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The double helix meets the crystal lattice: The power and pitfalls of nucleic acid approaches for biomineralogical investigations

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ABSTRACT

The inability to culture the majority of microorganisms from natural habitats has been well documented. In response to this constraint, the development and application of culture-independent methods for studying microbial communities have been a focus of microbial ecology for the past several years. Molecular approaches, in particular, offer great potential to investigate the role of microorganisms in areas as diverse as biogeochemistry, pollutant remediation, and disease causation. The purpose of this review is to give a brief critique of nucleic acid-based techniques currently used in microbial ecology, to serve as a reference for those wishing to employ such techniques, and to examine the applicability of these techniques toward studying the biogeochemical roles of bacteria, especially as related to metal cycling and biomineralization. Although published reports on the application of molecular techniques to biogeochemical and biomineralogical problems are scarce, this review aims to familiarize biogeochemists and biomineralogists with the strengths and weaknesses of these approaches. Advances have been made in describing the diversity of uncultured microbial communities, detecting/identifying specific cells and functional genes in environmental samples, detecting in situ activities of specific microorganisms, and determining some of the factors controlling gene expression in in vitro studies. However, the current methods provide limited quantitative information about natural processes. Acknowledging these limitations can assist the development of methods to answer basic questions concerning the in situ distribution and activities of microorganisms. Studies relating these in situ measurements of microbial activity and distribution to the physical and chemical microenvironment will ultimately permit a better understanding of the importance of microorganisms in mineral formation and dissolution processes.

INTRODUCTION

Although microorganisms have been on this planet for some 3.8 billion years, we are just beginning to appreciate the complex interplay between biotic and abiotic processes in mineral formation and dissolution (Ehrlich 1995). Pasteur, Winogradsky, and Beijerinck were largely responsible for convincing the world that microorganisms are more than mere agents of disease; that they are capable of mediating an array of geochemical changes. Their pioneering work provided a stable foundation for elucidating the role of microorganisms in several important geochemical cycles on our planet. Although we have traveled great intellectual distances to accept the importance of bacteria in planetary geochemistry, we are still relatively unenlightened in our understanding of the relative contributions of abiotic and biotic processes in geochemical and mineral cycling.

The intimate relationship between metals and microorganisms has long been appreciated by microbiologists and biochemists who recognized that several metals (i.e., Na, K, Mg, Ca, Fe, Mn, Co, Ni, Zn, Cu, Mo) are known to be essential for life processes. Other metals exhibit toxicity or are not known to have a biological function (i.e., Cd, Al, Sn, Au, Hg, Pb, etc.), but their speciation and incorporation into complex minerals is often directly or indirectly influenced by microorganisms. Furthermore, microorganisms are known to play a major role in the formation of many solid phase minerals including metal oxides and sulfides, carbonates, silicates, and clays (Ehrlich 1995).

Biogenic mineral formation occurs by at least two distinct processes (Lowenstam 1981). The first includes biologically controlled processes in which the site of mineral formation is located within a biological compartment physically distinct from the extracellular environment. Minerals formed in this manner consist of well-ordered crystals that develop in a biologically formed-scaffold; the mineral type, orientation of crystal axes, and mineral structure are all under genetic control. These genetically controlled processes govern the formation of minerals

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such as intracellular magnetite and greigite in magnetotactic bacteria (Balkwill et al. 1980; Towe and Moench 1981; Mann et al. 1987a; Mann et al. 1987b; Bazylinski et al. 1994; Bazylinski et al. 1995) and the intracellular calcification and silicification by unicellular algae (see de Vrind-de Jong and de Vrind 1997 for review).

Alternatively, in biologically induced mineral formation, minerals form as the indirect result of microbial metabolism (i.e., modification of Eh or pH, development of charged surfaces upon which metals bind, or production of extracellular proteins that serve as nucleation centers). Minerals believed to be formed in this manner include magnetite produced by dissimilatory iron-reducing bacteria (Sparks et al. 1990), and carbonates produced by cyanobacteria (Thompson and Ferris 1990; Bazylinski et al. 1994; Ferris et al. 1994; Schultze-Lam and Beveridge 1994). In the case of the extracellular manganese oxides produced by several phylogenetically diverse microorganisms, it is unclear whether either of these two processes are involved. Although the manganese oxides are extracellular, amorphous, and variable in structure and oxidation state, in many instances, specific extracellular enzymes are involved in the actual oxidation of reduced Mn (see review by Nealson et al. 1988; Nealson et al. 1989; Ghiorse and Ehrlich 1992; Tebo et al. 1997). Therefore, the oxidation of manganese is under genetic control even if the structure of the resulting mineral does not appear to be controlled in this manner. In most instances, it is not clear what benefits organisms reap from this passive role in mineral formation. Many possible reasons for the extracellular oxidation of manganese have been proposed, and these are summarized in a recent review (Tebo et al. 1997).

From a microbiologist's perspective one expects that it is easier to study mineralogical processes in which distinct genes and gene products are directly involved in the formation of the minerals vs. processes that occur as the indirect result of other metabolic activities. Once the genes are identified and characterized, detailed investigations of their activities, rates, and conditions of expression can be studied in model systems. Such studies are underway in the investigation of the genetics underlying magnetite formation in magnetotactic bacteria (see review by Bazylinski and Moskowitz 1997), and manganese oxidation by bacteria (see review by Tebo et al. 1997). Although biogeochemical activities can often be measured via mineralization assays and radiotracer experiments in microcosms composed of natural materials, typically identifying the organisms and the genes responsible for these activities has been difficult or impossible. Our limited understanding of the role of microorganisms in mineral formation arises from the difficulty in studying environmentally significant bacteria. While most microorganisms in natural habitats cannot be cultured (see review by Amann et al. 1995), recent technological advancements in microscopic, spectroscopic, and molecular techniques permit investigation of organisms that have evaded culturing attempts (Barns and Nierzwicki-Bauer 1997; Beveridge et al. 1997; Amann et al. 1995). However, understanding in situ microbial activities and distributions of organisms still poses substantial challenges.

THE QUESTIONS POSED BY MICROBIAL ECOLOGISTS

The questions directing much of the research in microbial ecology are theoretically quite simple. (1) What are the numbers and identities of microorganisms present in a given sample; (2) what are their activities and their role in ecosystem maintenance; (3) what genes are present to encode the activities of interest; (4) are the genes being expressed (i.e., transcribed), and are those transcripts translated and processed into active proteins; and (5) what controls the rate of transcription and translation for environmentally significant genes, and can we measure these rates in situ?

