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Microbiological and Geochemical Heterogeneity in an In Situ Uranium Bioremediation Field Site

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The geochemistry and microbiology of a uranium-contaminated subsurface environment that had undergone two seasons of acetate addition to stimulate microbial U(VI) reduction was examined. There were distinct horizontal and vertical geochemical gradients that could be attributed in large part to the manner in which acetate was distributed in the aquifer, with more reduction of Fe(III) and sulfate occurring at greater depths and closer to the point of acetate injection. Clone libraries of 16S rRNA genes derived from sediments and groundwater indicated an enrichment of sulfate-reducing bacteria in the order *Desulfobacterales* in sediment and groundwater samples. These samples were collected nearest the injection gallery where microbially reducible Fe(III) oxides were highly depleted, groundwater sulfate concentrations were low, and increases in acid volatile sulfide were observed in the sediment. Further down-gradient, metal-reducing conditions were present as indicated by intermediate Fe(II)/Fe(total) ratios, lower acid volatile sulfide values, and increased abundance of 16S rRNA gene sequences belonging to the dissimilatory Fe(III)- and U(VI)-reducing family *Geobacteraceae*. Maximal Fe(III) and U(VI) reduction correlated with maximal recovery of *Geobacteraceae* 16S rRNA gene sequences in both groundwater and sediment; however, the sites at which these maxima occurred were spatially separated within the aquifer. The substantial microbial and geochemical heterogeneity at this site demonstrates that attempts should be made to deliver acetate in a more uniform manner and that closely spaced sampling intervals, horizontally and vertically, in both sediment and groundwater are necessary in order to obtain a more in-depth understanding of microbial processes and the relative contribution of attached and planktonic populations to in situ uranium bioremediation.

Uranium contamination of sediment and groundwater is a problem at many former uranium ore-processing sites (39) where residual radionuclides have leached into the subsurface and pose a serious threat to human health and the natural environment, both locally and through off-site transport of soluble U(VI). Natural attenuation rates of decades, the extent of contamination, and the inefficiency and cost of pump and treat methods have spurred efforts to define low-cost methods for assessment and remediation of the many uranium-contaminated sites where U(VI) in groundwater remains above applicable standards (3, 16, 36, 39). Stimulating microbial reduction of soluble U(VI) to insoluble U(IV) has shown substantial promise as a strategy for in situ bioremediation of uranium-contaminated subsurface environments. For example, the addition of acetate to either aquifer (3, 31, 33, 36) or groundwater and sediment samples incubated in the laboratory (10, 15) promoted the growth and activity of dissimilatory Fe(III)-reducing microorganisms and resulted in removal of soluble U(VI) from contaminated groundwater. In these studies, uranium immobilization was associated with the growth of microorganisms in the family *Geobacteraceae* and the concomitant stimulation of Fe(III) and U(VI) reduction (3, 15, 31).

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The Old Rifle site is located at a former uranium ore-processing facility in Rifle, Colo., which is now part of the Uranium Mill Tailings Remedial Action (UMTRA) program of the U.S. Department of Energy (39). Continued leaching from spent mill tailings at this site has resulted in residual contamination of both groundwater and sediment within the local aquifer. In a previous study at this site, acetate injection into the aquifer was found to stimulate growth of *Geobacteraceae*, Fe(III) reduction, and a decline in the U(VI) content of groundwater down-gradient of the injection site. Continued acetate addition resulted in a shift in the dominant terminal electron accepting process from Fe(III) reduction to sulfate reduction, as well as complete degradation of the injected acetate under sulfate-reducing conditions and an apparent decrease in the rate of removal of soluble U(VI) from groundwater (3). These results indicated that the maintenance of Fe(III)-reducing conditions was critical for sustaining reductive precipitation of U(IV), and highlighted the need to correlate U(VI) removal from groundwater with alterations in microbial community composition in the subsurface.

A key requirement for understanding in situ bioremediation processes is an ability to predict the in situ activity and distribution of metal-reducing microorganisms relevant to bioremediation (14, 19–21). Current technology limits routine monitoring of microbial populations during in situ uranium bioremediation to groundwater sampling. Sediment sampling is expensive, labor-intensive, and limited to relatively few cores

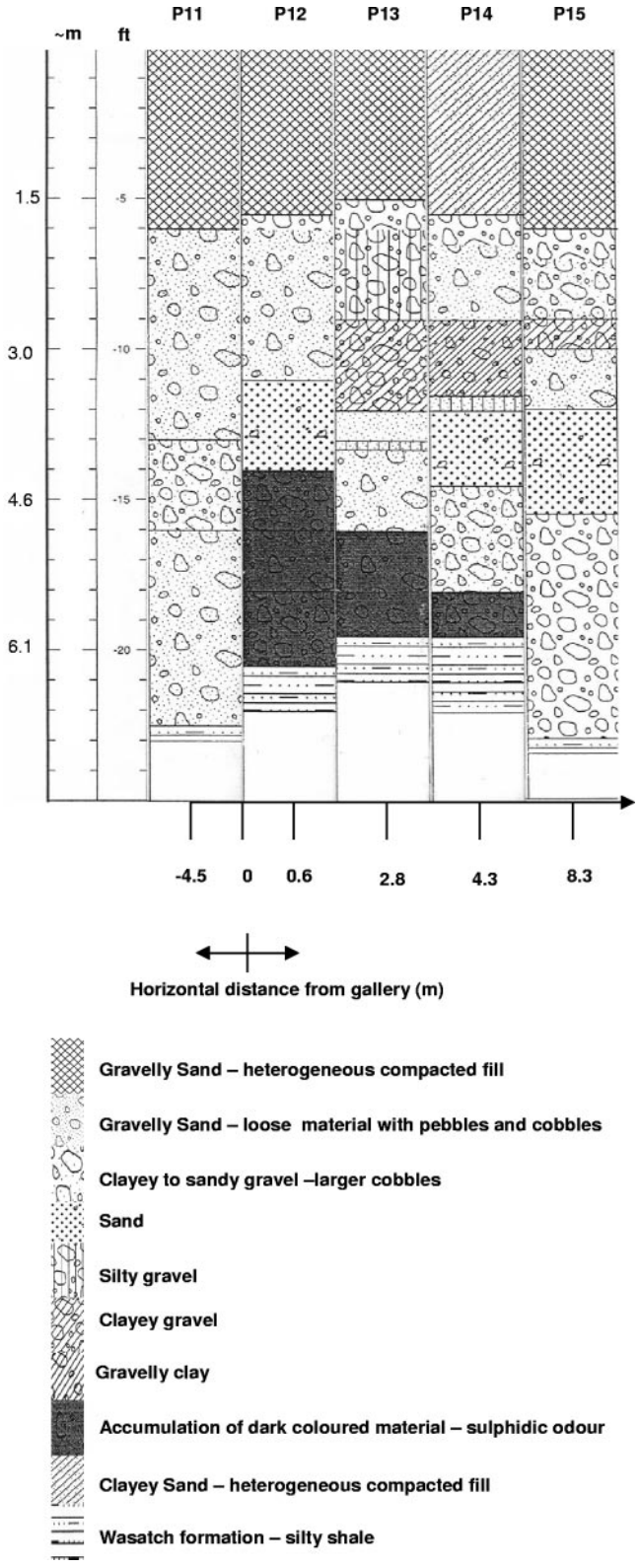

FIG. 1. Stratigraphy of borehole logs. Borehole logs collected from the in situ treatment plot installed at the Old Rifle site after 40 days of acetate injection.

