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ORIGINAL ARTICLE

Responses of microbial community functional structures to pilot-scale uranium *in situ* bioremediation

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A pilot-scale field test system with an inner loop nested within an outer loop was constructed for *in situ* U(VI) bioremediation at a US Department of Energy site, Oak Ridge, TN. The outer loop was used for hydrological protection of the inner loop where ethanol was injected for biostimulation of microorganisms for U(VI) reduction/immobilization. After 2 years of biostimulation with ethanol, U(VI) levels were reduced to below drinking water standard ($<30\,\mu g\,l^{-1}$) in the inner loop monitoring wells. To elucidate the microbial community structure and functions under *in situ* uranium bioremediation conditions, we used a comprehensive functional gene array (GeoChip) to examine the microbial functional gene composition of the sediment samples collected from both inner and outer loop wells. Our study results showed that distinct microbial communities were established in the inner loop wells. Also, higher microbial functional gene number, diversity and abundance were observed in the inner loop wells than the outer loop wells. In addition, metal-reducing bacteria, such as *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter* and *Shewanella*, and other bacteria, for example, *Rhodopseudomonas* and *Pseudomonas*, are highly abundant in the inner loop wells. Finally, the richness and abundance of microbial functional genes were highly correlated with the mean travel time of groundwater from the inner loop injection well, pH and sulfate concentration in groundwater. These results suggest that the indigenous microbial communities can be successfully stimulated for U bioremediation in the groundwater ecosystem, and their structure and performance can be manipulated or optimized by adjusting geochemical and hydrological conditions.
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Introduction

Uranium produced by mining and enrichment activities during the Cold War is a major soil and groundwater contaminant at US Department of Energy sites. In many instances, uranium in contaminated groundwater is in the U(VI) form, which is highly soluble and mobile in the subsurface environments. Under the appropriate conditions, the highly soluble U(VI) can be reduced to insoluble U(IV) and precipitated as mineral uranium by biotic and/or abiotic reactions (Hazen and Tabak, 2005; Tabak *et al.*, 2005). A promising strategy for preventing the spread of subsurface uranium contamination is by U(VI) bioreduction and immobilization (Lovley *et al.*, 1991; Bender *et al.*, 2000; Istok *et al.*, 2004; Wu *et al.*, 2007). In recent years, different scales of U(VI) bioreduction/immobilization have been tested (Finneran *et al.*, 2002; Holmes *et al.*, 2002; Anderson *et al.*, 2003; Istok *et al.*, 2004; Gu *et al.*, 2005; Wu *et al.*, 2006c, d), and a wide

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phylogenetic diversity of microorganisms have been found to be capable of reducing U(VI) and other metals in pure and mixed cultures (Lovley, 1991; Wall and Krumholz, 2006).

Following addition of an electron donor such as ethanol or acetate, U(VI) reduction is dependent on subsequent microbial activity and appropriate geochemical conditions. Various microorganisms may be important in uranium bioremediation through direct enzymatic reactions and/or indirect chemical reductions (Wu *et al.*, 2007). The reduction of U(VI) to U(IV) typically coincides with an increase in populations of Fe(III)-reducing and sulfate-reducing bacteria (SRB) (Holmes *et al.*, 2002; Wall and Krumholz, 2006; Cardenas *et al.*, 2008). However, U(VI) reduction is transient and the maintenance of microbial populations capable of U(VI) reduction is one of the key issues for a long-term reduction and stabilization of uranium *in situ* (Anderson *et al.*, 2003). During *in situ* bioremediation, the delivery of electron donor and the resulting reduction/oxidation reactions are also related to subsurface hydrology. Therefore, the impact of hydrological parameters on microbial populations and the U(VI) reduction process should be considered (Luo *et al.*, 2005, 2007). To date, most of the efforts to describe microbial communities during the remediation of uranium have been focused on phylogenetic composition (Nevin *et al.*, 2003; Brodie *et al.*, 2006; Akob *et al.*, 2007, 2008). Little research has been undertaken to determine the microbial community functional structure and its possible relationship to hydrogeochemical parameters during the *in situ* bioremediation of uranium.

One of the major challenges for linking the microbial community structure to ecosystem functioning is the extreme diversity and as-yet uncultivated status of many microorganisms. Functional gene arrays, which contain the genes encoding key enzymes involved in biogeochemical cycling processes, have been used to overcome such obstacles for studying microbial communities (Zhou, 2003). GeoChip 2.0, which covers more than 10 000 genes involved in critical microbially mediated biogeochemical processes, has been successfully used for tracking and studying the biogeochemical processes in different ecosystems including the dynamics of metal-reducing bacteria during *in situ* bioremediation in contaminated groundwater (He *et al.*, 2007; Van Nostrand *et al.*, 2009; Waldron *et al.*, 2009). Combined with multivariate statistical analyses (Ramette, 2007), several systematic experimental evaluations have indicated that GeoChip can be used as a specific, sensitive tool for detecting microbial populations and functional processes in environmental settings (Wu *et al.*, 2001; Rhee *et al.*, 2004; Gentry *et al.*, 2006; Zhou *et al.* 2008; Wang *et al.* 2009).

