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ANALYSIS OF MICROBIAL COMMUNITIES IN A CONTAMINATED AQUIFER UNDERGOING URANIUM BIOREMEDIATION

By

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ABSTRACT

ANALYSIS OF MICROBIAL COMMUNITIES IN A CONTAMINATED AQUIFER UNDERGOING URANIUM BIOREMEDIATION

By

Erick Cardenas Poire

Contamination by metal and radionuclides is a problem for the Y-12 National Security Complex in Oak Ridge, Tennessee, and at other Department of Energy (DOE) facilities and waste sites. In contrast to organic compounds, metals cannot be degraded so their bioremediation may be possible by controlling their bioavailability. Iron-reducing bacteria such as *Geobacter* and sulfate-reducing bacteria such as *Desulfovibrio,* among others, are known to utilize metals and radionuclides as terminal electron acceptors. Uranium (VI) reduction decreases its solubility therefore its movement in water can be controlled.

In this work, microbial communities of a uranium and nitrate contaminated aquifer undergoing bioreduction, and located at the Field Research Center (FRC) Area 3 at Oak Ridge, Tennessee, were analyzed through comparative 16S rRNA gene analysis using both traditional and pyrosequencing of clone libraries. Clone libraries of this gene revealed sequences belonging to genera known to contain uranium reducers and whose closest relatives are known to reduce U(VI) and utilize either ethanol, the carbon source injected as electron donor; acetate, a by-product of ethanol oxidation; or methanol, an impurity of the injected ethanol solution. The genera detected in clone libraries known to reduce uranium included *Desulfovibrio*, *Geobacter*, *Acidovorax, Anaeromyxobacter*, and

Desulfosporosinus, among others. Hydrologic characteristics helped to explain the higher levels of activity of sulfate reducers (SRB), iron reducers (FRB), and nitrate reducers in certain zones of the site.

Pyrosequencing, a novel tool for microbial ecology studies, was used in the analysis of the microbial communities of the treatment location in the FRC Area 3. Differences in community structure along the bioremediation zone were related mostly to chemical oxygen demand, an indication on how much of the stimulatory carbon source reached a sampled well. Sequences belonging to genera with U(VI)-reducing ability were detected throughout the Area 3. The genera detected were the same as in the clone libraries but the increased sampling depth revealed previously undetected *Shewanella* and *Deinococcus* sequences.

Diversity-based clustering showed four clusters of sequences that were related to their bioactivity (SRB, FRB, and denitrifiers levels). Indicator species analysis in addition to the clustering showed several groups that were significantly higher in abundance in the most active uranium reducing zones. The most significant indicator species were related to *Desulfovibrio, Desulfosporosinus,* and *Anaeromyxobacter.* Based on indicator species analysis, patterns of abundance, and metabolic ability of their closest relatives, sulfate-reducing bacteria, especially *Desulfovibrio,* appear to play the most important role in uranium reduction at this location at the time of the sampling.

This thesis is dedicated to my family for their support,

To my grandparents, I am sure you are proud

To Eliana, my wife, who started me with the idea of the Ph.D.

and to Luciana, my daughter, for the inspiration to finish it

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Carlos Rodriguez-Minguela, Claribel Cruz-Garcia and Veronica Gruntzig helped me adapting to the lab, to the sometimes-cold Michigan, and to the research world, I will never forget that.

I would like to thank the Ribosomal Database Project team with whom I worked for over a year. They really amazed me with the hard work they do and taught me much of the comparative 16S rRNA analysis I know. My thanks to Jim Cole, its director, one of the brightest people I have ever met.

Nowadays, research in environmental microbiology is a multidisciplinary team effort, and this work was not an exception. This thesis would have not been possible without the contribution of scientists at the Department of Energy Field Research Center, Wei-min Wu and Craig Criddle from Stanford University, and Jizhong Zhou from Oklahoma University.

I would also like to thank my labmates for providing a stimulating and fun work environment.

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PREFACE

Parts of the work for this thesis (chapters two and three) are part of a team effort between Michigan State University, Stanford University, and the Field Research Center (FRC) at Oak Ridge National Laboratory.