As discussed here, much of the recent progress in the field of microbial ecology has been limited to addressing the first question above: determining the identity of the microorganisms present in a given community. However, a complex issue that mineralogists are trying to address is the relative role of biotic and abiotic processes in mineral formation and dissolution. Because genetic control is at the foundation of all biotic processes, identifying the relevant genes and understanding how these genes are regulated within the constantly changing environmental milieu is essential for elucidating the contribution of biotic processes to mineral formation and dissolution. Although these goals remain out of reach, the emerging techniques allow us to begin addressing some interesting questions.

The purpose of this review is to give a brief critique of nucleic acid-based techniques currently used in microbial ecology and to serve as a reference for those wishing to employ such techniques in their system of interest. Furthermore, this review examines the applicability of these techniques to study the biogeochemical roles of bacteria especially as related to metal cycling and biomineralization. Indeed, there are only a few published reports of these techniques being applied to biogeochemical and biomineralogical problems. However, this review aims to familiarize biogeochemists and biomineralogists with the strengths and weaknesses of these approaches so that they may soon be applied to specific systems of biomineralogical interest.

THE MOLECULAR WORLD

Why use RNA for culture independent studies of microbial diversity and identity?

The field of microbial ecology has come farthest in the ability to address the first question listed above: What microorganisms are present in our sample of interest? This ability to address questions regarding identity of the community members has largely come through application of ribosomal RNA (rRNA) approaches. Several excellent reviews discuss the overall schemes of a nucleic acid approach to characterize microbial communities (Olsen et al. 1986; Pace et al. 1986; Ward et al. 1992; Amann

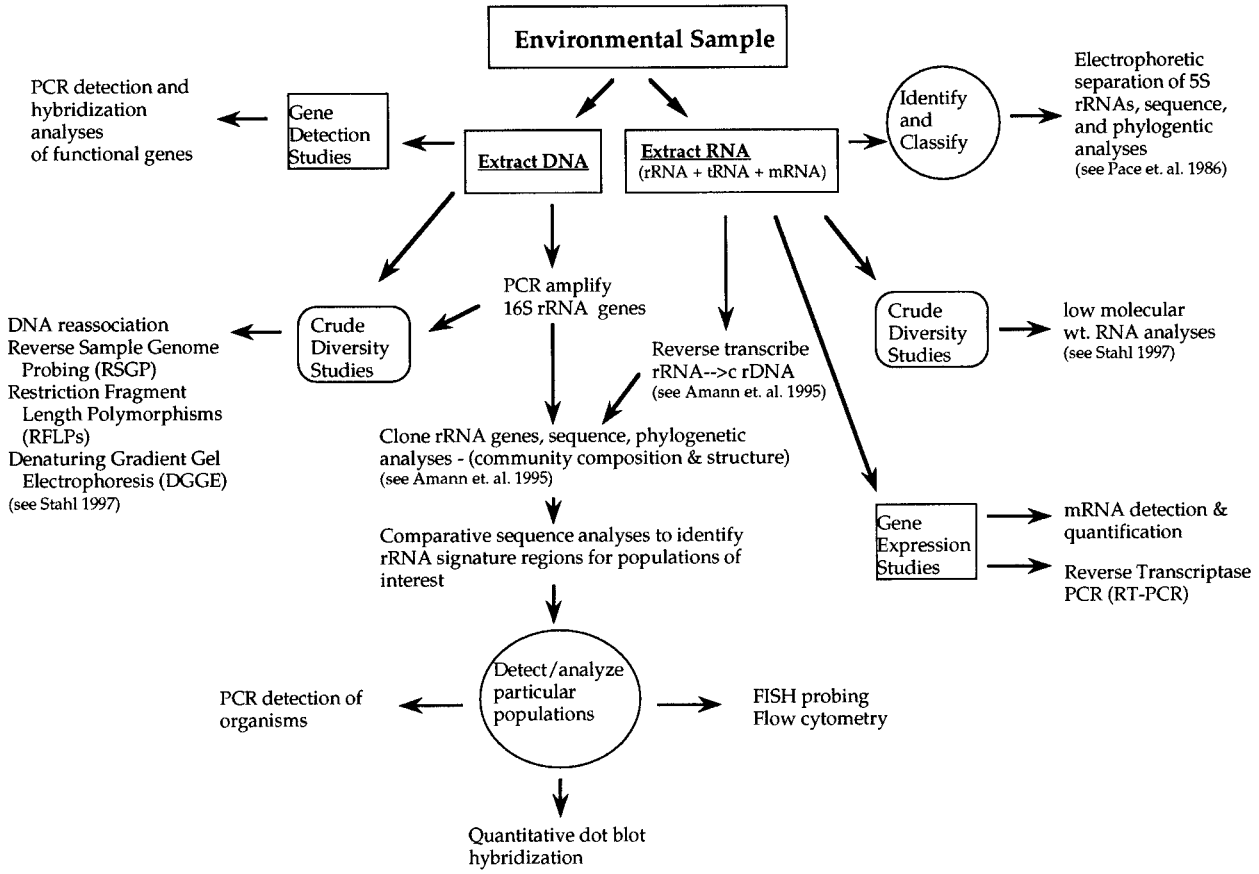

FIGURE 1. Flow chart of possible applications of nucleic-acid approaches to examine microorganisms in environmental samples. Once extracted from the environment, RNA and DNA can be used in investigations of microbial identity and taxonomic characterization, crude diversity studies, or an investigation of function by detection of functional genes (DNA), and/or the detection of gene expression (RNA). See the indicated references and text for details.

et al. 1995; Brockman 1995; Amann et al. 1997). and some of the salient features of these approaches are summarized in Figure 1. Culture-independent studies have used rRNAs in studies of microbial communities for several reasons: (1) They occur in all organisms and have the same essential function in all organisms. (2) This conservation of function dictates a conservation of structure such that most of the molecule is conserved among the most divergent of organisms. (3) Different portions of the molecule evolve at different rates resulting in highly conserved domains and hypervariable domains. More domains are included in the larger 16S and 23S rRNAs (vs. the smaller 5S rRNAs), and hence, these molecules are more commonly used in such studies. (4) rRNAs are abundant in actively growing cells ($\sim 10^4$ ribosomes per actively growing *E. coli* cell), and are therefore easy to detect, isolate, and sequence, and (5) rRNAs are not laterally transferred among organisms. As indicated in Figure 1, nucleic acid approaches permit investigation of community diversity, identification and/or detection of specific microorganisms, and functional studies of gene detection and expression.