A pilot-scale field test for *in situ* bioremediation of U(VI) has been conducted in Area 3 of the Department of Energy's Oak Ridge site (Wu *et al.*, 2006c, d, 2007). After 2 years of bioremediation by intermittent injection of ethanol into the inner loop, U(VI) concentrations in the groundwater decreased from the initial concentration of 50 mg l^{-1} to below the US Environmental Protection Agency maximum contaminant level for drinking water ($<30\,\mu g\,l^{-1}$), and a new microbial community had been established in both sediments and groundwater (Wu *et al.*, 2007; Cardenas *et al.*, 2008; Hwang *et al.*, 2009). The objective of this study was to characterize the functional structure of the sediment microbial communities at the Oak Ridge site after 2 years of successful bioremediation using GeoChip-based metagenomic technologies.

Materials and methods

Experimental design and sampling procedures

The pilot-scale treatment system for uranium bioremediation was located in Area 3 at the Integrated Field Research Site field site adjacent to the former S-3 Pond at the Y-12 National Security Complex, Oak Ridge, TN. This test system was composed of an outer loop for hydraulic protection and an inner loop for bioreduction by injecting ethanol with three multilevel sampling (MLS) wells for routine monitoring (Supplementary Figure 1). The full description of the hydraulic connection between the wells is reported elsewhere (Luo *et al.*, 2006, 2007). Biostimulation with ethanol was started on 7 January 2004 (day 137) and continued by injection of ethanol (1.07–1.34 mM) at a flow rate 0.45 l min^{-1} into FW104 over a 48 h period each week (Wu *et al.*, 2006d). After 2 years of treatment, aqueous U concentrations fell below the US Environmental Protection Agency maximum contaminant level ($<30\,\mu g\,l^{-1}$; Wu *et al.*, 2007). On day 774, the total amount of ethanol injected was 4100 g, and sediment samples were collected from 11 wells by the surge block method as previously described (Wu *et al.*, 2006d), including outer injection well (FW024), outer extraction well (FW103), inner injection well (FW104), inner extraction well (FW026) and seven MLS wells in different depths below ground surface (bgs) with three outer loop monitoring wells: FW100-2 (13.7 m bgs), FW100-3 (12.19 m bgs) and FW100-4 (10.67 m bgs) and four inner loop monitoring wells: FW101-2 (13.7 m bgs), FW101-3 (12.19 m bgs), FW102-2 (13.7 m bgs) and FW102-3 (12.19 m bgs). After sediment sampling, we characterized the hydrology of the treatment zone by injecting a conservative tracer (sodium bromide) together with ethanol and monitoring the mean travel time (MT) and bromide recovery (BR) as described by Luo *et al.* (2007).

Analytical methods

Previous reports (Wu *et al.*, 2006c, d, 2007) have given detailed descriptions about the source and

quality of chemicals used at the field site, methods used to measure sulfate, sulfide, nitrate-N, cations (Fe, Mn, U and so on), ethanol and acetate, the use of a kinetic phosphorescence KPA-11 analyzer for U analysis (Chemchek Instruments, Richland, WA, USA), and groundwater and sediment sample collection.

DNA extraction, amplification and labeling

Sediment DNA was extracted by freeze-grinding mechanical lysis and purified by Wizard DNA Clean-up System (Promega, Madison, WI, USA) as described previously (Zhou *et al.*, 1996). Rolling circle amplification of 50 ng purified DNA was carried out using the TempliPhi kit (GE Healthcare, Piscataway, NJ, USA) and the products were labeled with cyanine-5 using random priming with modified protocols described by Wu *et al.* (2006a). Labeled DNA was purified using QIA Quick Purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, measured on a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and then dried down in a SpeedVac (ThermoSavant, Milford, MA, USA) at 45 °C for 45 min.

Microarray hybridization

A 50mer functional gene array, also called GeoChip 2.0 (He *et al.*, 2007) was used to dissect the functional structure of the sediment microbial communities from different wells. All hybridizations were carried out in triplicate at 45 °C with 50% formamide for 10 h after a 45 min prehybridization with a prehybridization solution (5 × SSC, 0.1% SDS and 0.1% bovine serum albumin) as described by Waldron *et al.* (2009). Following hybridization, the slides were then washed three times at 45 °C for 1 min and one time at room temperature (RT) for 30 s with a 1.5 min soaking using wash buffer I (1 × SSC, 0.1% SDS), one time at RT for 1 min with a 1.5 min soaking using wash buffer II (0.1 × SSC, 0.1% SDS), four times at RT for 1 min using wash buffer III (0.1 × SSC), one time at RT for 1 min using water and then dried at RT under a slow stream of nitrogen gas. All prehybridization, hybridization and washing processes were performed using a HS4800 Hybridization Station (TECAN US, Durham, NC, USA). After hybridization, the arrays were imaged by ScanArray Express Microarray Scanner (PerkinElmer, Boston, MA, USA) and analyzed using ImaGene version 6.0 (BioDiscovery, El Segundo, CA, USA). Raw data output from ImaGene was submitted to Microarray Data Manager in our website (http://ieg.ou.edu/microarray/) and was analyzed using a GeoChip 2.0 data analysis pipeline. A signal to noise ratio of $\geqslant$1.5 was considered as a positive signal. For the GeoChip 2.0 design, in most cases three probes target the same gene, or the same group of highly similar genes with few targets only having one or two probes (He *et al.*, 2007). Because three replicates (up to nine data points) were conducted for each sample in this study, at least 0.34 time of the final positive spot (probe) number (minimum of two spots) was required for each detected gene.