Stanford University designed and managed the groundwater treatment system used at the Field Research Center Area 3 and mentioned in this work. This group also characterized the hydrology of the site. Wei-min Wu, Craig Criddle, Jian Luo, Matthew Ginder-Vogel, and Peter Kitanidis formed the Stanford team.

Oak Ridge National Laboratory provided us the site, performed biological and chemical analysis, and retrieved the sediment samples used for microbial community analysis. Jizhong Zhou, Terry Gentry, Jack Carley, Sue Carroll, David Watson, Baohua Gu, and Phil Jardine were part of the Oak Ridge team. Christopher Hemme, also from Oak Ridge, provided the DNA I used to construct one of our gene libraries, and helped us with useful discussion with his preliminary data from a metagenome constructed for that sample.

Michigan State University was in charge of the microbial community analysis. Mary Beth Leigh, Terence Marsh, James Tiedje and I were part of the team. Tony Gaca helped with the construction of the clone libraries as well as extracting DNA from sediment samples.

Finally the staff from the Ribosomal Database Project (RDP) provided the website used for the pyrosequencing analysis, technical support, and custom java scripts

used for this work. Jim Cole, Benli Chai, Qiong Wang, Siddique Kulam-Syed-Mohideen, and Ryan Farris were valuable members of the RDP for this project.

A portion of this work was previously published in here (1) . This part is not included in this thesis.

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INTRODUCTION

Uranium Contamination and Strategies for its Bioremediation

1. Physicochemical characteristic of uranium and its applications

Uranium (U) is the natural element with the highest atomic number, 92 (protons in its nucleus), and one of the densest (19.1 $g \cdot mL^{-1}$). Uranium occurs naturally as a mixture of several isotopes, mostly ^{238}U (99.28%) followed by ^{235}U (0.71%) and ^{234}U (0.0054%). The half-life for these isotopes are 4.47×10^9 years, 7.0×10^6 years and 2.55×10^5 years, respectively. Because of its size, uranium's nucleus is unstable and decays naturally in a known series of elements emitting alpha particles. Uranium ranks 42nd in abundance in the Earth crust, and it is distributed worldwide and even present in the oceans. Uranium is exploited mostly in mining operations in Canada, Australia and Russia.

The main applications of uranium exploit two of its features: 1) its high density and 2) the ability of its nucleus to be broken. In the military world, uranium is used for armor plating and high penetration bullets. By being naturally fissible, it can be used for energy production in nuclear reactors, and for military purposes in bombs. Other uses include painting, radioisotope dating and transmission electron microscopy.

In order to make uranium usable for energy production, uranium has to be transformed to increase the proportion of ^{235}U isotope relative to ^{238}U. This process generates depleted uranium [typically less than 0.2 % of ^{235}U, low enriched uranium (0.2 to 3% of ^{235}U) and enriched uranium (typically 3 to 5% ^{235}U)]. Uranium enrichment creates a high proportion of depleted uranium depending on the natural ^{235}U concentration and the targeted final concentration. e.g., enriching for 3.6% ^{235}U for fuel in a nuclear plant will need approximately 8 kg of natural uranium per kg of enriched uranium.

2. Uranium contamination

Uranium it is a contaminant in facilities associated with uranium mining and enrichment. Uranium mining is the most frequent industry that has uranium contamination problems. In these sites, human exposure to uranium is due to inhalation and skin contact and the risks relate primarily to the chemical toxicity of U (as a heavy metal) and secondly to its radioactivity. However, ^{222}Ra, a byproduct of U decay, is associated with lung cancer (7).

Depleted uranium contamination is associated with uranium enrichment facilities as well as to the military industry where uranium is used in ammunition or heavy armor plating. The most frequent exposure route for depleted U is inhalation followed by ingestion through water (4). Acute exposure to uranium leads to renal toxicity, while

chronic exposure can problems in lungs, the central nervous system, intestines, and kidneys (3, 7). Depleted uranium is 60% less radioactive than naturally occurring uranium, and its toxicity is more related to its heavy metal characteristics.