throughout the course of an in situ experiment due to cost and the potential to disrupt subsurface groundwater flow path and monitoring. However, it is unclear to what extent community analyses of groundwater samples reflect subsurface microbial communities and terminal electron-accepting processes present in sediment.

Here we report on an analysis of the microbial community and related geochemistry in both the groundwater and sediments of the Old Rifle site. The results demonstrated that microbial activity and distribution were highly impacted by the manner in which injected acetate moved through the site, which is highly heterogeneous along the groundwater flow path leading from the injection site. Our findings strongly emphasize the need for close interval sampling in future studies for understanding microbial partitioning and modeling the processes influencing in situ uranium bioremediation.

MATERIALS AND METHODS

Site description and plot design. Detailed descriptions of the geology and hydrogeology of the Old Rifle site as well as a comprehensive description of the in situ treatment plot installed at this site as part of a previous study have been presented elsewhere (3, 39). The Rifle aquifer represents a heterogeneous alluvial deposit composed mostly of unconsolidated clay, silt, sand, gravels, and cobbles, which 2.4 to 4 m of weathered claystone of the Wasatch formation underlies (Fig. 1). The floodplain deposits extend approximately 6.1 to 6.9 m below land surface, with saturation in the treatment plot occurring at a depth of approximately 4 m (range, 3.6 to 4.2 m). The test plot encompasses an area of 24 m by 16 m with three background monitoring wells placed 3.7 m upstream of an injection gallery positioned perpendicular to groundwater flow (Fig. 2). Three rows of five equally spaced down-gradient monitoring wells have been placed at distances of 3.7 m, 7.3 m, and 14.6 m from the injection gallery (Fig. 2). Previous biostimulation work at the site had involved introduction of sodium acetate (Sigma Chemical Company, St. Louis, Mo.) into the aquifer in quantities sufficient to achieve a target in situ concentration of 1 to 3 mM for approximately 90 days.

Prior to the initiation of the second phase of acetate amendment reported here, the site had been under natural groundwater flow without amendments for ~8 months. Groundwater analysis over this time showed no significant increase in dissolved oxygen subsequent to cessation of acetate addition. At the beginning of this study, some of the monitoring wells showed trace levels of nitrate (50 to 300 μM). Uranium levels in groundwater at time zero ranged from 1.51 to 1.06 μM in the background to 1.4 to 0.19 μM within the test plot. Incoming sulfate concentrations ranged from 7.6 to 9.5 mM. Groundwater flow at the site was ~0.85 m/day. Native groundwater amended with sodium acetate (and potassium bromide as tracer) was prepared and injected into the subsurface as previously described (3).

Liquid phase sampling. Acetate injection into the aquifer was continuous over a 4.5-month period from the end of June to mid-October and all monitoring wells were sampled at regular intervals prior to, during, and after the injection of acetate into the subsurface. Groundwater sampling for geochemical analysis was performed at all 18 monitoring wells, four of which (B02, M03, M08, and M13) (Fig. 2) were equipped with multilevel samplers set with semipermeable membrane sample cartridges at 0.3- to 0.4-m intervals of depth to the base of the unconfined aquifer layer. The four multilevel sampling wells will be the primary focus of geochemical groundwater results presented in this paper. The multilevel sampler cylindrical cartridges (2.5 by 7.5 cm) enabled exchange with the surrounding groundwater (26, 29, 35). Sampled cartridges were recovered from the subsurface at depths of 4 m, 4.3 m, 5 m, 5.3 m, 5.7.m, and 6.1 m.

Groundwater was pumped to the surface using a peristaltic pump (Cole-Palmer, Vernon Hills, IL) and water samples were taken after approximately 12 liters of water had been purged from each well. Groundwater was filtered (0.2-μm Millex filters; Millipore Corporation, Bedford, MA) and placed into 15-ml conical tubes for U(VI) and anion analyses. Single-depth well samples were collected from a depth of 5 m below the ground. Filtered groundwater samples were also collected for analysis of Fe(II), bromide, acetate, and other parameters. (Long et al., in preparation). The Fe(II) samples were preserved by addition of 50 μl of 12 N HCl. Samples for acetate analysis were collected into scintillation vials and preserved with 1 ml of 0.1 N H_2SO_4. Geochemical samples

