be used to demonstrate the potential for biodegradation in a particular hydrogeologic environment (e.g., Strevett et al. 2002; Struchtemeyer et al. 2005; Lookman et al. 2005). The laboratory microcosm approach can be especially valuable in distinguishing biotic from abiotic chemical reactions,

looking at toxicity effects, and evaluating the impact of changing electron acceptor or donor availability on biodegradation rates. Bacteria collected from microcosms can be analyzed for abundance and composition or cultured in order to further understand their physiology (for a review, see Madsen 2005). Limitations to using laboratory approaches for studying biogeochemical processes include that the collected sample may not be representative of the aquifer and the microbial community may be disturbed during sample collection or change over time within the microcosm itself. The presence of a large and diverse microbial community as well as variable complex geochemical parameters also make the execution of reproducible laboratory studies difficult. As a result, some investigators prefer field-based experimental approaches, such as in situ microcosms, to determine biodegradation potentials.

An advantage of using an in situ approach is that the complexities of the natural environment are incorporated into the study and these complexities are often difficult to reproduce in the laboratory. One type consists of a cylinder encasing a volume of the aquifer (on the order of 2 L) that is loaded with ground water spiked with compounds of interest (Gillham et al. 1990; Mandelbaum et al. 1997). This in situ microcosm allows the isolation of a small region of the aquifer for study while minimizing the disturbance to microbial populations. Substrata such as surrogate geological materials (Reardon et al. 2004; Bennett et al. 2000) or Bio-sep® beads (Microbial Insights, Inc., Rockford, TN) (Geyer et al. 2005; Kastner et al. 2006) can also be deployed to investigate the in situ microbial community. These materials can be incubated in a ground water well and a portion subsampled and analyzed over time. Though the subsequent collection of the biofilms on



**Figure 1.** A flowchart of possible microbial and molecular techniques that might be incorporated into aquifer biogeochemical studies.

**Table 1**  
**Overview of Different Microbial and Molecular Methods Used to Study Microorganisms in Contaminated Aquifers, Separated by the Goal of the Researcher**

Methodology	Advantages	Limitations	References
<b>I. Enumeration of total microorganisms</b>			
Direct counts on microscope	Relatively low tech; can determine total number and live/dead and active/inactive with special dyes	Little information on identity or physiological role of organisms; sediment contains an uneven distribution of organisms that are difficult to separate and enumerate	Kepner and Pratt 1994; Bhupathiraju et al. 1999
Flow cytometry	Automation leads to higher efficiency and increase in processing speed	Same as direct count method	Collier and Campbell 1999; Gruden et al. 2003
PLFA analysis	Total viable biomass can be estimated along with some indication of community structure and stress	Conversion factor between PLFA and microbial biomass may be inaccurate; PLFA concentrations vary in response to physical and chemical conditions	Green and Scow 2000; Ludvigsen et al. 1997
Culturing on nonselective media	Easy and robust; ideal for isolation of pure culture	Many environmental microbes cannot be cultured, especially those in oligotrophic environments	Christensen et al. 2000a; de Liphay et al. 2004
Hybridization/ amplification targeting universal 16S rRNA genes	No culturing needed; "snapshot" of entire community; rapid and reliable	Numerous copies of 16S rRNA gene in one cell; bias in extraction procedure; crosshybridization with nontarget sequences	Spiegelman et al. 2005; Stapleton et al. 1998; Fry et al. 1997
<b>II. Enumeration and identification of specific microorganisms</b>			
Culturing on selective media	Easy and robust; ideal for isolation of pure culture; can be used to examine growth under varying conditions	Inability to culture some organisms; anaerobic media are complex to make and maintain; organisms grow slowly and require subsequent analysis for identification	Bekins et al. 1999; Ludvigsen et al. 1999
Hybridization (including microarrays)	FISH requires no prior DNA extraction; detection of specialized groups, 16S rRNA, and functional genes can be targeted; use of RNA to assess activity	Interference from sediment matrix; low-biomass environments makes it difficult; must have known sequence targets	Amann et al. 1997; Zarda et al. 1998; Detmers et al. 2004; Gentry et al. 2006
qPCR	Rapid detection and quantification of 16S rRNA genes and functional genes	Differences in gene copy require standardization; short amplification products are necessary; lack of satisfactory primers for many genes	Stults et al. 2001; Sublette et al. 2006
Cloning and sequencing	Detects novel sequences and dominant species; species identified through comparing sequence data to databases	Extraction bias; time intensive; many clones must be created to ensure a representative sampling of the community	Dojka et al. 1998; Kane et al. 2001
PLFA	Qualitative identification of broad groups of organisms as well as specific ones such as SRB	Many phylogenetic and physiological groups do not have specific lipid biomarkers; requirement of specialized equipment and databases	Green and Scow 2000; Pombo et al. 2002
<b>III. Determine microbial community structure/diversity</b>			
Community-level physiological profiling (Biolog, Inc., Hayward, CA)	Commercially available; provides information on metabolic diversity in a short period of time	Relevance to environmental substrates and communities is unknown; high amount of generated data requires complex statistical analyses	Konopka et al. 1998; Röling et al. 2000
PLFA	Total lipids can be analyzed to create a "fingerprint" for the community	PLFA profiles may be influenced by stress on community and physiological status	Green and Scow 2000
DNA fingerprinting	Rapid snapshot of main species present in community; can be combined with cloning and sequencing to reveal identify	Smaller groups of organisms difficult to detect; qualitative information with limited resolution; can require sophisticated equipment	Spiegelman et al. 2005; Madsen 2000; Rooney-Varga et al. 1999; Haack et al. 2004
<b>IV. Measurements of activity</b>			
RNA analyses	Provide information on actively expressed genes; expression can be linked to function	Small amounts that biodegrade very quickly, hard to extract	Ogram et al. 1995; Alfreider et al. 2003
SIP	Connection of geochemical activity to organism facilitating process; can couple to a variety of geochemical and molecular methods	Used only in studies of carbon compounds; isotope can be expensive; difficulty in separation of isotope in biological molecules	Madsen 2006; Pelz et al. 2001; Geyer et al. 2005
Geochemical assays	Water samples can be collected over time; captures in situ concentrations and estimates microbial activity; can be coupled to other types of measurements	Sediment samples difficult to collect and cannot be resampled; bulk measurements may not adequately capture in situ heterogeneity; no direct info on organisms present	Reviewed by Cozzarelli and Weiss 2007

Note: SRB, sulfate-reducing bacteria.

the deployed materials may help survey the attached microbial community that is missed when only ground water is sampled, it is essential that researchers consider whether these samples are consistently representative of the spatial and temporal distribution of the *in situ* microbial community.

