Environmental Microbiology (2009) **11**(10), 2611–2626 doi:10.1111/j.1462-2920.2009.01986.x

GeoChip-based analysis of functional microbial communities during the reoxidation of a bioreduced uranium-contaminated aquifer

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Summary

A pilot-scale system was established for *in situ* biostimulation of U(VI) reduction by ethanol addition at the US Department of Energy's (DOE's) Field Research Center (Oak Ridge, TN). After achieving U(VI) reduction, stability of the bioreduced U(IV) was evaluated under conditions of (i) resting (no ethanol injection), (ii) reoxidation by introducing dissolved oxygen (DO), and (iii) reinjection of ethanol. GeoChip, a functional gene array with probes for N, S and C cycling, metal resistance and contaminant degradation genes, was used for monitoring groundwater microbial communities. High diversity of all major functional groups was observed during all experimental phases. The microbial community was extremely responsive to ethanol, showing a substantial change in community structure with increased gene number and diversity after ethanol injections resumed. While gene numbers showed considerable variations, the relative abundance (i.e. percentage of each gene category) of most gene groups changed little. During the reoxidation period, U(VI) increased, suggesting reoxidation of reduced U(IV). However, when introduction of DO was stopped, U(VI) reduction resumed and returned to pre-reoxidation levels. These findings suggest that the community in this system can be stimulated and that the ability to reduce U(VI) can be maintained by the addition of electron donors. This biostimulation approach may potentially offer an effective means for the bioremediation of U(VI)-contaminated sites.

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Introduction

Uranium (U) is a common groundwater contaminant at US Department of Energy (DOE) sites from cold war era nuclear weapons research and production (Riley *et al*., 1992). Due to its prevalence and potential toxicity, the DOE is interested in remediation strategies to stabilize the U(VI) and minimize the risk of transport off-site. Remediation of U-contaminated sites can be achieved by a change in oxidation state of soluble U(VI) to the less soluble U(IV) to limit transport off-site via biological reductive metal precipitation (Lovley, 1995; Gu *et al*., 2005). Reduction of U(VI) is facilitated by a number of different microorganisms including sulfate- (SRB) (Lovley and Phillips, 1992; Lovley and Phillips, 1994; Tebo and Obraztsova, 1998) and Fe(III)-reducing (FeRB) (Lovley *et al*., 1991; Wu *et al*., 2006b) bacteria as well as other microorganisms, such as *Clostridium* sp. (Francis *et al*., 1994) and *Deinococcus radiodurans* (Fredrickson *et al*., 2000). Many of these microorganisms have been found at U-contaminated sites, including the Oak Ridge Field Research Center (Oak Ridge, TN; OR-FRC) (North *et al*., 2004; Brodie *et al*., 2006; Nyman *et al*., 2006; Amos *et al*., 2007; Cardenas *et al*., 2008), and so could be useful for bioremediation efforts at these sites.

At OR-FRC, U contamination originated from the S-3 waste disposal ponds. These four unlined ponds collected

waste, primarily nitric acid, metals and radionuclides (U, Tc) until 1983 (Gu *et al*., 2003). The ponds have since been neutralized, denitrified, capped, and are currently covered with an asphalt parking lot. Groundwater in the surrounding area has a low pH (3.4–3.6) and high concentrations of U (50 mg l^{-1}) and nitrate (8–12 g l^{-1}) (Oak Ridge Field Research Center, 2007). A pilot test facility designed to examine the applicability of *in situ* U(VI) bioremediation of the groundwater was constructed adjacent to the former S3 ponds at the OR-FRC (Luo *et al*., 2006; Wu *et al*., 2006c,d). Bioreduction and immobilization of U *in situ* has been achieved by intermittently injecting ethanol to the subsurface. The U concentration in the groundwater decreased to below the US Environmental Protection Agency (EPA) maximum contaminant limit (MCL) for drinking water (< 0.03 mg l^{-1}) (Wu *et al*., 2007) and U speciation in sediment samples showed the reduction of U(VI) to U(IV) (Wu *et al*., 2007; Kelly *et al*., 2008).

Knowledge on microbial community structural and functional gene changes during bioremediation and system permutations can improve our understanding of these processes and improve bioremediation strategies. However, one of the major challenges in examining microbial communities is that only an estimated 1% of all microbes are cultivated (Whitman *et al*., 1998). As such, culture-independent molecular tools are necessary to examine these populations. Functional gene arrays (FGA) contain probes for genes that encode enzymes or proteins involved in specific functions of interest (Wu *et al*., 2001; Gentry *et al*., 2006; He *et al*., 2007). The most comprehensive FGA available to date is the GeoChip 2.0, which targets ~10 000 genes involved in the geochemical cycling of N, C and S, metal reduction and resistance, and in organic contaminant degradation (He *et al*., 2007). The GeoChip has been used in a variety of studies to examine the functional structure of microbial communities (Rodríguez-Martínez *et al*., 2006; Wu *et al*., 2006a; He *et al*., 2007; Yergeau *et al*., 2007; Mason *et al*., 2009; Zhou *et al*., 2008; Liang *et al*., 2009; Wang *et al*., 2009), probe pure culture isolates for specific gene functions (Van Nostrand *et al*., 2007), and in stable isotope probing experiments (Leigh *et al*., 2007).

In the current study, we tested the stability of the bioreduced pilot-scale test zone located near the S3 ponds and used the GeoChip to examine microbial community changes in the bioreduced subsurface during the resting (no ethanol injection), reoxidation (introduction of DO) and recovery (restoration of reducing conditions by ethanol injection) periods to better understand how changes to the functional microbial community affect U stability. Specifically, we addressed the following questions: (i) Did the microbial community change during the reoxidation period and were functional abilities affected? (ii) How did ethanol additions affect the community? (iii) Which experimental phase had the greatest effect on the microbial community? and (iv) Were bacteria responsible for U(IV) oxidation? Our results indicated that while the community structure was dramatically altered by introduction of ethanol to the system, the microbial community demonstrated a functional stability and maintained the ability to reduce U(VI) even under conditions of no ethanol addition or reoxidation.

Results

Field operation and geochemical changes during the test periods

The microbial community structure was examined over four experimental periods after low U concentrations were achieved (Fig. 1): (i) a resting period during which ethanol injection was halted (days 713–753), (ii) biostimulation by ethanol injection (days 754–805), (iii) a reoxidation period during which DO was added to the subsurface with suspension of ethanol injection [days 806–884, ethanol was injected on days 866 and 867 to evaluate recovery of Fe(III), sulfate and U(VI) reduction], and (iv) a recovery period during which ethanol was injected intermittently to restore reducing conditions (days 884–992) (Wu *et al*., 2007).

The electron donor, ethanol, was injected weekly at a rate of 35 or 50 g day^{-1} for 2 days except days 713–753 (resting period), 804–865 and 868–883 (reoxidation period) (Fig. 1A). Ethanol was chosen as the electron donor based on previous experiments (Wu *et al*., 2006d) and was monitored using chemical oxygen demand (COD). During ethanol injection, the COD in FW104 (inner loop injection well) was 140–150 mg l^{-1} and dropped to < 10 mg l^{-1} when injection stopped. The COD concentration in FW101-2 was 60–80% of that in FW104 and FW102-3 was 30–50% (data not shown). This is likely due to the longer mean travel time from FW104 to FW102-3 than to FW101-2 and subsequent electron donor consumption. Acetate, the intermediate of ethanol degradation, was the primary COD component (> 90%) in FW101-2 and FW102-3 during ethanol injection (data not shown). The DO in the inner loop injection well (FW104) was maintained at < 0.15 mg l^{-1} and near zero in the multilevel monitoring (MLS) wells except during the reoxidation period. The changes of DO in injection well FW104 and the two MLS wells are described in Fig. 1B. After DO was introduced to the subsurface, DO gradually increased to 2.0 mg l^{-1} in FW101-2, but only to 0.5 mg l^{-1} in FW102-3 (Fig. 1B). Sulfide concentrations increased during ethanol injection and decreased when ethanol injection stopped (Fig. 1C). Fe(II) was detected in FW102-3 throughout the test period, but decreased to near zero in FW101-2 during the reoxidation period (Wu *et al*., 2007; data not shown).

