Title: **Bacterial Community Succession During in situ Uranium Bioremediation: Spatial Similarities Along Controlled Flow Paths**

Running Title: Bacterial Succession During Uranium Bioreduction

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Abstract

Bacterial community succession was investigated in a field-scale subsurface reactor formed by a series of wells that received weekly ethanol additions to re-circulating groundwater. Ethanol additions stimulated denitrification, metal reduction, sulfate reduction, and U(VI) reduction to sparingly soluble U(IV). Clone libraries of SSU rRNA gene sequences from groundwater samples enabled tracking of spatial and temporal changes over a 1.5 y period. Analyses showed that the communities changed in a manner consistent with geochemical variations that occurred along temporal and spatial scales. Canonical correspondence analysis revealed that the levels of nitrate, uranium, sulfide, sulfate, and ethanol strongly correlated with particular bacterial populations. As sulfate and U(VI) levels declined, sequences representative of sulfate-reducers and metal-reducers were detected at high levels. Ultimately, sequences associated with sulfate-reducing populations predominated, and sulfate levels declined as U(VI) remained at low levels. When engineering controls were compared to the population variation via canonical ordination, changes could be related to dissolved oxygen control and ethanol addition. The data also indicated that the indigenous populations responded differently to stimulation for bio-reduction; however, the two bio-stimulated communities became more similar after different transitions in an idiosyncratic manner. The strong associations between particular environmental variables and certain populations provide insight into the establishment of practical and successful remediation strategies in radionuclide-contaminated environments with respect to engineering controls and microbial ecology.

Introduction

Uranium contamination from extraction processes for nuclear weapons production remains a

significant environmental problem, and the use of depleted uranium and other heavy metals in non-nuclear weapons is an additional environmental hazard. Depleted uranium is weakly radioactive and can damage mammalian kidneys due to heavy metal toxicity (Craft *et al*., 2004), and the oxidized form of uranium [i.e., U(VI)] is soluble, and thus mobile in groundwater. Particular microorganisms can use metals and metalloids such as U(VI) as an electron acceptor (Lloyd, 2003), and the formation of U(IV) via reduction forms a less soluble precipitate that is much less likely to contaminate water supplies. Populations within the community respond differently to disturbances and processes, and it is the cumulative effect of multiple populations directly or indirectly connected that facilitates overall activity. Therefore, work is needed to understand the relationships between biotic and abiotic parameters in the context of bioremediation.

The Field Research Center (FRC; http://www.esd.ornl.gov/orifrc/) on the Oak Ridge Reservation in eastern Tennessee was established by the U.S. Department of Energy (DOE) to evaluate *in situ* strategies for the long-term treatment of radionuclide wastes. Mixed wastes are difficult and expensive to remediate effectively with current physical and chemical technologies, but bioremediation with indigenous microorganisms holds promise as a cost effective and comparatively unobtrusive technology for *in situ* remediation (Iwamoto and Nasu, 2001). Many aspects of bioremediation must be better understood for successful and efficient applications including: the complex relationships between the microorganisms involved in contaminant removal, changes in pollutant concentration, and geochemical and hydrological conditions.

The study site had an initial pH of approximately 3.5 and was contaminated with nitrate, toxic metals, and organic contaminants that resulted from waste disposal over a30 year period (Fig. 1). During the course of the above study, we characterized changes in bacterial

community structure over time and space at the injection well (intermittent ethanol additions); at the extraction well used to capture and recycle water; at wells within the treatment zone (between the injection and extraction well); and at up- and down-gradient wells. The results demonstrate how system hydraulics and engineering control measures can impact structure and function and alter community dynamics within U(VI)-reducing communities.

Materials and Methods

Site description and biostimulation tests

The well and pump system consisted of an outer groundwater recirculation loop with a nested inner recirculation loop (Fig. 1). The outer loop, with recirculation between wells FW103 and FW024, created a hydraulic barrier that reduced the amount of contaminated groundwater from entering the nested inner loop (Fig. 1). Within the inner loop, the carbon/electron donor (ethanol) was injected weekly into well FW104 and extracted at well FW026, and FW101 and FW102 (13.7 m depth) were the stimulated zone. Ethanol was selected based upon previous batch microcosm experiments with field samples (Wu *et al*., 2006). The recirculation flow rates in the inner loop and outer loop (from FW103 to FW024) were 0.45 l/min. Additional clean water was injected at well FW024 at 0.7 to 0.9 l/min to minimize entry of ambient groundwater, increase the pH, and remove nitrate. Detailed descriptions are described elsewhere (Luo *et al.,* 2006; Hwang et al., 2006; Wu *et al.,* 2006a, 2007, http://www.esd.ornl.gov/orifrc/). Stimulation of bacterial growth with ethanol began on day 137 (January 7, 2004) and ended on day 754 (September 15, 2005) (Wu *et al.,* 2006b; 2007). The ethanol solution (industrial grade,88.1% ethanol, 4.7% methanol, and 7.2% water w/w) had a chemical oxygen demand (COD) to weight ratio of 2.1. The solution was prepared at a COD of 6.9 to 9.8 g/l in a storage tank and injected weekly at FW104 over a 48-hour period to give 120 to 150 mg/l of COD at FW104 except during a starvation experiment from days 713 to day

754. The groundwater temperature fluctuated between 12°C and 21°C due to seasonal variation over the course of the study (data not shown). For later time points, dissolved oxygen (DO) was maintained at low levels (<0.03 mg/l) as previously described (Wu *et al*., 2007).

Analytical methods

Groundwater samples were collected from the injection, extraction and monitoring wells to track biogeochemical changes. Anions (nitrate, sulfate, bromide, etc.) were analyzed with an ion chromatograph (Dionex DX-120) as previously described (Wu *et al.,* 2006b). Chemical oxygen demand (COD) was used to monitor the consumption of electron donor (ethanol) and metabolic by-products (e.g., acetate). COD, sulfide and Fe^{2+} were determined using a Hatch DR2000 spectrophotometer (Hatch Chemical). U(VI) concentration was determined by kinetic phosphorescence analysis using a KPA-11 analyzer (ChemCheck Instruments).