Metals and radionuclides are frequent contaminants in the US Department of Energy facilities and waste sites (31). One of these sites, the Y-12 National Security Complex in Oak Ridge, Tennessee, USA, has groundwater contaminated with depleted uranium as well as nitrate, sulfate, and heavy metals. The main sources of contamination are four ponds (S-3 ponds) where the Y-12 uranium enrichment plant stored its wastes for over 32 years. These unlined ponds had a 9.5 million liter capacity each and receive mainly acidic uranium nitrate as well as solvents, and wastes from other DOE facilities. In 1983, the ponds were neutralized with limestone, and their nitrate was removed by denitrification. Treated water was pumped out of the site and the ponds with their contaminated sediments were capped with a Resource and Conservation Recovery Act (RCRA) cover, and paved with asphalt to construct a parking lot (5). Nevertheless, the areas surrounding the ponds were already contaminated, and intrusion of groundwater to the ponds promoted the movement of soluble uranium from them (30, 40). Groundwater at this site is highly acidic (pH 3.5) and contains high levels of inorganic contaminants such as nitrate and sulfate; radionuclides such as uranium and technetium; and solvents such as acetone, trichloroethene and tetrachloroethene (41). This site is now used to develop and study uranium remediation strategies.

3. Uranium reduction as bioremediation strategy

One of the earliest definitions of bioremediation was " the utilization of microorganisms for the destruction of chemical pollutants" (1). Though this definition works fine for the degradation of organic compounds, it does not apply for metals as they cannot be degraded. However, bioremediation of metals is possible by altering their bioavailability. This can be done by sequestering metals into organic matrices, by assimilating the metals into the cells, by changing the solubility of the metals via oxidation/reduction or complextion with organic compounds, and by modifying the chemicals associated with uranium in situ (e.g., dissolving insoluble phosphate-uranium precipitates) (17, 23, 36).

From these, one of the most promising strategies is dissimilatory metal reduction, a process by which microorganism use metals (e.g., Fe(III), Mn (IV), and Cr (VI)) and radionuclides (e.g., U(VI) and Tc(VII)) as terminal electron acceptors (18). The electrons can come from the breakup of organic compounds and, in some special cases, directly from electrodes (10). Electrons are then transferred from the organisms to the metals through direct contact such in *Geobacter* spp. (26); through electron shuttles such in *Shewanella* spp. (22); or through reduced products such as hydrogen sulfide (13), reduced humic acids (16, 27), nitrogen oxides (32), and reduced Fe(II) such as green rust(29) (Figure 1.1). While oxygen is usually the favored electron acceptor for microbial communities, these metals can be reduced in anaerobic conditions.

Changes in the oxidation state can trigger changes in solubility in both directions. Reduction can yield more soluble products (e.g., Fe(III) to Fe(II)) or more insoluble ones (Tc(VI) to Tc(V) and Tc(IV)). In the case of uranium the reduction is desirable because

reduced uranium (U(IV)) is less water soluble; thus, uranium movement through water can be controlled if populations capable of reducing uranium are stimulated.

Stimulation of uranium reducers can be achieved mainly by injecting an electron donor and/or carbon source into the aquifer and providing the right conditions for microbial activity in terms of pH, nutrients and minimizing competing electron acceptors such as oxygen and nitrate. More recently uranium reduction has been achieved through direct electron transfer by inserting electrodes, and electrodes coated with iron reducing bacteria (FRB) into aquifers (10).

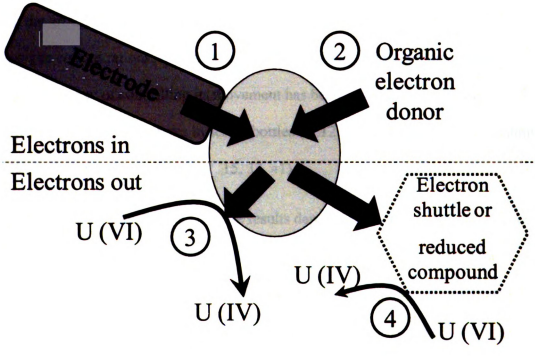

Figure 1.1. Mechanism for biological mediated Uranium (VI) dissimilatory reduction. Electron can be donated from electrodes directly (1) or from the decomposition of organic compounds (2). Form bacterial cells, electron can then be transferred to uranium by direct contact (3), or through electron shuttles of reduced compound such as humic acids or hydrogen sulfide (4).