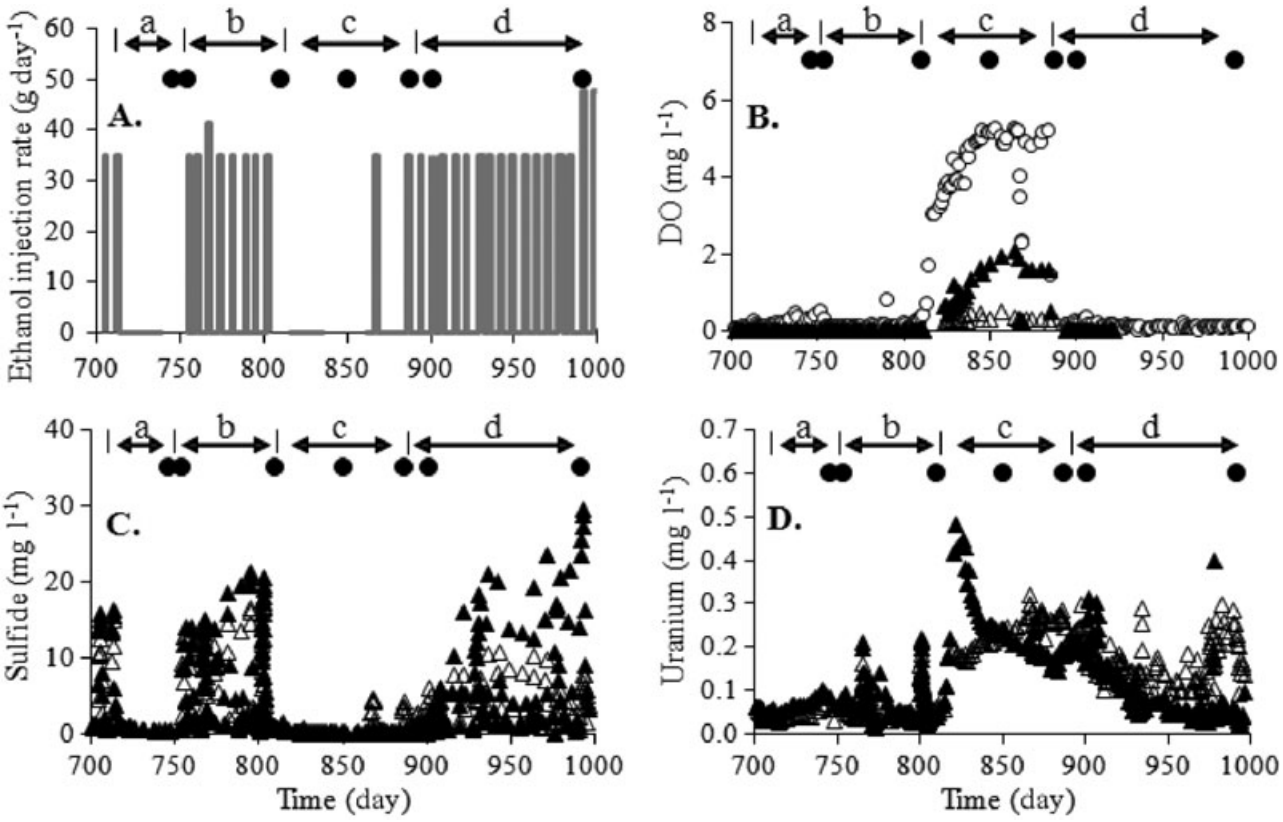

Fig. 1. Geochemical measurements taken during the experimental periods. Ethanol (A), DO (B), sulfide (C) and U (D) were monitored over the course of this study. Closed triangles represent FW101-2, open triangles represent FW102-3, open circles represent FW104 (inner loop injection well, DO panel only) and black circles indicate time points at which samples were taken; a, resting phase; b, normal operation; c, reoxidation phase; d, recovery phase.

Low U concentrations in the MLS (< 0.03 mg l^{-1}) were observed until day 715 when ethanol injection was halted (Fig. 1D). U concentrations began to increase during the resting period due to U from the recirculating groundwater. When ethanol injection resumed, the U concentration decreased. During reoxidation, U concentrations increased significantly in FW101-2 due to the reoxidation of U(IV). A similar response occurred in FW102-3, but lower levels of U were remobilized. The U(VI) concentrations returned to below 0.03 mg l^{-1} in FW101-2 on day 971 and in FW102-3 on day 1207 (data not shown). Reduction of Fe(III), sulfate and U(VI) was stimulated by injection of ethanol on days 867–869 (Fig. 1B, data not shown). Nitrate concentrations were low (0.2 mg l^{-1} as N or lower) when DO was introduced but near zero during ethanol injection (data not shown). The pH was maintained between 6.0 and 6.2 by occasional K_2CO_3 injection. The temperature of the subsurface varied from 12°C to 22°C during summer and winter respectively.

Changes in functional gene diversity

Community functional gene diversity, richness and evenness in the two wells were assessed based on microarray results (Table 1). During the resting and reoxidation periods, when no ethanol was added, the diversity and richness in both wells were relatively stable with approximately 100–200 genes detected at each time point from day 746–850. Increased DO levels did not appear to have a profound effect on diversity, although this could be due to a lack of sensitivity of this index. Well FW101-2 showed an initial decrease in richness and diversity immediately following resumption of ethanol injections (day 887), but showed an increase in richness and diversity as ethanol injections continued. Gene numbers and diversity immediately began to increase in well FW102-3 once ethanol injections resumed. Well FW102-3 showed an almost 10-fold increase in the number of genes detected on day 887 as compared with days 746–850. FW101-2 showed a more modest fourfold increase in gene number on day 901 compared with days 746–850.

Gene overlap between samples was also calculated. Higher percentages of shared genes were observed between time points having similar DO and ethanol treatments. For example, in well FW101-2, about 30% of the genes were shared between days 810 and 850, both during the reoxidation period, while only ~16% of the genes were shared between days 754 and 850, during

Table 1. Gene overlap (italicized), uniqueness (bold) and diversity indices of FRC samples.[a]

Operational Period									
Day	Ethanol	DO	746	754	810	850	887	901	992
				FW101-2					
746	N	N	**1.03%**	*16.51%*	*43.66%*	*34.30%*	*16.67%*	*6.07%*	*11.95%*
754	Y	N		**0.64%**	*19.46%*	*15.75%*	*22.49%*	*14.74%*	*28.87%*
810	N	Y			**0.93%**	*29.73%*	*25.60%*	*7.90%*	*13.95%*
850	N	Y				**9.94%**	*12.68%*	*7.65%*	*16.52%*
887	Y	N					**0.00%**	*4.65%*	*8.78%*
901	Y	N						**1.64%**	*28.31%*
992	Y	N							**0.90%**
Total genes detected			93	150	102	174	48	886	316
Shannon–Weaver *H*			4.20	4.79	4.34	4.82	3.58	6.59	5.48
Shannon–Weaver evenness			0.93	0.96	0.94	0.94	0.93	0.97	0.95
				FW102-3					
746	N	N	**0.00%**	*20.45%*	*43.36%*	*41.91%*	*5.93%*	*3.77%*	*2.83%*
754	Y	N		**2.53%**	*28.80%*	*22.29%*	*6.85%*	*3.66%*	*2.91%*
810	N	Y			**2.44%**	*40.29%*	*6.53%*	*4.00%*	*2.91%*
850	N	Y				**0.00%**	*6.84%*	*5.02%*	*4.07%*
887	Y	N					**1.65%**	*45.56%*	*39.46%*
901	Y	N						**14.58%**	*54.13%*
992	Y	N							**29.59%**
Total genes detected			74	76	76	106	993	1737	2321
Shannon–Weaver *H*			4.05	4.14	4.07	4.38	6.64	7.26	7.42
Shannon–Weaver evenness			0.94	0.96	0.94	0.94	0.96	0.97	0.96

a. Genes were used as 'species' and abundance was indicated by the average normalized signal intensity data of each gene from triplicate arrays × 10. DO, dissolved oxygen; N, no; Y, yes.

resting and reoxidation periods. Three samples had ≥ 10% unique genes: FW101-2, day 850 and FW102-3, days 901 and 992.