Sample collection, DNA extraction, and DNA purification

Groundwater was collected (approximately 2 to 5 liters), filtered, and the filters stored at –80°C until DNA could be extracted. Biomass collected on the filters (Nucleopore, polycarbonate, 0.2 μm) was washed, vortexed, and centrifuged with 1X PBS buffer prior to DNA extraction. Samples were suspended in 1X PBS buffer and the cells were disrupted through two cycles of freeze-thaw and grinding in sterile sand as described previously (Zhou *et al.,* 1996). DNA was extracted and purified from all samples with a PowerSoil™ DNA Isolation Sample Kit (MO BIO). Based upon total cell counts and an assumed DNA amount per cell (~5 fg), recovery efficiency was estimated to be 30-40% for all samples.

SSU rRNA gene PCR amplification, cloning, and sequence determination

SSU rRNA gene sequences were amplified by PCR with universal bacterial primers FD1 (5’ AGA GTT TGA TCC TGG CTC AG 3’) and 1540R (5’ AAG GAG GTG ATC CAG CC 3’) as previously described (Fields *et al.,* 2005; 2006). Each 20 μl PCR reaction contained

approximately 5-10 ng/ul of DNA, 200 nM of each primer, 10 μl of Bulls Eye Taq DNA polymerase 2.0 mix (Midwest Scientific), and the adjusted volume of sterilized water. The lowest number of PCR cycles was determined for each sample to minimize PCR-induced artifacts and five PCR reactions were combined prior to cloning. PCR conditions were as follows: denaturation at 94ºC for 2 min, the number of optimal cycles of 94ºC for 30 s, annealing at 58ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. Negative PCR controls without DNA template were run concurrently for each sample. PCR products (5 μl) mixed with DNA loading buffer (2 μl) were visualized by agarose gel electrophoresis in a 0.8% agarose TAE gel stained with ethidium bromide. Clone libraries were constructed via methodology to minimize possible biases introduced by PCR as previously described (Fields *et al.*, 2005; 2006) and the predominant population in this study was verified via qPCR.

The PCR products were purified via a spin column purification kit (Promega). Clone libraries were constructed with the TOPO TA pCR®2.1 cloning kit (Invitrogen) according to the manufacturer's instructions. Transformants from each clone library were checked for inserts by PCR with M13 primers (30 cycles of the PCR conditions described above except an annealing temperature of 60ºC and a final extension time of 10 min). The amplification products were analyzed by agarose gel electrophoresis.

PCR products from each clone were purified with a Montage PCR_{u96} plate according to manufacturer's instructions (Millipore). DNA sequences were determined with a Big-DYE Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions at 1:4 dilution with an internal sequencing primer (529r) as previously described (Fields *et al.,* 2005; 2006). Sequence reaction products were run on an ABI 3730 DNA sequencer.

DNA sequence analysis

DNA sequences were analyzed with Sequencher™ (v.4.0, Gene Codes Corporation) and vector sequences were removed. The sequences were analyzed for chimeras with Chimera-Check (Cole *et al.,* 2003) and Bellerophon (Huber *et al.,* 2004) and, the chimeric sequences were removed from further analyses. SSU rRNA gene sequence identification was done via comparison to known sequences in GenBank with the BLASTN server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the RDP (http://rdp.cme.msu.edu/index.jsp). Unique SSU rRNA gene sequences were parsed into operational taxonomic units (OTUs) based on 97% sequence identity. All clone sequences and reference sequences were aligned with Clustal W (Thompson *et al.,* 1994) and phylogenetic trees were constructed with the Neighbor-joining method and Jukes-Cantor distance model with bootstrap values of 500 replicates within MEGA v.4.0 (Tamura *et al.,* 2007).

Quantitative-PCR (qPCR)

Based upon clone library data, quantification of bacterial DNA for *Desulfovibrio* and *Geobacter* populations was performed with a Rotor-Gene 3000 Real-Time PCR Detection System (Corbett Life Sciences) and Rotor-Gene Interface software (v6.0.31; Corbett Life Sciences). PCR primers Dsv691F/Dsv826R and Geo494F/Geo825R were used as previously described (Fite *et al.,* 2004; Holmes *et al.,* 2002). Amplification reactions were done with Platinum Quantitative PCR SuperMix-UDG following the manufacturers' instructions (Invitrogen). DNA concentrations were approximately 100 ng per reaction, and the DNA was purified prior to qPCR by the CTAB method. Primer concentration was optimized with genomic DNA from *Desulfovibrio vulgaris* and *Geobacter metallireducens*. The following temperature profiles were used for amplification: one cycle at 95^{o}C for 1 min, followed by 40 cycles of denaturation at 95^{o}C (10 s), primer annealing (15 s) at 62^{o}C, and extension (30 s) at 72^{o}C. Quantification

was performed with standard curves made from known concentrations of DNA. Melt curve analyses were done by heating the PCR mixtures from 60°C to 95°C (1°C per cycle of 10 s) with simultaneous measurements of SYBR Green™ signal intensities.

Statistical analysis

Rarefaction curves, OTU richness, bacterial community diversity measures (e.g., Shannon-Weiner (H') index, 1/Simpson's (1/D) index, and Chao1) and sample coverage for each clone library were determined by the software program DOTUR (Schloss and Handelsman, 2005) with a 3% difference in nucleic acid sequences, and the evenness index (E) was calculated within Krebs software as previously described (Brown and Bowman, 2001). The H' index considers the equitability of the OTU distribution, 1/D also considers both richness and abundance, and Chao1 is a non-parametric estimation of OTU richness. However, composition and abundance may not be completely epitomized by clone distribution; therefore, measurements and indices were used for relative comparisons (Yan et al., 2003; Fields et al., 2006). Comparisons between clone libraries were done with different approaches. LIBSHUFF (http://www.arches.uga.edu/~whitman/libshuff.html)) analysis was used to construct a pairwise comparison of the SSU rRNA gene libraries as previously described (Fields *et al.,* 2006). UPGMA and a neighbor-joining method were used to construct phylogenetic trees with distance matrices based upon correlation values of $\triangle C_{xy}$ values with MEGA v4.0 (Tamura *et al.,* 2007).