Tracer tests and push-pull tests are field-based methods that involve injecting an experimental solution into the aquifer and then measuring changes in chemistry and microbiology that occur over time and/or space. Advantages of these tests are that they target a relatively large volume of the aquifer that includes the abundant and functionally important attached bacteria (see subsequently) and involve less disturbance of the microbial populations. These approaches are well suited for calculating rates of terminal electron-accepting processes such as nitrate reduction (Schroth et al. 1998), sulfate reduction (Kleikemper et al. 2002), and methanogenesis (Kleikemper et al. 2005). Push-pull tests have been used to determine the presence and activity of different groups of microorganisms (Istok et al. 1997) and the overall community composition (Kleikemper et al. 2002). Tracer tests have been used to examine the spatial variability of *in situ* microbial activity (Sandrin et al. 2004).

#### Sampling Considerations

An appropriate sampling scheme is essential in ground water studies incorporating microbial measurements (Lehman 2007). The sampling scale must match the spatial and temporal scale of the variation in geochemical, hydrologic, and microbial processes of interest. Aqueous geochemical samples, commonly retrieved from sampling locations separated by greater than 1 to 10 m, may result in missed evidence of important biogeochemical reactions due to spatial and temporal heterogeneity (Cozzarelli et al. 2001; McGuire et al. 2000; Wilson et al. 2004). Steep gradients in biogeochemical parameters can result in a high degree of heterogeneity in the microbial community composition (Santoro et al. 2006). The presence of low-permeability zones and/or numerous mixing interfaces may require more intensive sampling in order to capture biogeochemical reactions and microbial communities in these zones. Some researchers have deployed pore water samplers and microelectrodes to examine such gradients and interfaces in detail (Lorah and Olsen 1999; Báez-Cazull et al. 2007).

Another factor to consider is the type of sample (i.e., ground water and/or aquifer sediment) to collect. In many long-term studies of contaminated sites, only ground water samples are collected because they are easier to obtain, and changes can be observed over time in the same sampling well. The ground water can harbor a higher diversity of bacteria (Lehman et al. 2001) and can contain functional groups of organisms different than those in the sediment (Godsy et al. 1992), particularly when sampled at a large (meters) scale (Bekins et al. 1999). However, many investigators have found higher percentages (i.e., 90% of total) of attached bacteria in contaminated aquifer systems, including those contaminated with petroleum (Pombo et al. 2005), creosote (Godsy et al. 1992), sewage (Harvey and Barber 1992), and landfill leachate (Albrechtsen 1994). The sediment is also an

important geochemical reservoir and possibly a source of contaminants, which is essential to accurately assess biodegradation potential in contaminated aquifers (Bjerg et al. 2003). These results underscore the importance of considering both matrices when studying biogeochemical transformations in aquifers.

The collection of aquifer samples for microbial analyses requires special precautions (for a review, see Phelps and Fredrickson 2002) in order to minimize the contamination and disturbance of the microbial community. Aseptic techniques and minimal deviation from the *in situ* conditions (i.e., pH, nutrients, redox, and temperature) are critical as the microbial abundance and composition can change rapidly after collection (Hirsch and Rades-Rohkohl 1988; Haldeman et al. 1995). Samples collected for subsequent molecular analyses are usually immediately frozen on dry ice or liquid nitrogen in the field.

#### Overview of Microbial and Molecular Methods Used in Contaminated Aquifers

This section is designed to introduce the different microbial and molecular methods that can be incorporated into large studies of aquifer biogeochemistry and contaminant degradation. Because these techniques have been extensively reviewed in the literature (e.g., Spiegelman et al. 2005; Madsen 2000), detailed discussion of methods is not repeated here. Table 1 presents an overview of the methods that may be employed in contaminated aquifers (modified from Goldscheider et al. 2006), including the advantages and limitations of each method and references to recent reviews and studies employing each technique in aquifers (where possible). The cited literature is designed to aid in the introduction of the different techniques and is not intended to be inclusive of all the research articles in this area.

There are two broad categories of techniques, those that are culture-based methods and those that are culture-independent methods, each having its own unique applications in environmental studies (Handelsman 2004). Culture-based methods rely on the ability to grow specific organisms that have been obtained from environmental samples under specific conditions (Madsen 2005). The total viable aerobic bacteria are commonly enumerated by plating the samples on general media including tryptic soy broth agar (Pickup et al. 2001), Bitek agar (de Liphay et al. 2003), and R2A agar (de Liphay et al. 2004). The most probable number (MPN) technique has been found to be much more suitable for growing microbes under anaerobic conditions (Christensen et al. 2000a). Ground water scientists have used culture-based approaches to qualitatively delineate the microbial redox zones in contaminated aquifers (Bekins et al. 1999; Ludvigsen et al. 1999). Furthermore, a technique known as community-level physiological profiling relies on microbial growth to measure the functional diversity of environmental communities (Garland and Mills 1991; Konopka et al. 1998). Some limitations of culture-based techniques include the inability to grow many groups of environmental bacteria on standard media (Madsen 2000), the variability in microbial community composition over

short vertical distances of 10 to 20 cm (Bekins et al. 1999), and the difficulty in extrapolating the activity of organisms grown in nutrient-rich media to their in situ biogeochemical contribution. Despite this, culture-based methods continue to serve an essential role in enumerating and isolating organisms and studying the factors that might control their in situ activity (Oremland et al. 2005; Lovley 2003).

Culture-independent techniques do not rely on an organism's ability to be cultured, therefore broadening the percentage of the microbial community that can be targeted. Such techniques include simple measures of total microbial abundance and/or biomass such as microscopy and flow cytometry (Bhupathiraju et al. 1999; Collier and Campbell. 1999). More commonly, the lipids and/or nucleic acids are targeted by phospholipid fatty acid (PLFA) or DNA/RNA analyses, respectively. Nucleic acid techniques include the sequencing of DNA or RNA fragments to determine the identity of members of the microbial community, the detection and enumeration of specific gene sequences through the use of hybridization techniques such as fluorescent in situ hybridization (FISH) and the polymerase chain reaction (PCR), and the molecular fingerprinting of the microbial community to determine relative abundance and diversity (Figure 1). Recent advances in nucleic acid techniques such as quantitative PCR (qPCR) have also enabled ground water scientists to collect numerical data on the copy number of both functional genes and 16S RNA genes in microorganisms that are associated with contaminant degradation (Hristova et al. 2001; Sublette et al. 2006). Several review articles have discussed the methods and limitations of nucleic acid-based techniques in environmental studies (Spiegelman et al. 2005; Siering 1998; Hill et al. 2000; Kelly 2003), including techniques that pertain specifically to the subsurface (Madsen 2000; Stapleton et al. 1998; Scow and Hicks 2005).