Changes in overall community structure

Detrended correspondence analysis (DCA) of all detected genes was also used to examine overall functional structure changes in the microbial communities (Fig. 2). Detrended correspondence analysis is an ordination technique that uses detrending to remove the arch effect typical in correspondence analysis (Hill and Gauch, 1980). The total inertia for the model was 1.588. Eigenvalues for the first two axes of the DCA were 0.560 and 0.194, respectively, and explained ~50% of the variance. The wells clustered into two main groups: one during resting and reoxidation and the other after ethanol injections were resumed (Fig. 2). The samples without ethanol addition clustered more tightly than the samples with ethanol. Three points did not fall into these clusters, days 754 (FW101-2 and FW102-3) and 850 (FW101-2). On day 754, the communities in both wells had experienced starvation (no ethanol injection) for 40 days and had just received ethanol. On day 850, FW101-2 had been exposed to higher levels of oxygen for 46 days, the community appeared to be adapting to the increased DO and was less

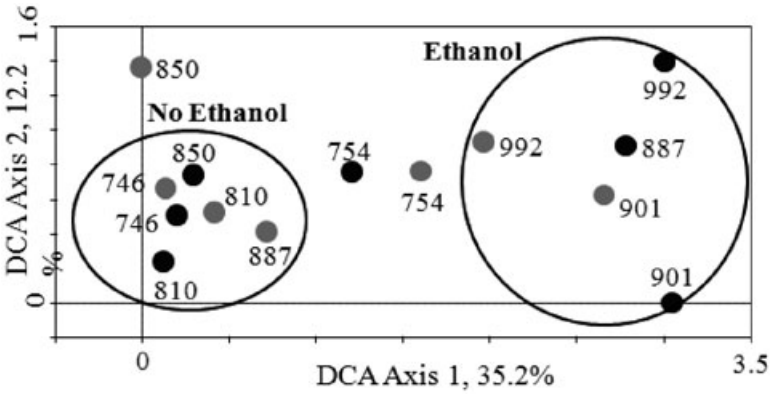

Fig. 2. Detrended correspondence analysis (DCA) of functional genes from FW101-2 and FW102-3. Functional genes detected using the GeoChip 2.0 were used for DCA. The numbers next to each symbol are the operational day corresponding to sample collection. Grey circles represent FW101-2 and black circles represent FW102-3.

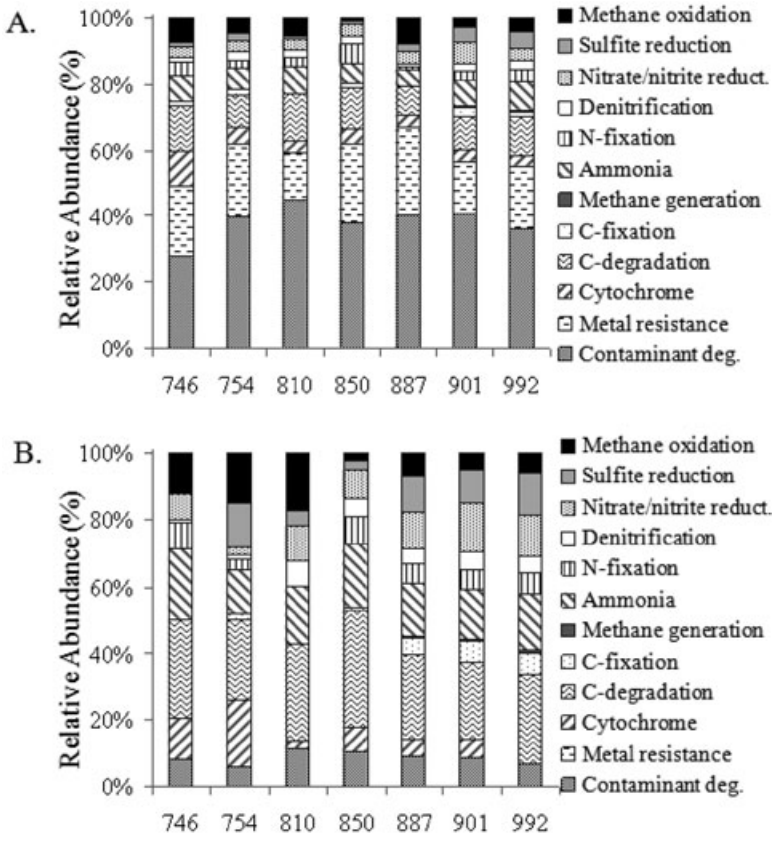

Fig. 3. Relative abundance of all functional gene groups detected. The total number of genes detected at each time point was used to calculate the relative abundance of each gene group from FW101-2 (A) and FW102-3 (B). Numbers along *x*-axis are operational day.

similar to the community on day 810. Alternatively, this could be due to the lower gene numbers detected during this period, although the communities on day 754, which had similar gene numbers to days 746, 810 and 850, clustered away from these time points, suggesting that there were differences in community structure. Detrended correspondence analysis results indicated that the microbial communities were significantly altered after ethanol addition, as indicated by the separate clusters before and after ethanol injections. This suggests that recovery from the stress of starvation and oxygen exposure occurred as indicated by increased gene numbers and decreased U and that the recovered communities differed from the communities during the resting phase.

Relative abundance of functional gene groups

The relative abundance of functional gene groups represented on the GeoChip was compared over the course of these experiments (Fig. 3). Contaminant degradation accounted for approximately 40% of all genes detected at each time point. Another 15–25% of the genes were involved in metal resistance. Carbon cycling genes accounted for 15–20%, with 10–15% associated with carbon degradation, 1–7% with methane oxidation, 0–3% with carbon fixation and < 0.5% with methane production. Methane oxidation genes decreased to 1% in both wells on day 850 and then increased again by day 887. Nitrogen cycling genes accounted for 15–20% of genes detected, with 5–10% associated with ammonification and ammonium assimilation (urease and glutamate dehydrogenase) and nitrification, 1–7% with nitrate and nitrite reduction, 0–6% with nitrogen fixation and 1–3% with denitrification. The remaining 1–5% was involved in sulfate reduction. Overall, the relative abundance of the various gene categories changed less obviously over time although there were changes in some gene categories. The relative abundance of dissimulatory sulfite reductase genes (*dsrAB*) did decrease during the period of increased DO, with a more pronounced decrease in FW101-2 compared with FW102-3 (Fig. 3 and Fig. S2). The relative abundance of metal resistance genes decreased in both wells after ethanol injections were restarted (Fig. 3 and Fig. S2). Overall, the relative abundance of the various gene groups remained similar over the study periods examined even though the number of genes detected varied considerably, suggesting the ability to retain functional ability after stress.