Ordination Analyses

Overall variation in bacterial community composition throughout the treatment process was characterized by Jackknife environmental cluster analysis and principal coordinates analysis (PCoA) within the UniFrac software package (Lozupone *et al.,* 2007) according to specified instructions given at the UniFrac website (http://bmf2.colorado.edu/unifrac/index.psp) and correspondence analysis (CA), detrended correspondence analysis (DCA), and canonical

correspondence analysis (CCA) were performed with Canoco v4.5 (Microcomputer Power Inc.). Detrended correspondence analysis (DCA), an indirect gradient analysis technique that detrends by segments, was used to obtain the length of the gradient in the species data to allow for the selection of the appropriate method (unimodal or linear). When comparisons were done between all wells, species data was reported in percent OTUs. When bacterial community profiles were compared between the injection and the two inner bioreduction wells only, species data was reported as percentage in bacterial families instead of percent OTUs. DCA revealed that the two data sets exhibited a unimodal (gradient length=6.74 and 3.51 for comparison between all wells and wells of the bioreduction zone, respectively) response to the explanatory variables. Thus, canonical correspondence analysis (CCA), a direct gradient analysis, was used to examine the relationship between bacterial community profiles and environmental factors (i.e. well characteristics, time, pH, sulfate, nitrate, and subsurface manipulations). CCA has been shown to provide a flexible and meaningful constrained ordination of ecological species abundance data with environmental variables (Anderson and Willis, 2003). Partial CCA (pCCA) was also used to discern patterns related to one set of variables while controlling for a different set of variables (co-variables; see Table 3).

A total of 14 explanatory variables were used when communities from all wells were analyzed: 6 dummy variables were assigned to characterize spatial locations (extraction, injection, and the inner and outer wells); 4 dummy variables were assigned to characterize engineered conditions of the subsurface (residual denitrification, ethanol stimulation without dissolved oxygen control, ethanol stimulation with dissolved oxygen control, and ethanol limitation with dissolved oxygen control); and pH, sulfate, nitrate, and time were each considered as a variable. A total of 14 explanatory variables were used when communities from the injection and the two inner bioreduction wells were compared: 3 dummy variables

were assigned to characterize spatial locations, 4 dummy variables for the engineered conditions of the subsurface as described above and pH, COD, uranium, nitrate, sulfide, sulfate, and time were each considered as a variable.

Variance partitioning as described by Anderson and Gribble (1998) and Muylaert et al. (2000) was performed to determine which variables (i.e. environmental, spatial, or temporal) could best explain the observed distribution of species composition (Table 3). The p values for the canonical axes were produced by Monte-Carlo permutation tests to determine the significance of microbial community changes in relation to environmental variables. CCA does not depend on parametric distributional assumptions and performs well with skewed species distributions (Palmer, 1993). Thus, species data were not transformed and explanatory variables were standardized.

Results

Biostimulation and U(VI) reduction

Nitrate concentrations dropped to below 0.05 mM after day 540 once stimulation was initiated on day 137 (Fig. 2). U(VI) concentrations decreased to below U.S. EPA Maximum Contaminant Limit for drinking water (0.03 mg/l) after day 615 in FW102. During ethanol injection, the COD concentration in wells FW104, FW101, and FW102 increased initially and then decreased as ethanol was consumed (Table 1). However, COD levels decreased to near zero when ethanol was not added from day 713 to day 754. The same trend was observed for sulfide concentrations and the opposite was observed for sulfate levels. Uranium concentrations in the injection well, FW104, decreased less rapidly than the two inner bioreduction wells, FW101 and FW102. A slight rebound in uranium concentration occurred after ethanol injection stopped, but U(VI) levels remained below 0.2 mg/l.

Tracer tests with bromide indicated that the outer- and inner-loops were hydraulically

connected. Approximately 9% of the water injected at well FW104 flowed to the outer-loop extraction well FW103, and approximately 17% of the water injected to the outer-loop well FW024 flowed to the inner-loop extraction well FW026 (Luo *et al.,* 2007). As a result, small amounts of ethanol injected into the inner-loop leaked to the outer-loop wells.

Bacterial diversity (richness and relative abundance)

A total of 23 clone libraries were constructed for the six tested wells. Approximately 80 to 100 clones (90±7; Table 2) were analyzed for each library and approximately 2,000 SSU rRNA gene sequences (approximately 500 bp in length) were analyzed to determine population distributions over the tested time period. When a 3% OTU cut-off was used, rarefaction curves for most clone libraries approached saturation (Fig. S1), and the percent coverage for sampled diversity ranged from 63% to 93%. The relative bacterial diversity and OTUs declined over time in well FW024 (Table 3). Although the number of sampled OTUs was initially stable in the injection well, FW104, an increase in sampled bacterial diversity (Table 2) and evenness (data not shown) was eventually observed. However, in the two bioreduction wells, FW101 and FW102, a fluctuation in diversity and evenness was observed from days 166 to 726. In general, diversity increased during periods of ethanol addition. Diversity decreased in FW101 prior to implementation of DO control (maintenance of low DO, <0.03 mg/l), but recovered to levels comparable to that of FW102 by day 622.

Bacterial composition and structure

For the purposes of this study, composition equates to richness and structure represents diversity. Phylogenetic analysis of the sampled SSU rRNA gene sequences revealed that bacterial communities in the groundwater changed in composition and structure throughout the different stages of treatment (Fig. 3A-D). Initially, β–*Proteobacteria* predominated, but as ethanol stimulation continued sequences indicative of other bacterial phyla such as δ-*Proteobacteria*,

Actinobacteria, *Acidobacteria*, and *Firmicutes* became prevalent (Fig. 3A). At day 166, β–*Proteobacteria* was the major group in the FW024, FW104, FW101, and FW102 libraries. However, as diversity increased, the β–*Proteobacteria* populations fluctuated in FW104, FW101, and FW102 (Fig. 3A). Predominant sequences within the β-Proteobacteria were closely related to *Herbaspirilllum, Zooglea*, *Dechloromonas*, *Ferribacterium*, *Hydrogenophaga*, *Curvibacter*, and *Acidovorax* spp. (Fig. S2B and C). Both *Zooglea* and *Acidovorax* sequences and isolates have been observed previously in FRC groundwater (Fields et al., 2005).