Data analysis

Data normalization was based on the mean signal intensity between replicates. After normalization, each hybridization/sample had a total of signal intensities from all detected probes. Total abundance of each sample scored as present was the sum of the normalized intensity of the sample on the microarray. To allow comparison across experimental samples, we calculated relative abundance values for each gene category by dividing the total normalized intensity of a certain gene category by the sum of the normalized intensity of the gene categories detected for the sample. Each probe on the GeoChip was mapped to its target, which is from a cultured or uncultured microorganism/taxa. If an organism had multiple probes, the average signal intensity was taken. The abundance for a specific taxa (for example, species, family, order, class) is the sum of signals from different organisms detected in this taxa. For comparing a particular functional gene from a certain organism in different samples, we calculated the average abundance of this gene by dividing the sum of normalized intensity of this gene by the number of detected gene sequences for this organism.

Principal component analysis (PCA), redundancy analysis (RDA) and variation partitioning analysis (Ramette and Tiedje, 2007) were performed with the package CANOCO 4.5 (Biometris/Plant Research International, Wageningen, The Netherlands) using functional gene communities and environmental parameters for each sediment sample as covariables and significance was tested by a Monte Carlo permutation test based on 999 random permutations. To identify patterns of variation among functional gene communities, we normalized environmental variables by subtraction of the mean and division by standard deviation before performing multivariable analyses. Significant Pearson's linear correlation (r) analysis was conducted in SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Correlations were considered significant at a $P<0.05$ baseline and considered to indicate a strong trend at a $P<0.10$ baseline. Hierarchical clustering was performed in Cluster 3.0 using the pairwise complete-linkage hierarchical clustering algorithm and trees were visualized using TreeView.

Results

Microbial functional gene diversities

After two 2 years of biostimulation, low levels of uranium ($<30\,\mu g\,l^{-1}$) were achieved and maintained

in the inner MLS located at the fast flow zone of the test system (Wu *et al.*, 2007; Cardenas *et al.*, 2008). The major geochemical parameters related to microbial activities on day 774 when the sediment samples were collected from these wells are presented in Table 1. Sulfide was detected in both groundwater and sediment in the inner loop due to SRB activities. The sediments from the inner loop wells were reduced with U(IV) (except for FW026) and higher Fe(II) content, but those in the outer loop were less reduced. An ordination plot constructed on the basis of the geochemical parameters separated the inner loop samples from the outer loop samples as two main clusters (Supplementary Figure S2). The microbial community compositions of the 11 sediment samples were analyzed with the GeoChip 2.0. More than 2350 genes in 138 gene families showed positive hybridization signals. Overall, the gene numbers and signal intensities detected revealed significant differences between the inner loop and outer loop wells. Most of the samples from the inner loop contained higher gene numbers and signal intensities than those from the outer loop. For instance, the number of genes with statistically significant positive signals in the outer loop injection well FW024 and extraction well FW103 was 25 and 123, whereas the number detected in the inner loop injection well FW104 and extraction well FW026 was 393 and 227, respectively. Furthermore, the overall genetic diversity detected in each of the sediment samples suggested that bioremediation treatment had strong effects on the microbial communities. Both Simpson's diversity index (*1/D*) and Shannon–Weaver index (*H′*) indicated that the levels of genetic diversity in the outer loop wells were much lower than those in the biostimulated inner loop wells. The *1/D* in the inner loop injection well FW104 and extraction well FW026 was 582.7 and 413.6, whereas the *1/D* in the outer loop injection well FW024 and extraction well FW103 was only 125.5 and 216.2, respectively.

The proportion of overlapping genes in different samples was consistent with the bioremediation treatment. For the inner loop injection well FW104, only 2.2% and 11.2% of the genes detected shared with the outer loop injection well FW024 and extraction well FW103, respectively, whereas higher proportions were shared with the genes from inner loop wells. These results suggested that microbial communities were effectively stimulated by the addition of electron donor, and similar community compositions were constructed within the same treatment loop.

Cluster analysis with the all microarray hybridization data indicated that 11 wells were separated into two broad groups (inner loop and outer loop) (Figure 1a). Five major gene groups can be visualized (Figure 1b), which seemed to well correlate with the location of the wells. In groups 1, 2 and 4, the average signal intensities in the samples from the outer loop wells were much higher than those

Table 1 Major geochemical properties of the treatment area after bioremediation (5 October 2005)