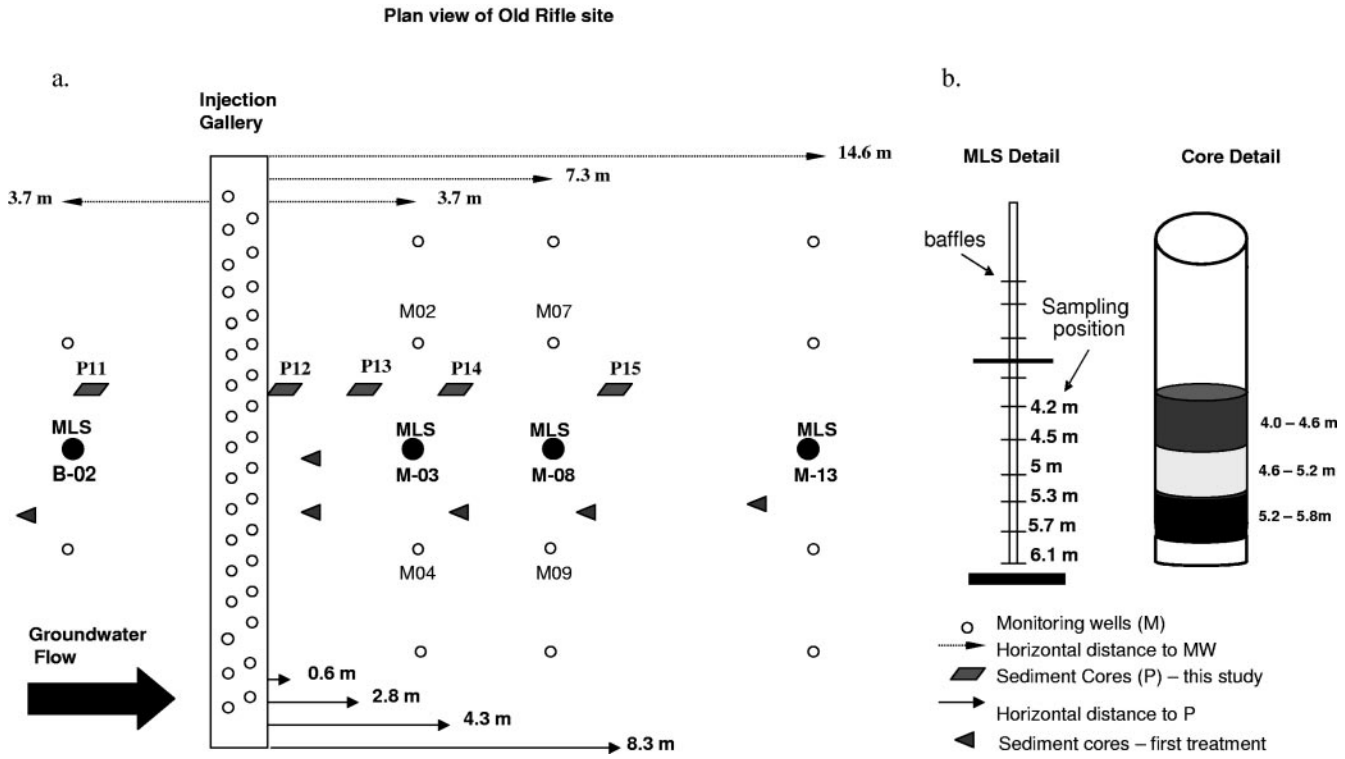

FIG. 2. Layout of the in situ test plot installed at Old Rifle. (a) Schematic layout of Old Rifle UMTRA site indicating location of monitoring wells and sediment cores. (b) Schematic layout indicating sampling depth positions on multilevel samplers (MLS) installed in monitoring wells B02, M02, M03, M08, and M13 and core subsections for collected sediment cores.

were shipped to the laboratory via overnight courier and stored at 4°C prior to analysis.

After a sampling, all cartridges were washed at least twice and then completely filled with distilled water and placed back into their sampling positions on the multilevel sampler well insert. The multilevel sampler insert was then slowly positioned back into the monitoring well for equilibration until the next sampling (minimum of one week). Single-depth samples were processed similarly to samples from multilevel sampler cartridges. Groundwater for molecular analysis was collected by filtering 1.5 liters of groundwater (after well purging) from the 5-m depth through 0.2-μm-pore-size Sterivex-GP filters (Millipore Corporation). Filters were immediately placed in dry ice, shipped to the laboratory by overnight courier, and stored at −80°C until analysis. These samples were collected from B02, M02, M03, M07, M08, and M13. Groundwater geochemistry and molecular analyses presented in this work were from day 38 of acetate injection.

Solid-phase sampling. A total of five sediment cores were collected at various distances from the injection gallery after 40 days of acetate injection. A background core sample, P11, was collected 4.5 m upstream from the injection gallery. Down-gradient cores, P12 to P15, were collected at 0.61 m, 2.8 m, 4.3 m, and 8.3 m, respectively, from the injection gallery (Fig. 2). Previous coring at the site had involved collection of four test site cores in a row between M03 and M04 and one core along the mid-line of the plot within the flow-path from the acetate injection gallery to M03 (Fig. 2). Coring was performed using a rotosonic drilling rig (Boart Longyear, Environmental Drilling Division, Little Falls, MN) to a depth of 5.8 to 6.4 m below land surface. Core samples were sectioned and logged by depth.

Samples from depths of approximately 4 to 4.6 m, 4.6 to 5.2 m, and 5.2 to 6.4 m were transferred to N_2-filled portable glove bags and subsampled into conical tubes, anaerobic pressure tubes, and Mason jars with no headspace. For simplicity, the shallower depth of each range will be used for identification of the three core depths (i.e., 4 m, shallowest core depth; 4.6 m, intermediate core depth; and 5.2 m, deepest core depth) evaluated in this paper. Efforts were made to remove larger cobbles (>3 cm) from samples. Samples for molecular analysis were immediately placed on dry ice. Anaerobic samples (for geochemical analysis) and Mason jars were packaged in coolers with ice. Samples were shipped to the laboratory by overnight courier and stored at −80°C (molecular) or 4°C (geochemical) prior to analysis.

Geochemical analysis. (i) Groundwater. Fe(II) concentrations were determined by previously described spectrophotometric methods (5, 25). Sulfate and bromide concentrations were measured with a Dionex DX-100 ion chromatograph (23). Uranium was measured by kinetic phosphorescence analysis as previously described (3, 10).

(ii) Sediment. Samples for iron and acid volatile sulfide analysis were handled anaerobically. Five replicate extractions were performed. Fe(II) was extracted with 0.5 M HCl, and the sample was subsequently treated with 6.25 N hydroxylamine to allow measurement of total iron (24, 25). Acid volatile sulfide determination was performed using an adaptation of a gas diffusion method (40). Briefly, S^{2-} was volatilized from sediment using 1 N HCl and trapped in 1 N NaOH and analyzed using a previously described spectrophotometric method (5).

16S rRNA gene-based community analysis. Bacterial 16S rRNA gene clone libraries were constructed from groundwater samples collected from up and downstream of the injection gallery at a series of monitoring wells (B02, M02, M03, M07, M08, and M13) located in parallel rows on either side of the collected sediment cores (Fig. 2). Sediment clone libraries were constructed for core samples collected from background (P11) and four different down-gradient locations within the treatment plot (P12-P15), at three different vertical depths (4, 4.6, and 5 m) (Fig. 2). DNA was extracted from groundwater filters and sediment subsamples (DNA from triplicate ~0.5-g samples was pooled) using the FastDNA SPIN kit (Bio101 Inc., Carlsbad, CA).