Changes of individual functional gene categories

Specific gene categories were further analysed using hierarchical clustering. The *dsr* genes, which are indicative of SRB, a group of microorganisms that have been

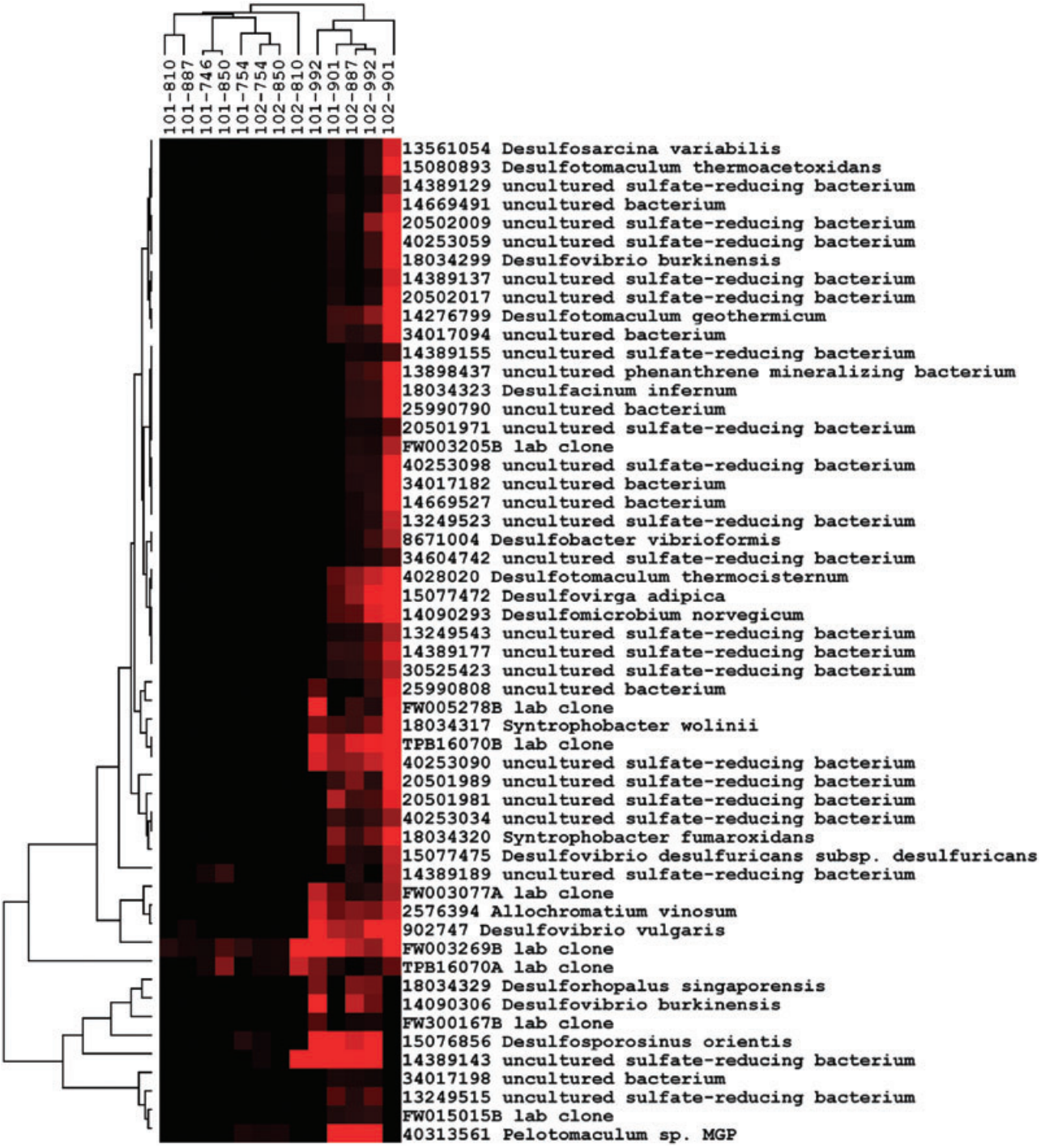

Fig. 4. Hierarchical cluster analysis of dissimilatory sulfite reduction genes. Genes that were present in at least three time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.

shown to reduce U(VI) (Lovley and Phillips, 1992; 1994; Lovley *et al*., 1993a; Tebo and Obraztsova, 1998), clustered based on before and after ethanol injections. Interestingly, while the relative abundance of *dsr* genes was reduced during the reoxidation period (Fig. S2), no separate cluster was obvious for the reoxidation period (Fig. 4). The most frequently detected *dsr* genes, other than from uncultured SRB, were those similar to *Desulfovibrio* and *Desulfotomaculum* species. The *dsr* genes were below detectable limits in FW102-3 on day 746.

Metal resistance genes showed a decrease in relative abundance after ethanol injections resumed (Figs S2 and S3). Genes for resistance to a variety of metals, including Al, Cd, Cu, As, Cr and Hg, were detected. The most frequently detected genes were for resistance to As, Cr, Hg and Te. A gene similar to Te resistance from *Rhodobacter sphaeroides* was detected in both wells at all time points. Genes similar to Cr and Te resistance from *Leuconostoc mesenteroides* and to As resistance from *Campylobacter jejuni* were detected in both wells at most time points. While metal concentrations (maximum concentrations detected in parentheses) were low in both wells, Al (0.26 mg l^{-1}), As (0.007 mg l^{-1}), Cr (0.002 mg l^{-1}), Zn (0.055 mg l^{-1}), Cu (0.003 mg l^{-1}), Pb (0.002 mg l^{-1}) and Ni (0.003 mg l^{-1}) were detected in both. Cd was below detection limits in both wells.

While the relative abundance of carbon degradation genes remained stable over the course of this study (Fig. 3), the number of genes detected sharply increased once ethanol injections resumed (Fig. S4). During the periods when no ethanol was being injected, the carbon degradation genes were dominated by the microorganisms containing cellulase (45%), chitinase (25%) and mannose (20%). Most of these genes were detected throughout the experimental periods. After ethanol injections resumed, the system was dominated by the microorganisms containing cellulase (50%), chitinase (21%), polygalacturonase (20%) and laccase (18%). In addition, two acetyl-CoA synthetase (*acsA*) genes derived from *Leptospira interrogans* (gi 46448414) and *Staphylococcus aurea* (gi 14324459) were detected after ethanol injection resumed. Only a few genes involved in methanogenesis were detected (Fig. S5). With the exception of FW101-2 on day 850, these genes were only detected after ethanol injections resumed and diversity had started to increase (FW101-2 days 850, 901 and 992; FW102-3 days 887, 901 and 992). Most of the detected genes were similar to those found in uncultured microorganisms. A gene similar to *mcr* from *Methanococcus vannielii* (gi 126868) was the only methanogenic gene detected on day 850. A gene similar to *mcrA* from *Methanothermobacter thermautotrophicus* (gi 7445687) was detected on all days except 850 and was the most abundant strain based on signal intensity.

Like the carbon degradation genes, most of the denitrification genes were detected after ethanol injection resumed (Fig. S6). Four denitrification genes, similar to *norB* from *Nitrosomonas europaea* (gi 34391466), *nirK* from *Pseudomonas chlororaphis* (gi 287907), *nirK* from an uncultured bacterium (gi 27125563) and *nirK* from *Alcaligenes* sp. (gi 29466066), were detected at most time points. Five genes involved in nitrogen fixation were detected across most time points (Fig. S7). These genes were similar to the *nifH* from unidentified or uncultured microorganisms (gi 3157698, 780713, 29293420, 1255496, 6523533 and 3157512). Three other *nifH* genes similar to those from unidentified or uncultured microorganisms (gi 3157722, 21586771 and 3157610) were only detected in FW101-2 during time points that did not have ethanol addition. Several *nifH* genes, including those derived from *Calothrix* sp. (gi 1698856 and 1698854) and *Serratia marsescens* (gi 11875063), were only detected in FW102-3.

A variety of organic contaminant degradation genes were detected throughout the course of this study (Fig. S8). Several genes were detected at almost all time points (five to six) in both wells. These included genes similar to those involved in the degradation of 2,4-D (*Bradyrhizobium* sp., gi 17298107; *Wautersia eutropha*, gi 567073), benzoate (*Acinetobacter* sp., gi 2352826), chloroacrylic acid (*Pseudomonas pavonaceae*, gi 10637971), phenylpropionate (*Nostoc punctiforme*, gi 23126645), protocatechuate (*R. sphaeroides*, gi 22975204), vanillin (*Corynebacterium efficiens*, gi 25027190), catechol (*Helicobacter pylori*, gi 15645359), cyclohexanol (*Acinetobacter* sp., gi 9965287), dibenzothiophene (*Thiobacillus* sp., gi 14149089), nitrotoluene (*Burkholderia cepacia*, gi 17942394) and toluene (*Clostridium tetani*, gi 28210648).

Cytochrome *c* genes, which are involved in electron transport and U(VI) reduction (Lovley *et al*., 1993b), were also examined from a limited number of microorganisms of interest. The average signal intensities for the different species covered by these probes on the arrays were calculated (Fig. 5). Most of the cytochrome genes detected were from *Desulfovibrio*-, *Geobacter*-, *Rhodopseudomonas*- and *Shewanella*-like microorganisms. Cytochromes from two other genera, *Chlamydophila* and *Mycobacterium*, were also detected. No genes from *Anaeromyxobacter* were detected.