Dissimilatory uranium reduction was first demonstrated for the FRB *Geobacter metallireducens*, and the sulfate-reducing bacteria (SRB) *Shewanella putrefaciens*, both of them belonging to the Delta class of the *Proteobacteria* (20). Since then, the ability to reduce uranium has been detected in at least 18 other genera (38) whose affiliations range from *Firmicutes, Deinococcus, Thermus, Actinobacteria,* Beta-, Delta- and Gamma-*Proteobacteria* as well as *Archaea* (38). In terms of microbial diversity the ability to reduce uranium seems widespread through the tree of life; however, for management purposes the widespread distribution can create challenges in the stimulation of the desired populations. In addition, is not clear what differences in reduction efficiency exist among the groups, and how this efficiency relates to environmental conditions such as pH, oxygen concentrations, and electron donor availability among others. Uranium reduction as a way of controlling its movement has been tested in pure cultures of FRB and SRB (19, 21), batch cultures in serum bottles (8, 12, 43), microcosms (28), columns (11, 39) and in pilot scale projects (2, 15, 37, 41).

Field studies have yielded diverse results depending on the carbon source used, the delivery method, and the characteristics of the site. In a former uranium mine, microcosm studies using contaminated sediments found the SRB *Desulfosporosinus,* and the fermenter *Clostridium* associated with uranium reduction (35). The medium used contained ethanol, lactate, acetate, benzoic acids, glucose, yeast extract and peptones (35). At a former uranium ore processing facility at Rifle, Colorado, in situ stimulation via acetate injection promoted U reduction by FRB *Geobacter*, but in a second phase, SRB not capable of U reduction outgrew *Geobacter* (2, 37). Recently it was reported that uranium removal at Rifle continued even after acetate amendments stopped (24), and that

this removal was associated with adsorption of uranium to the biomass of a *Firmicutes*-related bacteria that likely grew on dead cells from previously stimulated populations (24). At the Field Research Center at Oak Ridge, Tennessee, a pilot scale project showed associations between uranium reduction with SRB *Desulfovibrio* (14, 42, 43), FRB *Geobacter* (14, 42), FRB *Anaeromyxobacter* (6), SRB *Desulfosporosinus* (6, 14), and denitrifying *Acidovorax* (28).

4. Microbial communities involved in uranium bioremediation

In order to study the microbial communities involved in the uranium reduction in situ, culture independent methods have been predominantly used. The preferred methods to track specific populations involved the use of the 16S rRNA gene in combination with terminal restriction fragment length polymorphism (TRFLP), restriction fragment length polymorphisms (RFLP), denaturing gradient gel electrophoresis (DGGE), and clone libraries. Restriction enzyme-based methods are simpler and cheaper and can provide a profile for the whole community that can be later consistently compared. However, it is hard to associate peaks or bands with specific populations unless a clone library for the same site is constructed. DGGE provides bands that can be tracked over samples and later sequenced for their phylogenetic information. However, the variability between and within gel gradients as well as a limitation in how many bands can be resolved (usually 20 to 40), can make this method challenging to analyze (25). Finally, clone libraries provide the most sequence information (usually 500 to 800 bases per clone). This information can be use for phylogeny inference and community reconstruction. However,

library construction is time consuming, expensive, limited in scope, and in some occasions bias can be introduced in the cloning step (34). All PCR-based methods such as, TRFLP, DGGE and clone libraries can be biased due to primer coverage, differences in copy number of a gene in a genome, and extraction of DNA (9).

One recent alternative that is both high throughput and provide sequence information is massive parallel sequencing. By sequencing hypervariable regions of the 16S rRNA gene, thousand of reads can be generated per sample, and the structure of the microbial community reconstructed by comparing the sequences to known databases (33).

In this thesis I use comparative 16S rRNA gene studies of both traditional and pyrosequencing clone libraries, to study the diversity and structure of the communities associated with uranium bioremediation at the Oak Ridge National Laboratory Field Research Center, Area 3. The focal research questions are 1) what are the main populations associated with U reduction, and 2) how does their abundance, and presumably the features favoring their selection, relate to the geochemical conditions present at this site?