FW103 at day 535 was predominated by *Rhodocyclaceae* populations (49%), and the sequences were similar to clones from FW024 on the same sampling day. Like FW024, the sampled community in FW103 also shifted to *Comamonadaceae* populations (51%) at 747 day (Fig. 3B). Most of the clone populations in the *Comamonadaceae* family had 98% sequence identity to *Rhodoferax ferrireducens,* while others had 98% sequence identity to *A. delafieldii* (Fig. S2B and C). Clone libraries from FW104 and FW101 were predominated by an *Oxalobacteraceae* population at day 166 and the clone sequences had $\geq$97% sequence identity to *Herbaspirillum* spp. (AJ012069) (Fig. S2B). FW104 shifted from predominance by an *Oxalobacteraceae* population (40%) in the residual denitrification phase to predominance by *Rhodocyclaceae* population (most closely related to an uncultivated *Thauera* spp. clone, DQ426920) in the uranium reduction phase prior to DO control (Fig. 3B). The *Rhodocyclaceae* populations declined while *Comamonadaceae* populations increased (22%) during the initial phase of uranium reduction with DO control and the majority of the clone library had 98% sequence identity to *R. ferrireducens* (Fig. 3B and Fig. S2B). *Hydrogenophilaceae* populations increased (16%) by day 747 (Fig. 3B) and the clone populations had $\geq$97% sequence identity to an uncultivated *Thiobacillus* sp. clone (EF413894) (Fig. S2A) and these results might coincide with the infiltration of oxygen.

FW101 shifted from predominance by *Oxalobacteraceae* (68%) to *Comamonadaceae* populations (25%) (Fig. 3B), and the majority of the clone population had >97% sequence identity to *A. delafieldii* (Fig. S2C). *Gallionellaceae* populations were detected at 20% and 8% of the population at days 278 and 558 respectively, but were not detected once the well entered the phases of uranium reduction with DO control and ethanol limitation. Community dynamics in well FW102 was unlike FW101, with predominance by *Burkholderiaceae* (43%) and *Rhodocyclaceae* populations (17%) at day 166. Despite the differences in the bacterial community at 166 day, the two wells had a similar overall community structure by days 641 and 622 for FW101 and FW102, respectively (Fig. 3B).

After initiation of weekly ethanol additions, δ-*Proteobacteria* populations were detected by day 278 in wells FW101 and FW102 and by day 535 in FW104 (Fig. 3C). Phylogenetic analysis of the δ-*Proteobacteria* at the family level revealed differences in each well at the respective sampling days. However, *Desulfovibrionaceae* and uncultivated *Desulfovibrionaceae* populations predominated in both wells at the end of the experiment (Fig. 3C and 4). For comparison, δ-*Proteobacteria* sequences were detected at <3% in FW024 (outer loop injection well), FW103 and FW026 (down-gradient wells).

While δ-*Proteobacteria* bacteria were predominant in both FW101 and FW102, the sampled communities within these wells changed over time (Fig. 3A). The δ-*Proteobacteria* populations in FW101 shifted from predominance by *Geobacteraceae* populations (25%) during earlier stages of uranium reduction (278 day) to predominance by *Desulfovibrionaceae* during DO control (54%). *Geobacteraceae* were also detected in FW102 at day 278 (5%) and at a higher percentage on day 622 (25%) during the uranium reduction phase (no DO control), but declined at the later time points (<3%). *Desulfovibrionaceae* populations increased (to 56%) during the uranium reduction phase with DO control, but declined during the ethanol limitation

phase (Fig. 3C). The majority of *Desulfovibrionaceae* clones had 98% sequence identity to *Desulfovibrio magneticus* (D43994) (Fig. 5). The majority of the *Geobacteraceae* clones had 100% sequence identity to an uncultivated *Geobacter* clone (AY780563) (Fig. 5). To validate these results, qPCR was used to quantify *Desulfovibrio* spp. and *Geobacter* spp. from the same samples. The qPCR results demonstrated an increase in gene copy number for *Desulfovibrionaceae* (Fig. 4), and a similar trend for these populations correlated to the clone library analyses (r^2=0.9).

Acidobacteria, *Actinobacteria* and α-*Proteobacteria* populations were more consistently observed for the tested wells during the time course, whereas *Firmicutes* populations were detected in certain wells at certain days (Fig. 3D). The highest level of α-*Proteobacteria* (33% of clones) occurred in FW024 on day 535. The majority of α-*Proteobacteria* clones had 97% sequence identity to *Sphingobium herbacidovorans* (EF065102) (Fig. S3). *Sphingobium* sequences were previously detected at an uncontaminated background site (Fields *et al.,* 2005).

Acidobacteria populations were detected at low levels in FW024 and FW102 at all time points. In FW101, *Acidobacteria* peaked on day 278 (25% of clones) and declined thereafter (Fig. 3D). The majority of the clone populations had 97% sequence identity to *Geothrix fermentans* (U41563) (Fig. S4). *Actinobacteria* populations were consistently detected and represented 7% of the clones for FW026 on day 535 and lower levels at FW024, FW104, and FW103 throughout the experiment. Changes in the *Actinobacteria* population were especially pronounced in FW101 and FW102 (Fig. 3D). The majority of these clones had 98% sequence identity to an uncultivated *Actinobacterium* clone (AJ888538) (Fig. S5). A study by Gremion et al. (2003) suggested that *Actinobacteria* populations were a major metabolically active group in a heavy-metal contaminated soil.

Firmicutes were detected at <3% of clones between days 535 and 712 in FW104 and did not

change significantly (<5% of clones). In both FW101 and FW102; however, *Firmicutes* populations increased to 25% during the ethanol limitation phase (day 713-754). The clone populations of the *Firmicutes* family in these two wells during this phase had 96%-98% sequence identity to an uncultivated *Desulfosporosinus* spp. clone (AY607216) (Fig. S6).