Phylogenetic diversity

Although the GeoChip 2.0 does not contain specific phylogenetic markers, some information regarding what microorganisms are present can be obtained. The probes on the GeoChip were designed to be as specific as possible for a particular gene from a particular microorganism (Wu *et al*., 2001; Rhee *et al*., 2004; He *et al*., 2007). Over all sampling points, genes were detected from 272 genera and 712 species/strains or microorganisms similar to these strains. Most of the microorganisms detected were present during the recovery period after the functional gene diversity increased. Microorganisms similar to *Desulfovibrio, Pseudomonas*, *Geobacter*, *Wautersia* and *Rhodospirillum* were detected at all time points in both wells. Several genes from organisms similar to genera shown to be capable of U reduction, including *Clostridium*, *Deinococ-*

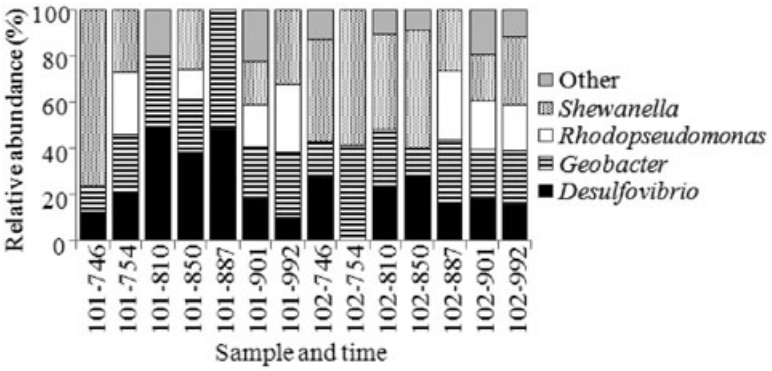

Fig. 5. Relative abundance of cytochrome-containing genera. The relative abundance was calculated based on the total signal intensity of all cytochrome genes originating from each genera. 'Other' includes *Chlamydophila* and *Mycobacterium*.

cus, *Desulfomicrobium*, *Desulfosporosinus*, *Desulfovibrio*, *Geobacter*, *Pseudomonas*, *Pyrobaculum*, *Salmonella*, *Shewanella*, *Thermoanaerobacter* and *Thermus* (Wall and Krumholtz, 2006), were detected. Of these, genes for organisms similar to *Clostridium*, *Deinococcus*, *Desulfovibrio*, *Geobacter*, *Pseudomonas*, *Salmonella* and *Shewanella* were detected in both wells at all or most time points. Genes from a microorganism similar to the Fe(II) oxidizer *Thiobacillus* and *Clostridium* were also detected at almost all time points. We were particularly interested in *Geobacter* and *Desulfovibrio* sp. as these have been shown to be important in U(VI) reduction. Comparable amounts of genes from organisms similar to both *Desulfovibrio* and *Geobacter* were detected at all time points, based on the average signal intensity of all genes detected (Fig. 5).

Relationships between community structure and environmental variables

To determine the most significant geochemical and environmental variables affecting microbial community structure, canonical correspondence analyses (CCA) of both wells were performed for each of the seven environmental variables [sulfate, sulfide, U, Fe(II), COD, pH and temperature]. Five of these variables were chosen based on the *P*-values generated during these analyses: temperature ($P = 0.072$), COD ($P = 0.176$), sulfate ($P = 0.102$), Fe(II) ($P = 0.152$) and U ($P = 0.160$). Both sulfide and pH had *P*-values above 0.35.

Initially, these five environmental variables and all detected functional genes from both wells were used for CCA. The variance inflation factors (VIFs) for sulfate and Fe(II) were greater than 20, indicating there was significant correlation between the environmental variables (multicollinearity) (data not shown). So, Fe(II), which had the highest VIF, was removed and the CCA repeated. The VIFs for the remaining variables decreased to less than 5. In addition, the wider angles between arrows indicate there is less correlation between these environmental variables. The specified CCA model with four environmental variables (sulfate, COD, U and temperature) explained 72% of the variation (sum of all eigenvalues, 1.588) and was significant ($P = 0.002$) (Fig. 6; Table 2). The first canonical axis is positively correlated with sulfate concentration and temperature and negatively correlated with COD and U concentration. The second axis is negatively correlated with COD. The length of the arrow in the biplot indicates the importance of each environmental variable (ter Braak, 1986). As such, COD appears to be the most important environmental parameter, while U, temperature and sulfate appeared to have lesser, but equivalent correlation to community structure shifts. Shifts in FW101-2, on day 992, FW102-3, on day 754, and both wells on day 901, were positively correlated with COD and negatively correlated with temperature. In contrast, shifts in FW101-2, on days 746, 754, 850 and 887, and FW102-3, on days 746, 850 and 992, were positively correlated with temperature and negatively correlated with COD. Shifts in well FW102-3 on day 887 were positively correlated with U, and shifts in FW101-2 on days 810 and 992 and

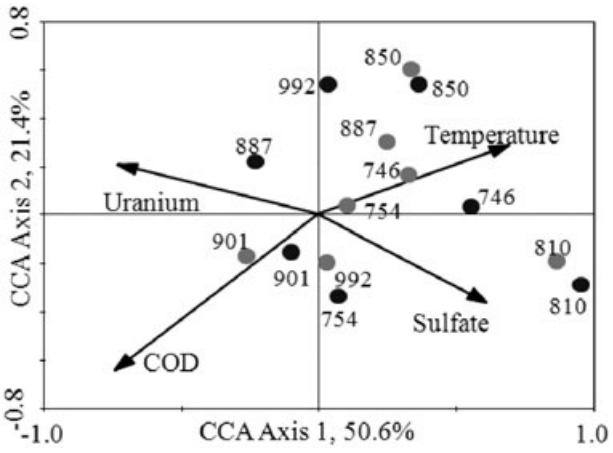

Fig. 6. Canonical correspondence analysis (CCA) for FW101-2 and FW102-3. Analysis was performed using all functional genes detected (symbols) and environmental variables (arrows) from FW101-2 and FW102-3. Environmental variables were chosen based on significance calculated from individual CCA results and variance inflation factors calculated during CCA.

Table 2. Summary of canonical correspondence analysis results.

Axes	1	2
Eigenvalues	0.455	0.193
Species–environment correlations	0.929	0.966
Cumulative percentage variance		
Of species data	28.7	40.8
Of species–environment relation	50.6	72.0
Sum of all eigenvalues	1.588	
Sum of all canonical eigenvalues	0.900	
Summary of Monte Carlo test		
Test of significance of first canonical axis	0.455	
F-ratio	3.617	
P-value	0.008	
Test of significance of all canonical axes	0.900	
F-ratio	2.940	
P-value	0.002	

FW102-3 on days 754 and 810 were positively correlated with sulfate.

Next, variation portioning analysis (VPA) (Økland and Eilertsen, 1994; Ramette and Tiedje, 2007) was performed to better understand how much each environmental variable influenced the functional community structure (Fig. 7). The same variables used for CCA were used for VPA. When geochemistry (sulfate and U) and temperature were held constant, there was a significant correlation between community structure and COD ($P = 0.016$). Similarly, when COD and temperature were held constant, geochemistry (U and sulfate) showed a significant correlation ($P = 0.006$) with the community composition. Temperature showed a nearly significant correlation ($P = 0.056$) with community structure when COD and geochemistry were held constant. Chemical oxygen demand was able to independently explain 13.5% of the variation observed while geochemistry explained 25.5%, and temperature explained 9.3%. Interactions between the three variables appeared to have more influence in this system than individual environmental variables (30.4% total). About one-third of the variation (35.9%) was unexplained. These results suggest that the variables selected greatly influenced the functional gene composition.