The 16S rRNA gene was amplified with universal bacterial primers 8F (AGA GTTTGATCMTGGCTCAG) and 907R (CCGTCAATTCMTTTRAGTTT) (9, 18). PCR mixtures contained a 1× concentration of Q and *Taq* polymerase buffers (QIAGEN, Valencia, CA), 0.5 μg bovine serum albumin, 200 μM deoxyribonucleoside triphosphates (Sigma-Aldrich Co., St. Louis, MO), 25 pmol forward and reverse primers (Sigma Genosys, The Woodlands, TX), and 1.25 U of *Taq* polymerase (QIAGEN). PCR tubes containing all reaction components except the template and *Taq* polymerase were UV irradiated for 7 min to ensure sterility. Reactions were performed with a Peltier thermal cycler (PTC 200; MJ

TABLE 1. Diversity indices based on phylotypes in 16S rRNA gene clone libraries[a]

Location	*H*	Evenness	*D*	D/D_{max}
P11_15	2.29	0.92	0.88	0.97
P11_17	2.09	0.84	0.87	0.95
P12_13	2.12	0.85	0.86	0.94
P12_15	1.84	0.74	0.78	0.85
P12_17	2.18	0.88	0.87	0.94
P13_13	1.87	0.75	0.81	0.88
P13_15	2.15	0.87	0.87	0.94
P13_17	2.18	0.88	0.88	0.96
P14_13	2.11	0.85	0.88	0.95
P14_15	2.17	0.87	0.87	0.95
P14_17	1.94	0.78	0.88	0.96
P15_13	2.1	0.85	0.87	0.95
P15_15	2.14	0.86	0.86	0.94
P15_17	1.99	0.8	0.85	0.93
B02	1.8	0.73	0.77	0.84
M02	1.95	0.79	0.82	0.89
M03	1.78	0.72	0.81	0.89
M07	1.51	0.61	0.73	0.8
M08	1.88	0.76	0.82	0.89
M13	1.06	0.43	0.48	0.53

[a] Indices were determined as described in the text.

Research Inc., Waltham, MA) beginning with a 5-minute denaturation at 95°C and then 25 to 30 cycles of 94°C (30 s), 45°C (1 min), 72°C (1 min), and a final 10-minute elongation at 72°C. For most samples, two different reactions with ~5 ng and ~1 ng template DNA were performed. PCR products were isolated by gel extraction (QIAGEN), mixed, cloned into the TOPO TA vector pCR2.1, and introduced into chemically competent *Escherichia coli* TOP10 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Inserts of the 16S rRNA gene from at least 30 clones from each clone library were amplified with M13 forward and reverse primers (30, 41). PCR products were cleaned with the QIAquick PCR purification kit (QIAGEN) and sequenced with the M13F primer.

Phylogenetic analysis. Sufficient numbers (≥30) of clones from each library constructed from groundwater and sediment samples were analyzed to ensure that coverage of 16S rRNA gene clone libraries was 80% or greater. Although the possibility of bias in DNA extraction and PCR amplification cannot be ignored, all samples were treated equivalently, suggesting that observed intersample comparisons should be valid. Sequences were compared to those compiled in GenBank with the BLAST suite of programs (http://www.ncbi.nlm.nih.gov/BLAST/) (1, 2). The Chimera check program (http://rdp.cme.msu.edu/index.jsp) of the Ribosomal Database Project (RDP-II) (27) was used to screen for chimeric artifacts (17).

Alignments using ClustalW and similarity identity matrices were performed in Bioedit (11). Representative 16S rRNA gene sequences for tree construction were obtained from the RDP. Phylogenetic analysis was performed using the Wisconsin Package, 10.2 (Genetics Computer Group, Madison, WI). Trees were constructed using neighbor joining distance methods with bootstrap analysis of 100 replicates.

Calculation of diversity indices. Species richness and evenness are considered the compositional and structural components of diversity, respectively. A number of indices have been developed to attempt to quantify the comparison between community diversities. The Shannon diversity index (*H*) is more sensitive to richness than evenness and hence rare types may have a disproportionately large influence on the magnitude of the index (28). Simpson's diversity index (*D*) is relatively less sensitive to richness and places more weight on the common species (37). The Shannon diversity index was calculated as follows: $H = -\Sigma p_i [\ln(p_i)]$, where p_i represents the proportion of the *i*th phylotype (7, 28). Simpson's diversity was calculated by the formula $D = 1 - \Sigma(p_i)^2$ (37). Evenness was calculated from H/H_{max} where H_{max} is equal to $\ln(S)$ and S is the total number of phylotypes (7). D_{max} was determined from the equation $1 - 1/(S)$. Indices are summarized in Table 1.

RESULTS AND DISCUSSION

Sediment composition profiles. The alluvial sediments at the Rifle site are physically heterogeneous (Fig. 1). The overlying vadose zone consists of an approximately 1.75 m layer of compacted fill (gravelly sand and silt with variable amounts of clay). This material was brought onto the site to replace mill tailings and the contaminated upper layer of alluvium after they were removed. From ~1.8 m down to the Wasatch formation base of the aquifer at between 5.9 to 7 m the sediment cores were comprised of a mix of heterogeneous layers of gravely sand, silty or sandy gravel, clayey gravel, or sand variably interspersed (0 to 80%) with cobbles (between 10 and 15 cm in diameter) and pebbles.

Geochemical profiles. After 38 (groundwater) to 40 (sediments) days of acetate addition to the treatment plot, there were sharp horizontal and vertical gradients in the geochemistries of both the groundwater and the sediments. The distribution of the tracer bromide suggested that one reason for the observed gradients was nonuniform delivery of acetate throughout the treatment zone. As expected, no bromide was detected in the up-gradient background wells (Fig. 3). Although M03 was the well closest to the injection gallery, bromide concentrations in the groundwater at M03 were lower than in down-gradient wells. This was not the case during the previous year's treatment (3). These results suggest that coring operations at the end of the previous field experiment resulted in groundwater diverting around this area. In the down-gradient wells, there was a significant increase in acetate and bromide concentrations with depth, indicating that much of the injectate was delivered to the deepest portions of the treatment zone.

The concentrations of electron acceptors and reduced products found with depth at each site suggested that there were also steep depth gradients in the microbial processes. It should be noted that the geochemical gradients represent an integration of microbial processes that took place during the acetate injection the previous summer, the intervening 8.5 months of no acetate injection, and the ca. 40 days of acetate injection just prior to groundwater and sediment sampling in this study. At day 40, analysis of HCl-extractable Fe(II)/total iron ratios suggested that, for sediments close to the injection gallery (particularly P12), much of the readily reducible Fe(III) had been reduced at the lower depths (Fig. 4). However, there were substantial gradients in the Fe(II)/total iron ratio with high percentages of HCl-extractable Fe(III) available at the shallower depths and in more down-gradient sediments (Fig. 4). Sediments from P14 exhibited an intermediate Fe(II)/total iron ratio indicative of a balance between Fe(III) reduction and bioavailable Fe(III) concentrations, and sediments from these cores exhibited the greatest accumulation of U(IV) (32).