Nucleic acid extraction from environmental samples

The initial procedure in applying nucleic acid approaches involves a direct extraction of nucleic acids from the environmental samples (Fig. 1). Several methods have been developed for the extraction of DNA (Fuhrman et al. 1988; Barns et al. 1994; Moré et al. 1994; Borneman et al. 1996; Zhou et al. 1996), or RNA (Pichard and Paul 1991; Fleming et al. 1993; Moran et al. 1993; Jeffrey et al. 1994; Ogram et al. 1995) from indigenous microorganisms in environmental samples. The quality of the extracted nucleic acids may be compromised by problems of shearing, degradation due to the presence of contaminating nucleases, or contamination with humics or other substances known to inhibit subsequent molecular biological manipulations. Extracting high quality nucleic acids from environmental samples is not trivial, and techniques must be optimized for each type of sample. It is unfortunate that methods for extraction of nucleic acids from environmental samples lack a quantitative component; little data exist on the efficiencies by which indigenous bacteria can lysed, and how these lytic efficiencies are af-

fected by the complex matrix of biological and non-biological material within different sample types. Furthermore, quantitative removal of *all* nucleic acids from that complex of biotic and abiotic material is neither possible nor predictable in most cases. The efficiencies of purification of nucleic acids away from nucleases and compounds known to be inhibitory to subsequent molecular manipulations (i.e., humic acids) have not been investigated. Although such quantitative studies are needed in the future, significant advances have been made in the ability to extract nucleic acids from a variety of natural samples and use them in these culture-independent studies.

Considerations when applying PCR to a mixture of nucleic acids

Once nucleic acids are extracted from samples there are several routes one may take depending on the questions of interest (Fig. 1). Often, the initial goal is to survey the microorganisms present in the samples. The first step usually involves the polymerase chain reaction (PCR) (Dieffenbach and Dveksler 1995; Pepper 1997) to amplify 16S rRNA genes or functional gene sequences from the bulk nucleic acid extracts. Unfortunately, PCR amplification of target gene sequences from a mixture of nucleic acids does not necessarily occur in an accurate and unbiased fashion. A primary concern in amplifying 16S rRNA genes from mixed samples is the formation of chimeric sequences from the artifactual joining of 16S rRNA gene sequences of two organisms (Liesack et al. 1991; Kopczynski et al. 1994) or from distinct copies of rRNA genes within the genome of a single organism (Wang and Wang 1997). Such chimeric sequences occur at variable frequencies ranging from 4.1–20% (Robison-Cox et al. 1995) to 8.8–32% (Wang and Wang 1997) and, therefore, should not be ignored. Although there are computational methods available to detect these artifacts (Maidak et al. 1997; Robison-Cox et al. 1995; Komatsoulis and Waterman 1997) , all methods fail to detect some chimeras (especially those from closely related sequences) or misclassify nonchimeras as being chimeric. However, by using several available methods, instead of a single method, such inaccuracies can be decreased (Komatsoulis and Waterman 1997).

Another issue in applying PCR to amplify rRNA genes within a mixture of nucleic acids is that the PCR reaction does not amplify the genes from all organisms equally (Reysenbach et al. 1992; Suzuki and Giovannoni 1996). This is one of the major drawbacks to developing quantitative PCR methods that enable us to estimate the prevalence of particular sequences in the original samples. This template bias is sometimes due to variable energetics in primer annealing and DNA denaturation due to G + C content in template or primer DNA. In other instances, the causes of template bias have not been identified, and it is not yet possible to accurately predict the existence and extent of preferential amplification by examination of template sequences (Suzuki and Giovannoni 1996). However, progress is being made in trying to understand and model the intricacies of these biases so that accurate quantitative PCR may eventually become a reality (Morrison and Gannon 1995; Raeymaekers 1995; Suzuki and Giovannoni 1996). Both genome size and the number of different copies of rRNA genes within a given genome also have been shown to result in differential amplification of rRNA genes from mixed community DNA (Farrelly et al. 1995). Because these parameters are unknown for the majority of organisms present in a given sample, Farrelly and coworkers (1995) contend that it is impossible to accurately quantify compositions of microbial communities by analyzing clone libraries from amplified 16S rRNA genes.

Estimation of microbial diversity and identity using PCR amplified rRNA genes

Once 16S rRNA genes have been amplified from the community DNA, the genes may be cloned and sequenced (see Fig. 1) for a phylogenetic analysis of the microbial components (Olsen et al. 1986; Pace et al. 1986; Ward et al. 1992; Amann et al. 1995). In virtually all instances where this cloning approach was used with amplified 16S rRNA genes from nucleic acids extracted from natural samples, sequences corresponding to previously uncharacterized, and often unexpected lineages were found (e.g., Schmidt et al. 1991; Liesack and Stackebrandt 1992; Barns et al. 1994; Giovannoni et al. 1996; Kuske et al. 1997; Suzuki et al. 1997; Wise et al. 1997). Phylogenetic information does not necessarily impart information on the functional potential or in situ activities of microorganisms. Furthermore, when sequences from previously undescribed lineages have been retrieved in this manner, the need for characterizing these novel genera in pure culture becomes even more apparent if we are to understand their functional role in the ecosystem. This has been demonstrated for the novel Korarchaeota lineage of the Archaeal domain discovered by applying this approach to characterize the microbial inhabitants of a hot spring (Barns et al. 1994; Burggraf et al. 1997); the role of these microorganisms in the hot spring ecosystem is not yet clear.

Rather than a detailed survey of organisms present, the researcher may be interested in a crude index of microbial diversity. Indices of diversity may be of use when one is trying to follow changes in microbial communities that result from natural community succession or environmental or anthropogenic perturbation. Some techniques that are used for obtaining crude diversity estimates include: DNA reassociation experiments, reverse sample genome probing, total cellular fatty acid analyses, restriction fragment length polymorphism (RFLP) of amplified 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA genes, low molecular weight RNA analyses, and fluorescent in situ hybridization (FISH) (e.g., reviews by Tiedje 1995; Stahl 1997). However, any estimates of microbial diversity must acknowledge the inability of microbiologists to satisfacto-

rily define a bacterial species (see discussions by Colwell et al. 1995; Stahl 1997).

Although microbial diversity is of great interest to microbial ecologists, biomineralogists and biogeochemists may be more interested in the ability to detect particular organisms considered important in a particular geochemical transformation. In this regard, 16S rRNA gene sequence information may also be used to direct analysis of a sample by FISH, flow cytometry, or PCR detection of particular microorganisms.

