

## Utilization of Microbial Biofilms as Monitors of Bioremediation

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### Abstract

A down-well aquifer microbial sampling system was developed using glass wool or Bio-Sep beads as a solid-phase support matrix. Here we describe the use of these devices to monitor the groundwater microbial community dynamics during field bioremediation experiments at the U.S. Department of Energy Natural and Accelerated Bioremediation Research Program's Field Research Center at the Oak Ridge National Laboratory. During the 6-week deployment, microbial biofilms colonized glass wool and bead internal surfaces. Changes in viable biomass, community composition, metabolic status, and respiratory state were reflected in sampler composition, type of donor, and groundwater pH. Biofilms that formed on Bio-Sep beads had 2–13 times greater viable biomass; however, the bead communities were less metabolically active [higher cyclopropane/monoenoic phospholipid fatty acid (PLFA) ratios] and had a lower aerobic respiratory state (lower total respiratory quinone/PLFA ratio and ubiquinone/menaquinone ratio) than the biofilms formed on glass wool. Anaerobic growth in these systems was characterized by plasmalogen phospholipids and was greater in the wells that received electron donor additions. Partial 16S rDNA sequences indicated that *Geobacter* and nitrate-reducing organisms were induced

by the acetate, ethanol, or glucose additions. DNA and lipid biomarkers were extracted and recovered without the complications that commonly plague sediment samples due to the presence of clay or dissolved organic matter. Although microbial community composition in the groundwater or adjacent sediments may differ from those formed on down-well biofilm samplers, the metabolic activity responses of the biofilms to modifications in groundwater geochemistry record the responses of the microbial community to biostimulation while providing integrative sampling and ease of recovery for biomarker analysis.

### Introduction

*In situ* intrinsic accelerated bioremediation in many situations is the most cost-effective means to rectify groundwater pollution. As noted in a recent National Research Council Report [24], the key to demonstrating the effectiveness of bioremediation at a site is establishing cause-and-effect relationships, which provide evidence that the desired bioprocesses are occurring, or are likely to occur. Therefore, as a central component of such strategies a comprehensive groundwater monitoring program is implemented to attempt to ascertain these relationships. Conventionally monitored indicators of bioremediation include the distribution of contaminants, the occurrence of diagnostic metabolic products, and spatial and temporal correlation of these with various geochemical parameters indicative of microbial processes [e.g. CH<sub>4</sub>, Fe(II)]. However, using this approach it is often difficult or impossible to demonstrate convincingly that bioprocesses are occurring. We propose that a definitive signature of bioremediation can be obtained by analyzing the viable biomass, community composition,

Dedication: The authors dedicate this paper to Professor Peter Hirsch, whose infectious enthusiasm for beautiful mushrooms, Antarctica, and most of all weird microbes he has isolated and characterized has greatly enriched our lives as microbiologists with the wonder of what lies beyond our lab-rat cultures. Once Peter visits your lab, looking through your microscope will be a different experience. Happy birthday and thanks for so enriching our lives and profession.

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**Table 1. Push-pull manipulations in wells at the NABIR Field Research Station, Oak Ridge, TN**

Well	Target pH	Electron donor	Type
FW21	—	—	Source
GW835	—	—	Source
FW019	6.5	50 mM Acetate	Neutral water/neutral sediments
FW034	6.8	30 mM Ethanol	Neutral water/neutral sediments
FW033	5.9	20 mM Glucose	Neutral water/acidic sediments
FW031	5.7	None (control)	Neutral water/acidic sediments
FW032	5.2	20 mM Glucose	Acid water/acid sediments
FW027	5.4	None (control)	Acid water/acid sediments

200 L of groundwater was withdrawn from the source wells, 100 mg/L Br<sup>-</sup> tracer and HCO<sub>3</sub><sup>-</sup> added for pH adjustment. Acetate, ethanol, or glucose was added for electron donor as indicated.