Location		*Outer loop wells*					*Inner loop wells*					r[a]	P[b]
Parameters	*Abbreviation*	*024*	*103*	*100-2*	*100-3*	*100-4*	*104*	*026*	*101-2*	*101-3*	*102-2*	*102-3*		
Groundwater														
pH	pH	5.87	5.92	5.50	5.87	5.80	5.75	5.74	6.23	6.10	6.45	6.23	**0.752**	**0.008**
Sulfate (mg l^{-1})	G-sulfate	151.8	145.3	148.1	139.3	138.6	113.0	114.9	102.7	114.9	96.6	105.8	**−0.963**	**<0.001**
Sulfide (mg l^{-1})	G-sulfide	0.016	0.29	0	0	0	9.6	1.18	13.8	3.46	5.17	11.43	**0.874**	**<0.001**
Nitrate-N (mg l^{-1})	G-nitrate	2.1	1.37	3.36	4.48	313.6	0	1.37	0	0.59	0.70	0.14	−0.313	0.349
U(VI) (mg l^{-1})	G-U(VI)	0.022	0.047	0.233	0.078	0.358	0.136	0.125	0.036	0.026	0.020	0.014	−0.468	0.146
Fe (mg l^{-1})	G-Fe	0.16	0.21	0.14	0.15	0.18	1.74	0.34	1.9	0.29	2.2	2.5	**0.910**	**<0.001**
Mn (mg l^{-1})	G-Mn	0.75	1.18	2.57	1.48	4.51	3.98	3.93	4.05	3.45	4.26	4.56	**0.710**	**0.014**
Sediment														
U(IV)/U	S-U(IV)/U	0	0	0	0	0	61	0	54	51	17	30	**0.743**	**0.009**
U(VI) (mg kg^{-1})	S-U(VI)	371	658	980	1098	1501	10300	1220	574	894	429	612	0.049	0.886
Total Fe (g kg^{-1})	S-Fe	50.8	44.9	32.4	36.2	34.4	199.1	47.1	43.3	29.4	33.7	36.4	0.182	0.592
Fe(II) (g kg^{-1})	S-Fe(II)	11.13	0.23	4.10	2.30	1.87	105.5	6.97	11.86	8.64	10.41	10.45	0.309	0.356
Sulfide (g kg^{-1})	S-sulfide	0.07	0	0	0	0	23	0.034	0.67	0.27	0.56	0.43	0.245	0.468
Trace test[c]														
Mean travel time (h)	MT	7.8	7.8	223	43	211	0	8.1	2.9	17.9	11.6	3.7	−0.507	0.111
Bromide recovery (%)	BR	4.5	10	12	18	8	100	50	93	60	94	94	**0.901**	**<0.001**

Abbreviations: BR, bromide recovery; MT, mean travel time.
[a]Pearson's linear correlation between PC1 scores and geochemical parameters.
[b]*P*-value for two-tailed significance test, bold values are statistically significant ($P<0.05$).
[c]MT is the mean travel time between injection well and monitoring well. High bromide recovery indicated better hydraulic connection to inner loop injection well.

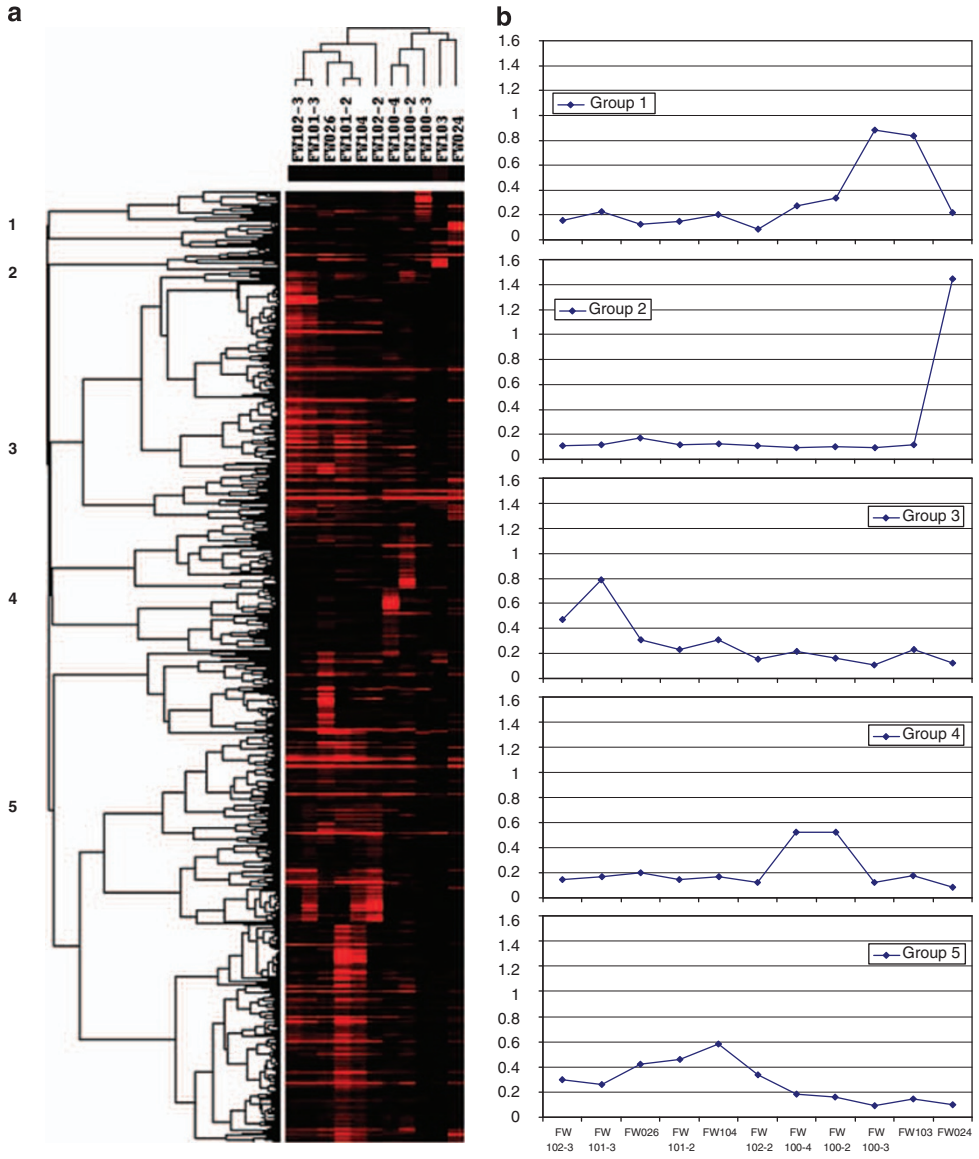

Figure 1 Cluster analysis of all genes detected by GeoChip 2.0. The figure was generated using Cluster and visualized in TreeView. Black indicates signal intensities below the threshold value and red indicates a positive hybridization signal. The color intensity indicates differences in signal intensity. Eleven samples were clearly separated into two groups: outer loop and inner loop. Five gene patterns were observed and indicated by numbers in the tree (**a**), and also illustrated in the graphs (**b**).