Using rRNA sequence information to detect particular organisms in an environmental sample by fluorescent in situ hybridization

The development and application of the FISH technique in microbial systems (Giovannoni et al. 1988; Delong et al. 1989; Amann et al. 1990; Amann et al. 1991) provides a way to detect and enumerate microorganisms in natural systems without culturing. FISH is a technique whereby fluorescently labeled DNA probes are annealed to a target sequence in nucleic acids of fixed cells. The target may be in a viral genome, a particular protein-encoding mRNA, a particular sequence on a chromosome, or a signature region of an rRNA molecule (Woese 1987) . This technique can be used in environmental studies in which samples are obtained from a field site, ''fixed'' with formaldehyde to inhibit further metabolic activity, and probed with a suite of probes capable of identifying bacteria at varying levels of taxonomic hierarchy. However, as is described below, there are limitations to employing this technique in many biogeochemical and biomineralogical systems of interest.

The small size of bacteria limits the size of a probe that can efficiently get inside a whole cell without destroying the morphological integrity of that cell. For bacteria, probes are generally restricted to short pieces of DNA (15–50 base pair oligonucleotides) that contain a covalently bound fluorescent molecule attached to one end of the piece of DNA. This single label per probe means that there must be a large amount of target present in the cell to be able to detect it. Therefore, initial applications of FISH to identify bacteria have targeted ribosomal RNA sequences, because ribosomes are abundant in actively growing cells ($\sim 10^3$–10^5/cell). However, in a given organism, the number of ribosomes per cell can vary significantly dependent on the growth rate (Bremer and Dennis 1987; Delong et al. 1989; Kemp et al. 1993). There are signature regions within the rRNA molecule specific for each level of taxonomic hierarchy (Woese 1987); it is these signature regions that define the probe sequence.

A detailed description of the FISH technique, and considerations for its application are discussed elsewhere (Stahl and Amann 1991; Manz et al. 1992; DeLong 1993; Amann et al. 1995; Amann et al. 1997; Zheng et al. 1996). The Ribosomal Database Project (Maidak et al. 1997) is a valuable tool for any laboratory doing FISH studies. It contains a wealth of useful information including a database of aligned rRNA sequences, probe-checking, chimera-checking, and tree-drawing tools, and access to a variety of useful web sites and servers. Similarly, the Oligonucleotide Probe Database (Alm et al. 1996) provides a centralized source for information on probe design, nomenclature, and application.

Although many laboratories successfully applied FISH to analyze microbial populations in situ (Amann et al. 1992; Wagner et al. 1994; Alfreider et al. 1996; Glöckner et al. 1996; Harmsen et al. 1997; Siering and Ghiorse 1997), low and variable signal strength, rapid fading of fluorescent signal, and autofluorescence of non-target cells and acellular material were often problematic. The application of laser scanning microscopy and digital video image processing alleviates some of these problems (Wagner et al. 1994; Ghiorse et al. 1996; Møller et al. 1996; Siering and Ghiorse 1997). However, it is still difficult to obtain consistently bright signals with many bacteria in natural samples.

A novel approach employing treatment of sample with chloramphenicol for time periods ranging from 30 min to 4 h prior to fixation resulted in increasing the number of fluorescently labeled cells in a FISH study of marine samples (Ouverney and Fuhrman 1997). This method relies on the ability of chloramphenicol to inhibit the protein synthesis, RNA degradation rate, and cell division (growth) of many bacteria. However, because rRNA transcription continues in the presence of chloramphenicol, the amount of rRNA per cell increases with time. This approach may be useful in systems where low growth rates result in low signal intensities, and thus, a failure to detect all the cells present. Because chloramphenicol specifically inhibits organisms in the domain Bacteria, it will not be applicable to FISH analyses of Archaea or Eukarya. However, other protein synthesis inhibitors (i.e., cyclohexamide for Eukarya, and diphtheria toxin for Archaea) may potentially be used in a similar fashion.

Additional studies have focused on increasing signal intensity through the application of brighter fluorescent labels, multiple probes, or by using indirect detection systems. An indirect detection system was successfully used in recent studies (Rapp et al. 1995; Lebaron et al. 1997; Schönhuber et al. 1997). In these reports, the oligonucleotide probes are labeled with horseradish peroxidase; after hybridization, a fluorescein-tyramide substrate is added. Cleavage of the substrate by the bound horseradish peroxidase results in a bright fluorescence, which can be analyzed by either epifluorescence microscopy or flow cytometry. Although the generated signals are brighter than those obtained by standard FISH protocols, this system has variable success with different genera and, therefore, is not currently generally applicable to environmental samples (Schönhuber et al. 1997).

In the biogeochemical realm, FISH probes have been employed to quantify methanogens in bioreactors (Raskin et al. 1994; Sørenson et al. 1997), examine the distribution of sulfate-reducing bacteria in biofilms (Amann et al. 1992; Ramsing et al. 1993) and in stratified water col-

umns (Ramsing et al. 1996), determine the abundance and organization of nitrifying bacteria in activated sludge and biofilm samples (Mobarry et al. 1996), and determine the distribution of a toluene-degrading bacterium in a biofilm (Møller et al. 1996). FISH probes for the detection of iron- and manganese-oxidizing bacteria related to *Leptothrix* spp. have detected these organisms in wetland water samples (Siering 1996; Siering and Ghiorse 1997). However, the procedures required to inhibit fading of fluorescent signal resulted in the apparent reductive dissolution of the oxides of interest (Siering 1996; Siering and Ghiorse 1997). More recently, Shrenk and coworkers (1998) have used FISH to investigate the distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* in relation to acid mine drainage.

Rather than using fluorescently labeled probes, DiChristina and DeLong used radio-labeled rRNA-targeted oligonucleotide probes to detect a dissimilatory iron- and manganese-reducing organism in nucleic acid extracts from lake water and sediment samples (DiChristina and DeLong 1993). They utilized quantitative dot blot hybridization (see discussions by Amann et al. 1995) in which a radio-labeled *Shewanella putrefaciens*-specific probe and a radio-labeled universal probe were each hybridized to filters containing equal amounts of total RNA extracted from environmental samples. The relative abundance of *S. putrefaciens* RNA in the sample was determined by dividing the radioactivity (in counts per minute) derived from the bound *S. putrefaciens* probe by the counts derived from the universal probe. This method provides a crude estimate of the relative physiological contribution of a particular population, because the amount of *S. putrefaciens*-specific RNA is a combined measure of the number of cells present and how fast they are growing.

