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Microbial Communities in Contaminated Sediments, Associated with Bioremediation of Uranium to Submicromolar Levels▽

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Microbial enumeration, 16S rRNA gene clone libraries, and chemical analysis were used to evaluate the in situ biological reduction and immobilization of uranium(VI) in a long-term experiment (more than 2 years) conducted at a highly uranium-contaminated site (up to 60 mg/liter and 800 mg/kg solids) of the U.S. Department of Energy in Oak Ridge, TN. Bioreduction was achieved by conditioning groundwater above ground and then stimulating growth of denitrifying, Fe(III)-reducing, and sulfate-reducing bacteria in situ through weekly injection of ethanol into the subsurface. After nearly 2 years of intermittent injection of ethanol, aqueous U levels fell below the U.S. Environmental Protection Agency maximum contaminant level for drinking water and groundwater (<30 μg/liter or 0.126 μM). Sediment microbial communities from the treatment zone were compared with those from a control well without biostimulation. Most-probable-number estimations indicated that microorganisms implicated in bioremediation accumulated in the sediments of the treatment zone but were either absent or in very low numbers in an untreated control area. Organisms belonging to genera known to include U(VI) reducers were detected, including *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus*, and *Acidovorax* spp. The predominant sulfate-reducing bacterial species were *Desulfovibrio* spp., while the iron reducers were represented by *Ferribacterium* spp. and *Geothrix* spp. Diversity-based clustering revealed differences between treated and untreated zones and also within samples of the treated area. Spatial differences in community structure within the treatment zone were likely related to the hydraulic pathway and to electron donor metabolism during biostimulation.

Metals and radionuclides are common groundwater contaminants at facilities and waste sites of the U.S. Department of Energy (DOE), occurring in more than 50% of these locations (30). One of these sites, the former Y-12 National Security Complex at Oak Ridge, TN, contains uranium in concentrations as high as 60 mg/liter (252 μM) in groundwater and 800 mg/kg in sediments (46). To control the migration of the uranium, microbial reduction of U(VI) to sparingly soluble and immobile U(IV) has been proposed as a promising approach (1, 2, 11, 15, 20, 40, 46). Bioreduction of U(VI) to U(IV) by pure and mixed cultures of iron(III) reducers (FeRB), such as *Geobacter* spp., sulfate reducers (SRB), such as *Desulfovibrio*, *Desulfosporosinus*, and *Desulfotomaculum* spp., and denitrifying bacteria, such as *Acidovorax* spp., among others, has been reported (43).

In recent years, researchers have evaluated this approach through laboratory-scale experiments using batch serum bottles (9, 12, 49), microcosms (27), and sediment columns (10, 44). Reduction and immobilization of uranium in the mentioned laboratory experiments have been observed and confirmed by X-ray near-edge absorption spectroscopy (XANES) analysis (10, 27, 38, 39, 44). *Geobacter* spp. and *Geothrix* spp. were found to be associated with Fe(III) and U(VI) reduction under field conditions (5, 12, 44, 48). Control of the microbial community structure may be one of key issues for the long-term reduction and stabilization of uranium in situ (1). While bench-scale tests provide valuable information about the feasibility of bioreduction, they cannot replicate all of the heterogeneity and complexity of the subsurface. Hydrogeology is a factor that can contribute to the complexity of field treatments by creating gradients of resources in situ, affecting the microbial diversity and thus potentially the remediation process. Little is known about the effect of groundwater flow on microbial community diversity during and after bioremediation, even when the kinetics of U(VI) reduction should be dependent on the microbial community and geochemical conditions. The ef-

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fect that hydrogeology has on microbial diversity should be addressed.

Pilot studies are the next step in demonstrating the feasibility of the in situ uranium remediation approach (11, 12, 15, 35, 46, 47). DOE does not provide specific uranium target levels, but the U.S. Environmental Protection Agency (EPA) regulates the maximum contaminant level (MCL) for drinking and groundwater at 30 μg/liter (0.126 μM). At the Field Research Center (FRC) site of the Environmental Remediation Sciences Program of the DOE, Oak Ridge, TN, test facilities have been constructed for the remediation of uranium-contaminated groundwater and sediments using various approaches (1, 15, 47). Prior to the tests, the microbial community structure was characterized by 16S rRNA gene clone libraries made from groundwater samples in Areas 1 and 3 and the majority of the sequences (>73% in Area 1 and >65% in Area 3) were found to be from proteobacteria belonging to the genera *Azoarcus* and *Pseudomonas* (8). Microbial community analyses of Areas 1 and 2 identified *Geobacter* spp. and *Anaeromyxobacter* spp., among others, as the potential uranium reducers (26). In these areas of moderate uranium contamination (0 to 5.8 μM), removal of nitrate and aqueous U(VI) was stimulated by the injection of ethanol or glucose into the subsurface using a push-pull approach (15). In the extremely contaminated Area 3, a long-term (>2 years) bioremediation test has been performed. This area is located near former S-3 Ponds and contains high levels of nitrate (up to 200 mM) and U (up to 250 μM), aluminum (12 to 13 mM). and calcium (22 to 25 mM) (46, 47). Prior to remediation, the microbial levels in groundwater of this area were extremely low, probably due to low pH (3.6) and high levels of nitric acid. A combination of remediation approaches was used to remove U(VI) reduction inhibitors (nitrate and Ca) and condition the area for metal reduction by raising the pH and providing a carbon source. Using these approaches, low U(VI) concentrations below MCL were achieved and a new microbial community capable of uranium reduction was established (46–48).

The objective of our study was to characterize the microbial community arising in an earlier study where successful U(VI) bioremediation was demonstrated (49). We used 16S rRNA gene sequence analysis to determine the structure of the bacterial consortia present in the treatment area where low uranium levels were achieved during biostimulation and in control samples from outside the treated area. The community data were integrated with geochemical and hydraulic data to provide insight about environmental variables that profoundly influence the remediation process. We were able to identify key bacterial groups associated with successful reduction of U(VI) in the subsurface and correlate their spatial relationship with hydrogeology and geochemistry in the treatment zone.

MATERIALS AND METHODS

Site description and bioremediation test. The bioremediation test was performed in Area 3 of the DOE FRC at the Y-12 National Security Complex, Oak Ridge, TN, as previously reported (22, 46). The field system consisted of an outer groundwater recirculation loop (injection well FW024 and extraction well FW103) that isolates an inner groundwater loop (injection well FW104 and extraction well FW026), preventing penetration by highly contaminated groundwater from the source zone (Fig. 1). The hydraulic control afforded by this system created a controlled in situ bioreactor. Reduction of U(VI) to U(IV) was accomplished through ethanol injection into the inner loop. Injection and extraction wells (FW104 and FW026) had a 10.2-cm diameter and a depth of 14.6 m below ground surface (bgs) with 2.5-m screened intervals between 11.28 and 13.77 m. Multilevel sampling (MLS) wells FW100, FW101, and FW102 were used to monitor hydrogeology and remediation performance. The MLS wells contained seven separate sampling tubes (diameter, 1.9 cm) at different depths bgs. In this study, MLS wells FW101-2 (13.7 m bgs), FW101-3 (12.2 m bgs), FW102-2 (13.7 m bgs), and FW102-3 (12.2 m bgs) were selected for routine monitoring of remediation performance because of their hydraulic connection to FW104 (22, 46). The recirculation flow rates in the inner loop were 0.45 liters/min (injection at FW104; extraction at FW026). The rates for the outer loop were 1.35 liters/min (injection at FW024) and 0.45 liters/min (extraction at FW103). Additional clean water was injected into FW024 at 0.7 to 0.9 liters/min in order to minimize entry of ambient groundwater (46). This clean water was a mixture of tap water and groundwater treated by an aboveground system to remove nitrate via a bioreactor (46). The remediation test was started on 23 August 2003 by preconditioning the site (day 0). During the initial 137 days, water was pumped from the subsurface, pH adjusted, treated to remove aluminum, calcium, and nitrate (this by a denitrification bioreactor), and then reinjected. Calcium and nitrate were removed to avoid formation of stable Ca-U-CO3 products, U(IV) reoxidation by nitrites, and nitrate competition as a terminal electron acceptor. Ex situ treatment was used to avoid clogging by nitrogen gas, biomass (due to denitrification), and calcium and aluminum precipitates (due to pH adjustment) (46).