It would be expected that, in the absence of microbially reducible Fe(III), sulfate reduction would be the predominant terminal electron-accepting process at the depths at which acetate was available (4, 22, 34). There was little depletion of sulfate from the groundwater at M03, except at the lowest depth, consistent with the poor delivery of acetate to this portion of the treatment plot (Fig. 3). The elevated concentrations of sulfide in the sediments collected close to the injection gallery (Fig. 1 and 4) may reflect some recent sulfate reduction (Fig. 3) as well as sulfate reduction that was stimulated in the acetate injection study the previous year (3). Substantial decreases in sulfate concentration were observed in M08 (as high as 83% at 5.7 m) while no sulfate reduction was seen in the

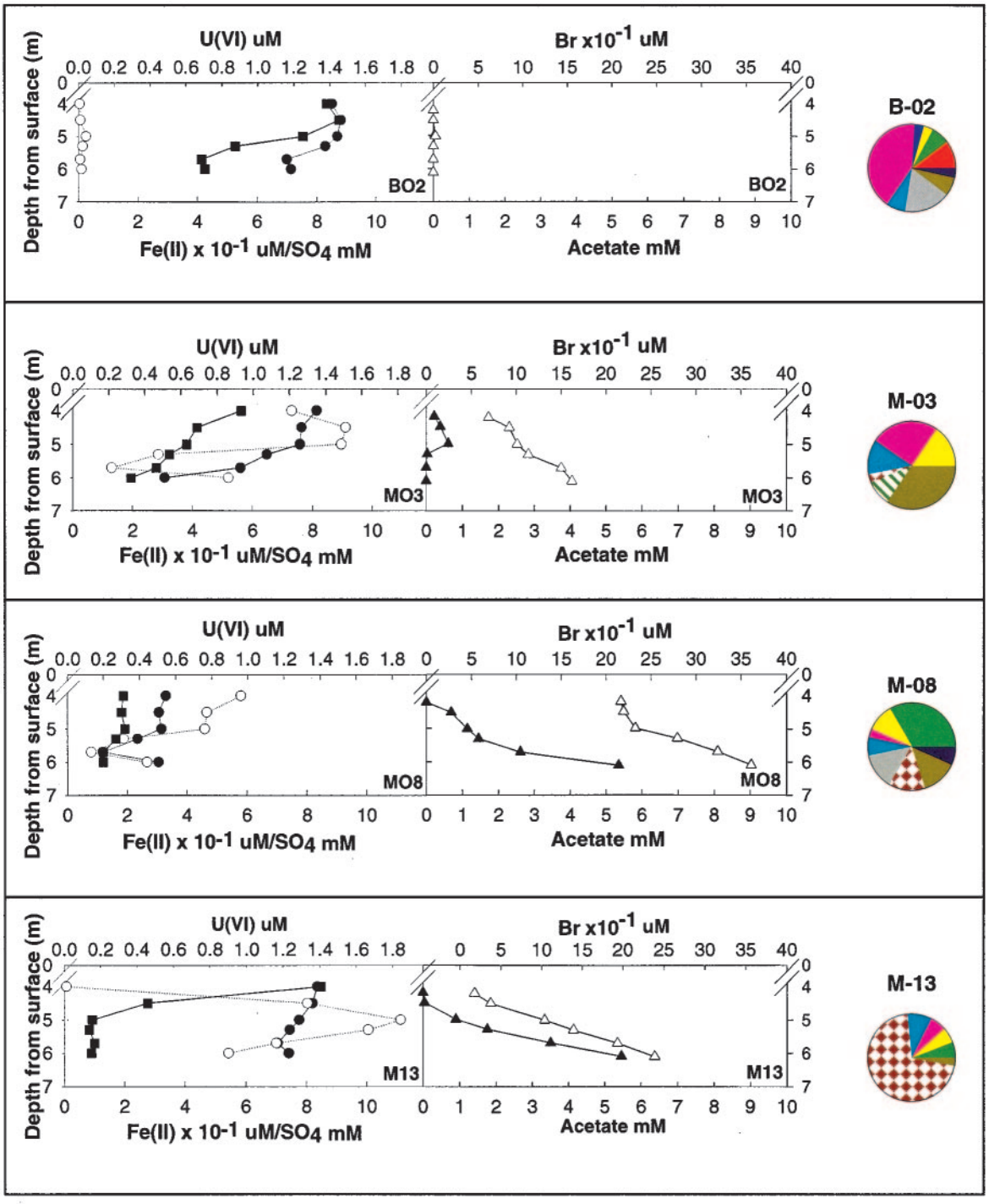

FIG. 3. Geochemical and molecular analysis of groundwater. Concentrations of SO_4, ● (mM); Fe(II), ○ (μM); U(VI), ■ (μM); acetate, ▲ (mM); and bromide, △ (μM) in multilevel sampler wells (B02, M03, M08, and M13) at day 38 of acetate injection in the Rifle aquifer (depths of 4.2, 4.5, 5, 5.3, 5.7, and 6.1 m from surface). For simplicity the zero acetate measure in the background well was omitted from the figure. Pie charts show percentages of total 16S rRNA genes in the corresponding wells sampled from 5 m below the ground surface. Locations and sampling detail were as shown in Fig. 2 and described in the text. The various bacterial taxa are represented based on their relative occurrence in groundwater samples. The designation "other δ-proteob." was applied to 16S rRNA gene sequences that fell into the *Deltaproteobacteria* but did not group with the *Geobacteraceae*, the *Desulfovibrionaceae*, or the three families comprising the order *Desulfobacterales* (*Desulfoarculaceae*, *Desulfobacteraceae*, and *Desulfobulbaceae*). The designation unclassified indicates sequences that could not be unequivocally classified with any one group due to insufficient similarity.