from the inner loop wells, whereas in groups 3 and 5 the samples with high signal intensities were all from the inner loop wells. Functional genes for organic contaminant degradation and metal resistance were the main groups, especially in groups 3 and 5. The biggest group, group 5, was clustered into two subgroups, which were mostly contributed by the samples from inner loop injection well (FW104) and extraction well (FW026), respectively. Metal resistance genes were more enriched in FW104, such as for arsenic resistance (*arsB*) (gi.3095051), cadmium, zinc and cobalt resistance (gi.1749680), and chromate transport (gi.23012809). In addition, high average signal intensities were also detected from several important U(VI)-reducing bacteria (for example, *Anaeromyxobacter* sp., *Desulfovibrio* sp., *Geobacter* sp., *Shewanella* sp.) in sample FW104.

Principal component analysis was used to examine overall patterns of variation among functional gene communities of these 11 sediment samples. Similar to the PCA results based on geochemical data, the inner loop microbial communities are well separated from the outer loop microbial communities along PC1, which explained 25.8% of the total variance (Figure 2). These results suggested that the microbial community functional structures were significantly altered in the inner loop by bioremediation efforts.

Characteristics of individual functional gene groups relevant to U(VI) reduction

Overall, the relative abundances of different gene categories in the inner loop samples were more consistent than those in the outer loop samples (Figure 3). Total signal intensities of the functional gene categories in the inner loop were much higher than the outer loop, including *dsr* genes, cytochrome *c* genes, metal resistance genes, denitrification genes and organic remediation genes. For example, the organic remediation genes were the most abundant genes detected among all samples, ranging from 33.9% to 35.6% in the inner loop samples and 28.9–36.2% in the outer loop samples, respectively. The abundance of the genes involved in metal resistance varied from 11.8% to 15.5% in the inner loop samples and 9.6–15.3% in the outer loop samples, respectively.

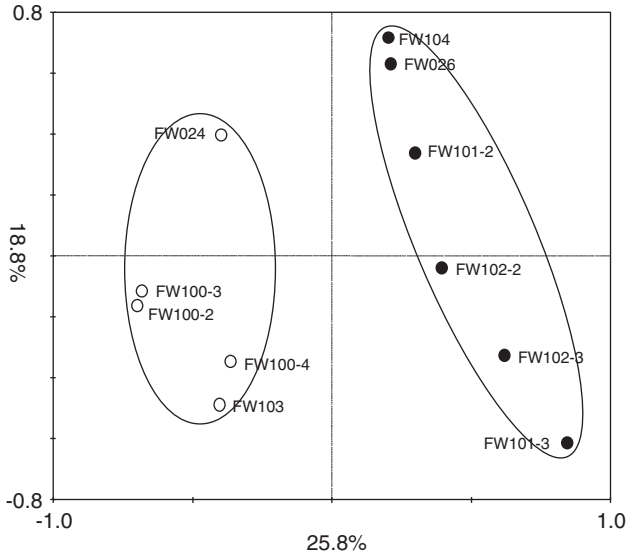

Figure 2 Ordination plot produced from principal component analysis (PCA) of all of genes detected by GeoChip 2.0. Open circles represent samples collected from five outer loop wells and solid circles represent samples collected from six inner loop wells.

Most of the genes involved in aromatic and chlorinated compound (TCE) degradation were abundant in the inner loop wells (Supplementary Figure S3A). The benzoyl-CoA reductase gene from *Rhodopseudomonas palustris* (gi.2190579) was abundant across most samples (Supplementary Figure S3A). For sulfate reduction genes, the samples from the inner loop and the outer loop were also well separated along PC1 axis, which explained 32.7% of the total variance (data not shown). Most of *dsrA/B* genes were dominated in inner loop injection well (FW104), extraction well (FW026), MLS wells FW101-2 and FW102-2. A positive correlation ($r = 0.567$, $P = 0.069$) was observed between signal intensities of SRB detected and uranium concentrations in sediments. Based on hierarchical cluster analysis, 75% of the *dsr* genes detected by GeoChip were based on the probes from environmental libraries and most of them were originally found at the Integrated Field Research Site (Supplementary Figure S3B). The Integrated Field Research Site groundwater clone TPB16009B