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CHAPTER TWO

Cardenas, E., W. M. Wu, M. B. Leigh, J. Carley, S. Carroll, T. Gentry, J. Luo, D. Watson, B. Gu, M. Ginder-Vogel, P. K. Kitanidis, P. M. Jardine, J. Zhou, C. S. Criddle, T. L. Marsh, and J. M. Tiedje. 2008. Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels. Applied and Environmental Microbiology 74:3718-29.

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

ABSTRACT

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

samples of the treated area. Spatial differences in community structure within the treatment zone were likely related to hydraulic pathway and electron donor metabolism during biostimulation.

INTRODUCTION

Metal and radionuclide are common groundwater contaminants at facilities and waste sites of the US Department of Energy (DOE) occurring in more than 50% of these locations (32). One of these sites, the former Y-12 National Security Complex at Oak Ridge, Tennessee, contains uranium in concentrations as high as 60 mg/l (252 μM) in groundwater and 800 mg/kg in sediments (48). To control the migration of the uranium, microbial reduction of U(VI) to sparingly soluble and immobile U(IV) has been proposed as a promising approach (1, 2, 12, 17, 22, 42, 48). Bioreduction of U(VI) to U(IV) has been reported by pure and mixed cultures of iron(III)-reducers (FeRB) such as *Geobacter* spp., sulfate-reducers (SRB) such as *Desulfovibrio*, *Desulfoporosinus*, and *Desulfotomaculum* spp. and denitrifying bacteria such as *Acidovorax* spp. among others (45).

In recent years, researchers have evaluated this approach through laboratory-scale experiments using batch serum bottles (10, 14, 51), microcosms (29), and sediment columns (11, 46). Reduction and immobilization of uranium in the mentioned laboratory experiments has been observed and confirmed by X-ray near-edge absorption spectroscopy (XANES) analysis (11, 29, 40, 41, 46). *Geobacter* spp. and *Geothrix* spp. were found to be associated with Fe(III) and U(VI) reduction in field conditions (5, 14,

46, 50). Control of the microbial community structure may be one of key issues for the long-term reduction and stabilization of uranium *in situ* (1). While bench-scale tests provide valuable information about the feasibility of bioreduction, they cannot replicate all of the heterogeneity and complexity of the subsurface. Hydrogeology is a factor that can contribute to the complexity of field treatments by creating gradients of resources *in situ*, affecting the microbial diversity and thus, potentially the remediation process. Little is known about the effect of groundwater flow on microbial community diversity during and after bioremediation, even when the kinetics of U(VI) reduction should be dependent on the microbial community and geochemical conditions. The effect that hydrogeology has on microbial diversity should be addressed.

Pilot studies are the next step in demonstrating the feasibility of the *in situ* uranium remediation approach (12, 14, 17, 37, 48, 49). DOE does not provide specific uranium target levels, but US EPA regulates the maximum contaminant level (MCL) for drinking and groundwater at 30 μg/l (0.126 μM). At the Field Research Center (FRC) site of the Environmental Remediation Sciences Program (ERSP) of the DOE, Oak Ridge, TN, test facilities have been constructed for the remediation of uranium contaminated groundwater and sediments using various approaches (1, 17, 49). Prior to the tests, the microbial community structure was characterized by 16S rRNA gene clone libraries made from groundwater samples in Areas 1 and 3 and the majority of the sequences (>73% in Area 1 and >65% in Area 3) were found to be *Proteobacteria* belonging to the genera *Azoarcus* and *Pseudomonas* (9). Microbial community analyses of Areas 1 and 2 identified *Geobacter* spp. and *Anaeromyxobacter* spp. among others as the potential uranium reducers (28). In these areas of moderate uranium contamination (0 to 5.8 μM),

removal of nitrate and aqueous U(VI) was stimulated by the injection of ethanol or glucose to the subsurface using a push-pull approach (17). In the extremely contaminated Area 3, a long-term (> 2 years) bioremediation test has been performed. This area is located near former S-3 Ponds and contains high levels of nitrate (up to 200 mM) and U (up to 250 μM), aluminum(12-13mM) and calcium (22-25mM) (48, 49). Prior to remediation, the microbial levels in groundwater of this area were extremely low probably due to low pH (3.6) and high levels of nitric acid. A combination of remediation approaches was used to remove U(VI) reduction inhibitors (nitrate and Ca) and condition the area for metal reduction by raising the pH and providing a carbon source. Using these approaches, low U(VI) concentrations below MCL were achieved and a new microbial community capable of uranium reduction was established (48-50).