Spatial and temporal analysis of the bacterial community

The temporal and spatial differences in the wells were further analyzed using hierarchical clustering with an UPGMA algorithm and principal coordinates analysis (PCoA) (Fig. 6A and 6B). The three wells most affected by groundwater recirculation and ethanol addition, FW104, FW101, and FW102, clustered together at day 166, which indicated that these wells were initially similar in community composition. The up-gradient well, FW024, did not have a major shift in bacterial community structure or composition over the tested time period. Moreover, the bacterial communities in the three wells FW104, FW101, and FW102 were similar to that of FW024 on day 166 (i.e., prior to ethanol addition), which suggested that the initial communities were similar before the treatment strategy was implemented.

FW024 at day 712 clustered with FW103 at day 747 (>99.9% confidence) (Fig. 6A). These two wells were the outermost up- and down-gradient wells, respectively. Similarly, the two down-gradient wells, FW026 and FW103, clustered together at day 535 with >99.9% support. FW101 at days 278, 535, and 622 clustered with FW104 on day 641, 712, and 746. These data suggested that the injection well lagged in bacterial community development compared to the communities at the down-gradient monitoring wells that were most directly affected by ethanol addition. Community structure for monitoring wells FW101 and FW102 clustered at days 641 and 622 (90 to 99% confidence) and days 726 and 670 (99% confidence). These results indicated that the bacterial communities from the wells most directly impacted by ethanol addition were altered in different ways and ultimately became more similar to one

another than the other wells.

Correspondence analysis resulted in similar results to the PCoA that clustered the inner wells (Fig. S7). In addition, lineage specific analyses revealed that clustering at earlier time points was due to the shared presence of several denitrifying bacterial populations. Samples from FW026 and FW103 at day 535 clustered because of the shared presence of both iron-oxidizing and iron-reducing populations, and samples from the two inner bioreduction wells clustered due to the shared presence of sulfate-reducing populations (Fig. 6B). LIBSHUFF analyses of the injection and two bio-stimulation wells revealed similar results in which the bacterial communities developed changes in parallel while the injection well lagged ($p<0.001$) (data not shown). These results indicated that the bacterial communities within treated wells diverged sharply from those in up- and down-gradient wells, and that the community changes could be correlated to changes in geochemical conditions (e.g., electron acceptors) during stimulation for bioremediation

Bacterial community structure and groundwater variable relationships

When CCA was done based upon population distributions with time and space as the variables, treatment wells (ethanol delivery) could be easily distinguished from outer and inner-loop wells, and injection wells and treatment wells displayed a trajectory that followed a temporal vector (data not shown). The CCA identified major species-environment correlations, and F-ratios were used to determine the variance for the independent variables as previously described (ter Braak and Smilauer, 2002). The test of significance (global permutation tests) based upon all canonical eigenvalues ranged from 0.002 to 0.04 and indicated that the relations between the species and the measured environmental variables were highly significant.

When both the bacterial community structure and physicochemical variables were compared via pCCA with engineering controls and geochemistry as variables and spatial location

as co-variables, the association of FW101 and FW102 at later time points was observed (Fig. 7 and 8). There was a shift in community structure between times with and without DO control, irrespective of ethanol addition. Upon examination of the inner bioreduction wells only, the pCCA suggested that environmental variables (with space as a co-variable) could explain approximately 63% of the species data (p=0.018) (Table 3). U(VI), nitrate, COD, sulfide, and sulfate strongly associated with the shifts in bacterial community patterns, and the correlations were meaningful in a biogeochemical context (e.g., nitrate decline corresponded to decline in denitrifiers; sulfide correlated to the predominance of sulfate-reducing bacteria).

At day 166, FW104, 101, and 102 had high concentrations of nitrate and uranium and the associated bacterial communities were predominated by phyla common for nitrate-reducing bacteria (*e.g.*, β-*Proteobacteria* and γ-*Proteobacteria*) (Fig. 8). Proceeding in a direction opposite to the gradient for nitrate and uranium indicates a negative correlation between nitrate and uranium and other bacterial communities. By contrast, nitrate-reducing species correlated positively to increasing chemical oxygen demand (COD), a measure of the available ethanol. The ordination diagram indicated that patterns of bacterial community were strongly associated with the spatial and temporal differences in the geochemistry of the groundwater and that these markers could represent good indicators for radionuclide reduction potential and monitoring. For clarity, higher divisions of bacterial classification were used in the comparisons, and associations of particular populations could be discerned for the bio-stimulated wells. Thus, as the *Acidobacteria*, *Geobacteraceae*, *Actinobacteria*, *Desulfovibrionaceae*, and *Firmicutes* populations increased, the levels of nitrate had declined and uranium declined. *Desulfovibrionaceae* and *Firmicutes* populations were also strongly associated with the sulfate (decline) and sulfide (increase) levels (Fig. 8).

It is interesting to note that *Acidobacteria* and *Geobacteraceae* populations were positively

correlated with higher COD levels (Fig. 8). The results suggested that these populations were more prevalent when ethanol levels were higher during earlier and intermediate time points. In contrast, the SRB sequences (namely, *Desulfovibrioaceae*) predominated at later time points along with sequences for *Firmicutes*, namely *Desulfosporosinus* spp. and uncultivated *Clostridium* species (Fig. 8).

Samples that had a decrease in nitrate and U(VI) levels were clustered, and the phases of DO control with and without ethanol had similar community structure and composition (Fig. 7 and 8). The relationships between the samples based upon community structure and the engineering controls were also highly significant ($p<0.01$). However, the samples that represented time points when DO control was implemented compared to no DO control (with ethanol delivery) had a shift in community structure and the associated populations were *Desulfovibrio*, *Desulfosporosinus*, and *Hydrogenophilaceae* species as well as *Bacteroidetes* and *Caulobacteraceae*. The pCCA data also suggested that the DO control phases had similar community structure and composition independent of ethanol addition. These results indicated that stimulant levels could be decreased or stopped at least for the tested time period without major shifts in population composition or distribution.