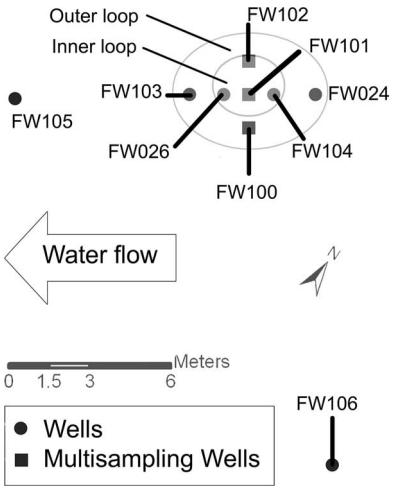

FIG. 1. Map of the Area 3 treatment zone depicting the location of the sampled wells and the control well. FW024, outer-loop injection well; FW104, inner-loop injection well; FW026, inner-loop extraction well; FW103, outer-loop extraction well. Wells FW100, FW101, and FW102 are multilevel sampling wells. FW106 is a control well. The contamination source is approximately 20 m to the right. (Adapted from a map created by Oak Ridge National Laboratory [http://public.ornl.gov/nabirfrc/FRCMaps/Area3_Inset.jpg].)

As result, the pH increased from 3.6 to around 6.0, and nitrate and U(VI) concentrations decreased to around 0.5 mM and 5 μM, respectively (46). After the conditioning phase, ethanol was added as an electron donor to stimulate U(VI) bioreduction starting on 7 January 2004 (day 137) (47). An ethanol solution (industrial grade, containing ethanol [88.12%], methanol [4.65%], and water [7.23%] [wt/wt]) with a chemical oxygen demand (COD)-to-weight ratio of 2.1 was prepared in a storage tank with 6.9 to 9.8 g COD/liter. This solution was normally injected at FW104 over a 48-h period each week to give a COD of 120 to 150 mg/liter at FW104.

Sampling for community analysis. Sediment samples for microbial community analysis were collected on day 774 (5 October 2005) from the inner-loop injection well FW104, extraction well FW026, and two MLS wells at two depths, FW101-2, FW101-3, FW102-2, and FW102-3. To collect the samples, a smooth polyvinyl chloride surge block (10 cm or 1.8 cm in diameter) was inserted into the well and then lifted up and down in a rapid plunging motion. This motion drew sediment from the soil matrix surrounding the well screens into the well. The surge blocks were rinsed with groundwater in between samples to remove attached solids. The sediment slurry that settled to the bottom of the wells was pumped to a 2-liter glass bottle under an Ar atmosphere, and the bottle was then

TABLE 1. Chemical properties of groundwater in wells of treatment area before and after remediation and in control well FW106

Well[a]	Status[b]	Chemical composition of groundwater											
		pH	Nitrate (mM)	Sulfate (mM)	Cl^- (mM)	S^{2-} (mM)	Al (mM)	Ca (mM)	Mg (mM)	Mn (mM)	Fe (mM)	U (μM)	% U(IV)[d]
FW104	A	3.83	208.00	31.00	12.00	0.00	13.60	59.10	11.40	NA	0.11	139.00	0
	B	5.75	0.00	1.18	2.34	0.30	0.02	0.65	0.32	0.07	0.03	0.57	61
FW101-2	A	3.78	182.00	18.20	9.70	0.00	16.70	57.40	12.50	NA	0.11	135.00	0
	B	6.23	0.00	1.07	2.32	0.43	0.00	0.67	0.30	0.07	0.03	0.15	51
FW102-3	A	3.80	137.00	3.20	8.80	0.00	NA[c]	27.90	6.70	NA	NA	150.00	0
	B	6.23	0.00	1.10	2.31	0.36	0.01	0.62	0.31	0.08	0.05	0.06	17
FW102-2	A	4.20	271.00	3.62	9.90	0.00	NA	57.90	11.96	NA	NA	47.00	0
	B	6.45	0.00	1.01	2.32	0.16	0.01	0.80	0.31	0.08	0.04	0.08	30
FW101-3	A	3.95	113.00	31.00	6.40	0.00	6.30	36.70	7.50	NA	0.09	134.00	0
	B	6.10	0.00	1.20	2.30	0.11	0.01	0.67	0.35	0.06	0.01	0.10	53
FW026	A	3.22	159.00	6.20	8.00	0.00	18.20	25.10	6.80	2.27	0.17	158.00	0
	B	5.74	0.01	1.20	2.36	0.04	0.02	0.64	0.32	0.07	0.01	0.53	<10
FW106	Control	3.60	61.90	25.40	13.30	0.00	NA	8.96	1.05	NA	0.03	128.00	NA

[a] Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies.
[b] A, before remediation (samples were taken in February to April, 2002) except for FW106 (day 278); B, after biostimulation on day 773 (4 October 2005).
[c] NA, data not analyzed.
[d] Detection limit for U(IV) is 10% of total U in XANES (48).

sealed with a rubber stopper. The slurry was transferred to the laboratory and centrifuged to separate the sediments from the water. The pellets were frozen at −80°C prior to being shipped on dry ice to Michigan State University. Fresh sediment slurry was also collected anaerobically in a 27-ml glass pressure tube for the most-probable-number (MPN) enumeration. Samples taken by this method are a mixture of the sediment along a 1-m depth of the well (screened interval) and could contain sediment in the deep matrix where an electron donor did not reach.

Before remediation, bioactivity in the treatment zone was extremely poor due to low pH (3.2 to 3.6) and high levels of nitric acid and uranium. DNA extraction from untreated sediments repeatedly failed. In order to compare microbial communities before and after biotreatment, DNA was collected by filtering 1,700 liters of groundwater from the FW106 well, which is located 12 m away from the treatment zone in parallel with treated sediments. Groundwater from FW106 has a composition similar to that in the treatment zone before remediation, i.e., high levels of nitric acid and uranium and low pH (Table 1).

Microbial enumeration. Numbers of denitrifying bacteria, FeRB, and SRB were estimated using the MPN technique with five tubes for each dilution. Anaerobic pressure tubes (27 ml) containing 10 ml basal medium were sealed with butyl rubber stoppers with aluminum caps. The basal medium contained the following components (per liter): NH_4Cl, 0.5 g; NaCl, 0.4 g; $NaHCO_3$, 0.55 g; and mineral solution, 100 ml. The mineral solution contained the following (per liter): $MnCl_2$, 0.4 g; $MgSO_4$, 1.5 g; $CaCl_2$, 0.5 g; and yeast extract, 0.02 g. The medium was prepared under an N_2-CO_2 (99:1 [vol/vol]) atmosphere and distributed to each pressure tube (10 ml per tube). After autoclaving, a sterile trace element solution (0.4 ml) and a sodium trimetaphosphate solution (50 mM, 0.025 ml) were added to each tube to obtain a pH of 7.0. The trace element solution contained the following (per liter): HCl (12 N), 6.4 ml; $FeCl_2 \cdot 4H_2O$, 0.3 g; $ZnSO_4 \cdot H_2O$, 0.1 g; $MnSO_4$, 0.085 g; boric acid, 0.06 g; $CoCl_2 \cdot 6H_2O$, 0.02 g; $CuSO_4$, 0.004 g; $NiSO_4 \cdot 6H_2O$, 0.028 g; and $NaMoO_3 \cdot 2H_2O$, 0.04 g. The electron acceptor solution was added to the tubes to obtain a final concentration of 5 mM Fe(III)-citrate for FeRB, 8.76 mM of sodium thioglycolate and 33 mM of $FeSO_4$ for SRB, and 9.9 mM of KNO_3 for denitrifiers. Ethanol solution (1 M) was added to each tube to give a final concentration of 10 mM. Groundwater from FW106 was pumped from the wells into anaerobic pressure tubes prefilled with nitrogen gas. Sediment slurries were collected in pressure tubes under anaerobic conditions. The sample was then serially diluted in MPN tubes. The tubes were incubated at room temperature for 2 months. Tubes were compared to controls for scoring as positive or negative for production of gas in denitrifying tubes, color change in FeRB tubes, and production of black Fe(II) sulfide precipitates in SRB tubes.