nutritional/physiological status, and respiratory state of the extant microbial community. Herein we describe an efficient means to rapidly collect and amplify the aquifer microbial community by monitoring biofilms that develop on microbial samplers deployed in monitoring wells. The biofilms are analyzed for lipid and DNA biomarkers that define metabolic capabilities and activity at the cellular level. Central to our paradigm is the recognition that access to solid (sediment) samples from the subsurface is expensive and, in some cases, not readily available. The high spatial variation of subsurface sediments on distance scales of centimeters illustrates a potential problem of sampling a “zone of influence” after biostimulation [31]. This imposes a severe restriction on the ability to interrogate the subsurface microbial community both spatially and temporally. Groundwater samples collected repeatedly overtime during a biostimulation experiment can provide an important temporal dimension, but are limited because of bias toward the ephemeral nonattached (planktonic) portion of the microbial community [15]. The underlying advantage of the monitoring approach described here is that it can be deployed in existing boreholes over a wide range of depths.

In this study simple *in situ* microbial sampling devices were used that acted as sensitive recorders of the microbial community response to biostimulation tests investigating the kinetics of U and Tc reduction in a shallow unconfined aquifer at the Oak Ridge National Laboratory. The null hypotheses for these experiments were (i) there are no differences in the microbial community biomass, composition, physiological status, or respiratory state of biofilms colonizing aquifer samplers between donor-amended and nonamended wells and (ii) there are no differences in the microbial parameters caused by choice of support matrix (glass wool or Bio-Sep). The biofilm samplers consisted of either glass wool or Bio-Sep beads. These devices were suspended in the screened interval of six monitoring wells during push-pull tests. In these tests three electron donors (Table 1) were added to the subsurface to stimulate microbiological activity and create anaerobic conditions that favored the

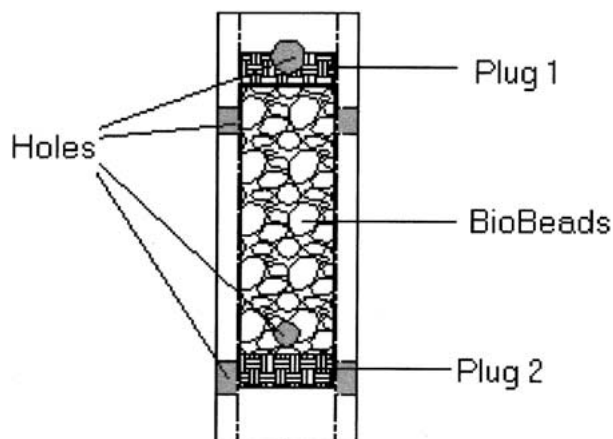
reduction of U(VI) and Tc(VII) to the less soluble and less mobile U(IV) and Tc(III). After a 6-week period of deployment the samplers were recovered and the resultant biofilms analyzed for the presence and viability of microbial biomass, the presence of microorganisms known to immobilize metals, and the oxidation/reduction (redox) status of the system.

## Methods

**Push-Pull Tests.** Field experiments were conducted under neutral and acidic conditions to stimulate denitrification and U and Tc reduction in the subsurface at this site (Table 1). In these tests, ~200 L of site groundwater was extracted and amended with sodium bicarbonate for pH adjustment, a bromide tracer, and acetate, glucose, or ethanol to serve as electron donors. The prepared test solution was then injected into each well and periodically sampled to monitor changes in groundwater chemistry resulting from donor addition (additional details about the design and methodology of these types of field tests is described in Istok et al. [13]).

**Biofilm Samplers.** Biofilm samplers (Fig. 1) were constructed from 1.25-cm O.D. Teflon PFA (perfluoroalkoxy) tubing cut into 4-cm lengths and loaded with glass wool or Bio-Sep beads [21]. Bio-Sep beads consist of 2 to 3-mm spherical beads engineered from a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (PAC). The bulk density of the beads is approximately 0.16 g cm<sup>-3</sup> with a porosity of 74%. The median pore diameter is 1.9 μm; however, large macropores (>20 μm) also exist inside the beads. The internal adsorptive capacity is >600 m<sup>2</sup> g<sup>-1</sup>. Bio-Sep beads are surrounded by an ultrafiltration-like membrane with pores of 1–10 μm. Bio-Sep beads and glass wool were purged of fossil organic residues by baking at 300°C for 4 h. Samplers were fastened to a tether and suspended down well for a period of 45 days during push-pull biostimulation tests (Table 1). Once recovered the biofilm samplers were frozen on-site with dry ice and shipped to the CBA laboratory for processing.