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

MATERIAL AND METHODS

Site description and bioremediation test. The bioremediation test was performed in Area 3 of the DOE FRC at the Y-12 National Security Complex, Oak Ridge, TN as previously reported (24, 48). The field system consisted of an outer groundwater recirculation loop (injection well FW024 and extraction well FW103) that isolates an inner groundwater loop (injection well FW104 and extraction well FW026) preventing penetration by highly contaminated groundwater from the source zone (Figure 2.1). The hydraulic control afforded by this system created a controlled *in situ* bioreactor. Reduction of U(VI) to U(IV) was accomplished through ethanol injection to the inner loop. Injection and extraction wells (FW104 and FW026) had a 10.2 cm diameter and a depth of 14.6 m below ground surface (bgs) with 2.5 m screened intervals between 11.28 and 13.77 m. Multilevel sampling (MLS) wells FW100, FW101 and FW102 were used to monitor hydrogeology and remediation performance. The MLS wells contained seven separate sampling tubes (diameter =1.9 cm) at different depths bgs. In this study, MLS wells FW101-2 (13.7 m bgs), FW101-3 (12.2 m bgs), FW102-2 (13.7 m bgs) and FW102-3 (12.2 m bgs) were selected for routine monitoring of remediation performance because of their hydraulic connection to FW104 (24, 48). The recirculation flow rates in the inner loop were 0.45 l/min (injection FW104, extraction FW026). The rates for the outer loop were 1.35 l/min (injection at FW024) and 0.45 l/min(extraction at FW103). Additional clean water was injected to FW024 at 0.7-0.9 l/min in order to minimize entry of ambient groundwater (48). This clean water was a mixture of tap water and groundwater treated by an aboveground system to remove nitrate via a bioreactor (48). The remediation test was started on August 23, 2003 by preconditioning the site

(day 0). During the initial 137 days, water was pumped from the subsurface, pH adjusted, treated to remove aluminum, calcium and nitrate (this by a denitrification bioreactor) and then re-injected. Calcium and nitrate was removed to avoid formation of stable Ca-U-CO3 products, U(IV) re-oxidation by nitrites, and nitrate competition as terminal electron acceptor. *Ex situ* treatment was used to avoid clogging by nitrogen gas, biomass (due to denitrification), and calcium and aluminum precipitates (due to pH adjustment) (48).

As result, the pH increased from 3.6 to around 6.0, and nitrate and U(VI) concentrations decreased to around 0.5 mM and 5 μM, respectively (48). After the conditioning phase, ethanol was added as an electron donor to stimulate U(VI) bioreduction starting on January 7, 2004 (day 137) (49). An ethanol solution (industrial grade, containing ethanol 88.12%, methanol 4.65% and water 7.23%, w/w) with a Chemical Oxygen Demand (COD) to weight ratio of 2.1 was prepared in a storage tank with 6.9-9.8 g COD/l. This solution was normally injected at FW104 over a 48-hour period each week to give a COD 120-150 mg/l at FW104.