Tracer test. A tracer study was performed to characterize the groundwater flow in the treatment zone. The hydraulic flow path is expected to affect the delivery of nutrients and thus affect the metabolism of ethanol in the groundwater and the microbial community diversity. Sodium bromide, a conservative tracer, was injected through the FW104 well together with ethanol from day 801 to day 803. An ethanol-NaBr tracer solution with COD/Br^- of 2.46 g/g was prepared and injected into the recirculation line of the inner loop, resulting in injected concentrations in well FW104 of 50 mg/liter bromide and 1.0 mM of ethanol. The tracer test continued for 50.75 h. Samples were periodically taken from the injection, extraction, and MLS wells for analysis of Br^-, COD, ethanol, and acetate.

DNA extraction and community analysis. DNA was extracted from 0.5 g of sediments with the Fast soil prep kit (MoBio Inc., San Diego, CA), following the manufacturer's instructions. DNA was used to amplify 16S rRNA genes using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAF-3′) and 1392R (5′-ACGGGCGGTGTGTRC-3′) in a Stratagene thermal cycler (Stratagene, La Jolla, CA). The PCR (50 μl) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.1 μg/μl bovine serum albumin, 10 pmol of each primer, 0.2 mM (each) deoxynucleoside triphosphate, 1.25 U *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA), and 20 ng of purified DNA. The PCR cycling conditions were as follows: 95°C for 5 min; 95°C for 1 min, 59°C for 1 min, 72°C for 1 min, 40s, for 28 cycles; and 72°C for 10 min. PCR products were analyzed in a 1.5% (wt/vol) agarose Tris-acetate-EDTA gel to confirm the size of the product. Four replicate PCRs were generated for each DNA extract and then were compiled to address variability that may be introduced by PCR bias. The PCR products (200 μl) were concentrated to 30 μl with a PCR purification kit (Qiagen Inc., Valencia, CA). The concentrated products were then run in a 1% agarose gel, excised, extracted with the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA), and eluted to 30 μl with EB buffer (10 mM Tris-Cl, pH 8.5). An additional step was taken to add polyadenine overhangs to the PCR products to facilitate cloning (10 min at 72°C, 18.8 mM Tris-HCl [pH 8.4], 47 mM KCl, 0.93 mM dATP and 0.5 U of *Taq* polymerase; Qiagen Inc., Valencia, CA). Products were cloned using the Topo TA cloning kit for sequencing, following the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Single-extension sequencing was conducted by Macrogen Inc. (Seoul, Korea) from the internal primer 27F.

Raw sequencing files (Ab1) were submitted to the *my*RDP application of the Ribosomal Database Project (RDP), where the nucleotide sequence was determined, the quality (*Q* score) of each base stored, and vector sequence removed (7). The resulting sequences were aligned to the RDP model, and rRNA distance matrices were generated with the Jukes-Cantor distance correction. Operational taxonomic units (OTUs) were defined at 97% sequence identity. The distance matrices were used to calculate alpha diversity indices (Chao1, Simpson, and Shannnon) and rarefaction curves using the DOTUR (distance-based operational taxonomic unit and richness determination) program (33). The diversity indices were rarified to account for differences in number of sequences per library.

The distribution of the OTUs in the different libraries was used in the EstimateS software program (version 7.5 [http://purl.oclc.org/estimates]) to make comparisons based on diversity patterns using Bray-Curtis and Sørensen beta diversity indices. These indices were used to cluster the samples according to their distances using MEGA 3.1 (18). The composition was normalized to account for differences in numbers of clones in the different libraries. The ∫-LIBSHUFF software program (34, 36) was used to compare the clone libraries according to Good's homologous and heterologous coverage. This approach

provides a quantitative comparison of 16S rRNA gene clone libraries from environmental samples (36).

A mask using the quality value ($Q > 20$) was used, and the resultant sequences were used for classification with an RDP classifier using an 80% confidence value. The Mallard software program (4) was used to detect sequences with anomalies, such as chimeras. The putative chimeras were later reevaluated using the RDP Sequence Match with a suspicious-free and near-full-length data set and with the Pintail program (3). Sequences confirmed as anomalous with Pintail were excluded from the analyses.

Phylogenetic trees were constructed using distance and maximum-likelihood methods. Aligned sequences were downloaded from the RDP and aligned manually, and the nonmodel positions were masked from the alignment. The Jukes-Cantor distance correction, full gap deletion, and a bootstrap test using 10,000 replicates and random seeds were used to construct the trees in the MEGA v3.1 software program (18).

Chemicals and analytical methods. The source and quality of chemicals used in the field test were described previously (46, 47). COD was used as an overall indicator to monitor the consumption of electron donors (ethanol, its metabolite acetate, and others). COD, sulfide, and Fe(II) were determined using a Hach DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Anions (including NO_3^-, Br^-, Cl^-, SO_4^{2-}, and PO_4^{3-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale, CA), metals (Al, Ca, Fe, Mn, Mg, U, K, etc.) were determined using an inductively coupled plasma mass spectrometer (Elan 6100; Perkin Elmer), and the U reduction state was determined using XANES as described elsewhere (47, 48). Ethanol and acetate were determined by using an HP5890A gas chromatograph equipped with a flame ionization detector and an 80/120% Carbopack BDA column (Supelco Division, Sigma-Aldrich Corp., St. Louis, MO), using helium as a carrier gas.

Nucleotide sequence accession numbers. The sequences determined in this work were submitted to GenBank under accession numbers EF692646 to EF693732.

RESULTS

In situ biostimulation test. After 1 year of biostimulation, the nitrate concentration in the treatment zone dropped to a level of nearly zero. An increase in the sulfide concentration and the appearance of Fe(II) in groundwater suggested that sulfate and iron reduction were occurring (48). Microbial activity during biostimulation was determined by monitoring changes in the aqueous concentration of the electron donor added (as COD), sulfide, and uranium. A representative time course of biostimulation is shown in Fig. 2. On day 704, ethanol was injected into FW104, causing an increase in COD concentrations in all major MLS wells (Fig. 2A). The COD in FW104 was mainly attributed to ethanol (>80%) and a small amount of acetate (<30 μM), while in the MLS and FW026 wells, the COD came mainly from acetate (data not shown). No ethanol was detected in the MLS wells and the FW026 well, and methanol concentrations were below the detection limit (<50 μM) in all cases. On day 706, ethanol injection was stopped and the COD concentrations in the MLS wells decreased rapidly. The same pattern was observed again on days 711 to 713, when ethanol was reinjected. Sulfide concentrations in all MLS wells increased after ethanol was injected and decreased after injection stopped (Fig. 2B) but remained at detectable levels (>20 μM). Sulfate concentrations in MLS wells decreased when ethanol was injected and rebounded when the injection stopped (data not shown), indicating the presence of SRB activity in the subsurface. The uranium concentration in all MLS wells decreased after ethanol was delivered and slightly rebounded when ethanol was not injected (Fig. 2C). However, the uranium levels after the rebound were lower than those before ethanol injection. This rebound was likely due to uranium being carried in the recirculated water (~0.5 μm) and to