It is unfortunate that few applications of the FISH technique have appeared in biomineralogical research. This may be because many mineralogists are interested in processes occurring on mineral surfaces. The primary techniques for examination of biological material on mineral surfaces are transmission electron microscopy, scanning electron microscopy, atomic force microscopy and environmental scanning microscopy (see Barns and Nierzwicki-Bauer 1997 for recent applications of these techniques). None of these microscopic techniques imparts the ability to detect and quantify fluorescent signals. In the future it may be possible to conjugate gold directly to rRNA-targeted oligonucleotide probes and detect the site of probe binding by energy dispersive X-ray analysis (EDS) during atomic force microscopy or environmental scanning microscopy. Although gold-labeled antibodies are commonly used as probes for surface and intracellular targets of eukaryotes, the gold colloids used for conjugation to antibodies are quite large, and in situ hybridization of bacteria with gold-labeled oligonucleotide probes would likely require smaller colloids and additional permeation steps.

Alternative uses of rRNA signature regions to detect and/or quantify particular organisms

Hybridizations with fluorescently labeled oligonucleotides may also be detected and analyzed by flow cytometry. Flow cytometry involves passing particles suspended in a liquid through an illuminated chamber at a particular flow rate (i.e., 1000 cells/s). Detectors measure the amount of light scattered (and at what angle it is scattered), and can also detect particular wavelengths of emitted light as the particles pass through the chamber. The history, underlying principles, and general applications of this technique are superbly reviewed elsewhere (Shapiro 1995; Davey and Kell 1996). Flow cytometry imparts the ability to discriminate cells on the basis of size (by forward light scattering), presence of internal structures (by 90° angle scattering), or by emission of fluorescence of particular wavelengths (either innate, or probe-, stain-, or antibody-mediated) (Shapiro 1995; Davey and Kell 1996). This analysis can potentially enable estimations of biomass, growth and metabolism for the population of interest. Furthermore, since several hundred to several thousand cells can be analyzed per second, larger total sample volumes can be analyzed than with FISH, and in a fraction of the time. This is particularly useful if the organism of interest is present in small numbers (i.e., $<1\%$ of total population). Even with these potential advantages, the application of this technique to analyze natural mixed microbial populations is in its infancy.

Most successful applications of FISH probes in flow cytometry have been with aquatic samples (Amann et al. 1990; Lim et al. 1995; Simon et al. 1995; Wallner et al. 1997), and limited success has been achieved with activated sludge samples (Wallner et al. 1995; Wallner et al. 1997). Flow cytometry also has been applied to microorganisms that have been extracted from solids including: compost (Diaper and Edwards 1994), soil (Christensen et al. 1993), and a sand-based aquifer microcosm containing previously inoculated cells (DeLeo and Baveye 1996). However, the extraction efficiencies and the effect of extraction procedures on the microbial cells was not investigated in these studies.

Flow sorting during flow cytometry imparts the ability to physically separate cells on the bases of their above-described parameters. Recently, Wallner et. al. (1997) applied the principle of flow sorting to specifically separate rod-shaped magnetosome-containing bacteria from a magnetic enrichment of lake water sediment. Once sorted, the cells were subjected to 16S rRNA gene amplification, and the resulting PCR products (amplicons) were cloned and sequenced. They used FISH probes for specific bacteria to concentrate particular FISH probe-positive cells from activated sludge samples. Wallner et al. (1997) found that the purity of the sorted population was enhanced by employing an additional sorting step prior to molecular analyses (PCR amplification and cloning of 16S rRNA genes). The ability to physically separate noncultured cells present as minor members of a community

has far-reaching impact for the field of microbial ecology. In combination with the ability to PCR amplify sequences from small numbers of cells, flow sorting may be an extremely useful approach to identify and study particular microbial components of mixed natural samples.

One limitation in the application of flow cytometry to environmental samples is the presence of large amounts of cellular aggregates and/or noncellular particulate matter. Furthermore, most natural samples also contain a wide range of particle sizes, and many materials exhibit significant autofluorescence. Currently available flow cytometers have a lower sensitivity than most epifluorescent microscopes, thus making it more difficult to analyze bacteria smaller than 1 μm (Wallner et al. 1997). Many bacteria in the environment are smaller than this size, especially in oligotrophic environments. It is probable that the future will offer new flow cytometers with increased detection sensitivities and improved cell labeling techniques such as those currently being investigated for FISH.

Application of the FISH technique alone, or in combination with flow cytometry, may prove unsuccessful if any of the following conditions exist: (1) the organisms of interest grow at slow rates in situ (and thus contain few rRNA targets), (2) considerable background fluorescence exists as is often present in soil samples, and which may be present in mineralogical samples, or (3) structural and physiological features of the cells prevent adequate fixation and probe entry. In these instances, the same signature regions used for FISH probes can be used as PCR primers to facilitate detection of a particular organism of interest in nucleic acids extracted from environmental samples. However, due to the previously mentioned considerations associated with nucleic acid extraction and PCR amplification, this will not permit a quantitative estimate of the numbers of the target organism in the original sample. Furthermore, detecting the presence of an organism by PCR gives no information as to whether it is viable, or whether the organism is physiologically active. Although there are several methods being used for the detection and assessment of physiologic status of cells in situ, no method is without its limitations (Madsen 1996).

Are nucleic acid biosensors the future for microbial detection and diversity estimation?

Nucleic acid biosensors rely on the ability of single-stranded nucleic acids to specifically bind to their complementary sequence via Watson-Crick base pairing. The method of attachment of the nucleic acid to the detector, the ability to transduce binding into a detectable signal, and the applications of these sensors have been the subject of much recent research and are reviewed elsewhere (Bier and Fürste 1997). In a recent study, a 60 μm^2 biosensor (which the authors call an oligonucleotide microchip) was constructed that contained a matrix of bound oligonucleotide probes specific for signature regions in the ribosomal RNAs of several known nitrifying bacteria (Guschin et al. 1997). Each oligonucleotide was immobilized onto a different gel element within a polyacrylamide gel matrix bound to the surface of a glass slide. A specially designed robotic micromanipulator was used to prepare the microchip. RNA and DNA extracts containing single-stranded nucleic acids from a mixture of organisms were fragmented, labeled with fluorescent dyes, and allowed to bind to the microchip. Binding of the labeled DNA or RNA fragments from the extract, and washing of unbound nucleic acids was controlled and monitored with a Peltier thermotable, which was mounted on the stage of a specially designed epifluorescence microscope. Binding and dissociation events could be monitored in real time, and the hybridization kinetics could be determined under different conditions to allow discrimination between perfect duplexes and those containing one or more mismatches.