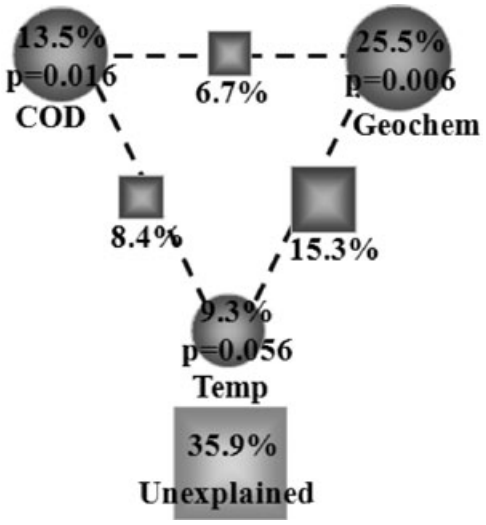

Fig. 7. Variance partitioning of environmental variables analysed by CCA. The diagram represents the relative effects of each variable upon the functional community in FW101-2 and FW102-3. The circles represent the effect of individual variables, by partitioning out the effects of the other variables. The squares between the circles represent the combined effect of the circles on either side of the square. Their was no combined effect of all variables. The square at the bottom of each figure represents the effect that could not be explained by any of the variables tested. Variables used in CCA were used for the VPA. COD, chemical oxygen demand; Temp, temperature; Geochem, geochemical variables (sulfate and uranium). *P*-values shown were generated during partial CCA.

Discussion

Effect of operational phase on the microbial community diversity and structure

The success of bioremediation strategies is in large part dependent on the microbial community at the contaminated site. First, it is vital to have microorganisms which are capable of performing the needed functions [e.g. U(VI) reduction]. Additionally, having a microbial community that is functionally diverse, stable, and capable of rebounding from adverse conditions is critical in establishing an effective and long-term bioremediation system. In the current study, genes from all of the major functional groups represented on the GeoChip were detected, including those that could be involved in U(VI) reduction during all experimental phases (Fig. 3). Thus, biostimulation of this system to increase U(VI) reduction should be a successful long-term approach. In fact, the community did recover after the absence of ethanol and introduction of DO to the system. Previous studies of communities at the OR-FRC have observed similar functional stability; for example, while diversity based on small-subunit rRNA sequences was affected by the level of contaminants present (i.e. less diversity with increased contaminants), the functional abilities, based on the presence of the respective functional genes, were similar (Fields *et al*., 2006).

During the reoxidation phase, an increase in DO was observed and some community changes could be linked to the presence of DO; however, all major functional gene categories were detected as were genes that could be involved in U(VI) reduction. The most obvious effect was a decrease in the relative abundance of *dsr* during the reoxidation period (Fig. 3 and Fig. S2). While very low concentrations of O_2 have been shown to inhibit growth of SRB (Marschall *et al*., 1993), some strains of *Desulfovibrio* are more tolerant to O_2 (Sigalevich *et al*., 2000), and many have mechanisms to utilize, limit or escape O_2

exposure (Le Gall and Xavier, 1996; Sass *et al*., 2002). However, another study at the FRC using artificial neural network modelling suggested that DO has little effect on *dsr* gene frequencies (Palumbo *et al*., 2004). An additional study of *dsr* gene diversity across a contaminant gradient at the FRC showed similar *dsr* composition in all wells examined, both contaminated and uncontaminated wells, suggesting a resiliency in the SRB community (Bagwell *et al*., 2006). In contrast to the *dsr* genes, the relative abundance of cytochrome *c* genes, which have previously been shown to be important for U(VI) reduction (Lovley *et al*., 1993b; Shelobolina *et al*., 2007), did not appear to be affected by the system perturbations. The cytochrome *c* genes detected during the reoxidation period were primarily from non-SRB (data not shown, Fig. 5) and may not have been as affected by the DO as the SRB. In addition, only a small portion of environmental cytochrome genes are covered on the array so other cytochrome genes that may be present in this system, but not on the array may be important.

In contrast to the response to DO, the microbial community was significantly affected by the ethanol amendments, with the most obvious effect being a rapid increase in gene numbers and concomitant increases in diversity (Table 1). These results are consistent with other studies showing that nutrient limitation reduces microbial activity and diversity and that introducing additional carbon sources increases microbial diversity and activity (Reardon *et al*., 2004; Fields *et al*., 2005; Brodie *et al*., 2006; Akob *et al*., 2007). While in general, infusion of nutrients increases microbial diversity and numbers, microcosm experiments using sediments from the OR-FRC showed an initial drop in diversity when ethanol was added (Luo *et al*., 2007b). This is similar to the initial decrease observed in functional gene diversity in FW101-2 on day 887 (Table 1). A reduction in diversity does not, however, necessarily indicate a decrease in function. Brodie and colleagues (2006) found an increase in microbial activity concomitantly with a decrease in biomass during the U(IV) oxidation phase of a soil column from Area 2 of the OR-FRC. While an initial decrease in gene number and diversity was observed, genes from all major functional categories were detected and gene numbers and diversity did increase by day 901.

Effects of environmental variables on functional community structure

A central goal of microbial ecology is to understand the influence of environmental factors on microbial community diversity, structure and function. Variation portioning analysis has been used to determine the contribution of multiple environmental parameters to the changes of microbial communities (Økland and Eilertsen, 1994; Ramette and Tiedje, 2007; Zhou *et al*., 2008). In the current study, COD, primarily acetate from ethanol degradation, appeared to be one of the most important environmental variables in determining the diversity and structure of the groundwater communities. Almost 65% of the total variation was explained by using COD, geochemical variables and temperature (Fig. 7). Ramette and Tiedje (2007) showed that environmental variables, plant diversity or spatial scale can explain 20–30% of the biological variation by the presence, abundance and genotypic similarity of *Burkholderia ambifaria* and 50–70% for abundance of *Burkholderia* spp. in agricultural soils; however, 34–80% of the variation could not be explained. Zhou and colleagues (2008) showed that environmental parameters were able to explain about 20% of the biological variation in a forest soil community; however, more than 50% of variation could not be explained by both environmental variables and geographic distance. The higher level of explanation obtained in this study compared with previous studies may be due to this being a much simpler system with lower diversity than would be expected for forest or agricultural soil communities.

U(IV) reoxidation

Previous studies have observed shifts in microbial community structure which correlated with remediation phases. For example, principal component analysis results of a 16S rRNA microarray (Phylochip) showed microbial communities within a soil column from the OR-FRC were separated based on experimental phase (original sediment, U reduction and U oxidation) (Brodie *et al*., 2006). Earlier studies of the system examined in this study revealed that changes in community structure and composition were correlated with specific remediation phases [e.g. active U(VI) reduction] (Wu *et al*., 2006c; He *et al*., 2007). However, by day 746, when the current study period began, U reduction rates had decreased and U concentrations had fallen to near or below EPA drinking water standards (0.03 mg l^{-1}) and appeared stable during normal operation (intermittent ethanol injections, DO control) (Wu *et al*., 2006d). As such, changes in the functional community structure observed in this study did not correlate with specific remediation phases. In other words, the community did not separate based on whether U(VI) reduction or U(IV) oxidation was occurring. However, changes in functional genes were associated with the different operating phases of the system (increased DO, cessation of ethanol addition). Regardless, the ultimate question is not whether the communities change under various conditions, but rather whether these changes affect their function. Previous studies on this system demonstrated an active U(VI)-reducing population (Wu *et al*., 2006a; He *et al*., 2007). However, an increase in U(VI) concentration during the reoxidation period was observed, indicating a reoxidation

of previously reduced U(IV) (Fig. 1D; Wu *et al*., 2007). Once DO levels were controlled, U(VI) reduction resumed and returned to pre-oxidation levels at the end of the study period. So, while changes in the overall community occurred, the community retained the ability to reduce U(VI) and sulfate.