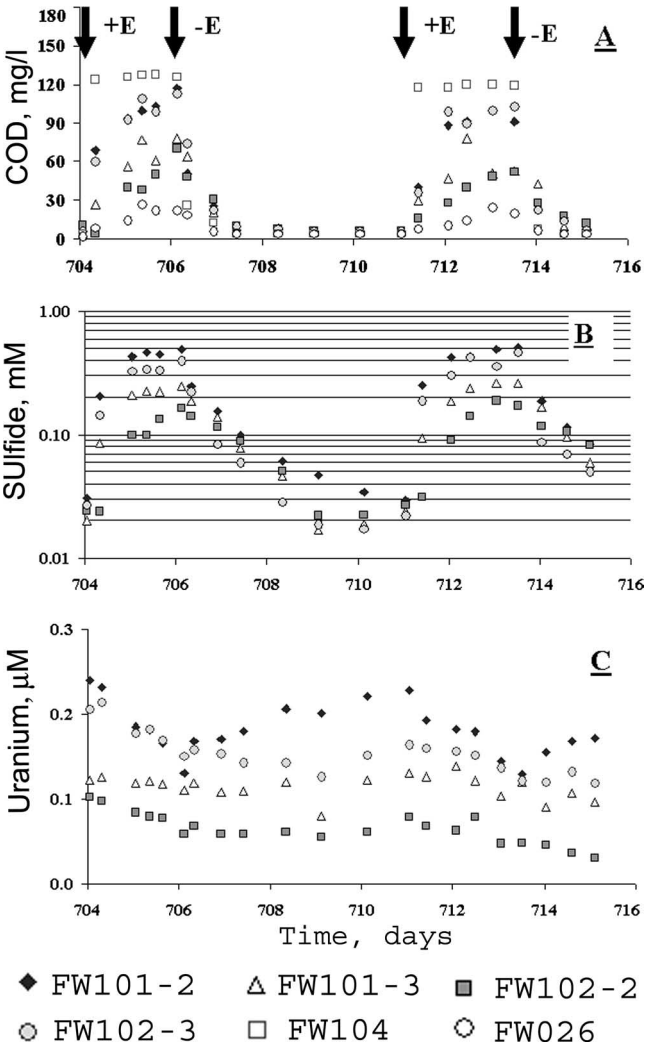

FIG. 2. Typical biostimulation of U(VI) reduction by injecting ethanol to the subsurface (days 704 to 714). (A) COD concentrations. (B) Sulfide concentrations. (C) Uranium concentration changes in MLS wells. The U concentration in injection well FW104 was 0.5 μM during this test period and is not shown due to scale. "+E" indicates the start and −E indicates the stopping of ethanol biostimulation.

the lack of an electron donor for U(VI) reduction when ethanol injection stopped. During the 10-day test shown in Fig. 2, the uranium concentration in injection well FW104 was around 0.5 μM (data not shown in Fig. 2C), which was much higher than those in the MLS wells. Uranium levels in FW101-3 and FW102-2 were near or below the EPA MCL (0.126 μM) throughout the 10 day-test period, while U levels in FW101-2 and FW102-3 dropped even below 0.126 μM after ethanol injection. The relatively higher U levels in the latter two wells are likely due to the lack of enough electron donor after ethanol injection stopped. Lower U concentrations in the MLS wells were achieved later, as shown in Table 1. Aqueous Fe(II) concentrations were 10 to 20 μM in the MLS wells before ethanol injection, dropped below 5 μM during ethanol injection as the sulfide concentration increased, and then slowly rebounded after ethanol injection stopped and the sulfide concentration decreased (data not shown). The decrease in the

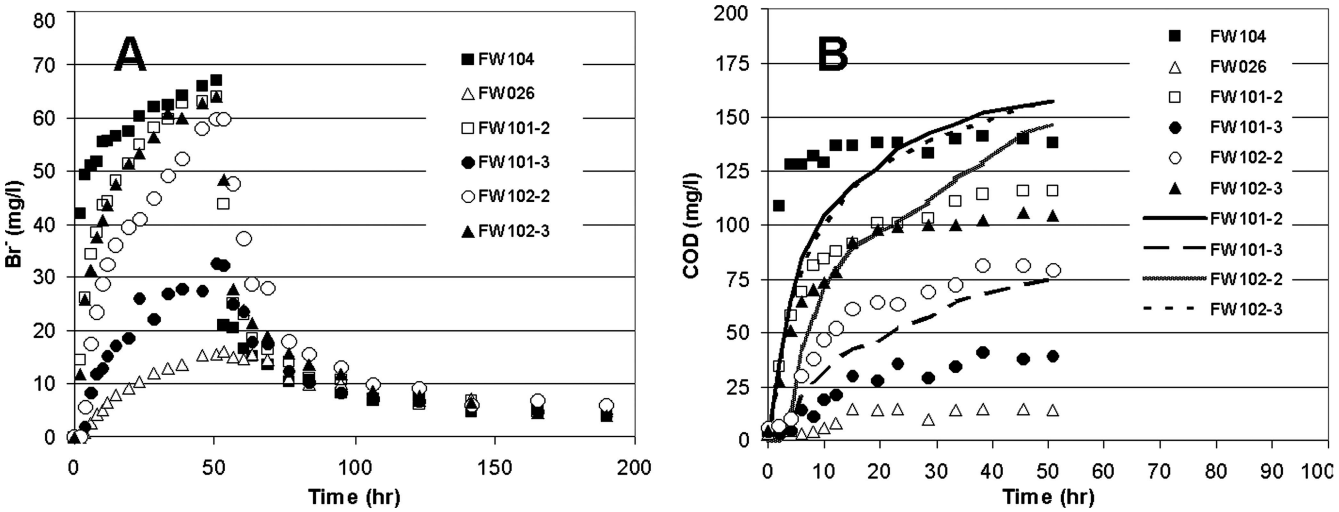

FIG. 3. A tracer test performed with Br^- on days 801 to 803 shows the hydraulic connection between injection well FW104, extraction well FW026, and MLS wells and biodegradation of ethanol. (A) Changes in bromide concentrations (21). (B) Comparison of measured COD (symbols) and calculated COD (lines) concentrations based on the COD-versus-Br^- ratio (2.46 g/g).

Fe(II) concentration during ethanol injection is likely due to formation of more FeS precipitates by reaction with the produced sulfide. The FeS precipitates likely accumulated in sediments.

The reduced-sediment samples showed a black color (FW104) or a dark green color (MLS). Reduced U(IV) was detected by XANES in the sediment samples from the injection well and the MLS (Table 1). In the FW026 well, the U(IV) content was below the detection limit of XANES (<10% of total U). The highest content of reduced uranium was found in injection well FW104 (48).

Groundwater flow pathway. The injection of the $NaBr^-$/ethanol solution to FW104 lasted for 50.75 h. Bromide concentration increased in the MLS and extraction (FW026) wells with different recovery ratio and mean travel time (Fig. 3). These results indicate that all MLS wells were hydraulically connected to FW104 (Fig. 3A). The Br^- recovery percentages were as follows: FW101-2, 93%; FW101-3, 60%; FW102-2, 94%; FW102-3, 93%; and FW026, 50% (Fig. 3A). FW101-2, FW102-2, and FW102-3 received more than 93% of water injected to FW104, while FW101-3 and FW026 received 50% and 60% of water from FW104, respectively. The rest of the water was from surrounding areas. Mean travel times from FW104 to the different wells were as follows: FW101-2, 2.84 h; FW101-3, 18.4 h; FW102-2, 11.6 h; FW102-3, 3.7 h; and FW026, 46 h (21). Therefore, groundwater injected into FW104 reached FW101-2 and FW102-3 more rapidly than the other two wells. The fraction of groundwater flow from the injection well to each MLS well and the mean travel time may significantly influence microbial communities in the MLS wells, as discussed below. During the tracer test, COD concentrations in the MLS and FW026 wells increased after ethanol injection (Fig. 3B). The measured COD concentrations in the MLS wells were significantly lower than those calculated based on the Br^- concentration and the COD/Br^- ratio in the NaBr-ethanol tracer solution used. The difference was likely due to biodegradation of ethanol and acetate in the subsurface. Ethanol was observed only in the FW104 well during the tracer test. The COD in MLS and FW026 was mainly from acetate (>80%), based on acetate versus COD measured. During the tracer test, sulfide concentrations increased in all MLS wells and U(VI) concentrations decreased in the same trend, as shown in Fig. 2 (also data not shown). Another separate tracer test, carried out by injecting bromide into outer-loop well FW024, indicated that the inner-loop extraction well FW026 received 17% of water injected into FW024 (21). The infiltration of water from the outer loop may also influence the microbial community.

Microbial enumeration. MPN results indicated that after biostimulation for nearly 2 years, increased levels of denitrifiers, SRB, and FeRB were present in the sediments in the bioreduced area in comparison with control well FW106 (Table 2). Well FW106 did not undergo stimulation and shares a similar geochemical composition with the active area wells (Table 1). After biostimulation, the microbial concentrations in the treatment area were as follows (in cells/g sediment): denitrifiers, 10^7 to 10^8; SRB, 10^6 to 10^8; and FeRB, 10^5 to 10^7. The highest levels for all three trophic groups were found in

TABLE 2. MPN for three major trophic groups in sediments of treatment zone (day 774) in comparison with groundwater from control well FW106[a]

Well	MPN estimate (cells/g sediment)		
	Denitrifiers	FeRB	SRB
FW104	7.23×10^8	9.40×10^7	1.53×10^8
FW101-2	1.54×10^8	2.05×10^7	2.06×10^8
FW102-2	2.39×10^7	5.48×10^5	1.87×10^7
FW026	1.10×10^7	1.93×10^6	1.07×10^6
FW106[b]	3.3[c]	ND	ND

[a] Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies. ND, none detected.
[b] Data on day 278 (47).
[c] No. of cells/ml.