This emerging technology provides promise for the analysis of the components of a microbial community. Hundreds to thousands of oligonucleotide probes can theoretically be bound to a single chip, and the same microchip can be used for 20–30 different hybridization experiments. Such an approach seems far superior to time intensive FISH analysis when information on spatial relationships among the bacteria is not desired. The ability to measure binding kinetics in real time at each probe position has the potential to avoid many of the problems associated with interpreting the results of FISH analysis. For instance, when applying FISH probes to natural samples, variations in signal intensity are common (see examples in Siering 1996; Siering and Ghiorse 1997). In FISH, there is no way to determine whether a dim signal is due to low growth rate or to a few positions of mismatch between the probe and rRNA target sequence; a fast growing organism containing 1–3 mismatches may still bind enough probe under high stringency conditions to fluoresce very brightly. The oligonucleotide microchip technique may eventually permit accurate quantification and discrimination of the microbial components of environmental samples, because the optimum conditions for hybridization and discrimination for each probe/target combination can be determined, and the concentration of oligonucleotides bound to each gel element can be varied over a wide range. Improvements are needed to minimize the technical limitations of this and other biosensor techniques, but such technology may play an important role in future microbial ecological studies.

Moving beyond organism detection and identification—are the genes present to encode the function of interest?

To understand the role of a microorganism in a geochemical process, detection and identification of the microorganism in an environment in which the process is occurring is essential. However, demonstrating the presence of an organism in an environment where the process is occurring does not mean the detected organism is important in the process of interest. One ultimately needs to correlate the distribution and abundance of the organisms

with the presence of the activity and the presence of any genes and gene products involved in the process. This analyses should also include characterization of the associated physical and chemical environment so that biotic and abiotic parameters can be related.

If genes known to be involved in a particular process have been identified, isolated, characterized, and sequenced, it is possible to use this sequence information to develop PCR primers for amplifying the gene of interest from indigenous bacteria in natural samples. Amplifying and sequencing functional genes from organisms present in environmental samples allows us to investigate the distribution, evolutionary relationships, and diversity of functionally analogous genes, Sequence analysis can also aid in determining whether the genes of interest have been transferred horizontally among the community members. Furthermore, in minor community members, the activities may be below the threshold level of detection, but the PCR-detectable functional gene may be present and actively transcribed/translated.

PCR amplification was used in the detection of the gene encoding the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) enzyme of the Calvin cycle from marine samples (Paul et al. 1990; Xu and Tabita 1996; Pichard et al. 1997) , the *amoA* gene involved in the oxidation of ammonia by autotrophic nitrifying bacteria from aquatic and soil samples (Sinigalliano et al. 1995; Rotthauwe et al. 1997), the *nahAc* gene involved in the initial step in catabolism of napthalene from sediments (Herrick et al. 1993), the *mer* genes encoding mercury resistance from soil and sediment samples (Bruce et al. 1995), and for the detection of a putative manganese-oxidation gene of *Leptothrix discophora* in wetland water samples (Siering and Ghiorse 1997).

However, in each of these cases, a significant amount of pure culture microbiological work, biochemistry, and genetics had to be done to define and characterize the genes of interest. Ideally, this characterization should be extended to assess the sequence diversity of the genes in all organisms known to possess them so as to determine conserved regions for primer design. Such efforts are usually not possible unless the gene(s) have been the subject of many years of productive research (as is the case for RUBISCO, *nah,* and *mer* genes). Even with data culminated from years of research, there are no guarantees that the designed primers will be capable of amplifying homologous genes containing some divergent sequence, or functionally analogous genes with very divergent sequences, or that the primers will be specific for the targeted genes of interest. These problems arise because of the greater uncertainties associated with the physiologic and genetic diversity of the uncultured microorganisms involved in biogeochemical transformations.

Furthermore, demonstrating the presence of a particular gene from bulk nucleic acid extracts provides no information at the individual cell or microscale level. The detected gene may not be actively expressed within the cell, and since DNA can persist sorbed to soil particles for extended periods of time, the amplified gene may be from cells that have long since expired. Furthermore, to determine the role of microorganisms in a particular process, we must be able to assess which microorganisms in a natural bacterial assemblage are able to carry out that process and how they are spatially related to one another and to their physical environment. As previously mentioned, detection of gene sequences by FISH requires multiple copies of the target sequence to enable detection. Because most functional genes in bacteria exist in only one or a few copies per cell, new methodologies are required.

Recently, major accomplishments were made in the application of prokaryotic in situ PCR methods with these goals in mind (Hodson et al. 1995; Porter et al. 1995; Jacobs et al. 1997) . In situ PCR involves: (1) cell fixation to preserve the cell structure during thermal cycling; (2) *limited* permeabilization, which permits entry of primers and PCR reagents into the cells without permitting the PCR products to leak out of the cell; (3) specific amplification of target sequence and incorporation of fluorescently labeled deoxynucleoside triphosphates (dNTPs); and (4) subsequent detection of the fluorescent product by epifluorescence microscopy or flow cytometry. Although improvements have been made in sensitivity and specificity of this technique (Hodson et al. 1995), general applicability of this approach to environmental samples is not yet a reality due to inconsistent reliability, lack of specificity, and low detection efficiency. This technique has been used to examine the expression of a gene involved in nitrogen fixation (*nifH*) from *Azotobacter vinelandii,* and a toluene dioxygenase gene (*todC1*) from *Pseudomonas putida* (see Chen et al. 1997). In situ PCR offers great promise in helping us to better understand the microscale interactions between bacteria, minerals, and the genes involved in transforming those minerals.

Are the functional genes expressed?

To prove a gene of interest is responsible for a process, one must be able to detect expression of the gene in situ and correlate changes in gene expression with changes in the environment, and changes in the associated activity. As indicated above, several methods have been developed to extract RNA from environmental samples. However, detecting or quantifying the presence of a particular messenger RNA (mRNA) transcript is considerably more challenging because most of the RNA within a cell is rRNA (not mRNA). Prokaryotic mRNAs are short lived molecules with typical half lives of less than a few minutes. Furthermore, studies of gene expression require a priori information of such features as transcript size and stability, as well as expected levels of transcript present. This information can only be obtained through comprehensive pure culture and biochemical studies. The best studied systems for the detection and quantification of gene expression in environmental samples are the RUBISCO (Pichard and Paul 1991; Pichard and Paul 1993;

Pichard et al. 1997) and *mer* systems (Jeffrey et al. 1994; Jeffrey et al. 1996).