Total variation of the species data was partitioned into six categories (Table 3). When all wells were considered, the environmental (i.e., geochemistry) and space variables accounted for the majority of variation (32.0% and 24.6%, respectively, Fig. 9). However, 36% of the biological variation was unexplained (Fig. 9). When only the bio-stimulated wells were considered, the environmental variable could account for approximately 50.1% of the variability, and the unexplained portion of the variability was 17.3% (Fig. 9).

Discussion

Diversity and community function

Microbial communities have been extensively characterized at uranium-contaminated sites (Abdelouas *et al.,* 2000; Anderson *et al.,* 2003; Chang *et al.,* 2001*,* 2005; Elias *et al.,* 2003; Nevin *et al.,* 2003; Vrionis *et al.,* 2005;) including the OR-FRC (Yan *et al.,* 2003; North *et al.,* 2004; Fields *et al.,* 2005, 2006; Akob *et al.,* 2007). In general, it is assumed that bacterial diversity will depend upon the degree of contamination and will change in structure and composition in response to geochemical changes (e.g., nutrients, pH, E_h, etc.). In our study, transitory trends were suggested from nitrate-reducing to iron-related redox metabolism to sulfate-reducing populations, and these results indicated that bacteria exhibited distributions at the landscape scale in agreement with predictable geochemical factors. During biostimulation, population distributions followed geochemical parameters (i.e., nitrate- to iron- to sulfate-reduction) with spatial differences that matched the expected geochemical changes; however, the bio-stimulated communities displayed idiosyncratic responses during the transitions (communities were dissimilar at intermediate time points). As described by Lawton et al. (1998), the idiosyncratic theory assumes that the relationship between species richness and ecosystem function does not follow a consistent pattern. In addition, the measured variables could only account for 50% of the biological variability in the stimulated wells.

The bioreduction wells (FW101 and FW102) had different streamlines passing from the injection well and progressed differently to a common community structure dominated by sulfate-reducing bacteria. The bioreduction wells displayed a variety of responses when diversity and uranium levels were compared as a measure of ecosystem process (i.e., uranium reduction), and the comparison of nitrate levels to diversity displayed a similar relationship (Table 2; data not shown). The results suggested that the population distributions depended on the particular conditions of the local environment. Further work is needed to discern the relationships between specific ecosystem processes and bacterial diversity, but the present study implied that

population distributions depended on the particular conditions experienced as opposed to a system that is insensitive to diversity or one in which diversity contributes to ecosystem processes in a unique but predictable fashion.

The observed increase in bacterial diversity could be attributed to the input of the energy and carbon source that allowed increased colonization. The diversity of the treatment wells increased during the greatest decline in soluble U(VI) levels but fluctuated when U(VI) levels further decreased below 0.03 mg/l. The diversity increase was more likely a consequence of nutrient addition rather than the decline in U(VI) levels since uranium is not the only toxic contaminant present in the OR-FRC groundwater (Fields et al., 2006). The increase in diversity most likely altered population distributions thereby changing the functionality of the community. It would appear that carbon/energy levels increased above a minimum required for survival (or at least to the limit of detection) of various species. This type of response has been observed for plant diversity as productivity increases from very low levels (Huston and DeAngelis, 1994).

Engineering controls

Multivariate analytic tools are commonly used in macro-ecology studies to identify diversity and distribution patterns of plants and animals in relation to environmental parameters. Ramette (2007) suggested that these tools can also be readily applied in microbial ecology to help reduce data set complexity as DNA sequence data is increasingly available due to high-throughput sequencing technologies. Indeed, microbial ecology studies have increasingly used multivariate analyses to relate environmental variation to bacterial composition in aquatic systems (Yannarell and Triplett., 2005; Kent et al., 2007; Rubin and Leff, 2007), to determine bacterial community dynamics in relation to temporal variation in marine ecosystems (Pringault et al., 2007; Sapp et al., 2007), to develop microbial communities as indicators of ecosystem stress (Cao et al., 2006;

Córdova-Kreylos et al., 2006; Fields et al., 2006), as well as to link geophysical signatures to microbial communities (Allen et al., 2007).

In our study, the use of pCCA enabled the identification of the crucial engineering controls for which bacterial community composition shifts appeared significantly related. We first compared bacterial community composition shifts between all wells and found that while the majority of the variation could be explained by spatial and environmental variables, a portion of the variance remained unexplained. Other ecological studies that used variance partitioning have also observed high levels of unexplained variation (Titeux et al., 2004). When we examined the bacterial community composition between the bioreduction wells only, the unexplained variation decreased. Tieteux et al. (2004) suggested that the high unexplained species assemblages could be due to unaccounted factors such as the fluctuations of communities along temporal and spatial scales, unmeasured environmental variables, and limitations in separating geographical factors that could be due to spatially structured processes. Our results suggested that other geochemical variables could be important indicators, and while it is difficult to measure everything, future efforts should attempt to include a range of variables to represent an even more holistic approach.

Changes in bacterial diversity were expected to result from engineering controls, and the pCCA indicated that bacterial populations shifted with DO control measures and carbon/energy source additions. When ethanol was added major shifts in sampled diversity and structure occurred, but when ethanol was withheld, DO control was needed to prevent further changes in the bacterial community. These results suggested that engineering controls impacted the development of bacterial communities with the desired metabolic activity. As pointed out by Kassen and Rainey (2004), microbiologists typically define function via mechanisms that involve genes and gene products, but a broadened perspective should include fitness of

individual populations. Fitness could be a measure of population survival and reproduction and is an ecological measure that relates genotype interactions with biotic and abiotic variables.

Other field studies observed bacterial population shifts during short-term push-pull stimulations that were predominated by iron-reducing populations (North *et al.*, 2004) or a mix of sulfate-reducing and/or iron-reducing populations (Abdelouas *et al.*, 2000; Vrionis *et al.*, 2005), while others observed predominance by SRBs, namely *Desulfosporosinus* (Nevin *et al.*, 2003), *Desulfosporosinus* and *Clostridium* (Suzuki *et al.*, 2003), *Desulfobacter* species (Vrionis *et al.*, 2005). Over the tested period in our study, the communities became dominated by sulfate-reducing populations closely related to *Desulfovibrio* species. It is likely that additional populations are directly and/or indirectly involved in U(VI) bio-reduction at this site, and it will become increasingly important to develop more sensitive molecular techniques as well as more robust cultivation methods to better understand remediation strategies.