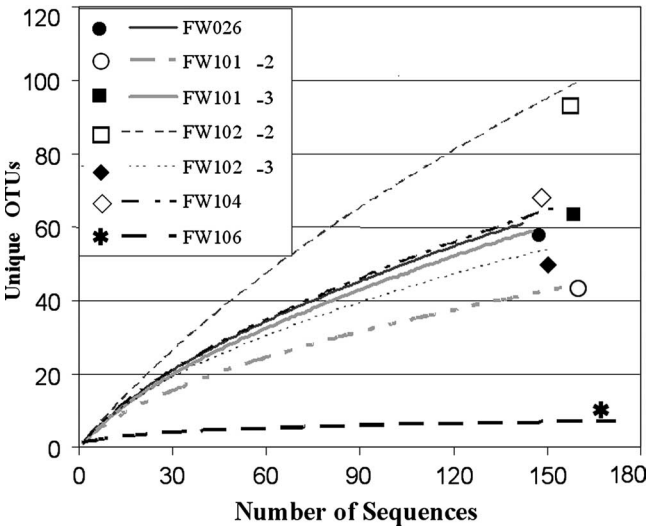

FIG. 4. Rarefaction curves of the 16S rRNA gene libraries constructed. OTUs were defined at 97% sequence identity. The library from the untreated control (FW106) was close to complete sampling (93% coverage).

inner loop injection well FW104, where ethanol was injected. Relatively low levels were found in extraction well FW026. Consistent with the tracer study, FW101-2 (more connected to injection well FW104 based on the tracer study) showed higher bacterial counts than FW102-2 (less connected) (Table 2). MPN counts from FW106 (groundwater) showed levels of denitrifier as low as 3.3 cells/ml groundwater (47). Neither SRB nor FeRB were found in FW106. Comparison with sediment MPN at day 453 showed a decrease in SRB and denitrifiers and no change for FeRB in FW104. The MPN count at day 774 for all three groups remained around 10^8 cell/g sediment in FW104 (47). Integration of MPN at day 774 and three earlier time points (days 278, 354, and 453) for FW101-2 showed an increase in the counts over time, with microbial levels at day 774 around 10^7 cell/g sediment, one order lower than levels for FW104. Conclusions from the MPN analysis must be qualified, because we contrast sediments with groundwater samples. However, prior MPN studies using samples from the same well show a one-log difference in counts in sediment versus groundwater samples (data not shown).

Sequence analyses. After discarding putative chimeric sequences, an average of 155 sequences per sample were used for each library. Rarefaction analysis at 97%-similarity levels (Fig. 4) showed that the estimated coverage (rarified number of OTUs divided by rarified Chao1 estimator) ranged from 36 to 58% in the stimulated area and was 93% in the background area (FW106). Even with low coverage in the biostimulated zone, clear differences in diversity were observed. The highest diversity was found in all the wells of the biostimulated area; working at 97% similarity for OTUs, the number of OTUs ranged from 41.2 to 91.4 (rarified values). Only 6.6 OTUs were found in the background area, FW106 (rarified value).

Microbial community structure and major groups detected. All libraries were dominated by members of the phylum *Proteobacteria*, with *Deltaproteobacteria* and *Betaproteobacteria* being the most dominant proteobacterial classes in the biostimulated zone (Fig. 5). In contrast, the background area was

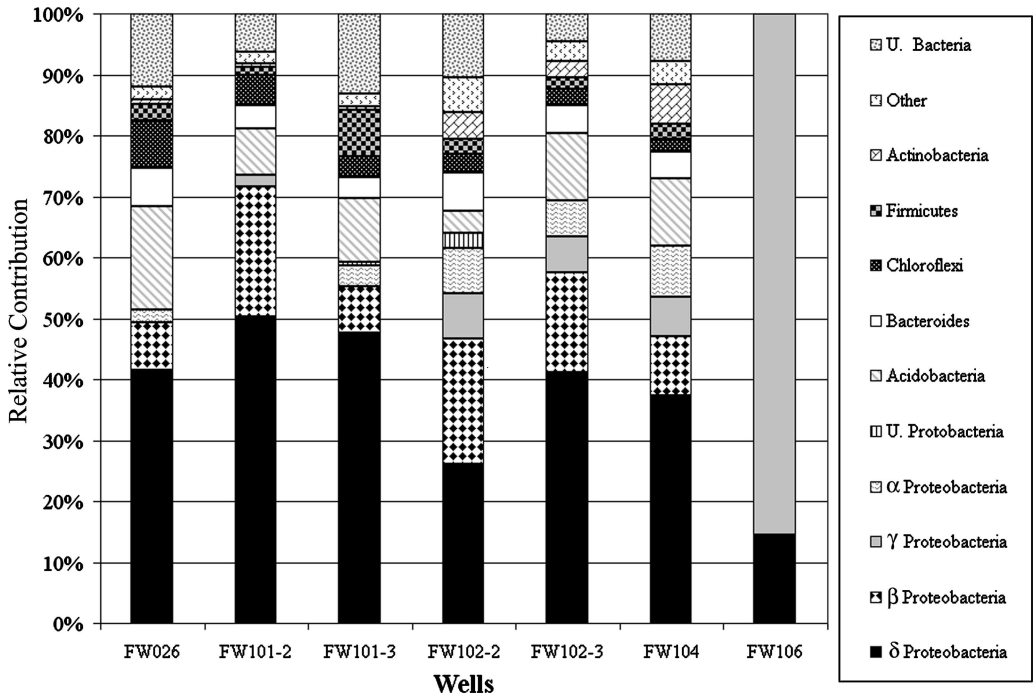

FIG. 5. Microbial composition of the clone libraries based on the RDP Classifier. The "other" group category includes the phyla *Spirochaetes*, *Gemmatimonadetes*, *Verrucomicrobia*, *Chlamydia*, *Planctomycetes*, *Nitrospira*, *Cyanobacteria*, and the proposed phyla OP11, OP10, BRC1, and TM7. Bacteria that could not be assigned with the 80% confidence bootstrap value were included in an artificial "Unclassified Bacteria" (U. Bacteria) taxon. Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies.