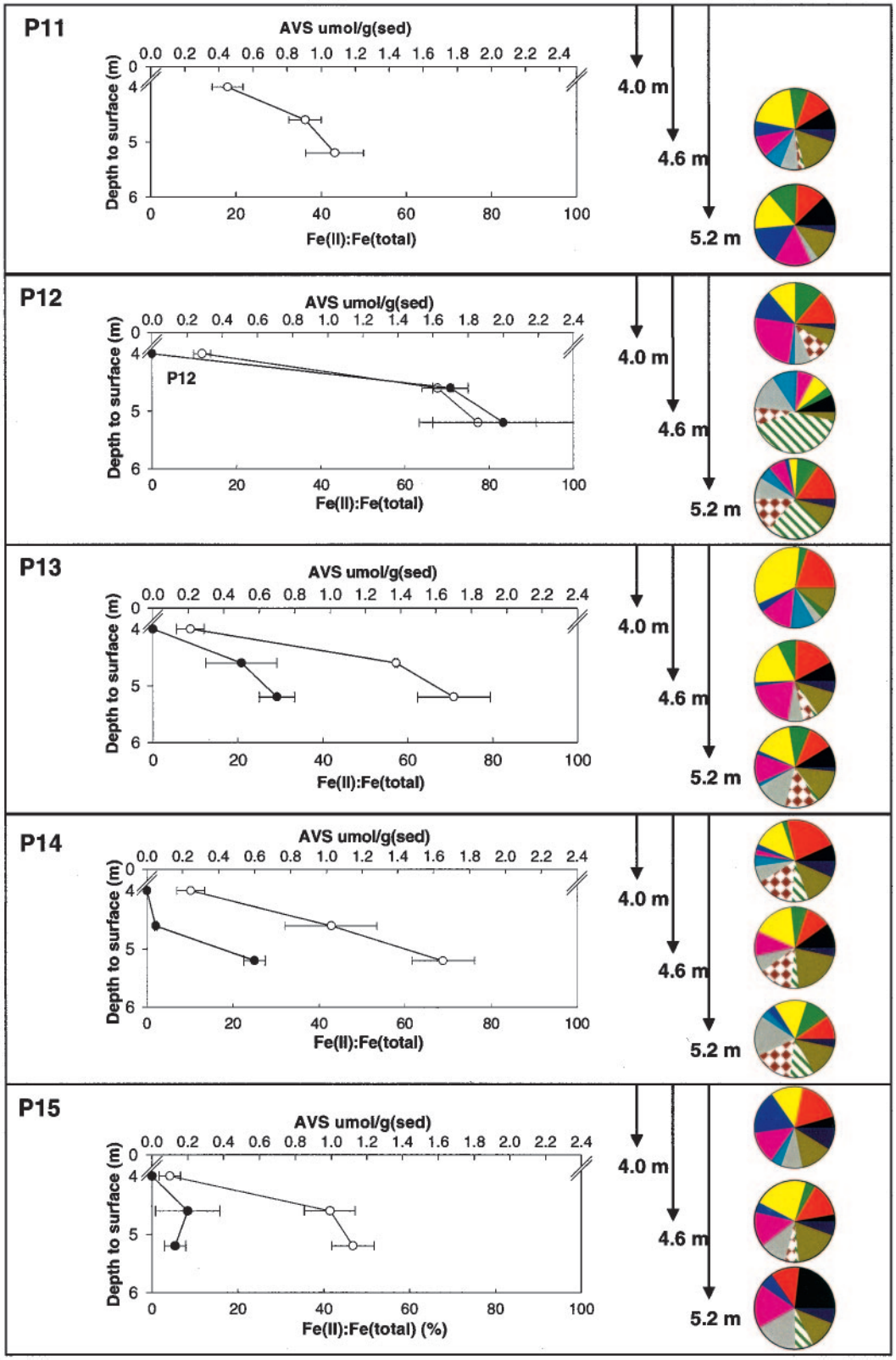

FIG. 4. Geochemical and molecular analysis of sediment. Acid volatile sulfide (AVS) (●; μmol/g) and Fe(II)/Fe(total) (○) in cores P11 to P15 (at depths of 4, 4.6, and 5 m from the surface) collected on day 40 of acetate injection in the Rifle aquifer (see Fig. 2 and text for details). The key for designations of bacterial taxa and groups within the pie charts are the same as for Fig. 3.

furthest down-gradient well M13. In well M13, sulfate concentrations were comparable to those in the up-gradient background well but there were elevated levels of dissolved Fe(II) at depths where acetate was available. Unfortunately, sediments from this zone were not available, but the water chemistry was consistent with Fe(III) reduction rather than sulfate reduction being the most important terminal electron accepting process at this site.

Initial U(VI) concentrations were lower than those in the background well throughout the treatment zone and decreased further by day 38. The most significant declines in U(VI) were in the intermediate well (M08) and at the lower depths of the most down-gradient well (M13), where U(VI) concentrations fell below the UMTRA-prescribed limit of 0.18 μM (39).

Gradients in composition of the microbial communities. There were also substantial differences in the composition of the microbial communities with distance from the injection gallery reflected in the groundwater and sediment samples and with depth in the sediment cores (Fig. 4). The microbial communities from the two sediment depths sampled from the background site were relatively comparable. However, the composition of the microbial community in the groundwater sample from the background well which integrated groundwater along the screened interval differed from that in the sediments (Fig. 3). Both types of samples revealed a high degree of 16S rRNA gene diversity (Fig. 5 and 6), with sequences from sediment exhibiting a more even distribution among the various microbial groups than those detected in groundwater as reflected in the Shannon-Weaver index (*H*), 1.8 versus 2.29, and evenness, 0.73 versus 0.92, measures for background groundwater and sediment, respectively, (Table 1.).

In the groundwater, betaproteobacteria were the predominant (41%) members of the microbial community (Fig. 3), as has been previously observed (3), while in the background sediments, betaproteobacterial sequences comprised only 9 to 15% of all 16S rRNA gene sequences and sequences resembling those of green sulfur/nonsulfur microbes were the most prevalent (19% [Fig. 4]). In sediment, *Acidobacteria*, *Alphaproteobacteria*, and *Actinobacteria* comprised ca. 10 to 15% of the total 16S rRNA gene sequences in background sediments, respectively (Fig. 4). In contrast, in groundwater, only the *Actinobacteria* organisms were observed to an appreciable (10%) extent (Fig. 3). Consistent with the lack of significant rates of anaerobic respiration in the absence of added acetate, *Geobacteraceae*-like sequences and sequences of deltaproteobacterial sulfate reducers of the *Desulfobacterales* order comprised only 1% of all the background sediment 16S rRNA gene sequences, respectively (Fig. 4 and 6) and were not detected in groundwater from the background well (Fig. 3).