The choice of technique will largely be influenced by the questions being asked and the environment in which the research is occurring. Investigators needing a simple estimate of microbial abundance and diversity may perform assays such as direct counting, plating of samples onto a general media, PLFA analysis, simple DNA fingerprinting technique, or amplification of DNA with universal primers. Conversely, investigating a specific biogeochemical process like sulfate reduction might require the use of very specific culture media, primers targeting either certain groups of sulfate reducers or the dissimilatory sulfate reductase gene, and using a combination of stable isotopes and PLFA to identify the organisms facilitating this biogeochemical process.

The geochemistry of the aquifer will also drive some methodological decisions influenced by the ability of different techniques to detect and characterize certain groups of organisms. For example, FISH and PLFA analysis have been found to be most successful in sulfate-reducing aquifers (Detmers et al. 2004; Kleikemper et al. 2002). The type of contamination may also influence the choice of methods and the ability to resolve and identify microbial communities. While culture-based methods and molecular fingerprinting best distinguished microbial

communities in an aquifer contaminated with landfill leachate (Röling et al. 2000), data collected in an aquifer contaminated with herbicide using similar techniques indicated few changes in microbial community composition (de Liphay et al. 2004).

## Emerging Technologies

Enumeration and community characterization techniques can provide information on the numbers, identity, diversity, and potential activity of microorganisms that may be important in biogeochemical studies of aquifers. However, as noted in the text and in Table 1, there are limitations to each of these methods. Furthermore, the suitability of these methods is influenced by the hydrogeology of the aquifer and the dominant biogeochemical processes.

However, as noted in the text and in Table 1, there are limitations to each of these methods as well as environments where one or more of the described techniques do not appear suitable. Furthermore, scientists remain interested in developing more definitive techniques to determine the role of microbial communities in degradation and the overall function and health of an ecosystem. Such emerging techniques include RNA analyses, genomics, stable-isotope probing (SIP), and numerical approaches.

### RNA Analyses

Both rRNA and mRNA concentrations have been used to correlate bacteria with their in situ activity. The advantages of targeting actual rRNA molecules as opposed to DNA genes encoding for rRNA production is that cellular ribosome content can be correlated with general metabolic activity and growth rates (Madsen 2000). Because mRNA concentration is reflective of genes that are actively transcribed, its isolation and manipulation is useful for monitoring the activity of a functional group of microorganisms responsible for facilitating a particular process (Fleming and Saylor 1995). However, detecting and quantifying mRNA is difficult because of its low concentrations and half-life of less than a few minutes (Siering 1998; Wilson et al. 1999).

The first study to isolate and characterize RNA from the subsurface was performed in an uncontaminated aquifer in Hanford, Washington, upgradient from a disposal site of organic and inorganic chemicals (Ogram et al. 1995). Extracted rRNA was converted to cDNA by reverse transcriptase PCR, followed by hybridization with probes for the three phylogenetic domains (Archaea, Eucarya, and Bacteria). The results indicated that the bacterial domain was the most metabolically active although there was a surprising abundance of archaeal rRNA as well, suggesting that these might be an important functional component of the microbial community. mRNA was also isolated from the same samples and studied through PCR primers and hybridization probes designed to detect sequences highly related to a gene encoding toluene-4-monooxygenase. By extracting mRNA from ground water collected at a shallow contaminated aquifer in New York, Wilson et al. (1999) found that naphthalene dioxygenase genes were actively transcribed in the

ground water where naphthalene degradation was occurring at the time of sampling. mRNA analyses have also been used to examine the expression of genes involved in chlorobenzene degradation (Alfreider et al. 2003) and methanotrophy (Cheng et al. 1999) in aquifers.

mRNA concentrations of functional genes can be used for relating actively expressed genes to in situ biogeochemical rates (Schneegurt and Kulpa 1998). In contaminated soils, naphthalene dioxygenase mRNA transcript levels were positively correlated with [<sup>14</sup>C] naphthalene mineralization rates and soil naphthalene concentrations (Fleming and Sayler 1995). mRNA levels for *omcB*, a gene involved in Fe(III) reduction, recently was found to be positively correlated with Fe(III) reduction rates in anaerobic continuous cultures (Chin et al. 2004). In the only study to date to use mRNA to assess subsurface environments during bioremediation, the metabolic state of *Geobacter* was inferred from levels of mRNA of *nifD*, a gene used in N fixation, in a petroleum-contaminated aquifer (Holmes et al. 2004).

The recent development and application of newer mRNA techniques coupled to qPCR has led to quantitative estimates of expressed genes involved in trichloroethene (TCE) degradation (Johnson et al. 2005), the combination of mRNA with FISH techniques to detect expression on a single-cell basis in aquifer samples (Bakermans and Madsen 2002b), and the capacity to perform simultaneous FISH of mRNA and rRNA involved in aerobic CH<sub>4</sub> oxidation in sediment samples (Pernthaler and Amann 2004). A recently developed technique, called catalyzed reporter deposition-fluorescence in situ hybridization, uses rRNA concentrations to quantify live prokaryotic cells in the deep seafloor (Schippers et al. 2005). Such an approach may be possible in aquifers, where FISH has proven difficult due to low microbial biomass.

### Genomics

Since the initial application of molecular techniques to ecological and biogeochemical studies, the collection of such data has broadened into the field of genomics, also known as metagenomics (Handelsman 2004). Genomics involves the study of microorganisms' genome through sequence-based and functional analysis. In genomic studies on environmental samples, DNA is isolated, cloned into a suitable vector, screened for transformation, and then analyzed through high-throughput sequencing. The rapid screening and sequencing of a large number of clones is known as whole-genome shotgun sequencing (Chen and Pachter 2005), an approach that has been employed in the Sargasso Sea (Venter et al. 2004) and an acid mine drainage site (Tyson et al. 2004). Data collected with this method allow researchers to detail the composition and diversity of the community and to assemble near-complete genomes of prevalent organisms. Such information can provide insight into the in situ metabolic role of the target organism (Handelsman 2004). For example, a metagenomic sequence analysis at the acid mine drainage site discovered that a group of *Leptospirillum* sp. contained genes similar to those involved in nitrogen fixation, suggesting

that this subspecies was responsible for providing the microbial community with nitrogen (Tyson et al. 2004). Because shotgun sequencing requires the "piecing" together of short DNA sequences, some researchers have started using large insert vectors such as the bacterial artificial chromosome (Deutschbauer et al. 2006). To our knowledge, large metagenomic libraries have not been created from microbial communities in contaminated aquifers, although there has been success in recovering sequence data from DNA that has been amplified by PCR (Erwin et al. 2005; Abulencia et al. 2006). Further advances in high-throughput sequencing, cloning, and amplification technology will likely increase our understanding of microbial community composition as well as how individual members of the community contribute to its overall activity and function (Allen and Banfield 2005; Roesch et al. 2007).