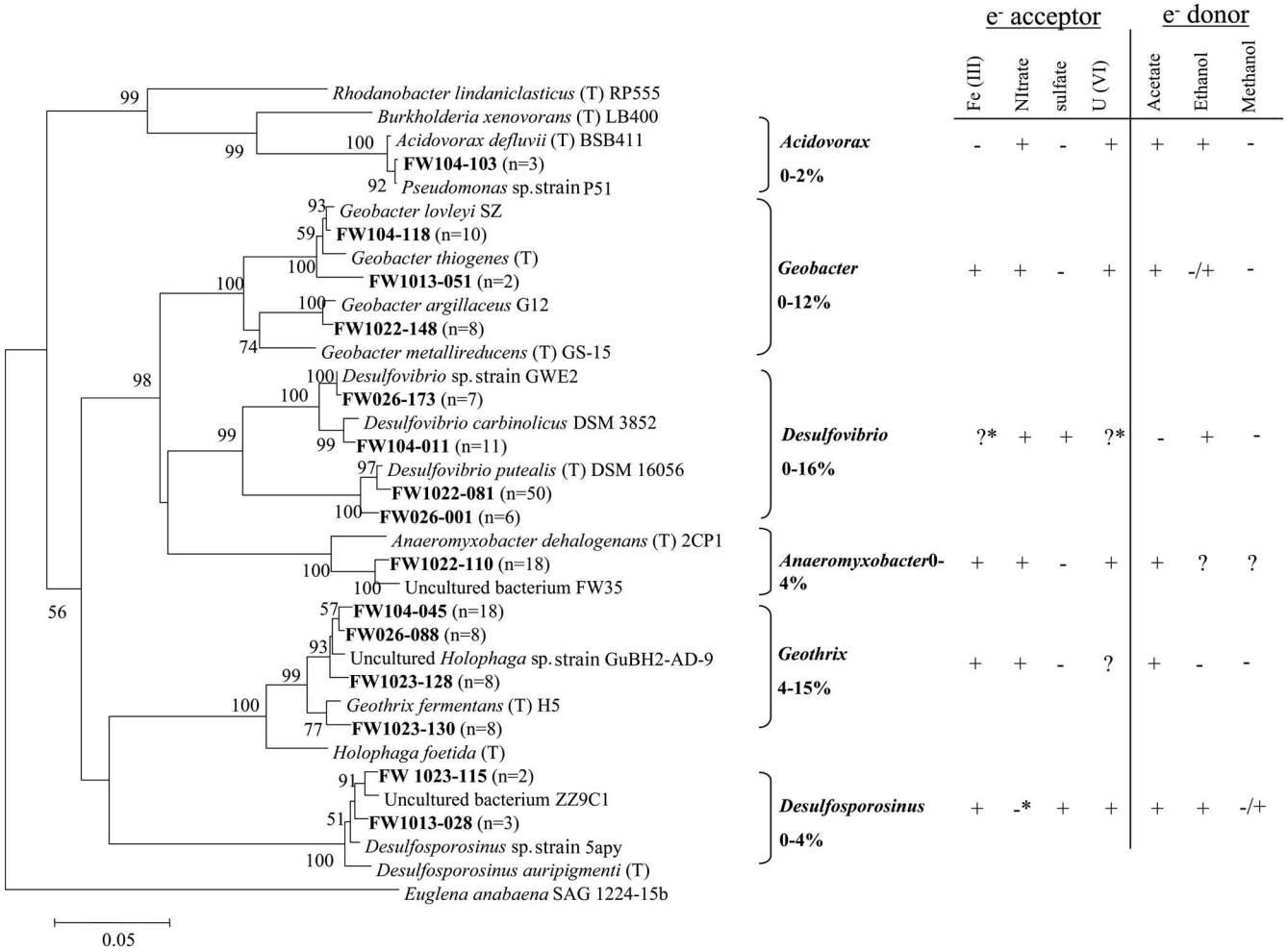

FIG. 6. Neighbor-joining tree showing the relationship of selected representatives (shown in bold) from the groups similar to known U-reducing bacteria. Metabolic abilities of the clones' closest cultivated relatives are indicated. Nonmodel positions from the 16S rRNA were masked and Jukes-Cantor distance correction used. Type strains have a "(T)" label. Bootstrap values (10,000 repetitions) are displayed if larger than 50%. +, present in closest relatives; −, absent in closest relatives; ∗, activity found some species of the genus; ?, unknown. The range of relative contribution to the different samples is also shown.

dominated by *Gammaproteobacteria*. Bacteria belonging to groups known to include U(VI), nitrate, sulfate, and Fe(III) reducers were detected in the active area but not in the background area library.

Sequences from the genera *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Acidovorax*, and *Desulfosporosinus* were detected in the libraries. These genera are known to include U(VI) reducers and additionally can contribute with one or more of the following activities: iron(III), sulfate, and nitrate reduction (Fig. 6). The contribution of the putative species responsible for the uranium reduction and their richness (measured as the number of OTUs in a genus) were highly variable (Table 3). *Geobacter* and *Desulfovibrio* were detected in all the libraries of the active area. In five of the six samples from the active area, the relative contribution of *Desulfovibrio* was higher than that from *Geobacter* (Table 3). *Anaeromyxobacter*, *Acidovorax*, and *Desulfosporosinus* sequences were not present in all the libraries.

Nitrate reducers from different taxonomic lineages were present in the libraries of the active zone but not in the untreated area (FW106). Most of the nitrate reducers were members of the Proteobacteria phylum, e.g., *Ferribacterium* (2 to 27%), *Thiobacillus* (0 to 29%), *Sphingomonas* (0 to 6%), *Desulfovibrio* (4 to 16%), *Azoarcus* (0 to 5%), *Acidovorax* (0 to 4%), and *Pseudomonas* (0 to 1%), among others. The denitrifying acidobacterium *Geothrix* was also detected in all the libraries of the active area (4 to 17%). Nitrate respiration is not exclusive to these groups, and the mentioned bacteria are also know for using other electron acceptors.

Iron(III) reducers were represented by bacteria from three groups: *Deltaproteobacteria* (*Anaeromyxobacter* and *Geobacter*), *Betaproteobacteria* (*Ferribacterium* and *Thiobacillus*), and the acidobacterium *Geothrix*. *Ferribacterium* was the most abundant FeRB, contributing an average of 17% to the libraries.

Other commonly found soil bacteria, like members of the *Acidobacteria*, *Actinobacteria*, *Planctomycea*, and *Verrucomicrobiae*, were also present in the samples. These bacteria were present in the active area and not in the untreated area.

TABLE 3. Relative contributions of known uranium reducer genera in clone libraries from sediment samples in Area 3 wells[a]

Genus	No. of OTUs	Closest isolated relative(s)	Relative contribution per well (%)					
			FW104	FW101-2	FW102-3	FW102-2	FW101-3	FW026
Desulfovibrio	7	*D. putealis* *D. carbinolicus*	6	16	12	4	5	6
Geobacter	7	*G. argillaceus* *G. lovleyi* *G. thiogenes* *G. humireducens* *G. psycrophilus*	3	1	1	12	1	1
Anaeromyxobacter	2	*A. dehalogenans*	1	4	3	3	2	0
Geothrix	9	*G. fermentans*	11	8	10	4	10	15
Desulfosporosinus	7	*D. auripigmenti*, *D. orientis*	1	0	0	2	4	0
Acidovorax	3	*Pseudomonas* strain P51	2	1	0	0	0	1

[a] The number of unique OTUs (97% similarity) and closest isolate (Seqmatch at RDP) are reported.

Diversity analyses. All libraries from the stimulated zone showed greater diversity than those from the untreated zone based on both evenness and richness values, as indicated by the diversity indices used (Table 4). One single OTU dominated the untreated area sample (FW106). This member of the *Xanthomonadaceae* family was also detected in a metagenome experiment for well FW106, where it was shown to carry a variety of metal resistance genes (C. L. Hemme, T. J. Gentry, L. Wu, M. W. Fields, C. Detter, K. Barry, D. Watson, C. W. Schadt, P. Richardson, T. Hazen, J. Tiedje, E. Rubin, and J. Zhou, presented at the 106th General Meeting of the American Society for Microbiology, Orlando, FL, 21 to 25 May 2006).

Diversity-based clustering revealed that the FW106 community formed a cluster separated from the treatment-zone wells (Fig. 7). The two different clustering methods used resulted in congruent topologies. High similarity was observed between communities from FW102-3 and FW104, which were 53% similar according to a calculated Bray-Curtis index. An $\int$-LIBSHUFF comparison indicated that these two libraries were not significantly different ($P = 0.05$). All the other pairwise comparisons were nonsignificant (libraries were significantly different; $P = 0.05$). Additional comparison of FW104 and FW102-3 communities with the LIBCOMPARE function of the RDP showed nonsignificant differences ($P = 0.01$) at all the different levels of taxonomy from phylum to genus. No clear relation was observed between the alpha diversity indices and the hydrology.

TABLE 4. Diversity indices in treated and untreated (FW106) areas

Well	Index[a]		
	Chao1 (LCI, HCI)	Shannon (H′) (LCI, HCI)	Simpson (1-D)
FW104	125.5 (91.7, 200.6)	3.7 (3.5, 3.9)	0.97
FW101-2	76.5 (54.4, 137.9)	2.9 (2.7, 3.2)	0.90
FW102-3	87.8 (64.6, 147.7)	3.4 (3.2, 3.6)	0.95
FW102-2	250 (168.8, 415.1)	4.3 (4.2, 4.4)	0.99
FW101-3	114.3 (81.1, 192.9)	3.5 (3.3, 3.7)	0.95
FW026	124.9 (88.3, 210.4)	3.7 (3.5, 3.8)	0.97
FW106	6.0 (6.0, 6.0)	0.7 (0.5, 0.9)	0.31

[a] LCI and HCI are rarefied 95% lower and higher confidence intervals (provided by the DOTUR application). Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies.