## Biofilm Sampler



**Figure 1.** Diagram of a biofilm sampler. Plugs are made of glass wool, and the sampler is loaded with glass wool or Bio-Sep beads.

**Phospholipid Fatty Acid (PLFA) Analysis.** PLFA analysis was performed using previously reported precautions [30]. The glass wool and beads were extracted with the single-phase chloroform–methanol–buffer system of Bligh and Dyer [1], as modified by White et al. [29]. The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography [9]. The polar lipids were transesterified to the fatty acid methyl esters (FAMES) by a mild alkaline methanolysis [9], with the Mayberry and Lane [20] method to protect cyclopropane PLFA and release plasmalogen ethers as dimethylacetals. The FAMES were analyzed by gas chromatography/mass spectroscopy using an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass-selective detector with a 50-m nonpolar column (0.2-mm I.D., 0.11- $\mu$ m film thickness) with a temperature program of 100°C initial temperature, 10°/min to 150°C for a minute, 3°/min to 282°C for 5 min with injector temperature at 270°C and detector at 290°C. Total analysis time was 55 min.

**Respiratory Quinone Analysis.** The neutral lipid fraction of the Bligh and Dyer [1] extract after fractionation on silicic acid columns was examined for respiratory ubiquinone and menaquinone isoprenologues by high-performance liquid chromatography/atmospheric pressure photoionization tandem mass spectrometry (HPLC/APPI/MS/MS) [17, 18].

**DNA Extraction and PCR Amplification.** Nucleic acid was precipitated directly from the PLFA Bligh/Dyer aqueous phase [14] with an equal volume of isopropanol in an ice bath for 30 min. DNA was pelleted by centri-

fugation at 13,000 g at 4°C for 15 min., washed with 80% ethanol twice, air-dried, and redissolved in TE buffer (pH 8.0). The DNA extract was then purified and eluted in denionized water as described in Chang et al. [4]. PCR amplification of the 16S rDNA fragments prior to DGGE was performed as described by Muyzer et al. [23]. The primers targeted eubacterial 16S regions corresponding to *Escherichia coli* positions 341–534 [2].

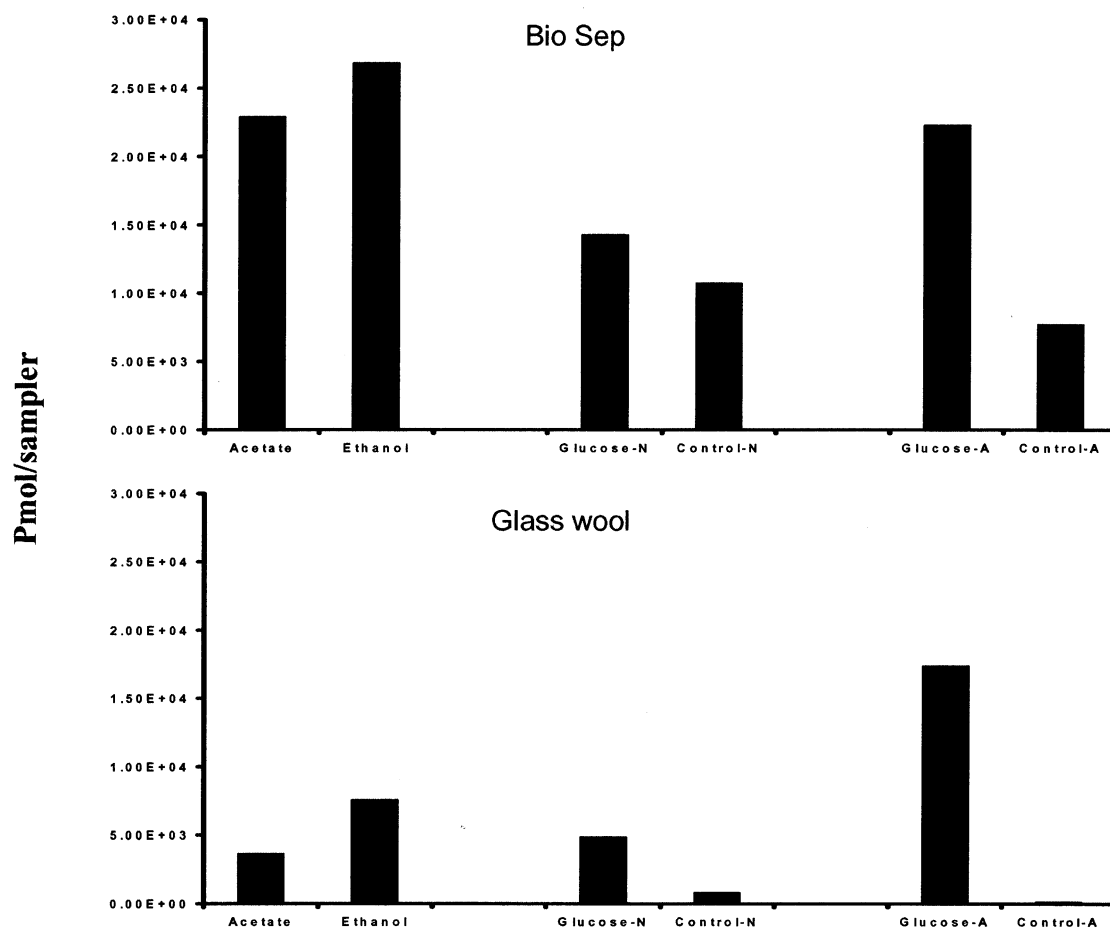
**DGGE and Sequence Analysis.** DGGE was performed using a D-Code 16/16 cm gel system (Bio-Rad, Hercules, CA) as referenced in [4]. Prominent bands were excised and subjected to sequencing. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information and “Sequence Match” facility of the Ribosomal Database Project [19], respectively, accession numbers AY245556 to AY245577. Sequences were assembled using “SeqPup Version 0.6” [8]. Phylogenetic algorithms (DNA-DIST, NEIGHBOR, and SEQBOOT) were operated within the ARB software environment [26].