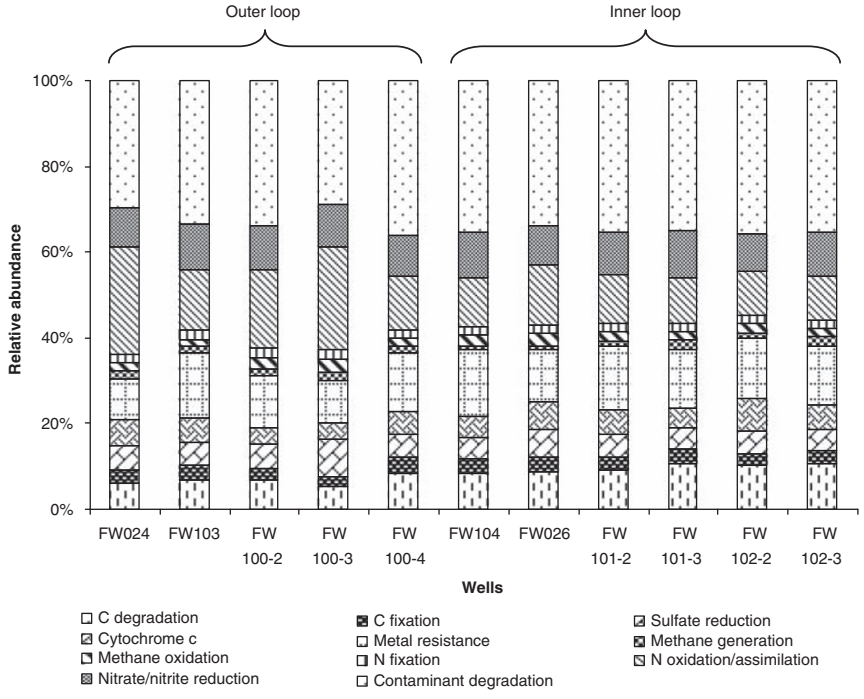

Figure 3 Relative abundance of all functional gene groups. The total signal intensity of genes detected for different wells was used to calculate the relative abundance of each gene group. The patterns represent the different gene categories detected.

was abundant in all samples except the outer loop injection well (FW024). These results also indicated that the GeoChip-based detection is reliable even though the detected number of genes is lower in some samples.

Over all sampling wells, the genes detected by GeoChip were based on the probes from 234 microbial genera. Microorganisms from or similar to α-proteobacteria (*Rhodopseudomonas*), γ-proteobacteria (*Pseudomonas* and *Shewanella*) and δ-proteobacteria (*Anaeromyxobacter*, *Desulfovibrio* and *Geobacter*) were detected in all samples. Interestingly, most of these microorganisms are capable of U(VI) reduction (Wall and Krumholz, 2006; Wu *et al.*, 2006d; Amos *et al.*, 2007) and those from δ-proteobacteria have also been detected from the samples using 16S rRNA clone libraries (Cardenas *et al.*, 2008). However, a significant change was observed in the average intensities of these microorganisms based on the number of cytochrome *c* genes detected (Figure 4). The average signal intensities of these microorganisms detected from inner loop wells were much higher than those from outer loop wells. Comparing the genera detected from different wells, we observed less than 30% overlap between the inner loop injection well FW104 and the outer loop injection well FW024, whereas more than 61% overlap was detected between the injection well FW104 and exaction well FW026 in the inner loop. Much higher signal intensity derived from uncultivated bacteria was detected from FW104 than FW024. More than 10 times higher total intensities of these populations were detected from inner loop injection and extraction wells than those from outer loop where electron donor was limited.

Relationship of microbial community functions and hydrogeochemical parameters

The difference in microbial community structure among the different wells could be influenced by hydrogeochemical parameters. Six hydrogeochemical parameters, groundwater sulfate concentration (G-sulfate), groundwater nitrate concentration (G-nitrate), pH, sediment Fe(II) content [S-Fe(II)], MT and BR (Table 1), were selected based on a forward selection procedure and variance inflation factors with 999 Monte Carlo permutations. The forward selection for RDA models provided the evaluation of the six parameters with the following order from the most to the least explicative variable of the microbial functional gene data: G-sulfate > pH > MT > S-Fe(II) > G-nitrate > BR. The variance inflation factors of these six parameters were all less than 20.

The relationship between the functional gene communities and the six parameters in the RDA ordination plot (Figure 5) was very consistent with the PCA ordination patterns. The first axis of the RDA explained 35.0% of the variation in functional gene communities, and is positively correlated with the samples from the outer loop wells and negatively correlated with the samples from the inner loop wells. Interestingly, the injection well from