Biotic associations

Similar to previous studies, we observed nitrate to be a major factor that influenced U(VI) reduction. After nitrate removal, a major shift in bacterial communities was observed with continued ethanol additions. Community structure first transitioned to nitrate-reducing and then populations of iron-oxidizers (*G. ferruginea*) and iron-reducers (*Geothrix* spp., *Geobacter* spp., *Holophaga* spp.). *G. ferruginea* is able to live in low-oxygen conditions (Anderson and Pedersen, 2003) and has been found in many acidic metal-rich waters (Kim *et al*., 2002; Bruneel *et al*., 2006; Hallberg *et al*., 2006). *Geothrix* spp. have been isolated from a hydrocarbon contaminated aquifer (Coates *et al*., 1999) and were also enriched when exposed to petroleum compounds in laboratory experiments (Abed *et al*., 2002). *Geothrix*-like sequences were also detected in the stimulated treatment zones of FRC sediments (Cardenas *et al.*, 2008). *Geobacter* spp. have been found in metal-polluted freshwaters (Cummings *et al.*, 2003) and

detected in high numbers at sites treated for U(VI) remediation (Holmes *et al.*, 2002; Anderson *et al.,* 2003). The metabolic potential of iron-oxidizing and iron-reducing populations could have further implications on the stability of U(VI) immobilization as iron-oxidizing populations can potentially re-oxidize U(IV) and/or oxidize Fe(II) in a nitrate-dependent manner. The dynamics between iron-oxidizers, iron-reducers, and sulfate-reducers warrants further investigation at contaminated field sites.

Geobacter sequences were observed throughout the experiment but were highest when COD levels (i.e., organic carbon) were elevated. The *Geobacter* populations appeared to associate with *Acidobacteria* populations (e.g. *Geotherix* spp,. and *Holophaga* spp.), but the nature of possible interactions between these populations is unknown. Further work is needed to examine possible direct or indirect interactions. It is not known if niche complementarity would lead to greater stability in bioremediation processes although studies have shown that it can explain positive relationships between diversity and function in prairie grasslands (Tilman *et al.*, 1996).

Diverse *Acidobacteria* have been detected in different uranium-contaminated sites (Barns *et al*., 2007); however, little is known regarding physiology or ecology. The abundance of *Acidobacteria* in groundwater suggested an ability to tolerate high levels of metal- and nitrate-contamination, but the data also indicated that the *Acidobacteria* did not continue to compete with sulfate- and metal-reducing populations. Further analysis by pCCA revealed that the prevalence of *Geobacter* and *Acidobacteria* populations was more strongly correlated to the availability of ethanol rather than low U(VI) levels. These results emphasized the importance for a better understanding of population interactions leading to competition and/or mutually beneficial use of nutrient and energy sources.

In terms of the ecosystem process of interest in our study, multiple species could be

classified into the same 'functional group' (i.e., heavy-metal reduction). For instance, both iron- and sulfate-reducers could be considered at least partially substitutable with respect to contribution to the ecosystem process of interest (i.e., U(VI) reduction); however, iron- and sulfate-reducers can be very different organisms with respect to biogeochemistry and community interactions. Different studies have demonstrated the utility and predominance of iron- and sulfate-reducers at respective sites. Further work is still needed to determine how environmental conditions at respective sites can be controlled to optimize community structure, diversity, and function within the context of desired ecosystem processes.

When DO control measures were compared to community composition, the results suggested that *Geobacter* spp. were more oxygen tolerant as the populations were detected at higher levels when dissolved oxygen was not being controlled and that *Desulfovibrio* spp. were able to adapt to more anoxic conditions. An alternative explanation might be an interaction between *Geobacter* and an oxygen-tolerant microorganism. However, as mentioned previously, the COD levels were also higher when DO was not specifically controlled. Separate studies at the OR-FRC showed that metal-reducing populations such as *Geobacter* spp. and *Anaeromyxobacter* spp. predominated in contaminated sediments following *in situ* biostimulation of microbial populations during push-pull tests (Petrie *et al.*, 2003; North *et al.*, 2004; Michalsen *et al.*, 2007). However, different electron donors and engineering controls were used, and these comparisons also support the notion that there is an idiosyncraticrelationship between population distribution and ecosystem process in terms of biostimulation for heavy-metal reduction.As ethanol was being limited, *Firmicutes* were detected at increasing levels in the groundwater and were especially pronounced in FW102. The *Firmicutes* sequences were closely related to the fermenting bacteria, *Clostridium* spp., the sulfate-reducing, *Desulfosporosinus* spp, and other sequences closely related to the

Peptococcaceae family.

Research has shown that both *Clostridium* spp. and *Desulfosporosinus* spp. can be stimulated in uranium mine sediments (Suzuki *et al.*, 2003) and *Desulfosporosinus* spp. have also been enriched in high-salinity subsurface sediment stimulated for U(VI) reduction (Nevin *et al.*, 2003), as well as heavy metal column experiments (Geets *et al.*, 2006). *Clostridium* spp. were shown to be able to reduce U(VI) (Francis *et al.*, 1994; Madden *et al.*, 2007) and similar populations were also detected in other OR-FRC sediments using glycerol-amended enrichments (Petrie *et al.*, 2003). *Clostridium* populations were also suggested to be active members of the OR-FRC microbial community through a study of RNA-derived clone libraries (Akob *et al.*, 2007) and low G+C bacteria have been isolated from OR-FRC groundwater (Fields *et al.*, 2005).

pCCA analysis indicated that bacterial communities were similar in the DO control phases regardless of electron donor availability. Indeed, *Desulfovibrio* populations were still predominant during this phase despite the fact that the subsurface condition may have started to favor fermentative metabolism by *Firmicutes*. The presence of populations that have both sulfate- and metal-reducing capabilities at the later phases of our study suggested a potential for continued uranium-reduction during the periods when soluble electron donor is not provided. This was further observed via pCCA in which *Firmicutes* and *Desulfovibrio* populations were negatively correlated with U(VI) levels.