It is not possible to make direct comparisons between the microbial communities recovered in the groundwater from the treatment zone and from the sediment cores because the steep gradients in geochemistry with distance from the injection gallery indicate that only sediments that were taken at the same point as the groundwater could be reliably compared. *Geobacteraceae* became progressively more abundant with increasing distance from the injection gallery (Fig. 3 and 4), consistent with the observation that microbially reducible Fe(III) close to the injection zone had been depleted, but was still plentiful further down-gradient. The relative proportion of *Geobacteraceae* in each phase could not be readily attributed to the relative availability of Fe(III) because of the inability to compare relative numbers of microorganisms and iron ratios in common units without making assumptions regarding pore volumes, surface areas, and transient partitioning between attached and unattached lifestyles (19). The greatest enrichment of *Geobacteraceae* in sediments was seen in core P14 which was also the core containing the greatest proportion of U(IV) to total uranium (32). Elevated numbers of *Geobacteraceae* in M08 and M13 were accompanied by decreases in groundwater U(VI) concentration.

Close to the injection gallery where geochemical measurements indicated depletion of microbially reducible Fe(III) and accumulation of sulfide (Fig. 1 and 4), sequences most closely related to *Desulfobacterales* predominated in the intermediate and deepest sediment samples and were elevated relative to background in groundwater from M03. The sediments which had the highest concentrations of sulfides also had the highest proportion of *Desulfobacterales*. In the absence of electron donor limitation, Fe(III) and sulfate reduction can occur simultaneously, as seen in the geochemistry at this site (Fig. 3 and 4) and also evidenced by the cooccurrence of members of both the *Geobacteraceae* and the *Desulfobacterales* within the same sample(s) (Fig. 3 and 4). With the exception of minor fluctuations (1 to 2%) and an apparent bloom of *Firmicutes* sequences in M08, all sequences other than those from within the *Deltaproteobacteria* occurred in proportions less than or equivalent to those in the background in both groundwater and sediment.

In the analysis of microbial structure and observed community shifts in this work, an effort was made to distinguish between changes related to acetate amendment and those that simply reflected natural fluctuation within indigenous populations of microbes. The occurrence of certain microbial sequences is a reflection of native conditions at a site. Extensive work on sediments from the southwestern United States have shown that organisms related to *Betaproteobacteria* and *Acidobacteria* are ubiquitous throughout these sediments and that together with *Verrucomicrobiales* and *Planctomycetales* are often the dominant indigenous microorganisms in subsurfaces of this region (3, 6–8). The ubiquity of the sequences of these organisms in both background and treatment samples from the Rifle aquifer suggests that fluctuation in their relative proportions was a function of site heterogeneity and not necessarily a response to exogenous nutrient additions. Elevated numbers of sequences related to gram-positive, spore-forming, sulfate-reducing *Desulfosporosinus* and *Desulfotomaculum* spp. were seen in the first bioremediation effort at this site (3), as well as in M08 during this study, but did not consistently coincide with samples exhibiting high U(VI) loss.

Implications for conducting and monitoring bioremediation. The finding of sharp gradients in the geochemistry and microbiology during in situ uranium bioremediation has important implications for future attempts at in situ uranium bioremediation and for monitoring this process. For example, the tendency of the injectate to move primarily downward instead of horizontally in the aquifer must be taken into account and remedied in order to more evenly distribute the electron donor throughout the zone of uranium contamination. A more even distribution of electron donor may reduce some of the steep vertical geochemical and microbiological gradients that were observed. Acetate could be delivered in a

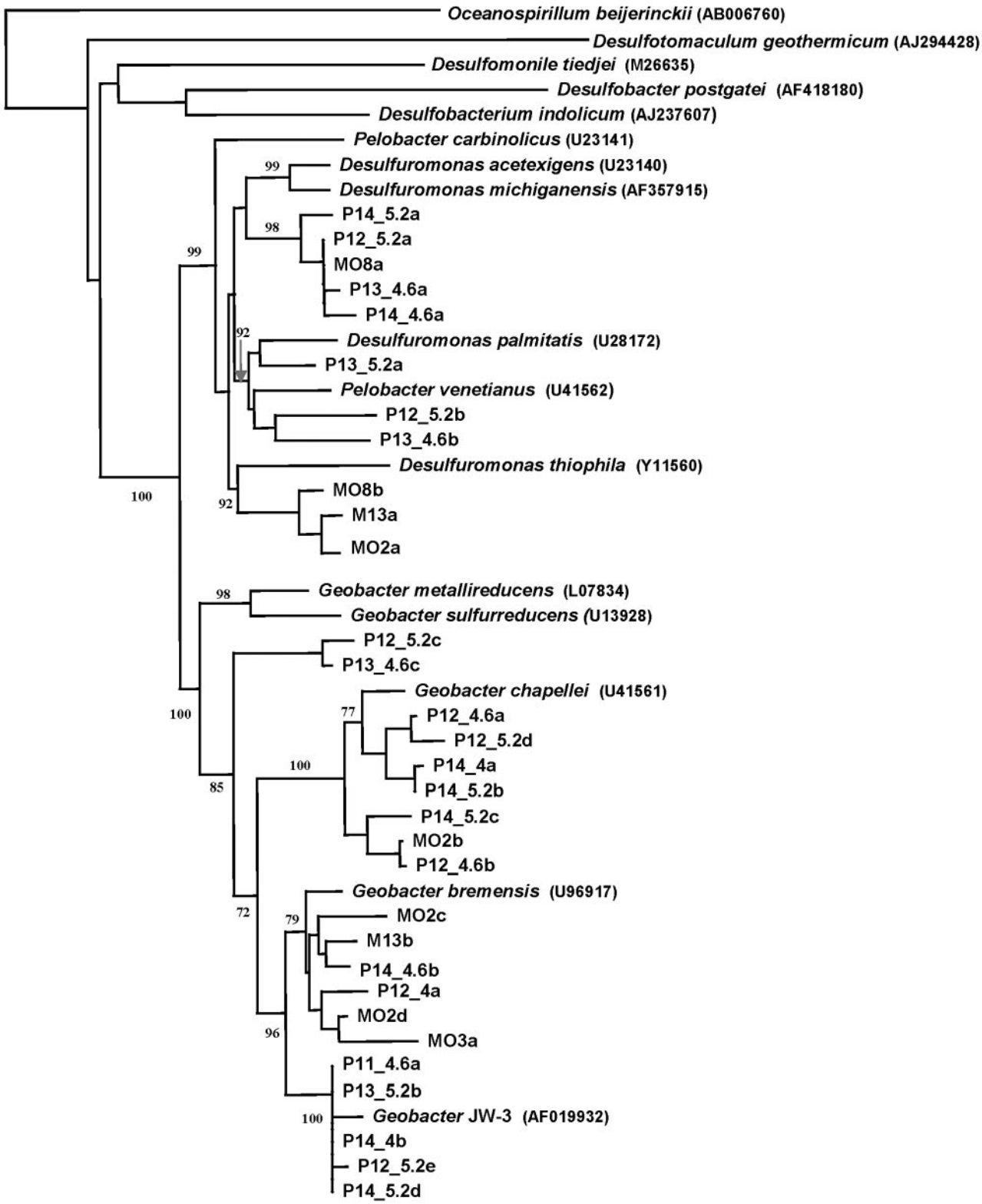

FIG. 5. Phylogenetic tree showing the relationship of *Geobacteraceae* clones to 16S rRNA gene sequences of previously described representative sequences. For clarity, one representative sequence for each location (i.e., core/depth or monitoring well) within each cluster was selected. Branch points were supported by maximum-likelihood and neighbor-joining distance analysis. Bootstrap values were calculated for 100 replicates, and values for key branch points are shown. The scale bar represents the expected number of changes per sequence position.