As mentioned above, an increase in U(VI) concentrations during the reoxidation period was observed in this system, indicating a reoxidation of previously reduced U(IV). This reoxidation could be attributed mainly to chemical mechanisms by oxygen but microbial activity cannot be ruled out (Wu *et al*., 2007). *Thiobacillus*, an Fe(II)-oxidizing bacterium that is able to oxidize U(IV) in the presence of nitrate (Beller, 2005), was detected in sediment samples from this site (Cardenas *et al*., 2008) and low levels of nitrate (0.02–0.03 mM) did enter the bioreduced area from outside. Genes from a microorganism similar to *Thiobacillus* (carbon fixation and organic contaminant degradation genes) were detected in the current study as well. Brodie and colleagues (2006) observed a decline in major/efficient metal-reducing bacteria during a U reoxidation phase using a sediment column from FRC sediment, although the authors suggested that reoxidation was not the result of changes in the microbial community in that system. In the current study, the increase in U(VI) during reoxidation corresponded with a decrease in *dsr* genes (Figs 1 and 3, and Fig. S2). However, there was not a significant correlation between *dsr* genes and U concentrations. This presumptive decrease in SRB is most likely due to inhibition by DO and lack of electron donor in the absence of ethanol injection. Previously, a significant correlation was observed between U(VI) concentrations and the amount of cytochrome genes detected ($r = 0.73$, $P < 0.05$) in the U(VI) reduction phase of this system, implying the importance of these metal-reducing microorganisms in U(VI) reduction (He *et al*., 2007). However, in this study, a decrease was not observed with cytochrome *c* genes, nor was significant correlation between U and cytochrome genes observed. He and colleagues (2007) examined this system during the active U(VI) reduction phase (days 191–713); while, in the current study, U(VI) levels had stabilized to below 0.03 mg l^{-1} and the community was simply maintaining that level of U(VI). Higher levels of U(VI) occurred in MLS wells closer to the injection well where DO levels were higher (Fig. 1D, Wu *et al*., 2007). It is not clear whether biotic or abiotic processes are most responsible for the reoxidation in the presence of DO.

Several U(VI) reducers, including *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus* and *Acidovorax* and FeRB (*Geothrix* and *Ferribacterium*), have been detected previously in the sediments from FW101-2 and FW102-3 on day 773 using 16S rRNA clone libraries (Cardenas *et al*., 2008). Hwang and colleagues (2009) reported the presence of *Geobacter* spp., *Desulfovibrio* spp. and *Acidovorax* in groundwater during active U(VI) reduction. High levels of *Geobacter lovleyi* strain SZ were detected in the groundwater at this site after biostimulation (Amos *et al*., 2007). Previous studies using microcosms suggested that SRB, especially *Desulfovibrio* spp., may be a primary microorganism reducing U(VI) (Nyman *et al*., 2006). In the current study, GeoChip detected genes from organisms similar to *Desulfovibrio* and *Geobacter*. Both were detected at all time points examined.

While there were significant changes within the community during the different operational phases, genes from the major functional gene groups, including those associated with U(VI) reduction, were observed at all time points. GeoChip results demonstrated that ethanol additions resulted in an increase in diversity and overall gene numbers detected as well as changes to the overall functional microbial community. However, while there were significant changes within the community, the community was able to recover and reduce U(VI). This suggests stability of the community's functional capabilities, which supports the sustainability of a long-term biostimulation approach for remediation of U-contaminated sites. These findings provide information on how changes in environmental conditions can affect the functional structure of microbial communities, information that is necessary to design effective remediation strategies. However, while DNA-based GeoChip analysis is able to provide information regarding the functional capabilities of microbial communities, knowledge on active communities is not provided if the size of a population does not change. Additional studies will be required using mRNA-based GeoChip hybridizations or stable isotope probing to identify active community members.

Experimental procedures

Field treatment system

The system set-up and design are described in detail elsewhere (Luo *et al*., 2006; Wu *et al*., 2006c; 2007). Briefly, the system consists of two injection, two extraction and three MLS wells in a nested design (Fig. S1). The outer loop was designed to protect the inner loop from adjacent water flow and stabilize the water flow, minimizing introduction of contaminated groundwater (Luo *et al*., 2006; Wu *et al*., 2006c). Injection of clean water (nitrate-free, treated groundwater plus tapwater or tapwater alone) to the outer loop maintained a flow field that protects the inner loop from invasion of contaminated groundwater. On day 137 (7 January 2004), after preconditioning to remove bulk nitrate and Al and increase pH to 5.7–6.1, an ethanol solution, prepared with industrial grade ethanol (88.12% ethanol, 4.65% methanol, w/w), was intermittently (2–3 days per week) injected into the inner loop injection well (FW104) at a concentration mea-

Table 3. Major geochemical concentrations at each time point examined.

Operational day	746	754	810	850	887	901	992
			FW101-2				
Sulfate (mg l^{-1})	111.5	101.4	120.1	56.0	68.8	95.2	41.4
Sulfide (mg l^{-1})	0.3	1.0	0.9	0.02	0.2	1.5	23.5
Uranium (mg l^{-1})	0.08	0.07	0.04	0.23	0.18	0.16	0.03
COD (mg l^{-1})	3	44	3	3	39	126	130
Fe(II) (mg l^{-1})	1.26	1.2	1.33	3.95	3.22	0.82	2.09
DO (mg l^{-1})	0	0	0.1	1.71	0	0	0
pH	6.00	5.98	5.83	6.00	5.97	6.25	6.69
Temperature (°C)	19.7	19.6	17.30	14.3	14.3	13.7	16.1
			FW102-3				
Sulfate (mg l^{-1})	112.5	96.3	136.2	64.4	36.5	67.4	61.5
Sulfide (mg l^{-1})	0.3	2.3	0.4	0.2	3.5	6.0	6.1
Uranium (mg l^{-1})	0.06	0.06	0.06	0.24	0.24	0.16	0.14
COD (mg l^{-1})	7	52	3	3	57	67	15
Fe(II) (mg l^{-1})	2.52	2.5	0.57	4.0	3.45	2.5	3.76
DO (mg l^{-1})	0	0	0	0.32	0	0	0
pH	6.00	5.83	5.73	5.63	5.43	5.99	5.85
Temperature (°C)	19.7	19.6	17.30	14.3	14.3	13.7	16.1

COD, chemical oxygen demand; DO, dissolved oxygen.

sured as the COD at 120–150 mg l^{-1} to provide electron donors for U(VI) reduction. The recirculation flow rates between the injection and extraction wells of both the inner and outer loops were 0.45 l min^{-1}. The injection flow rate of clean water was 0.9–1.2 l min^{-1}. DO in the aquifer is naturally low (0–0.3 mg l^{-1}), but, during the initial 638 days of operation, DO penetrated the treatment area via injection of clean water into the outer loop. To prevent DO penetration, since low DO (1–2 mg l^{-1}) was introduced into the outer recirculation loop, Na_2SO_3 (0.9 mM) was added to the clean water in the storage tank to consume DO ($Na_2SO_3 + O_2 \rightarrow Na_2SO_4$) from days 638, except for the reoxidation period (days 806–884). Groundwater samples were taken to monitor pH, COD, HCO_3^-, U(VI), sulfate, sulfide, Fe(II), nitrate and metals.

Resting state and DO reoxidation periods

From days 713 to 754, ethanol addition ceased and the system remained anoxic in order to study how a resting state affected the system. Ethanol injections were then resumed and the system was allowed to recover for 58 days. From days 811 to 884, air-saturated tap water (9–12 mg l^{-1} DO) was introduced into the outer recirculation loop, allowing an increase in DO to occur within the inner loop (Wu *et al.*, 2007). During this period ethanol injections ceased. This allowed for the examination of the effects of DO on the stability of bioreduced U(IV), and reoxidation of U(IV) in this system. Subsequent control of the DO was restored on day 884 by addition of Na_2SO_3 to the stored tap water, and ethanol was again injected to FW104 weekly.

Monitoring wells and groundwater sample collection

Two MLS wells (FW101-2 and FW102-3; 13.7 and 12.2 m below ground respectively) were selected to monitor changes in the functional structure of the microbial communities based on the results of hydrological tests using Br^- as a tracer. A full description of the hydraulic connection between the injection well (FW104) and the MLS wells is reported elsewhere (Luo *et al.*, 2006; 2007a). Both MLS are in the fast flow zone connected to the inner loop injection well FW104. Tracer tests showed that up to 90% of the Br^- was recovered from these two wells. During this test period, the mean travel time from FW104 to FW101-2 and FW102-3 was 2.8 and 3.7 h respectively (Luo *et al.*, 2007a).