The presence of an mRNA transcript does not indicate that induction of gene expression has occurred. For example, many genes (especially catabolic genes) are constitutively transcribed at low levels, and their expression is greatly increased in the presence of the substrate (or other inducer). Furthermore, the quantity of a particular mRNA transcript present at any given time is the product of several factors including increased gene expression, increased mRNA stability, or increase in the number of gene copies (gene dosage). To show that the increased amount of transcript is due to induction of gene expression, it is necessary to normalize the specific mRNA concentration to the concentration of the corresponding DNA template. Such a measurement of gene expression per gene dose has been successfully demonstrated for RUBISCO expression in marine samples (Pichard and Paul 1993). Although this study was elegant in its approach and methodological developments, it may not be generally applicable to investigating the environmental expression of many genes. RUBISCO has been touted as the most abundant protein on the earth (Ellis 1979), and therefore its transcript should be easier to detect than that of less abundant proteins. Furthermore, the nonquantitative nature of nucleic acid extractions (as discussed above) currently limits this idealized approach.

Recent advancements to increase detection sensitivities of gene expression rely on a form of PCR known as reverse transcriptase-PCR (RT-PCR). In this technique, reverse transcriptase is first used to synthesize a single stranded DNA copy (cDNA) of the RNA template; then, the complementary strand of the cDNA is synthesized by the *Taq* DNA polymerase, and the double-stranded DNA molecule is subsequently amplified by normal PCR amplification Although this approach was used to detect and/or follow gene expression in artificially seeded samples (Selvaratnam et al. 1995; Bogan et al. 1996; Klein and Juneja 1997), the application of RT-PCR to detect transcripts from indigenous bacteria in environmental samples is lacking. However, RT-PCR was recently used to detect the presence of transcripts from a gene involved in napthalene degradation (*nahAc*) in nucleic acids extracted from coal tar-contaminated ground water (Wilson 1998; Wilson et al., unpublished data). Interestingly, the diversity of these *nahAc* transcripts expressed in this single field site corresponded to the diversity of all known *nahAc* genes as previously determined by culture studies at several different field sites (Wilson 1998).

However, like most PCR-based approaches, this study lacked an essential quantitative component. There have been many attempts to quantify gene expression by a technique referred to as competitive RT-PCR (Dieffenbach and Dveksler 1995; McCulloch et al. 1995) in which an internal standard is coamplified with the target using a common set of primers. If the efficiency of amplification of both templates is identical, then the final yields of each product should theoretically correspond to the initial concentration of each template. Clearly, the largest impediment to this approach is our lack of understanding of template bias during PCR (see above). However, if all the essential control experiments are done to determine that both templates amplify with equal efficiency, this approach may be useful in applications correlating gene expression with other parameters such as specific activity levels of the corresponding enzyme, loss or transformation of a catabolic substrate, and gene dosage (see above). Such an approach was used to correlate manganese peroxidase transcript abundance with enzyme activity, and loss/transformation of polyaromatic hydrocarbon (PAH) substrates in benchtop PAH-amended soil cultures of *Phanerochaete chrysosporium* (Bogan et al. 1996). Unfortunately, no attempt was made to correlate transcript abundance to gene dosage in this study, and the wide variability they found in mRNA levels of housekeeping genes (which should be expressed at equal levels throughout growth), made it impossible to reach definitive conclusions regarding patterns of gene expression.

Additionally, these RT-PCR approaches lack information at the individual cell level. An in situ RT-PCR method for bacteria was first developed to detect *Pseudomonas* cells expressing the *nahA* gene involved in naphthalene degradation (Hodson et al. 1995). This work used another technique referred to as RNA primer extension (RPE) to detect RNA sequences in multiple copies in a single cell. RPE employs a single primer to promote the arithmetic amplification of target sequences (vs. exponential amplification as with PCR), and may therefore be useful for a semi-quantitative assessment of gene expression. This technique can also be used for detection of slow growing bacteria in natural samples by targeting signature regions of the rRNA molecules as with FISH. However, both of these methods use thermal cycling of cells on a microscope slide, which dramatically increases the technical difficulty when compared to in situ hybridization procedures such as FISH that only involve incubation at the hybridization temperature.

A new technique called in situ reverse transcription (ISRT) has been developed for the in situ detection of RNA without the need for thermal cycling (Chen et al. 1997). ISRT involves the incorporation of multiple labeled nucleotides during the reverse transcription of the RNA targets, and since this can be done to samples collected directly on filters, ISRT may be more generally applicable to environmental samples than are methods requiring thermal cycling. This approach was used to detect and enumerate two lignin-degrading bacteria from complex enrichments, and *Pseudomonas* cells expressing a toluene degradation gene (*tod*) in seawater exposed to toluene vapor (Chen et. al. 1997). ISRT greatly enhances the sensitivity of detection of specific rRNA and mRNA sequences when compared to FISH (Chen et. al. 1997); hence, it may be especially useful for the in situ analysis of communities composed of slow growing organisms.

Do the expressed genes result in the activity of interest?

Just as the presence of a gene does not guarantee its in situ expression, gene expression does not necessarily indicate functionally active protein is being produced to yield the activity of interest. Several genes are constitutively transcribed and translated into active proteins at low levels (see above), and some activities are ultimately dependent on post-transcriptional or post-translational modifications. The ability to measure biogeochemical activities in situ is ultimately essential to understand the role of microorganisms in a given process. Methods are available to assess well-studied microbial activities such as those associated with respiration, nitrogen transformations (Capone 1997), catabolism of hydrocarbon pollutants to carbon dioxide, biomass and water, methanogenesis, and methanotrophy. Descriptions, strengths, and limitations of these techniques are detailed by Madsen (1996). Stable carbon isotope analysis is commonly applied to measure microbial activities associated with carbon cycling (Grossman 1997). Radioisotopic and non-isotopic methods are established to measure transformations of sulfur (Hsieh and Yang 1989; Hines et al. 1997; Ulrich et al. 1997), and phosphorus (Jones 1997). Extremely sensitive detection of several trace gases associated with many of these biogeochemical processes is now possible using laser photoacoustic technologies (Fink et al. 1996; Zuckermann et al. 1997). Advances have also been made in measurement of biologically mediated manganese-oxidation activity in natural samples (Tebo and Emerson 1985; Tebo and Emerson 1986; Sunda and Huntsman 1987; Sunda and Huntsman 1988; Juniper and Tebo 1995). However, the lack of information on the genetics and biochemistry of many reactions of biomineralogical interest still obstructs a comprehensive understanding of the biotic components associated with mineral formation and dissolution. Even with the existence of direct assays for known enzymatic activities, interpretation of data is often complicated by the lack of specific association of the activity with the cells, and separation of biotic and abiotic contributions to the measured activity.