DISCUSSION

Remediation of uranium and metals in the subsurface. Microbial reduction of metals including uranium has been the proposed alternative to control the mobility of the contaminants in the groundwater (43). In this field experiment, ethanol injection successfully created a reducing environment capable of achieving uranium remediation and immobilization to levels below the EPA drinking water MCL (47). The biostimulation process changed the structure of the microbial communities from small and low-diversity communities to more abundant and diverse communities, with bacteria capable of reducing the contaminants nitrate, sulfate, and U(VI) in situ.

Microbial communities. (i) Major trophic groups detected. Microbial enumeration together with clone libraries revealed that after biostimulation, viable FeRB, SRB, and denitrifiers

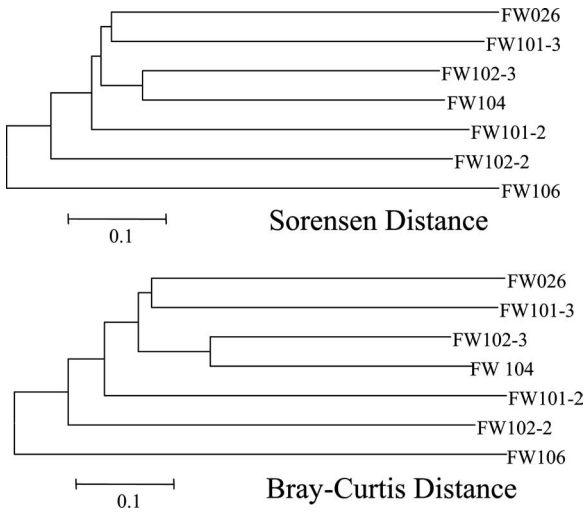

FIG. 7. Clustering of samples according to diversity patterns. The topology was similar when using the Sørensen index (presence/absence) or the Bray-Curtis index (presence/absence and abundance). The indices were normalized to account for differences in the total number of sequences per library.

had grown in the treated area where uranium, nitrate, sulfate, ferric compounds, and perhaps other compounds served as electron acceptors. At least three major microbial trophic groups appear to be involved in bioremediation of the area. The first group, denitrifiers, can remove nitrate and provide a favorable low-redox environment for U(VI) reducers, FeRB and SRB. The latter two groups may contribute to the remediation process by having members capable of reducing not only Fe(III) and sulfate but also U(VI). Microbial enumeration indicated that these three trophic groups were present in the sediments at high levels after biostimulation. In contrast, the untreated-area sample (FW106) showed the presence of low numbers of denitrifiers (3.3 cells/ml) in groundwater but neither SRB nor FeRB. Previous reports on the groundwater MPN for this stimulated area (same wells, earlier time points) also show these three groups, but the microbial counts were lower than that at our time point (47). The cell count in the untreated sediments is unknown, but it could be significantly low since DNA extraction was unsuccessful. Clone libraries permitted detailed speciation of these trophic groups.

(ii) Carbon source as selective agent and the community of ethanol degraders. The use of ethanol as a carbon source likely increased the diversity of taxa and physiologies. Injected ethanol provided both the carbon source and the electron donor in the forms of ethanol, acetate (metabolic intermediate), and methanol (an impurity in industrial ethanol). Electron donor consumption and metabolism were likely performed by denitrifiers, FeRB and SRB (Fig. 6). Based on groundwater analysis during the tracer study, we hypothesize that the SRB were mainly responsible for ethanol consumption and acetate release between the injection well and the MLS wells (where no nitrate was detected and sediments showed a black or dark-green color), while FeRB and denitrifiers utilized mainly acetate between MLS wells and the extraction well. At this well, there was evidence of groundwater infiltration from the outer loop and the sediments were green-yellow in color. Close relatives of the SRB detected (*Desulfovibrio* spp. and *Desulfosporosinus* spp.) utilize ethanol and not acetate when doing sulfate reduction, though a few strains of both groups can also use acetate (17, 31). The release of metabolic acetate can be used later by other species, including *Geobacter* spp.. This hypothesis is supported by sulfide accumulation during ethanol injection (SRB activity) (Fig. 2) and the detection of mostly acetate (and not ethanol) as a carbon source in less-connected wells. The sequential utilization of ethanol and release of acetate likely create a niche for acetate-consuming FeRB and acetate-consuming denitrifiers. The first group was represented in our libraries by *Geobacter* spp., *Geothrix* spp., and *Anaeromyxobacter* spp. These bacteria are capable of acetate and ethanol degradation, mainly by Fe(III) reduction, as well as by denitrification (2, 6, 32). The second group is represented by denitrifiers, such as *Acidovorax* spp. and *Thauera* spp. These *Betaproteobacteria* members utilize acetate and ethanol as electron donors and have been previously found in the aboveground denitrification reactor used to pretreat groundwater at this site (14).

Methanol utilization may play a minor role because of its small contribution (5% in the ethanol solution used); nevertheless it can be used for denitrification by almost all denitrifiers and also for sulfate reduction by some *Desulfosporosinus* spp. (31) and *Desulfovibrio carbinolicus* (23).

(iii) Other C sources and contaminants. In addition to the C sources used to stimulate the microbial activities, other C sources, such as aromatic and chlorinated compounds, humic acids, and cellular components, are present in the contaminated area.

Aromatic (phenols) and chlorinated compounds (trichloroethene) were detected in the groundwater prior to biostimulation but mainly removed during site preconditioning (46). These compounds are still present in the groundwater outside the treatment area and could have infiltrated to the inner loop (based on tracer test results) and provided additional carbon sources, electron donors, and acceptors to the underground communities. Several detected groups of different phylogenetic affiliations show some potential for dechlorination or aromatic-compound degradation. In the case of dechlorination, at least 50% of the *Geobacteraceae* sequences were related to *Geobacter lovleyi*, an isolate known to use chlorinated compounds such as trichloroethene and tetrachloroethene as electron acceptors (37). Close relatives of the chlorophenol respirer *Anaeromyxobacter dehalogenans* 2CP-1 (32) and the tetrachloroethene respirer *Desulfosporosinus meridiei* were also detected (31). Aromatic compounds can also be used as carbon sources by some bacteria of the *Acidovorax* genus which was detected in the sediments. The betaproteobacterium *Pseudomonas* sp. strain P51 can degrade chlorinated benzenes (41). Aromatic compounds and solvents present at the beginning of the bioremediation operation could have influenced the initial communities before the biostimulation began, though their concentration is generally low and it is not clear if they can support growth at levels present at the site.

Humic substances can be used as electron acceptors (19). Sequences related to the humic-acid reducers *Geothrix* sp.(6) and *Geobacter humireducens* (5a) were detected in the libraries. Reduced humic substances can potentially abiotically reduce U(VI) far from the bacteria, and they have been shown to be effective in increasing Fe(III) reduction in subsurface environments (25).

In addition, other bacterial groups not directly related to bioremediation, as far as we know, were found in the sediments, e.g., *Planctomycea*, *Chloroflexi*, *Actinobacteria*, etc. They are likely involved in the degradation or digestion of dead cells, soil humics, and extracellular substances produced during biostimulation in the subsurface. *Chloroflexi* members are known to grow in filaments and produce hydrogen and are proposed to be involved in dead-cell recycling (50). This group can potentially contribute to the stability of the bacterial community by promoting the formation of biofilms and can facilitate bioremediation by transferring electrons in the form of hydrogen to other groups more likely to be involved in bioremediation, such as *Desulfovibrio* spp. The *Chloroflexi* group was the only group not belonging to the FeRB, SRB, or denitrifiers present in all the libraries from the active zone.

(iv) Putative genera involved in U(VI) reduction. Analyses of the uranium oxidation state in sediments by XANES confirmed that the decrease in the groundwater U concentration was due to reduction of U(VI) to U(IV). Elevated total U and U(IV) percentages of total U (16, 47, 48) were found in inner loop injection well FW104 consistently with effective bioremediation (Table 1). In this study, we found a variety of previously

reported U(VI)-reducing bacteria present in the reduced sediments, including FeRB *Geobacter* spp. and *Anaeromyxobacter* spp., SRB *Desulfovibrio* spp. and *Desulfosporosinus* spp., and the denitrifier *Acidovorax* spp. Our results suggest that uranium reduction cannot be attributed to a single group, and it is very likely that this role is taken by several different bacteria.