Samples were taken from the two selected MLS wells on days 746, 754, 810, 850, 887, 901 and 992 (Table 3). Groundwater was pumped into sterilized glass bottles using a peristaltic pump and kept on ice until delivered to the laboratory. The groundwater (2 l) was filtered through a 0.2 μm filter to collect the biomass and the filters were stored at –80°C until ready for DNA extraction.

In this study, we examined only the microbial community accessible through groundwater sampling. Of course, there could also be microorganisms attached to the sediment or otherwise inaccessible which may be involved in the processes examined. Previous studies have shown that different environments and sample types have differing numbers of microorganisms for both active and quiescent cells (Haglund *et al.*, 2002; Reardon *et al.*, 2004). Due to the nature of our system, however, we focused on groundwater samples because they could be obtained frequently and in large volumes, and simply was not feasible for solids.

Analytical methods

The source and quality of chemicals used in the field test were described previously (Wu *et al.*, 2006c,d). Chemical oxygen demand was used as an overall indicator to monitor the consumption of electron donors (ethanol, its metabolite acetate and others). Chemical oxygen demand, sulfide and Fe(II) were determined using a Hach DR 2000 spectrophotometer (Hach Chemical). DO concentrations in the injection and extraction wells were determined directly using a HATCH Q10 DO meter while DO in the MLS wells was measured above ground by passing groundwater through a glass vial.

Anions (including NO_3^-, Br^- and SO_4^{2-}) were analysed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120). Metals (Al, Ca, Fe, Mn, Mg, U and K) were determined using an inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer ELAN 6100) as described elsewhere (Wu *et al*., 2006c). Ethanol and acetate were determined by a HP5890A gas chromatograph equipped with a flame ionization detector and an 80/120% Carbopack BDA column (Supelco Division; Sigma-Aldrich) using He as the carrier gas.

DNA extraction and amplification

Community DNA was extracted by the freeze-grinding method of Zhou and colleagues (1996). Extracted DNA was stored at –80°C until needed. Aliquots (50 ng) of DNA were amplified using the Templiphi kit (GE Healthcare) with the following modifications. Spermidine (0.1 mM) and single-stranded binding protein (267 ng μl^{-1}) were added to improve the amplification efficiency (Wu *et al*., 2006a). Reactions were scaled down to 10 μl of sample and reaction buffers and 0.6 μl of enzyme. Precautions described by Zhang and colleagues (2006) were followed to decrease the amount of background DNA amplified. Rigorous evaluation of the amplification strategy, in terms of representation, repeatability and quantification, was carried out by Wu and colleagues (2006a) and found to provide reliable, representational amplification. All samples were amplified, labelled and hybridized in triplicate. We were unable to obtain biological replicates due to the difficulties in obtaining multiple samples.

Microarray hybridization

Amplified DNA (~2 μg) was mixed with 20 μl random primers (octamers, 2.5×; Invitrogen BioPrime DNA Labelling kit), heated to 99°C for 5 min, and immediately placed on ice. The labelling master mix {8 μl [2.5 μl of dNTP (5 mM dAGC-TP, 2.5 mM dTTP), 1 μl of Cy-5 dUTP (25 nM; Amersham), 2 μl of Klenow (40 U μl^{-1}), 2.5 μl of water]} was added and the samples were incubated at 37°C for 3 h in a thermocycler. After the addition of Cy5, samples were protected from the light as much as possible. Labelled DNA was cleaned using a QIAquick purification kit (Qiagen) per the manufacturer's instructions and then dried down in a SpeedVac (45°C, 45 min; ThermoSavant).

A comprehensive 50mer FGA (GeoChip 2.0; He *et al*., 2007) was used to examine the functional gene diversity of the samples before, during and after the perturbation periods. The GeoChip contained 24 243 probes for functional genes involved in C, N and S cycling, metal reduction and resistance, and contaminant degradation (He *et al*., 2007). Microarray slides (UltraGAPS™ coated slides, Corning) were spotted with 50mer probes as described by Rhee and colleagues (2004) and cross-linked at 600 mJ (Stratagene UV Stratalinker 2400).

Samples were hybridized overnight (14–16 h) at 42°C. Labelled DNA was suspended in hybridization mix (40 μl; 50% formamide, 3× SSC, 0.3% SDS, 0.7 μg μl^{-1} Herring sperm DNA, 0.86 mM DTT) and incubated at 95°C for 5 min then maintained at 60°C until hybridization. Arrays were assembled and samples processed as described previously (Van Nostrand *et al*., 2007). After hybridization, GeoChips were imaged (ScanArray Express Microarray Scanner, Perkin Elmer) and analysed using the Imagene software (6.0 premium version, Biodiscovery). Raw data from Imagene were analysed using a GeoChip data analysis pipeline. A signal to noise ratio [SNR = (signal mean – background mean)/background standard deviation] of ≥ 1.5 was considered as a positive signal. While most studies use an SNR of ≥ 2, a recent study conducted in our lab using a mixed community culture containing genes complementary to probes on the GeoChip showed that an SNR threshold of 1.2 was required to detect all of the genes expected based on other approaches. In addition, similar relationships of overall community structure were observed for the current study using SNR values of ≥ 2 and ≥ 1.5. A positive signal in at least one-third of the probes for a particular gene (minimum of two probes) was required for a gene to be considered positive. Most of the genes had three probes on the array (He *et al*., 2005a,b; 2007).

Statistical analysis

Statistical analyses were performed using PC-ORD (Version 5.0 MjM Software; McCune and Mefford, 1999) or Canoco. Detrended correspondence analysis was performed using Canoco (Version 4.5, Biometris – Plant Research International, the Netherlands) with detrending by segments. Canonical correspondence analysis was also performed with Canoco. Environmental factors included sulfate, sulfide, U, COD, temperature, pH and Fe. Geochemical results were *z* transformed to convert all measurements to the same scale [$z = (x_i - \bar{x})/s$ where x_i is the sample value, and $\bar{x}$ is the mean of all samples; Sokal and Rohlf, 1994] prior to statistical analysis. The analysis was performed with focus on interspecies distance and significance was tested using the Monte Carlo permutation (499 permutations). Hierarchical clustering was performed in CLUSTER 3.0 using uncentered correlations and average linkage for both genes and samples and trees were visualized in TREEVIEW.

Acknowledgements

The authors especially thank Chuanmin Ruan and Kenneth Lowe for help with the analytical work. This research was supported by the US DOE under the Environmental Remediation Science Program and the Virtual Institute for Microbial Stress and Survival (http://VIMSS.lbl.gov) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics Program: GTL through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy, and by the Oklahoma Center for the Advancement of Science and Technology under Oklahoma Applied Research Support Program.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Groundwater recirculation system at OR-FRC. Arrows indicate groundwater flow. Circles with an x indicate injection (FW024 and FW104) and extraction wells (FW026 and FW103). Filled circles indicate monitoring wells (FW100, FW101 and FW102).
Fig. S2. Relative abundance of cytochrome *c*, *dsr* and metal resistance genes. The relative abundance of genes was calculated based on the total signal intensity of each gene group. The relative abundance of cytochrome *c* (A), *dsr* (B) and metal resistance (C) genes in FW101-2 (grey) and FW102-3 (black) are shown.
Fig. S3. Hierarchical cluster analysis of metal resistance genes. Genes that were present in at least five time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.
Fig. S4. Hierarchical cluster analysis of carbon degradation genes. Genes that were present in at least four time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.
Fig. S5. Hierarchical cluster analysis of methanogenesis genes. All genes were used for cluster analysis. Methanogenesis genes were only detected at time points shown in the figure. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.
Fig. S6. Hierarchical cluster analysis of denitrification genes. Genes that were present in at least four time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.
Fig. S7. Hierarchical cluster analysis of nitrogen fixation genes. Genes that were present in at least two time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.
Fig. S8. Hierarchical cluster analysis of contaminant degradation genes. Genes that were present in at least six time points were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red colouring indicates higher signal intensities.

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