## Results

**Microbial Biomass.** Microbial biomass PLFA results are shown in Fig. 2 and are expressed on a per sampler basis. In the beads, ethanol and acetate produced similar biomass responses. The addition of glucose, whether in the neutralized or acidic wells, stimulated higher biomass than in the associated controls. Biomass on the glass wool samplers showed the same trend as in the beads; however, the accumulated biomass was an order of magnitude less, except in the glucose (acid) treatment. This is most likely due to the higher surface area of the beads compared to the glass wool. Plasmalogen-derived dimethyl acetals (DMA) are found in *Clostridia* and close relatives as well as some Gram-negative bacteria [22]. Microbial biomass DMA was higher in the beads than on the glass wool samplers (Table 2) and was highest in the wells that had received donor. The glucose (acid) well had by far the most DMA biomass and was 2 $\times$  that of the acetate and glucose (neutral) treatments and 15 $\times$  that of the acetate and controls. The same DMA biomass trend was seen in the glass wool samplers but with much lower overall biomass levels.

**Community Structure PLFA.** Table 2 presents the results of the community PLFA analysis. The bead samplers contained a diverse community profile that consisted of all major PLFA structural groups. The dominant PLFA group was the monounsaturates that accounted for 58 to 65% of the PLFA profiles in the beads regardless of treatment. Comparisons between treatments showed no obvious differences except in the case of the glucose

## Biomass PLFA



**Figure 2.** Biomass PLFA of the biofilm samplers containing glass wool or Bio-Sep beads suspended down well during push-pull biostimulation tests.