Whole-genome sequencing can also be applied to pure cultures which can help researchers better understand organisms important in biogeochemistry, including those in contaminated environments (Lovley 2003). The most notable examples are different species of the genus *Geobacter*, organisms that serve important biogeochemical roles in many contaminated aquifers. Genomic research on *Geobacter metallireducens* has shown that, despite the long-term belief that it was nonmotile, its genome contains genes encoding flagella and pili that are expressed when the organism was growing on insoluble iron oxides (Childers et al. 2002; Mehta et al. 2005). Another species of *Geobacter*, *G. sulfurreducens*, was thought to be a strict anaerobe until genetic studies indicated an ability to use oxygen as a terminal electron acceptor (Lindroos et al. 2002). Thus, these findings have led to a new understanding of the relationship between the environment and *Geobacter* and the way in which *Geobacter* facilitates Fe(III) reduction (Lovley 2003; Oremland et al. 2005). Similar studies are ongoing for environmentally relevant organisms such as *Dehalococcoides ethanogenes* and *Shewanella oneidensis* (Lovley 2003).

Metagenomics also includes the use of microarrays, a hybridization technique in which numerous probes are used to detect the relative presence and expression of multiple organisms and/or genes (Gentry et al. 2006). Microarrays can also be used in conjunction with sequencing studies in order to help screen and identify clones (Sebat et al. 2003). DNA microarrays have been used to detect 16S rRNA genes of *Geobacter chapellei* and *Desulfovibrio desulfuricans* in soil extracts (Small et al. 2001) and to track gene expression of *G. sulfurreducens* under varying conditions (Methe et al. 2005). Microarrays have also been used to study functional genes, including those associated with the nitrogen cycle in marine sediments (Wu et al. 2001) and methanotrophy in landfills (Stralis-Pavese et al. 2004). In studies of microbial communities in contaminated environments, microarrays have been used to identify and track members of an enriched benzene- and toluene-degrading microbial consortia (Koizumi et al. 2002) and to examine 16S rRNA isolated from uranium-contaminated sediments (Chandler et al. 2006; Brodie et al. 2006). He et al. (2007) recently

developed and applied a novel comprehensive microarray containing 4150 functional gene groups involved in nitrogen, carbon, sulfur, and phosphorus cycling; metal reduction and resistance; and organic contaminant degradation. This microarray, called the GeoChip, was successfully used for tracking the dynamics of metal-reducing bacteria and associated communities for an in situ bioremediation project.

#### Stable-Isotope Probing

SIP allows researchers to “track the flow of atoms in isotopically enriched molecules through complex microbial communities into metabolically active microorganisms” (Madsen 2006). Specifically, an environmental sample is exposed to stable isotope-enriched substrates followed by recovery of the labeled biomarker (i.e., DNA, RNA, or PLFA) (Lee et al. 1999; Dumont and Murrell 2005). The labeled compounds can then be used to identify the microorganisms that are actively using the added substrate. SIP was originally performed with PLFA molecules, by using isotope ratio mass spectrometry to study and identify active sulfate reducers and methanogens (Boschker et al. 1998). DNA-SIP originated in studies of methane-oxidizing bacteria incubated with  $^{13}\text{CH}_4$  (Radajewski et al. 2000, 2002; Morris et al. 2002). RNA can also be used as a potentially more responsive biomarker due to its higher turnover rates (Manefield et al. 2002).

Over the past few years, several studies in contaminated environments have used SIP to identify key organisms involved in biodegradation (Madsen 2006). SIP of PLFA, combined with 16S rRNA sequencing, was used in aquifer microcosms to demonstrate the key groups of sulfate-reducing bacteria responsible for toluene degradation (Pelz et al. 2001). A novel study employed in situ biotrap with Bio-sep beads amended with  $^{13}\text{C}$ -labeled benzene or toluene followed by lipid extraction of the attached microbial community to verify biodegradation by indigenous microorganisms (Geyer et al. 2005). Bio-sep beads spiked with  $^{13}\text{C}$ -labeled acetate were used by Chang et al. (2005) to examine microbes responsible for U(VI) reduction. SIP can also be used to monitor the effects of changing environmental conditions on the activity of the microbial community. For example, a recent RNA-SIP study in gasoline-contaminated ground water showed that a phylotype affiliated with the genus *Azoarcus* specifically appeared in the  $^{13}\text{C}$ -RNA fraction only when nitrate was supplemented (Kasai et al. 2006).

Field-based SIP, in which the labeled substrate is injected into ground water or sediments, has also been used to link organisms to biodegradation processes. In a field study of naphthalene degradation in sediments contaminated with coal tar waste,  $^{13}\text{C}$ -labeled naphthalene was added to the surface sediments (Jeon et al. 2003). A combination of gas chromatography/mass spectrometry (GC/MS), molecular biology, and culture-based techniques was used to link an undiscovered group of bacteria with naphthalene degradation. Specifically, DNA that was enriched in  $^{13}\text{C}$  naphthalene was sequenced to determine the identity of the organisms using the substrate. In a parallel set of experiments, organisms capable of naphthalene

degradation were enriched; sequence data indicated that one of the enriched organisms was the same as that identified in the enriched DNA. Field-scale labeling of PLFA has also been used to trace acetate assimilation and mineralization in a petroleum hydrocarbon-contaminated aquifer as well as identify the keys microorganisms contributing to these processes (Pombo et al. 2005, 2002). These results indicate that SIP is a powerful new technique for identifying microorganisms that are actively involved in specific metabolic processes under conditions that approach those occurring in situ. Furthermore, there is the potential that data collected from SIP analyses can be used to predict and optimize conditions for microbial biodegradation of contaminants (Madsen 2006).

#### Numerical Approaches

The large amount of data collected with genomics as well as the need to relate different types of geochemical and molecular data have driven the need for numerical methods capable of data analysis and integration. Bioinformatics is a necessary tool in biogeochemical studies where a large amount of sequence and/or microarray data are collected and analyzed (Chen and Pachter 2005). This analysis is complicated by the need to piece together sequence data from different organisms whose abundance and diversity is complex (Allen and Banfield 2005). Aligning sequence data from even a single species to create a full genome sequence is a difficult task that requires exceptional computational skills and software (Oremland et al. 2005). Thus, realizing the potential for discovery from genomics will depend on advancing methods of data construction and analysis (Handelsman 2004).