Activity measurements on the microbial scale

In any measurement of activity, scale and sample size are obvious concerns. The importance of how one goes about getting a truly representative sample in light of the spatial and temporal heterogeneity in natural microbial assemblages cannot be underestimated. This becomes even more critical when applying molecular techniques in which only a minute sample is analyzed at a given time. Detailed and useful critiques of sampling concerns can be found elsewhere (Madsen 1996; Brockman and Murray 1997).

To a biogeochemist interested in methane emissions from a small wetland it may be possible to approximate emissions by placing collection vessels a few meters apart in a transect across the wetland and averaging the values. Conversely, a biomineralogist interested in corrosion on the surface of a metal may need to determine bacterial presence and activity on a scale of micrometers on mineral surfaces or in biofilm matrices. The in situ hybridization and PCR methods described above offer promise toward in situ detection of organisms, genes, and gene expression, but what about in situ measurements of microbial activities at the microscale?

A technique referred to as microautoradiography was first described by one of the modern-day founding fathers of microbial ecology, Thomas Brock (Brock and Brock 1966). During microautoradiography, cells are exposed to a radio-labeled substrate, and only cells that are actively growing will incorporate the substrate into biomass. The radioactivity is detected by covering the specimen with a thin photographic emulsion, and it results in the production of microscopically visible silver crystals upon development of the emulsion. When this technique is combined with immunofluorescence or some other method to identify particular cells (i.e., in situ hybridization), one can correlate the presence of the activity of interest to a particular cell of known taxonomic classification. In a review published some twenty years after the first description of this technique, Brock chastised the microbial ecology community for not embracing this method for its utility, and for avoiding it due to ''inertia, ignorance, and laziness'' (Brock 1987). Indeed, only a few studies using this technique have appeared in recent literature (Ward 1984; Madan and Nierzwicki-Bauer 1993; Ghiorse et al. 1996; Andreasen and Nielsen 1997). Hopefully, the usefulness of this method will drive the development of better microautoradiographic methods that yield more reproducible results.

Relating organisms and their activities to their microscale environments using microprobes and biosensors

It is ultimately essential to correlate in situ measurements (of organism, gene, activity, etc.) with a detailed physical and chemical characterization on the microscale level. The uniting of biology and engineering into the development of microprobes and biosensors has opened the way for such studies. For discussions on these technologies, see Damgaard et al. (1995); Burlage (1997); and Hanson and von Usedon (1997). Microprobes allow the measurement of a physical or chemical parameter (i.e., pH, O_2, temperature, Cl^-, etc..) within a microenvironment. Typical probe tips are within the size range of 1–10 μm to 10–50 μm in diameter, and the concentration of analyte at the probe tip is converted into a measurable current that is reported on an ammeter. Unfortunately, few of these are currently commercially available, and they are generally constructed by the laboratories interested in their use.

In an elegant study (Ramsing et al. 1993), microprobes were used to measure pH, O_2, and H_2S gradients in photosynthetic and non-photosynthetic biofilms containing indigenous sulfate reducing bacteria. Physical and chemical analyses of the biofilms combined with FISH probing

for sulfate-reducing bacteria allowed determination of the spatial relationship of the bacteria within the biofilm, and related the positioning of the sulfate-reducing bacteria to the physical and chemical analyses. In each hybridization experiment, reference genera were deposited next to the biofilm slices to determine hybridization efficiencies and specificities for each probing experiment. Using the collected data, they calculated sulfate-reduction rates, which indicated that most of the sulfate-reducing populations were counted by FISH probing. Ramsing et. al. (1996) applied a similar approach to investigate the vertical distributions of potential electron donors and acceptors in a stratified fjord, and these data were related to the presence of various taxonomic groups of bacteria by FISH probing. Their attempts to directly measure in situ respiration and sulfate reduction rates were unsuccessful and probably due to the rates being below the detection limits of their methods (Ramsing et. al. 1996). A similar study was also done in nitrifying biofilms in which the results from in situ hybridizations for nitrifying bacteria was combined with gradient analysis of O_2, NO_3^-, NO_2^-, NH_4^+, and N_2O (Schramm et al. 1996). An improved version of the nitrite microsensor was developed, which is less susceptible to interference from other ions (de Beer et al. 1997). These studies set a new trend in microbial ecology toward quantitative integration of the biology and chemistry of microenvironments, and this approach will certainly play a critical role in elucidating the importance of microorganisms in mineralization and dissolution processes.

Another type of probe being used in microenvironmental analysis is a biosensor in which a biological component (i.e., antibody, enzyme, nucleic acid, bacterial cells) interacts with an analyte, and upon interaction causes a measurable electronic signal to be generated by an electronic component. The biological component imparts specificity to the probe, and therefore, precise, nondestructive attachment of the biological component to the electronic component is essential. A biosensor for nitrite was developed that contained immobilized denitrifying bacteria in front of a N_2O microsensor (Larsen and Revsbach 1996), and this was used in the above mentioned study to measure NO_3^- profiles in biofilms (Schramm et al. 1996).

Conclusions

When can molecular approaches provide valuable insights into a biogeochemical or biomineralogical system? We have taken an immense step in our ability to detect and identify microorganisms present in a given environment. However, we have not been as successful in taking these techniques to the next level—i.e., what are these bacteria doing, and what role do they play in the ecosystem? The power and potential of molecular techniques is real, but we need to focus on more integrative and quantitative approaches to unravel some of the remaining questions. More investigations are needed which correlate the presence and distribution of microorganisms with a comprehensive analysis of the habitat at the scale in which microorganisms function. Such in situ studies will require microbiologists to work together with geologists, geochemists, geneticists, molecular biologists, biochemists, hydrologists, ecologists, and engineers if we are going to understand the daunting complexities of the interactions between microorganisms and minerals. Furthermore, I cannot help but wonder whether we should accept that only approximately 0.1% of bacteria are culturable, and therefore abandon traditional microbiology in light of the new molecular approaches. Or do microbiologists need to start working on culturing methods that better mimic the in situ physical and chemical parameters faced by microorganisms in the real world? Pure culture studies will ultimately be essential for understanding the genetic and biochemical bases of the biogeochemical processes in which we are interested.

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