The contribution to uranium reduction, based solely on the relative frequency in our libraries and the reported literature, ranks the contributor groups (in order of decreasing contribution) as follows: *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus*, and *Acidovorax*.

Desulfovibrio spp. were detected in all the samples of the active area, and their contribution peaked in wells with high percentages of reduced uranium. This group was the most abundant group with reported uranium-reducing abilities found in the sediment samples. Even though there are not reports of U(VI) reduction by the closest relatives of the cloned sequence, the high frequencies of these bacteria in the sediment clones (up to 16%) suggest a substantial role in the groundwater ecosystem.

Geobacter spp. were found in all sediment samples from the active area. Our sequences were closely related to the uranium reducer *G. lovleyi* (37) and to the humic-acid reducer *G. humireducens* (19). Additionally, *Geobacter* spp. was found to be associated with U(VI) reduction at sites contaminated with uranium and U ore where acetate was added (2, 26, 42).

Anaeromyxobacter sequences were detected in all sediment samples from the active area except for samples from FW026. These sequences were related to the known U(VI) reducer *A. dehalogenans* (45) and to a clone from a uranium mine sediment where uranium reduction was demonstrated (38).

Desulfosporosinus sequences were present in half the libraries of the active area, and the closest isolate to most of the sequences was *Desulfosporosinus orientis*, an SRB also known for reducing Fe(III), nitrate, and U(VI) (24, 38). Despite their relative small contribution to the total community, *Desulfosporosinus* spp. may play a minor role in U(VI) reduction and a bigger role in the long-term stability of the reduced uranium since they can form spores and survive under starvation conditions.

Acidovorax sequences were found in half the libraries of the active area. An *Acidovorax* sp. was shown to be capable of U(VI) reduction in microcosm tests with sediments from the FRC (27). Nitrate and nitrite have been shown to be able to reoxidize and remobilize Fe(III) (35), and the presence of this denitrifier could contribute to the removal of these competing electron acceptors and ensure the stability of the reduced uranium.

Geothrix sequences were found in all the libraries of the active zone. This iron(III) reducer can use humic acids as an electron acceptor and has been found during uranium reduction events in FRC Area 2 using 16S rRNA gene microarrays and enrichment studies (5). The *Geothrix* genus has not been characterized for U(VI) reduction, but based on the number of clones retrieved, it was an important member of the community. It is possible that it contributed to U(VI) reduction indirectly via reduced humic acids or reduced iron(II) compounds.

Abiotic U(VI) reduction may also play a role under our operational conditions (i.e., pH of 5.8 to 6.6 and an HCO_3^- content of <5 mM). Sulfide, the end product of SRB, and green rusts, products of FeRB, can both reduce U(VI) to U(IV) (13, 28). Indirect U(VI) reduction by reduced humic substances can also contribute an important piece given the constant presence of FeRB humic-reducing *Geothrix* spp. in all the tested sediments from the active area. Therefore, the activity of SRB and FeRB could also indirectly contribute to the reduction of U(VI) and maintenance of a stable and low level of uranium.

Dynamics of the community. (i) Patterns of diversity detected. Microbial diversity varied in the sediments of the treated zone (Table 4). The utilization of standardized techniques in all of our community analyses allows us to conclude that the observed differences were due to real differences in the community and not the consequence of PCR bias. The irregular pattern observed is likely due to the heterogeneity of groundwater flow and the distribution of contaminants, as shown by the tracer studies. Microbial enumeration analyses for the three trophic groups studied (denitrifiers, FeRB, and SRB) showed a decline in cell counts going from the injection well and its more-connected wells to the less-connected wells (Table 2). Even though replication did not allow statistical conclusions, some apparent trends were observed.

The different approaches used to study the diversity and to compare the communities yielded consistent results, and the clustering analyses showed a topology that was in agreement with the tracer studies depicting the groundwater flow. Because the flow of the water is not homogenous, gradients of electron donors are expected since the microbial communities consume and convert the injected ethanol solution. The hydrology clearly affected the microbial counts (MPN), but no clear relationship between the water flow and the alpha diversity indices was found. Though this may be due to incomplete sampling, it is more likely that the ethanol injection created a selective pressure for specific functions (like iron reduction, ethanol utilization, etc.) but not for specific bacteria. Bigger gradients and more-divergent communities would be expected in natural systems where the water flow is not controlled. Having more-divergent communities in an area of remediation adds additional layers of complexity that can make the monitoring of the performance more difficult. Thus, control of the hydrology is key to having a more homogenous response to the bioestimulation.

When the carbon and electron acceptors were analyzed, more ethanol-consuming organisms were detected in the more-connected wells. Consistent with the expected sequential electron acceptor utilization (47), more denitrifiers were present in highly connected wells and FeRB were more abundant in the wells with lower connectivity. The exception to this pattern was *Desulfovibrio*, an SRB that showed high relative abundance in more-connected wells, possibly due to its ability to utilize the injected ethanol. *Desulfovibrio* spp. were more abundant than spp. of *Desulfosporosinus*, also an SRB capable of using ethanol. The presence of *Desulfovibrio* in the aboveground bioreactor (14) could have given *Desulfovibrio* spp. an initial competitive advantage over *Desulfosporosinus* spp. by a continuous inoculation of *Desulfovibrio* spp. carried in the treated water from the bioreactor. On the other hand, the ability of *Desulfosporosinus* to sporulate and degrade methanol may account for its survival. Overall the microbial methods

were in agreement with the hydrological studies. However, more study is needed to understand the relation quantitatively.

(ii) Emergence of previously undetected populations. After preconditioning by pH adjustment and removal of inhibitors, the ethanol injection increased the microbial diversity of the subsurface, as shown by diversity indices that consider richness and abundance (Table 4). The activity of the new complex bacterial community created favorable conditions where FeRB, SRB, and denitrifiers thrive, in contrast with the untreated area, where they were either absent or at very low levels.

It is very unlikely that the microbial community observed was fully derived from indigenous species that survived the extreme conditions of the area. The most likely source of new colonists was the upper soil, where nitrate is low and pH neutral. These bacteria could have been transported by either natural groundwater infiltration or forced recirculation during the treatment. *Geobacter* and *Anaeromyxobacter* spp. have been found in FRC Areas 1 and 2 using iron reducer enrichments with acetate and lactate (29). *Geobacter* spp. and *Geothrix* spp. also have been detected in FRC Area 2, using high-density 16S rRNA gene arrays (5). *Pseudomonas* spp. and *Azoarcus* spp. have been detected in FRC Area 3 with 16S rRNA clone libraries prior to biostimulation (8). Therefore, these microorganisms are present in pH-neutral soils at the site.

A second likely microbial source could be the aboveground bioreactor system. This reactor worked for 400 days to remove nitrate from the groundwater, and the treated nitrate-free water was reinjected into the subsurface (46, 47). The reinjected water likely carried some bacteria to the treated area, although it was filtered. Sequences related to *Desulfovibrio*, *Thauera*, *Azoarcus*, *Ferribacterium*, and *Acidovorax* have been previously reported for the bioreactor (14) and were detected in the collected sediments of the active area in this study.

Our results suggest that biostimulation efforts successfully fostered communities comprised of a variety of bacterial groups (*Geobacter*, *Desulfovibrio*, *Geothrix*, *Anaromyxobacter*, *Desulfosporosinus*, and *Acidovorax*) involved in the groundwater remediation process. This study provides a detailed view of the differences and similarities among the microbial communities throughout the active area that correlated with the path of groundwater flow depicted by tracer studies. The results demonstrated that microbial communities can be established by in situ biostimulation with an electron donor to achieve successful reduction of the U(VI) concentration below the EPA MCL. These findings contribute to an improved understanding of the composition, variability, and controls on microbial communities in the subsurface associated with a successful bioremediation process and provide a foundation for future implementation and monitoring efforts applied to this and other contaminated sites.

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