It is also essential that these molecular data be integrated with the geochemical data collected in aquifer studies. Geostatistics, historically used to analyze spatial data collected from heterogeneous aquifers, is beginning to incorporate indirect or direct measurements of microbial diversity and activity (Franklin et al. 2002; Mohamed et al. 2006). Recent work has also demonstrated the usefulness of multivariate statistical methods for discerning relationships in complex data sets and for developing hypotheses regarding the relationship between community structure and measured environmental parameters. Statistical analyses of DNA fingerprints are commonly used to relate microbial community composition to pollutant concentration and redox processes (Röling et al. 2001; Haack et al. 2004). Mouser et al. (2005) used a multivariate statistical approach to integrate hydrochemical and microbial community profiles, resulting in a clear distinction between contaminated and uncontaminated sites. Recently, artificial neural networks (ANNs) have been found to be more useful than multivariate statistical techniques for analyzing data structures that are complex, nonlinear, fuzzy, probabilistic, and inconsistent such as DNA fingerprints (Noble et al. 2000). For example, ANNs can be used to identify sets of bands in fingerprints that indicate the potential for degradation (Röling and Van Verseveld 2002) and for the coupling of functional gene diversity and geochemical data from environmental

samples (Palumbo et al. 2004). The relationships revealed by these approaches form the basis for developing conceptual and numerical models, usually with the goal of predicting future biogeochemical reactions at the investigated or other similar sites.

### Comprehensive Integrative Studies

Traditionally, geochemists and hydrogeologists have used laboratory assays and field-based geochemical measurements and experiments to understand aquifer biogeochemistry and develop conceptual models for site remediation strategies. These methods could provide estimates of contaminant degradation rates and potential metabolic activity but offered limited insight into the microorganisms that facilitate the observed geochemical processes. On the other hand, microbiologists relied heavily on enrichment and molecular techniques to enumerate and characterize the microorganisms responsible for different biogeochemical processes. However, microbiologists rarely performed field studies to confirm the importance of the in situ microbial community and to relate the microbiology to the observed geochemistry. Because microbial and chemical processes interact to control the chemical evolution of ground water, the study of aquifer biogeochemistry in more recent years has broadened into an inherently multidisciplinary pursuit (Haack and Bekins 2000). Within the last decade alone, there has been a dramatic increase in the use of a comprehensive experimental design that incorporates a wide range of geochemical and microbiological techniques to study aquifer biogeochemistry. The goals of such studies have ranged from initial investigations of general site biogeochemistry to evaluating specific remediation strategies such as natural attenuation and biostimulation. In many cases, these innovative and integrative combinations of methods have provided compelling linkages between the microbial community and the observed biogeochemical processes. Collectively, these studies have illustrated that the inclusion of microbial and molecular methods into the experimental design can result in a better understanding of the processes occurring in contaminated aquifers.

Table 2 provides information on a subset of recent comprehensive integrative studies that used a variety of geochemical and microbiological approaches to significantly enhance the understanding of aquifer biogeochemistry in contaminated systems. These studies are divided into four different categories based on the overall objectives: (1) understanding the overall site geochemistry and microbiology, (2) finding useful indicators of contaminant degradation, (3) assessing the natural attenuation potential, and (4) considering the use of other remediation strategies such as biostimulation and bioaugmentation. In the subsequent sections, investigations in each category are discussed with a focus on the experimental objectives, the different techniques used, and the contribution of the microbial and/or molecular techniques to the overall findings/conclusions. Collectively, these studies provide a framework for incorporating microbial and molecular methods into studies of contaminated aquifers.

### Understanding Site Geochemistry and Microbiology

In the first category of studies (Table 2), the goal of the researchers was to assess whether the microbial community is capable of the degradation of a specific contaminant. In the work in an aquifer contaminated with landfill leachate, results of initial experiments suggested a clear difference in the microbial community and geochemistry within and outside of the contaminant plume (Röling et al. 2001, Lin et al. 2005). Though molecular techniques such as denaturing gradient gel electrophoresis (DGGE) were not able to link microbial samples with geochemical processes (Röling et al. 2001), both microbial and molecular measurements suggested that the Fe(III)-reducing population might be important in this environment. A follow-up study by Lin et al. (2005) targeted the presence, abundance, and diversity of *Geobacter* spp. and found that the community composition of this group was directly related to the occurrence of degradation processes and the hydrogeochemistry of the polluted water. The differences in *Geobacteraceae* community structure in the plume were reflected by an intense band in the DGGE profiles of samples taken from an iron-reducing zone close to the landfill. Statistical analysis showed that this band, as well as three other less-intense DGGE bands, was indicative of ground water pollution. DGGE was proposed as a potential tool for predicting and monitoring natural attenuation in similar environments. These researchers recently expanded on their use of culture-independent 16S rRNA gene-based methods by using culture-based techniques to enrich for iron-reducing consortia using a range of culturing media, with various electron donors and acceptors and varying incubation conditions (pH, temperature), and by applying dilution-to-extinction culturing (Lin et al. 2007). Interestingly, the dominant *Geobacter* subspecies that was previously identified in the DGGE analysis was not isolated. The isolation of another *Geobacter* strain and *Serratia*, *Clostridium*, *Rhodospirillum rubrum*, and *Desulfotobacterium* strains suggested the presence of a more diverse iron-reducing community, one that was best studied using a variety of different types of culture-dependent and independent techniques.

Several recent studies have been focused on understanding the relationship between the geochemistry and the microbial community of a hydrocarbon-contaminated aquifer (Pombo et al. 2005; Kleikemper et al. 2005). On the basis of geochemical evidence, this aquifer was actively undergoing natural attenuation, with contaminant degradation believed to be coupled to sulfate reduction and methanogenesis. The researchers used an integration of traditional geochemical techniques such as stable isotope analysis and push-pull tests with molecular analyses of PLFA and DNA to determine which microorganisms may be responsible for the observed biogeochemistry. In a study focusing on sulfate reduction, sulfate and labeled acetate were added in push-pull tests, followed by PLFA on the suspended and attached microbial community (Pombo et al. 2005). The researchers were able to identify that, despite differences in composition, both communities were actively degrading the added acetate, indicating that both populations could be important in the degradation of hydrocarbon in this aquifer. They also