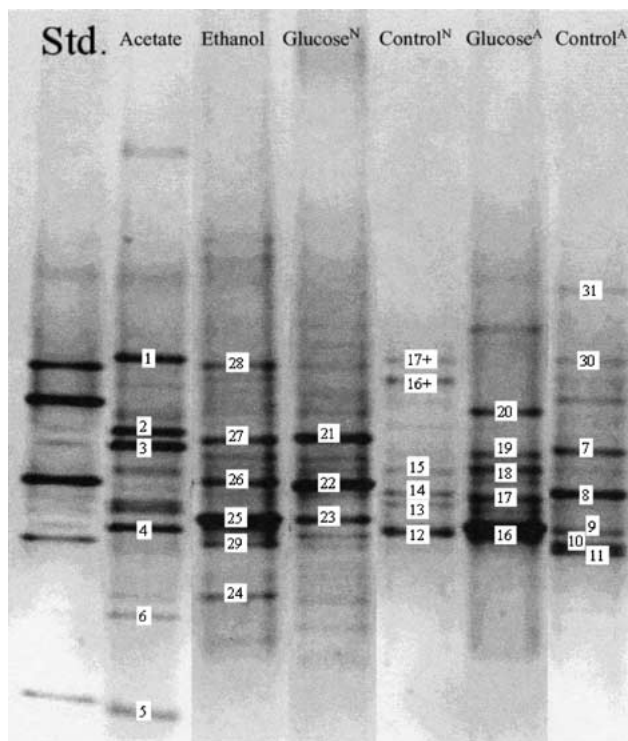
(neutral) treatment and its associated control. The PLFA profile of the glucose (neutral) treatment contained  $3 \times$  less polyunsaturated PLFA than the associated control well. The glass wool samplers contained less diverse PLFA profiles, but monounsaturates were also the dominant PLFA group. However, the monounsaturates accounted for substantially more of the PLFA profile when compared with the beads, except for the control (acid) treatment that contained appreciably more normal saturated PLFA.

**Community Structure 16S rDNA.** Microbial communities in the beads were profiled by DGGE of amplified 16S V3 rDNA fragments. Sequences with close homologies (>95%) to *Alcaligenes*, *Ralstonia*,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -, Proteobacteria, *Sphingomonas*, *Dechloromonas*, and *Geobacter* were detected. Several of these genera are capable of using nitrate as a terminal electron receptor (bands 1, 3, 7, 16, 24, 28 and 31, Fig. 3 and 4) and metal reduction (bands 5, 6, 18, 21, 22, and 27, Fig. 3 and 4).

Sequences affiliated with *Geobacter* (a known U reducer [16]) were only detected in the wells that received donor addition regardless of pH. Two sequences (2 and 29) could not be associated with a genus. Sequence 2 shared a close homology with an environmental clone detected in phosphorous removal system that was transitioning to nitrate respiration [6]. Sequence 29 had a close homology with an environmental clone detected in a hydrocarbon and chlorinated solvent contaminated aquifer [7].

**Physiological and Respiratory Status.** The lipid composition of microorganisms is a product of metabolic pathways and so reflects the phenotypic response of the microbe to the environment. Gram-negative bacteria make *trans* fatty acids to modify their cell membranes against environmental stress, as such the physiological status of Gram-negative communities can be assessed by ratios of specific PLFAs. The total *trans* to *cis* isomer ratio for 16:1 $\omega$ 7 and 18:1 $\omega$ 7 was higher in the wells that did not receive donor additions, and the same trend was





**Figure 3.** DGGE eubacterial community profile of the biofilm samplers containing Bio-Sep beads suspended down well during push-pull biostimulation tests. The portion of the gel shows the range of 30–52% denaturant, in which all visible bands were found. Numbered bands were excised and sequenced. The band numbers correspond to the last number on the tree diagram in Fig. 4. For example, band 1 on this figure is FW019-1 on Fig. 4.

dominated by UQ7, which accounted for at least 50% of quinone isoprenologues measured (data not shown). Control samples had a more even distribution of quinones.