While microbial communities adsorbed in the sediments can be significantly different from the planktonic populations in the aquifer, a recent study that characterized sediments at day 774 showed that *Desulfovibrio* spp. were the most abundant genus in the bio-stimulated zone (Cardenas *et al*., 2008), and our results with groundwater also showed a predominance of *Desulfovibrio* sequences at this time period. The Cardenas *et al*. (2008) study also detected *Desulfosporosinus*, *Geobacter*, *Anaeromyxobacter*, *Geothrix*, and *Acidovorax* related sequences

associated with the sediment, and similar sequences were detected in our temporal study of the groundwater albeit at different proportions. These results suggested that some predominant populations might inhabit both the solid- and aqueous-phases (e.g., *Desulfovibrio*), while others might be exclusive to solid-phases (e.g., *Acidobacteriaceae*). In the case of this test site, *Desulfovibrio* sequences predominated in both the sediments and groundwater; however, it is not known if the predominance of the same putative population(s) in the solid- and aqueous-phases would be unique to an engineered site and/or dependent upon the existing community composition. While not the focus of this study, future work is needed to discern the importance and functionality of surface-adhered versus aqueous-phase communities.

Conclusion

The development of certain bacterial populations corresponded to the manipulated groundwater geochemical variables and showed that it is possible to stimulate the desired function in an engineered system. In terms of U(VI) immobilization, the wells responded differently, but prolonged engineering controls were able to stimulate growth of bacterial communities with the desired U(VI) reduction capacity. The notion of niche development through the application of selective pressures provides a possible framework for use of molecular ecology to improve process performance. Questions remain about how diversity and stability are related in terms of biochemical function, whether all *in situ* engineered systems would behave similarly, and whether the niche exclusion and selection concepts can be applied to improve models of active transport.

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Table 1. Chemical properties of groundwater during the bioremediation process.

Sample number	Well	Days	COD (mg/l)	Sulfate (mg/l)	Sulfide (mg/l)	pH	Nitrate (mM)	U(VI) (mg/l)

1	FW024	166	9	31.6	ND	5.89	0.198	0.50
2		535	7	26.3	ND	5.92	0.190	0.07
3		712	<2	130	ND	5.75	0.015	0.017
4	FW104	166d	40.0	88.0	ND	6.16	0.811	1.053
5		535d	137	42.0	0.00	6.88	0.038	0.174
6		641d	137	40.6	1.52	5.93	0.0017	0.165
7		712d	119	58.7	9.34	5.70	<0.001	0.142
8		746d	4.00	111	0.01	5.77	0.011	0.137
9	FW101-2	166d	17.0	89.3	ND	6.51	0.211	0.791
10		278d	3.00	47.6	NA	6.43	0.0047	0.152
11		535d	70.0	39.3	2.23	6.35	<0.001	0.186
12		558d	95.0	17.9	2.74	5.88	<0.001	0.175
13		622d	96.0	2.23	7.48	6.03	<0.001	0.068
14		641d	108	2.78	9.90	6.02	0.001	0.042
15		726d	8.00	101	0.84	6.00	<0.001	0.062
16	FW102-2	166d	9.00	76.5	ND	6.33	0.711	0.696
17		278d	2.00	27.3	NA	5.97	0.047	0.027
18		622d	51.0	8.66	3.37	6.24	<0.001	0.021
19		670d	59.0	9.72	7.35	6.25	<0.001	0.002
20		726d	12.0	96.3	0.66	6.24	<0.001	0.023
21	FW026	535	32.0	41.6	0.04	6.11	0.085	0.242
22	FW103	535	10	27.1	ND	6.12	0.147	0.083
23		<2	135	ND	5.97	0.021	0.05	<2

NA denotes data not analyzed. ND: Not determined (below detection limit).

Table 2. Characteristics and diversity estimates for SSU rRNA gene clones from groundwater

samples at different time points.

Sample number	Well	Days	Number of clones[a]	OTU[b]	H'[c]	1/D[d]	Chao-1[e]	Evenness[f]
1	FW024	166	83	34	3.13	17.2	53.1	0.83
2		535	84	31	2.79	10.4	73.0	0.76
3		712	90	20	2.26	6.20	27.3	0.68
4	FW104	166d	84	24	2.58	9.35	69.5	0.74
5		535d	81	27	2.66	8.21	40.0	0.76
6		641d	80	24	2.93	14.3	29.2	0.80
7		712d	97	25	2.89	16.7	26.2	0.83
8		746d	89	41	3.37	28.4	95.2	0.84
9	FW101-2	166d	84	16	1.97	4.56	19.0	0.64
10		278d	85	16	2.24	7.83	19.0	0.73
11		535d	83	33	3.10	17.1	50.0	0.83
12		558d	98	22	2.25	5.21	29.5	0.66
13		622d	98	35	3.00	14.3	70.0	0.78
14		641d	87	20	2.55	11.4	32.0	0.78
15		726d	92	23	2.25	5.09	42.5	0.64
16	FW102-2	166d	103	28	2.30	4.81	47.4	0.63
17		278d	100	42	3.16	15.12	150.8	0.79
18		622d	98	39	3.16	18.35	126.8	0.81
19		670d	101	30	2.57	8.43	72.0	0.69
20		726d	95	52	3.61	32.13	101.6	0.93
21	FW026	535	84	36	3.10	16.60	72.1	0.80
22	FW103	535	85	24	2.33	6.46	48.0	0.67
23		747	90	28	2.49	6.337	58.6	0.67

[a]Number of clones sequenced from each library.

[b]Operational taxonomic unit (OTU) based on partial SSU rRNA gene sequences (≥97%).

[c]Shannon-Weiner index, higher number represents higher diversity.

[d]Reciprocal of Simpson's index, higher number represents higher diversity.

[e]Chao-1 estimates; [f]Evenness index