<b>Table 2</b> <b>Examples of Comprehensive, Integrative Investigations of Aquifer Biogeochemistry</b>					
<b>Question</b>	<b>Experimental Design/Techniques</b>	<b>Contaminant</b>	<b>Results</b>	<b>Role of Microbial Techniques</b>	<b>References</b>
I. Understanding site geochemistry Is there a relationship between microbial community structure (i.e., <i>Geobacter</i> spp.) and hydrogeochemistry and contaminant degradation?	Geochemical analyses; DGGE; cloning, sequencing of 16S rRNA gene; MPN-PCR for <i>Geobacteraceae</i> ; culturing of iron-reducing bacteria followed by sequence analysis	Landfill leachate	<i>Geobacter</i> presence and abundance was related to the occurrence of degradative processes and hydrochemistry of polluted water	A diverse and distinct Fe-reducing microbial community occurred in active degradation zones; a subset of molecular markers indicated the degree of contaminant degradation; <i>Geobacteraceae</i> were dominant, but culture-based analyses indicated a more diverse population of Fe reducers	Röling et al. 2001; Lin et al. 2005, 2007
What sulfate-reducing bacteria are actively degrading acetate in a contaminated aquifer?	Geochemical analyses; push-pull tests; FISH; SIP followed by PLFA analysis	Petroleum hydrocarbons	Petroleum degradation was coupled to sulfate reduction and methanogenesis; The main acetate utilizer in the ground water was <i>Desulfotomaculum acetoxidans</i> , and <i>Desulfobacter</i> sp. and <i>D. acetoxidans</i> were in the sediment	Molecular techniques revealed different microbial communities in the sediment and ground water as well as different ones in the field vs. those enriched in the laboratory	Pombo et al. 2005
What is the diversity and activity of methanogens in a petroleum-contaminated aquifer?	Geochemical analyses; push-pull tests; cell counts; FISH; DGGE; cloning and sequencing	Petroleum hydrocarbons	The methanogen community was able to consume a number of different substrates; methanogenesis may contribute significantly to petroleum mineralization	High abundances of an acetate-using methanogen ( <i>Methanosaeta</i> ) indicated that acetate was the main substrate for methanogens; molecular analyses also indicated that a large CO <sub>2</sub> -using population may coexist	Kleikemper et al. 2005
II. Indicators of contaminant degradation What in situ parameters can indicate petroleum degradation?	Geochemical analyses; MPNs; direct counts; sequencing of 16S rRNA genes	Petroleum hydrocarbons	Indicators of pollution degradation included the enrichment of anaerobic bacteria in the contaminant plume, the presence of putative metabolites, the alkalinity/hardness ratio, and 16S rRNA genes	The retrieval of a 16S rRNA sequence identical to that of an organism that degrades propylbenzene indicated that this biodegradation process occurs at this site	Eriksson et al. 2006
What is the response of the aquifer bacterial community to BTEX contamination? Do measurements of microbial abundance and activity indicate areas of active degradation?	In situ mesocosm study; geochemical analyses; DGGE on 16S rRNA genes and BTEX degradation genes followed by sequencing SIP; in situ biotrap; PLFA; geochemical analyses	Oil refinery materials (BTEX) Benzene, toluene	Upon contact with contamination, the microbial community rapidly evolved into one dominated by contaminant degraders Significant incorporation of <sup>13</sup> C into the microbial lipids indicated that the microbial community was active in degradation	Bacteria with degradative genes quickly colonized contaminated downstream areas; the degradative genes were excellent indicators of contamination SIP was a powerful technique for determining where biodegradation was occurring and to assess potential rates	Hendrickx et al. 2005 Geyer et al. 2005

Question	Experimental Design/Techniques	Contaminant	Results	Role of Microbial Techniques	References
<p>III. Natural attenuation potential</p> <p>Can the indigenous microorganisms support natural attenuation, which species are involved in the degradation?</p>	<p>Geochemical analyses; laboratory sediment microcosm assays; direct counts; PLFA and rRNA biomass analysis, MPNs, PCR of 16S rRNA genes, cloning, and sequencing</p>	<p>Chlorinated ethenes</p>	<p>The microbial community was actively degrading TCE and its daughter products</p>	<p>Anaerobic-halorespiring organisms and Fe reducers identified. Enrichment cultures demonstrated the importance of direct, non-cometabolic oxidation of cis-DCE and VC.</p>	<p>Davis et al. 2002</p>
<p>Can the indigenous microorganisms degrade coal tar waste?</p>	<p>Geochemistry; Laboratory microcosms; isolation of naphthalene-degrading bacteria, PCR and ARDRA of 16S rRNA and nahAc genes, SIP, push-pull tests</p>	<p>Coal tar waste</p>	<p>Biodegradation of coal tar constituents occurred via both aerobic and anaerobic terminal electron accepting processes (TEAPs)</p>	<p>Molecular techniques revealed a much larger diversity of degraders; SIP combined with sequencing was able to clearly identify the naphthalene degrader</p>	<p>Bakermans et al. 2002; Bakermans and Madsen 2002a; Jeon et al. 2003</p>
<p>What are the chemical and microbial factors that could result in natural attenuation?</p>	<p><sup>14</sup>C assays with phenol; laboratory microcosms with rate data; direct counts; isolation of culturable bacteria; PCR of 16S rRNA and functional genes</p>	<p>Tar acid</p>	<p>Despite evidence of potential degradation, discrepancies between geochemical data collected by field measurements and in microcosms indicate that natural attenuation was not occurring</p>	<p>There was a diverse microbial community capable of aerobic and anaerobic degradation under the observed geochemical conditions; factors other than the microbial community likely limited natural attenuation</p>	<p>Pickup et al. 2001; Williams et al. 2001</p>
<p>Can in situ microcosms be used to monitor biodegradation potential in different geochemical zones of a contaminated aquifer?</p>	<p>In situ microcosms (BACTRAP) with <sup>13</sup>C-labeled toluene, benzene; geochemical measurements; PLFA; DNA fingerprinting; sequence analysis of partial 16S rRNA genes</p>	<p>Toluene and benzene</p>	<p>The BACTRAPs were useful in obtaining qualitative information on the in situ biodegradation of contaminants; both geochemical and microbial measurements indicated that the microbial community was capable of degradation</p>	<p>PFLA indicated that microorganisms were actively degrading the contaminants; 16S rRNA gene analysis helped discriminate microbial communities and suggested the presence of an iron- and sulfate-reducing community</p>	<p>Stelzer et al. 2006</p>
<p>IV. Other remediation strategies</p> <p>How are the activity and composition of the microbial community affected by biostimulation?</p>	<p>Geochemical analyses; push-pull tests; PCR of 16S rRNA genes, cloning, and sequencing; MPN-PCR</p>	<p>Uranium and nitrate</p>	<p>Electron donor addition resulted in the reduction of nitrate, Fe(III), and U(VI). Fe-reducing bacteria were enriched during the biostimulation treatments</p>	<p>A close correspondence between microbial activity and an increase in 16S rRNA gene sequences suggested that Geobacter-type organisms were important metal reducers in the acidic subsurface at this site</p>	<p>North et al. 2004</p>
<p>Can electron donors be added to stimulate the microbial community to dechlorinate 1,2-DCA?</p>	<p>Geochemistry; laboratory microcosms; fingerprinting with automated ribosomal intergenic spacer analysis and DGGE; sequencing; PCR of functional genes</p>	<p>Chlorinated ethenes</p>	<p>Several electron donors were found to stimulate dechlorination; the microbial response was varied, depending on the sampling location</p>	<p>Through sequencing, bacteria similar to those enriched from other halogen-contaminated sites were detected; all the wells were found to host microbial communities capable of degradation</p>	<p>Marzorati et al. 2006</p>

demonstrated that labeled methane was produced during acetate degradation, implying that acetoclastic methanogenesis may play an important role in this environment. A detailed study by Kleikemper et al. (2005) found that, although methane was produced with the addition of a number of different carbon substrates, acetoclastic methanogenesis was likely the dominant methane production pathway due to high abundances of one genus of acetate-using methanogens. Thus, molecular techniques not only provided information about the identity of the microbial community but their likely activity and potential contribution to the biogeochemical processes controlling the fate of hydrocarbons.