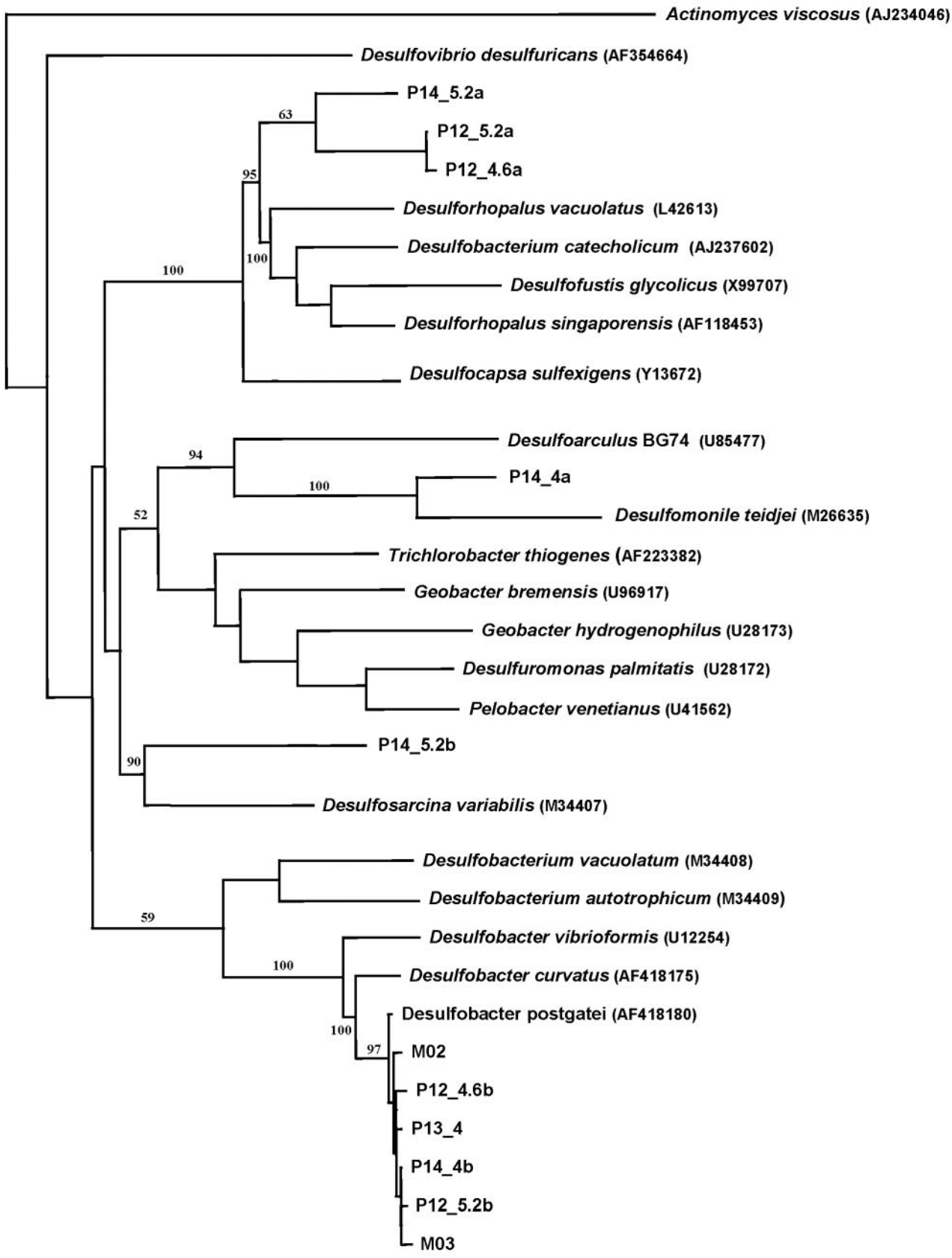

FIG. 6. Phylogenetic tree showing relationships between detected deltaproteobacterial sulfate-reducing bacterial sequences and representative sequences. Details are as for Fig. 5 and in the text.

more uniform manner if electricity was available at the site, which would permit using pumps that could mix acetate vertically and between injection wells. However, this will not eliminate variations due to fine-scale heterogeneities in the sediments as well as the horizontal gradients that may develop due to differences in permeation and availability of electron donors and acceptors with distance from the injection gallery.

The necessity for close interval sampling in order to document bioremediation processes has been noted previously (38). The appropriate sampling interval distance is determined by the steepness of the gradients themselves. The difference that sampling distances can make is reflected at the Rifle site by a comparison of averaged groundwater geochemical data obtained from depth-specific multilevel sampler samples with those obtained from point source-collected groundwater. In this study individual depth intervals revealed a geochemical gradient within the well but, when averaged, produced measurements which closely reflected those obtained by single-point sampling (data not shown).

The results of this work also demonstrate the need for more in-depth sampling of the microbial community during in situ uranium bioremediation. Many studies (12, 13, 19) as well as the current work suggest that the same microbes are not found in the same proportions in groundwater and sediment. In the Rifle aquifer, heterogeneity of the solid phase potentially resulted in substantially different exposures to soluble U(VI), sulfate, acetate, and insoluble Fe(III) minerals, factors that in turn influence both activity and diversity within each phase. However, there is little available information on the relative metabolic activity of the planktonic versus sediment-associated populations. For example, although it might be considered that *Geobacter* species attached to sediments are more metabolically active than planktonic cells, this may not be the case because the field experiments conducted to date have found that the highest U(VI) removal is associated with high proportions of *Geobacter* species in the groundwater (3; this study and D. E. Holmes, D. R. Lovley, and K. P. Nevin, manuscript in preparation). Future work evaluating differences in in situ gene expression in members of these two associated but distinct microhabitats will be useful in defining the contribution of each of these populations to U(VI) bioremediation.

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