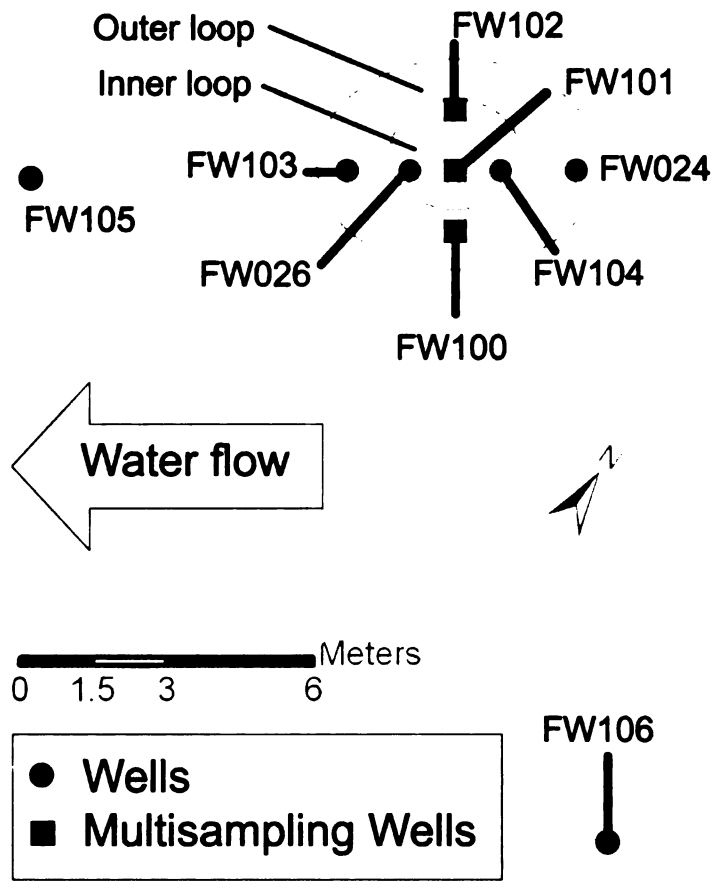

Figure 2.1. Map of the Area 3 treatment zone depicting the location of the sampled wells and the control well. FW024: outer loop injection; FW104: inner loop injection; FW026: inner loop extraction; FW103: outer loop extraction well. Wells FW100, FW101 and FW102 are multilevel sampling wells. FW106: control well. Contamination source is approximately 20 m to the right.

Sampling for community analysis. Sediment samples for microbial community analysis were collected on day 774 (October 5, 2005) from the inner loop injection FW104, extraction FW026, and two MLS wells at two depths FW101-2, FW101-3, FW102-2 and FW102-3. To collect the samples, a smooth PVC surge block (10 cm or 1.8 cm in diameter) was inserted into the well and then lifted up and down in a rapid plunging motion. This motion drew sediment from the soil matrix surrounding the well screens into the well. The surge blocks were rinsed with groundwater in between samples

to remove attached solids. The sediment slurry that settled to the bottom of the wells was pumped to a 2 L glass bottle under Ar atmosphere and the bottle was then sealed with a rubber stopper. The slurry was transferred to the laboratory and centrifuged to separate the sediments from the water. The pellets were frozen at -80 °C prior to being shipped on dry ice to Michigan State University. Fresh sediment slurry was also collected anaerobically in a 27-ml glass pressure tube for the most probable number (MPN) enumeration. Samples taken by this method are a mixture of the sediment along 1 m depth of the well (screened interval) and could contain sediment in the deep matrix where electron donor did not reach.

Before remediation, bioactivity in the treatment zone was extremely poor due to low pH (3.2-3.6) and high levels of nitric acid and uranium. DNA extraction from untreated sediments repeatedly failed. In order to compare microbial communities before and after biotreatment, DNA was collected by filtering 1700 liters of groundwater from the FW106 well which is located 12 m away from the treatment zone in parallel with treated sediments. Groundwater from FW106 has similar composition to that in the treatment zone before remediation, i.e. high levels of nitric acid and uranium and low pH (Table 2.1).

Table 2.1. Chemical properties of groundwater in the wells of treatment area before and after remediation and the control well FW106.

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

*A: before remediation (samples were taken in February-April, 2002) except for FW106 (day 278). B: after biostimulation on day 773 (Oct. 4, 2005). na: data not analyzed. **Detection limit for U(IV) is 10% of total U in XANES (50). Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies.

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

FeRB tubes, and production of black Fe(II) sulfide precipitates in SRB tubes.