#### Indicators of Contaminant Degradation

In addition to understanding the site geochemistry, ground water scientists are also interested in obtaining information about the presence and/or rates of biodegradation. Recent studies have examined the feasibility of using microbial and/or molecular parameters that might also serve as powerful tools for detecting and measuring in situ biogeochemical processes. Eriksson et al. (2006) incorporated the use of geochemical methods such as gas chromatography/mass spectrometry and analyses for alkalinity and hardness with MPN analyses and 16S rRNA gene sequencing in an aquifer contaminated with petroleum hydrocarbons. In addition to finding the alkalinity/hardness ratio and the chemical concentrations of some putative metabolites to be useful geochemical indicators, investigators recovered a large number of 16S rRNA sequences that were similar to organisms found in other petroleum-contaminated aquifers as well as a sequence identical to that of an isolated propylbenzene-degrading culture. These 16S rRNA gene sequences were suggested as possible markers for petroleum degradation.

In a study at an aquifer contaminated with benzene, toluene, ethylbenzene, and xylenes (BTEX) compounds at an oil refinery site in the Czech Republic, investigators tested the response of the microbial community to BTEX (Hendrickx et al. 2005). Specifically, functional genes were used to monitor the microbial community located in an in situ mesocosm system. Specific genes indicative of BTEX contamination were recovered only from the contaminated area. Such genes were suggested to be feasible metabolic markers for measuring microbial activity during bioremediation. Both this study and that by Eriksson et al. (2006) demonstrated that there were organisms capable of contaminant degradation in this aquifer, although it should be clearly noted that the recovery of gene sequences does not definitively indicate what the organisms' specific function or level of activity may be in situ.

A new method that is designed to better link geochemical and molecular indicators for biodegradation is the use of in situ sampling methods such as Bio-sep beads (see Experimental Design section). By using this technique in a BTEX-contaminated aquifer, Geyer et al. (2005) were able to demonstrate that toluene and benzene degradation was actively occurring and was facilitated by indigenous organisms under in situ conditions. In addition to obtaining reliable information about if and where biodegradation was occurring, SIP was used to determine

that different populations were active in the degradation of each hydrocarbon. Results obtained with techniques such as SIP may help determine which bacteria are actively degrading a contaminant and to subsequently develop suitable markers like gene sequences that could effectively detect and enumerate these organisms.

#### Natural Attenuation Potential

One of the most cost-effective treatment procedures for contaminated aquifers takes advantage of in situ bacteria that naturally degrade the contaminants over time. There are numerous reviews available on the potential microbial degradation pathways for organic contaminants commonly found in aquifers, such as alkylbenzenes (e.g., Spormann and Widdel 2000), n-alkanes (e.g., Rabus et al. 2001), and methyl tertiary-butyl ether (e.g., Deeb et al. 2000). Evidence to support natural attenuation by microbial processes in situ usually takes the form of geochemical data demonstrating contaminant loss and footprints of biodegradation and/or laboratory microcosms demonstrating degradative ability with less attention given to the actual organisms involved in the transformation process (Cozzarelli and Weiss 2007). The strongest evidence of microbially driven attenuation is obtained when there is a correlation between the bacteria present and the observed biogeochemical process. As a result, many recent geochemical studies of natural attenuation have incorporated microbial and molecular methods into their experimental design (Table 2).

Davis et al. (2002) investigated the presence and ability of the indigenous microbial community to support natural attenuation of chlorinated compounds in an anaerobic contaminated aquifer. They used a two-pronged approach consisting of culture based and molecular analyses of the dechlorinating community. Laboratory microcosms and pure culture studies indicated that organisms directly catalyzed the mineralization of high-order chlorinated ethenes. The recovery of sequences similar to known anaerobic-halorespiring organisms such as *Desulfitobacterium*, *Dehalobacter*, and certain groups of iron reducers provided qualitative support for a role of reductive dechlorination processes in the aquifer. Enrichment cultures demonstrated the importance of direct, non-cometabolic oxidation of dichloroethene (cis-DCE) and vinylchloride (VC) in natural attenuation, information that may not have been detected by examining site geochemistry. The incorporation of molecular analyses in this study allowed the identification of a functional linkage between the microbiology of the site and the natural attenuation processes.

At a coal tar waste site, geochemical analyses and laboratory microcosms were used to investigate whether the indigenous microbial community was degrading naphthalene and other organic coal tar constituents (Bakermans et al. 2002). A parallel study of the microbial community using molecular techniques found that community composition correlated strongly with the geochemical changes induced by coal tar waste (Bakermans and Madsen 2002a). Analyses of functional genes involved in naphthalene degradation also indicated a diverse population of naphthalene degraders. Jeon et al.

(2003) extended this research by using push-pull tests combined with SIP to identify the specific members of the microbial community actively degrading naphthalene and to quantify rates of naphthalene degradation. Moreover, using 16S rRNA sequencing, they were able to identify a naphthalene-degrading member of the microbial community and used isolation techniques to obtain a pure culture of this organism. In laboratory assays, the researchers were able to definitively demonstrate that this organism was indeed mediating naphthalene degradation. Collectively, these three related studies used a combination of techniques to establish that contaminant degradation was occurring and to identify which bacteria were specifically mediating the degradation process.

At an aquifer in the United Kingdom contaminated with coal tar and phenol, researchers examined if the chemical and microbial parameters could support natural attenuation (Pickup et al. 2001; Williams et al. 2001). By taking the geochemical (i.e., contaminant and electron acceptor concentrations) and microbial measurements of ground water samples, a preliminary conceptual biogeochemical model was developed to assess the controls on natural attenuation. Though the geochemical data indicated limited degradation, microbial measurements (i.e., enumeration of culturable bacteria and PCR and cloning of 16S rRNA and functional genes) suggested the presence of a diverse microbial community capable of both aerobic and anaerobic degradation. The researchers concluded that some other factor (i.e., phenol toxicity) might be limiting the microorganism's in situ activity. Thus, natural attenuation was not an effective remediation solution for this site.