## Discussion

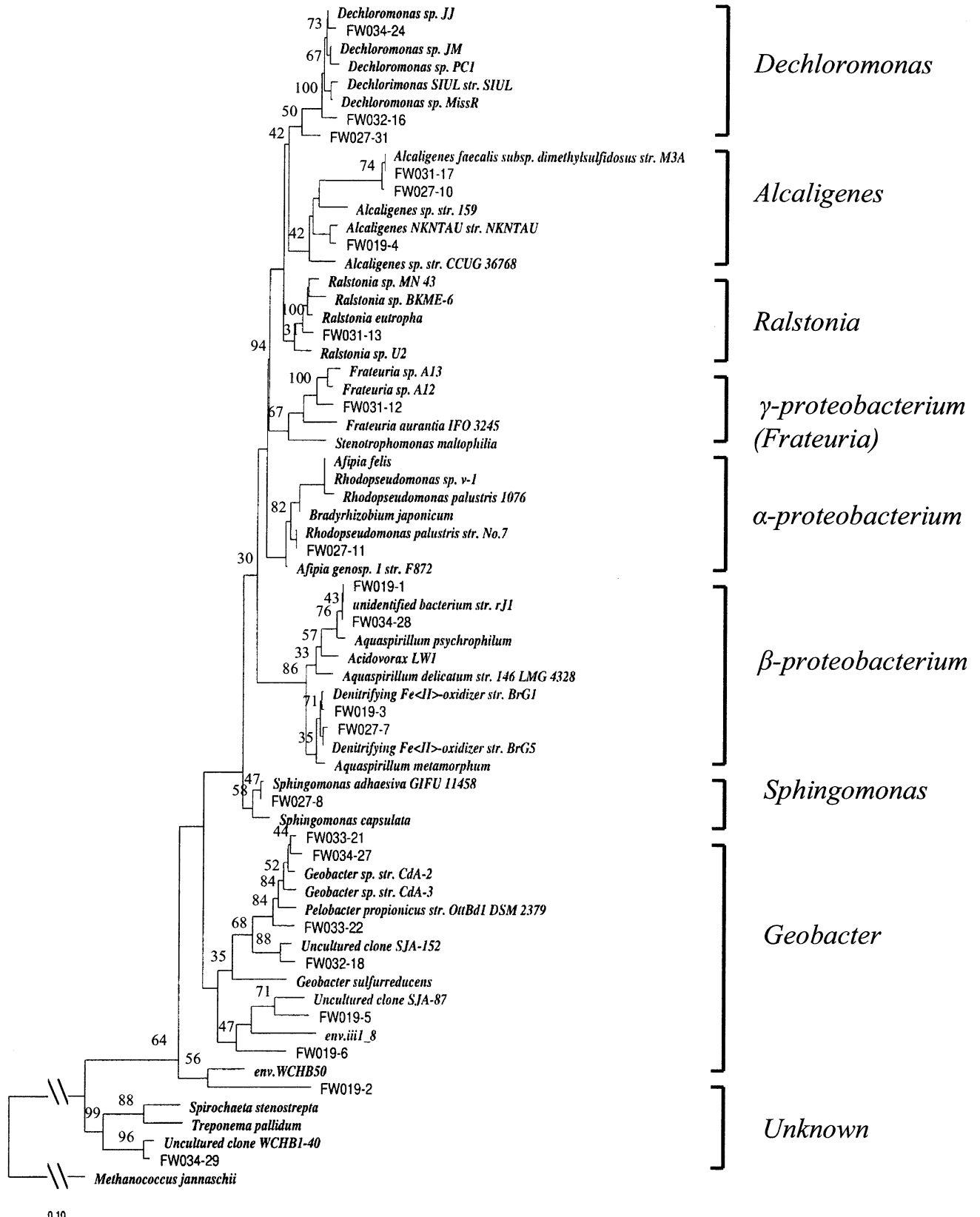
There were two main objectives in this project. The first was to ascertain if the biofilm samplers could be used as monitoring devices in the aquifer during biostimulation tests, and would the data be interpretable and relevant. The second objective was to explore which substrata (glass wool or Bio-Sep) would provide a better surface to support *in situ* biofilm formation. A microbial community response to the addition of electron donors in the push-pull experiments was detectable from the results of the combined lipid biomarker/DNA analysis. Biomass growth on the biofilm samplers was higher in the wells that received donor additions compared to the associated control wells. Samplers loaded with Bio-Sep beads contained more biomass per sampler than glass wool. DuPont originally marketed Bio-Sep as a biocatalyst support for packed-bed bioreactor systems treating lean

streams of hydrocarbon-contaminated groundwater [3, 25]. The adsorptive surface (powdered activated carbon) integral to Bio-Sep makes this support matrix especially well suited for low nutrient conditions such as those in groundwater.