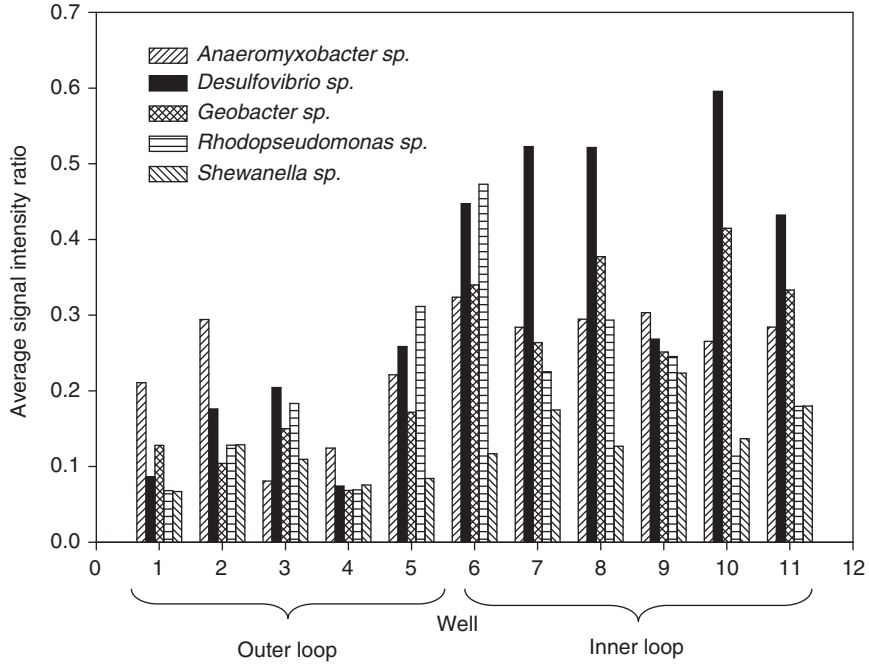

Figure 4 Relative abundance of cytochrome *c* genes detected from different microorganisms. The relative abundance of genes was calculated from different samples based on the average signal intensity of each microorganism. The patterns represent the different gene categories detected. 1, FW024; 2, FW103; 3, FW100-2; 4, FW100-3; 5, FW100-4; 6, FW104; 7, FW026; 8, FW101-2; 9, FW101-3; 10, FW102-2; 11, FW102-3.

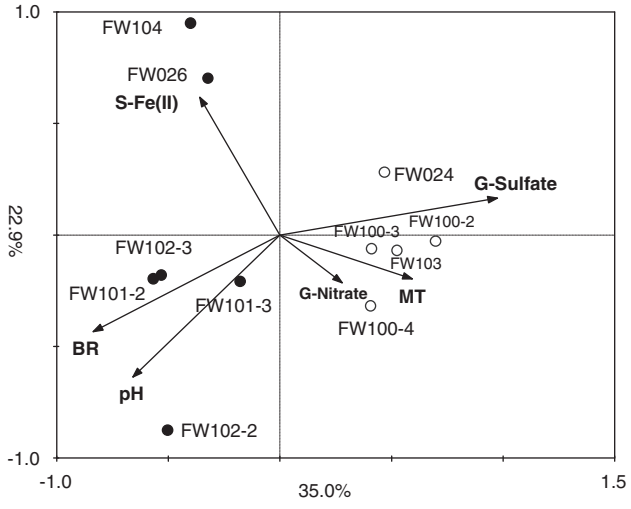

Figure 5 Biplot of redundancy analysis of entire functional gene communities of sediment samples from different wells on day 773. Open circles represent samples collected from five inner loop wells, whereas solid circles represent samples collected from six outer loop wells. Descriptors (arrows) are the concentration of three geochemical parameters (sulfate, pH and nitrate) in groundwater, mean travel time (MT), bromide recovery (BR) in the subsurface and Fe(II) content in sediment [S-Fe(II)].

inner loop (FW104) grouped closely with its extraction well (FW026), and the injection and extraction wells from outer loop (FW024 and FW103) grouped together in the RDA profile.

These six hydrogeochemical parameters explained 65.8% of the total variance. Within these six parameters, G-sulfate appeared to be the most important environmental parameter, which significantly explained 23% ($P=0.001$) of the variance. Among these six parameters, significant correlations were observed between G-sulfate and BR ($r=-0.868$, $P=0.001$), pH and BR ($r=0.829$, $P=0.002$), G-sulfate and pH ($r=-0.707$, $P=0.015$) and G-nitrate and MT ($r=0.647$, $P=0.031$).

To better understand how much each environmental variable influences the functional community structure, we performed variation partitioning analysis. Among the three important environmental parameters, G-sulfate, pH and MT, no significant correlations were observed between G-sulfate and MT ($r=0.499$, $P=0.118$); negative correlation was observed between G-sulfate and pH ($r=-0.707$, $P=0.015$), and pH and MT ($r=-0.560$, $P=0.073$). A total of 48.1% variations of microbial communities can be explained by G-sulfate, pH and MT, as well as by their interactions. G-sulfate was able to independently explain 17.1% of the variation observed whereas pH explained 14.2% and MT explained 12.4%. Interactions between the three variables appeared to have more influence in this system than individual environmental variables. These results suggest that pH, the concentration of sulfate and the hydraulic flow of groundwater appeared to be key factors in shaping microbial community functional structures in this system.

Discussion

Microbially mediated reduction of highly soluble uranium (VI) to insoluble uranium (IV) is a promising strategy for the potential remediation of uranium-contaminated groundwater. In this test system, a nested-well groundwater recirculation facility was used to achieve hydraulic control and a series of conditioning steps were accomplished to create a new microbial community for uranium bioremediation (Wu *et al.*, 2006c, d). After 2 years of performance, a low uranium level ($<0.30\,\mu g\,l^{-1}$) was achieved and maintained stably under anaerobic conditions (Wu *et al.*, 2007). This was the first demonstration that high-level uranium-contaminated groundwater can be successfully bioremediated *in situ* to the level below the maximum contaminant level.

Knowledge of microbial community structure and their functions in relation to environmental conditions is important for designing a successful bioredmediation strategy (Lovley, 2003). Microbial communities can be stimulated to be more effective in U(VI) reduction in response to ethanol injection. It is expected that those stimulated populations are able to grow with ethanol or/and use ethanol or its derived products as electron donors. In this case, distinct microbial communities could be formed between inner loop and outer loop wells due to the differences of ethanol and intermediate (acetate) concentrations. This is supported by our GeoChip data. First, high diversity and abundance of microbial populations and functional genes were observed in the inner loop wells, especially for microorganisms/genes involved in metal reduction/resistance and organic remediation. Also, although most of the known U(VI)-reducing bacteria were found in both inner and outer loop wells, little overlap in the U(VI)-reducing microbial communities was observed between those two zones. In addition, more than 10 times higher total signal intensity was detected from U(VI)-reducing bacteria in the inner loop injection well FW104 than in outer loop injection well FW024. These results indicated that microbial communities could be critical in reducing U to very low concentrations.