The use of in situ microcosms known as BACTRAPs® (Isodetect, Munich, Germany) was used to evaluate the natural attenuation potential in an aquifer contaminated with toluene and benzene (Stelzer et al. 2006). These microcosms were amended with <sup>13</sup>C-labeled toluene and benzene, and PLFA analysis was used to verify the assimilation of this substrate into the microbial community. This ability of the community to degrade the contamination was supported by the presence of secondary metabolites on the microcosms. Because the fatty acid analysis could not discriminate microbial communities at different geochemical zones in the aquifer, the cloning and sequencing of 16S rRNA genes was used. Results from this study indicated the potential of the microbial community to degrade the contaminant but also revealed a dominance of non-culturable bacteria related to previously described iron- and sulfate-reducing organisms. Thus, molecular analyses provided information on the microbial community that was not discernable by geochemical or culture-based methods.

#### Other Remediation Strategies

Molecular techniques are also starting to be used in geochemical studies examining remediation strategies other than natural attenuation (Table 2). In a study of an aquifer in which biostimulation was considered as a treatment option, North et al. (2004) used a combination of push-pull tests and molecular analyses to examine aquifer sediments cocontaminated with uranium and nitrate. The injection of electron donors stimulated the

reduction of the contaminants with a concomitant increase in the gene abundances of metal-reducing bacteria. These results, in conjunction with cloning and sequencing information, indicated that members of the  $\delta$ -Proteobacteria could be important metal reducers in acidic subsurface sediments.

Marzorati et al. (2006) used laboratory microcosms to examine whether electron donors could be used to stimulate the microbial community to dechlorinate 1,2 dichloroethane (DCA). By using DGGE fingerprinting followed by the sequencing of the dominant bands, they found that the response of the microbial community was heterogeneous, depending on the well sampled. All of the wells, however, were found to host microbial communities that could degrade 1,2 DCA including organisms similar to those enriched from or found in halogen-contaminated sites. They also found a novel sequence for a putative dehalogenase in the microcosms with the highest rates of dechlorination, possibly suggesting that this organism may be facilitating the degradation. Not only do these studies indicate that the microbial community and its response to biostimulation may be varied but they also reinforce the concept that specific gene sequences may serve as important predictors and indicators of biodegradation.

The studies in Table 2 have not only facilitated a holistic and detailed understanding of the basic biogeochemistry of contaminated aquifers but they have also shown that microbial and molecular techniques have a clear role as indicators of biodegradation and in evaluating potential remediation strategies. In each of these experiments, scientists have begun to assemble many convergent lines of biogeochemical evidence ranging from measurements of geochemical transformation and stable isotopic fractionation in the field to laboratory metabolic assays, and to extraction and analysis of phospholipids fatty acids and DNA.

#### The Future of Biogeochemical Studies in Aquifers

Perhaps, only a decade ago, there was a large divide between scientists using geochemical approaches to study contaminant degradation and those that were actively culturing and targeting the microbes that might be involved in this process. Even within the microbiological research community, the advent of molecular approaches had further splintered collaborations as there was the development of a strong bias against the use of culture-based approaches that some argued could only detect less than 1% of the microbial community. Molecular approaches, including sequencing, DNA fingerprinting, qPCR, and microarray technology, were heralded as being the best at generating information about the composition, abundance, and activity of the microbial community. Though few can now doubt the great potential for such techniques to resolve the genetic and metabolic potential of communities and species such as *Geobacter* sp., it has recently become clear that these techniques have limitations.

Molecular techniques have a methodological bias due to the extraction and amplification of DNA from samples, causing some potentially important organisms

to be underestimated or undetected. The molecular characterization of complex microbial assemblages is also challenging, exacerbated by the recovery of incomplete sequence data, an overall lack of reproducibility in results, and the inability of some techniques to resolve differences in the microbial community. Furthermore, comparing microbial communities through techniques such as DNA fingerprinting provides information on qualitative differences but requires additional methods to actually determine how those communities are different in terms of species composition. Even the targeting of specific groups of organisms through techniques such as real-time PCR and microarrays can require having prior knowledge of the community in order to obtain satisfactory results. This can lengthen the course of study and result in high supply and equipment costs. The success of genomic approaches also hinges on having adequate biomass to analyze, a real challenge in some aquifers that have a small microbial community. Finally, the analysis of data collected with genomics requires computational skills, adequate informational databases, and perhaps most importantly, an ability to use such data to answer ecosystem-level questions on biogeochemical processes and contaminant degradation. The discussion of such limitations does not negate the important and increasing role of molecular techniques in biogeochemical studies, for these methods will continue to be at the cutting edge of science, with methodological advances likely alleviating or eliminated some of the challenges.

The growing realization of the limitations of molecular techniques has led to a renewed interest in the use of culture-based techniques to detect and study microbial communities, particularly as part of a larger biogeochemical study. In fact, obtaining a “representative” pure culture of an organism that carries out an important biogeochemical process still remains a necessary first step in relating a microorganism to a specific function in the environment (Oremland et al. 2005; Madsen 2005). Culture-based techniques can be used to determine how organisms respond both physiologically and genetically to a range of culturing condition, with various electron donors and acceptors and varying incubation conditions (pH and temperature) (Lin et al. 2007; Methe et al. 2005). Culture-based approaches such as MPN analysis can also qualitatively delineate the microbial redox zones in contaminated aquifers. (Ludvigsen et al. 1999; Bekins et al. 2001). When such techniques are combined with molecular approaches, data can be obtained on the identity of numerically dominant organisms facilitating specific redox processes. For example, many studies using SIP have employed cultured-based methods to isolate and then study the organism that molecular data suggested was facilitating a specific biogeochemical transformation (Jeon et al. 2003; Stelzer et al. 2006). The many uses of culture-based approaches in biogeochemical studies necessitates the need for researchers to continue to develop media that will more appropriately mimic the natural environment, leading to a heightened recovery of “culturable” organisms.

As has been discussed in this paper, some of the most compelling biogeochemical studies in contaminated environments have used a variety of different methods to

consider contaminant degradation potential. The comprehensive studies further demonstrate that there is no single technique that will serve as the “magic bullet” for biogeochemical investigations in contaminated aquifers. These results have further underscored the need in the ground water research community to conduct qualitative and quantitative comprehensive studies in which different techniques are used in order to better understand the contaminated ground water system.

We urge researchers working in contaminated ground water systems to adopt a comprehensive, balanced methodological approach, in which molecular approaches are coupled with the more traditional geochemical and cultured-based techniques. We anticipate that the study of aquifer biogeochemistry will continue to grow as a collaborative science in which hydrologists, geochemists, microbiologists, and statisticians must work together.

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