Table 2 and Fig. 2 and 3 show that the biofilms that colonized the down-well samplers reflected the nature of the matrix (glass wool or Bio-Sep) as well as the pH and presence of nutrients (substrate addition) in the aquifer. This was detected in the composition of the microbial PLFA and DNA. In comparison to the glass wool, the microbial community that colonized the Bio-Sep was more diverse, as reflected in terminally branched saturated PLFA (largely Gram-positive bacteria), polyenoic PLFA characteristic of microeukaryotes, and the monoenoic PLFA characteristic of Gram-negative heterotrophs [30]. Change in community structure resulting from donor addition was also illustrated by 16S rDNA sequence analysis detection of *Geobacteraceae* (bands 18, 21, 22, 27, and possibly 5 and 6, Fig. 3 and 4) in the wells that received donor. Furthermore, sequences affiliated with nitrate reduction (nitrate averaged 2230 mg/L) were indicated in bands 7, 16, 11, 25, and 28 (Fig. 3).

Effects on the community were also detected by metabolic status biomarkers. There was a marked decrease in *trans/cis*, the PLFA ratio reflecting less exposure to toxic environment in the wells that received donor additions regardless of sampler matrix. However, the biofilms on the glass wool had a much higher *trans/cis* ratio than the microbial community contained in the Bio-Sep, suggesting that the community on the glass wool was under more metabolic stress. Although this is a complex system, and care should be taken when interpreting this ratio, such shifts have been detected previously in and correlated with various types of environmental contamination [28].

There were also changes in the respiratory state of the microbial community between sampler matrix and treatments as reflected in the UQ/MK ratio [17, 18] and total Q/PLFA. The UQ/MK ratio and the Q/PLFA ratio decreased in the microbial communities that colonized the Bio-Sep beads in contrast to the glass wool. This result suggests that the bead communities were not as active as those on the glass wool and contained more anaerobic character. This would be consistent with the 16S rDNA analysis that showed anaerobes were a significant proportion of the bead communities that received donor additions. Anaerobic activity in the donor-amended wells was also characterized by increased recovery of dimethyl acetals (DMA) from plasmalogen (vinyl-ether) phospholipids, characteristic of low-G+C Gram-positive *Clostridium*, *Lactosphaera*, and *Desulfosporosinus*. These plasmalogen-forming anaerobes were stimulated by substrate addition and represented 0.4 to 1.3% of the PLFA.



**Figure 4.** Neighbor-joining analysis of 16S V3 fragments retrieved from DGGE band excisions. Sequences prefixed "FW" were generated during this study. Reference sequences were obtained from the RDP database, except sequences of *Denitrifying Fe <II> oxidizer str. BrG1* and *BrG5* were from GeneBank with accession numbers V51101 and V51105. Numbers on the tree refer to bootstrap values on 1000 replicates; only values above 30 are given. Scale bar represents 10% estimated change.

The biofilm formed on these samplers integrated the experience of the community over the 6-week deployment period and may be more similar to sediments than to filtrates of transiently collected water samples. This sediment-like environment within the Bio-Sep (large surface area and numerous active sites) makes possible a time series of sampler collection, which would not be possible with the more difficult and costly sediment sampling. The underlying advantage of this monitoring approach is that it can be deployed in existing boreholes over a wide range of depths. Such an approach is virtually the only one that will be cost-effective at, for example, the 200 Areas at Hanford, Washington, where depths to the water table exceed 60 m and cost of new wells can easily exceed \$150K. In addition, recovery of the beads in the biofilm samplers is straightforward, requiring no specialized equipment. Biomarker recovery and processing are also considerably easier using samplers than with sediments for both the lipids and DNA because there is less interference from clays and humic materials.

The biofilms collected and analyzed from the samplers provided interpretable and relevant data regarding the microbial community biomass, structure, and metabolic status during biostimulation push-pull tests. These tests resulted in the reduction (bioremediation) of Tc (VII) and U (VI) (Istok et al., "In-situ Bioreduction of Technetium and Uranium in a Nitrate-Contaminated Aquifer," in review). Bio-Sep beads provided a larger surface area than glass wool and concentrated nutrients in the system which facilitated more biomass growth and a more diversified community structure. This type of sampling system can provide more comprehensive and interpretable data in contrast to transient membrane filter retentates of groundwater samples. Different types of solid phases may be used in the future to query the subsurface microbial populations and may aid in the characterization and manipulation of subsurface environments to clean up pollutants.

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