As distinct microbial communities were observed between the inner and outer loop well, it is expected that some microbial populations related to U(VI) reduction were stimulated or enriched in the inner loop wells. Indeed, we observed that the U(VI)-reducing bacteria, *Desulfovibrio* spp. (Lovley and Phillips, 1992; Payne *et al.*, 2002), were enriched in the inner loop. Also, in recent years, many studies have indicated that *Anaeromyxobacter* spp. are involved in U(VI) reduction in contaminated subsurface environments (North *et al.*, 2004; Wu *et al.*, 2006b;

Sanford *et al.*, 2007; Akob *et al.*, 2008; Cardenas *et al.*, 2008). In addition, although there is very little information about the metal reduction of the versatile photosynthetic bacterium *Rhodopseudomonas* spp., the complete genome sequence of *R. palustris* indicated that this strain contains several cytochrome *c* genes (Larimer *et al.*, 2004), which were detected frequently in the inner loop wells by GeoChip. Finally, the high proportion of *Pseudomonas* spp. genes detected could be due to the presence of other carbon sources such as aromatic, chlorinated compounds, which were detected in the groundwater before biostimulation (Wu *et al.*, 2006c) as well as degradation of dead biomass grown on ethanol. They could be important *in situ* U bioremediation by maintaining environments favoring U(VI) reduction. Those stimulated key microbial populations may be important directly or indirectly in U(VI) reduction and maintenance of a low uranium concentration in this system.

Microbial community structures and functions are affected by many environmental factors, such as electron donors and acceptors, concentrations of chemical compounds, environmental pH, and hydrological conditions in aqueous systems. It has been reported that the reduction of aqueous U(VI) can be enhanced by the presence of aqueous sulfate at laboratory, pilot or field scales (Spear *et al.*, 2000; Wu *et al.*, 2006d). Nyman *et al.* (2007) found that sulfate was required for the growth of U(VI)-reducing bacteria, and *Desulfovibrio*-like species were predominant organisms in U(VI)-reducing enrichments from U(VI)-contaminated sediment. Similar to our previous studies (Wu *et al.*, 2006a; He *et al.*, 2007), the signal intensities of many *dsrA/B* containing sulfate-reducing populations previously recovered from this site showed significant correlations with the bioremediation treatment. A high abundance of cytochrome *c* genes was also obtained from the important SRB, *Desulfovibrio* spp. The relative abundance of *Desulfovibrio* spp. from inner loop wells, where ethanol was added as an electron donor, was substantially higher than those from outer loop wells. Sulfate level could significantly explain 17.1% ($P = 0.028$) of the microbial community variations (Figure 5). It is highly possible that sulfate in the treated area supported the growth of U(VI)-reducing SRB and facilitated U(VI) reduction.

In addition to amendment with electron donor, pH adjustment is another key strategy for U(VI) reduction *in situ*. U(VI) adsorption is highly pH dependent and a slight pH change near the optimal pH (6.0) for U(VI) adsorption could cause a relatively large change in the U(VI) concentration (Bostick *et al.*, 2002; Liu *et al.*, 2005; Wu *et al.*, 2006d). On the other hand, pH has a key role in shaping microbial diversity and activity. During our field test, a bicarbonate buffer was used to raise pH in subsurface and higher pH (5.7–6.5) was obtained in the inner loop (Wu *et al.*, 2006d, 2007). Consistently, higher microbial diversity and functional gene abundance were obtained from inner loop wells (Figures 1, 3 and 4). RDA results also showed that pH was an important hydrogeochemical factor ($P = 0.017$) for the microbial community functional gene structures.

Although the geochemical factors for U(VI) bioremediation have been widely studied, little is known about the influences of hydrological factors. The hydrological condition during the bioremediation process influences fluid transport through the subsurface and the delivery or availability of electron donor, nutrients as well as migration of microorganisms. A high hydraulic connection indicated by a high BR in this study is essential to ensure delivering chemicals to the target (contaminated area), and a short MT means that electron donor is consumed less when it reaches the target area. A significant influence of the MT on the microbial functional gene structures was found in this study. The highest microbial diversity, gene number and community overlap between injection and extraction wells were measured for the MLS well FW101-2 that had the shortest MT and highest BR. Similarly, higher microbial diversity and gene number were also observed in outer loop extraction well FW103, where a small fraction of electron donor escaped from the inner loop, than in the injection well FW024 where clean water was injected.

In conclusion, the results of our study showed that a low level of aqueous uranium could be maintained under anaerobic conditions, and that U(VI)-reducing bacteria likely have a key role. U(VI) reduction can be achieved under controlled hydrogeochemical conditions, and the key factors to stimulate the U(VI)-reducing microbial community are related to the delivery of electron donor and hydrological conditions. The available electron acceptors, such as sulfate and nitrate, influence the microbial community structure. This study confirmed that the establishment of microbial community function was strongly correlated with geochemical conditions (such as sulfate level and pH) and hydraulic flow condition (which influences available electron donor) in the treatment zone. These findings allow us to better understand the linkage between microbial community structure and functions in the groundwater ecosystems, and provide relevant insights on the microbial role in *in situ* U bioremediation.

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