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

DNA extraction and community analysis. DNA was extracted from 0.5 g of sediments with the Fast soil prep kit (MoBio Inc., San Diego, CA, USA) following the manufacturer's instructions. DNA was used to amplify 16S rRNA genes using the universal primers 27F (5'- AGAGTTTGATCMTGGCTCAF-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') in a Stratagene thermal cycler (Stratagene, La Jolla, CA, USA). The PCR reaction (50 µl) contained 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.1 µg/µl BSA, 10 pmol of each primer, 0.2mM each dNTP , 1.25 U taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) and 20 ng of purified

DNA. The PCR cycling conditions were: 95°C for 5 min; 95 °C for 1 min, 59 °C for 1 min, 72°C for 1min 40s, for 28 cycles; 72 °C for 10 min. PCR products were analyzed in a 1.5% (wt/vol) agarose TAE gel to confirm the size of the product. Four replicate PCR reactions were generated for each DNA extract and then were compiled to address variability that may be introduced by PCR bias. The PCR products (200µl) were concentrated to 30 µl with a PCR purification kit (Qiagen Inc., Valencia, CA, USA). The concentrated products were then run in a 1% agarose gel, excised, extracted with the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and eluted to 30 µl with EB buffer (10 mM Tris-Cl, pH 8.5). An additional step was taken to add poly-adenine overhangs to the PCR products to facilitate cloning (10 min at 72 °C, 18.8 mM Tris HCl pH8.4, 47mM KCl, 0.93 mM dATP and 0.5 U of taq polymerase; Qiagen Inc., Valencia, CA, USA). Products were cloned using the Topo TA Cloning kit for sequencing following manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Single extension sequencing was conducted by Macrogen Inc (Seoul, Korea) from the internal primer 27F. The sequences were submitted to to the GenBank under accession numbers EF692646-EF693732.

Raw sequencing files (Ab1) were submitted to the *my*RDP application of the Ribosomal Database project where the nucleotide sequence was determined, the quality (Q score) of each base stored and vector sequence removed (7). The resulting sequences were aligned to the RDP model and rRNA distances matrices were generated with the Jukes-Cantor distance correction. Operational taxonomic units (OTUs) were defined at 97% sequence identity. The distance matrices were used to calculate alpha diversity indices (Chao1, Simpson and Shannnon) and rarefaction curves using Distance-Based

Operational Taxonomic Unit and Richness (DOTUR) determination program (35). The diversity indices were rarified to account for differences in number of sequences per library.

The distribution of the OTUs in the different libraries was used in EstimateS (8) to make comparisons based on diversity patterns using Bray-Curtis and Sørensen beta diversity indices. These indices were used to cluster the samples according to their distances using MEGA 3.1 (20). The composition was normalized to account for differences in number of clones in the different libraries. ∫-LIBSHUFF (36, 38) was used to compare the clone libraries according to Good's homologous and heterologous coverage. This approach provides a quantitative comparison of 16S rRNA gene clone libraries from environmental samples (38).

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

Phylogenetic trees were constructed using distance and maximum likelihood methods. Aligned sequences were downloaded from the RDP, aligned manually and the non-model positions were masked from the alignment. The Jukes-Cantor distance correction, full gap deletion and a bootstrap test using 10000 replicates and random seeds were used to construct the trees in MEGA v3.1 (20).

Chemicals and analytical methods. The source and quality of chemicals used in the field test were described previously (48, 49). COD was used as an overall indicator to monitor the consumption of electron donors (ethanol, its metabolite acetate and others). COD, sulfide and Fe(II) were determined using a Hach DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Anions (including NO_3^-, Br^-, Cl^-, SO_4^{2-} and PO_4^{3-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale, CA), metals (Al, Ca, Fe, Mn, Mg, U and K etc.) were determined using an inductively coupled plasma mass spectrometer (ICPMS) (Perkin Elmer ELAN 6100), and U reduction state was determined with XANES as described elsewhere (49, 50). 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 Corp., St. Louis, MO) using Helium as carrier gas.

RESULTS

***In situ* biostimulation test.** After one year of biostimulation, nitrate concentration in the treatment zone dropped to a level of nearly zero. Increase in sulfide concentration and appearance of Fe(II) in groundwater suggested that sulfate and iron reduction were occurring (50). Microbial activity during biostimulation was determined by monitoring changes in aqueous concentration of electron donor added (as COD), sulfide and uranium. A representative time course of biostimulation is shown in Figure 2.2. On day

704, ethanol was injected to FW104, causing increase COD concentrations in all major MLS wells (Figure 2.2A).

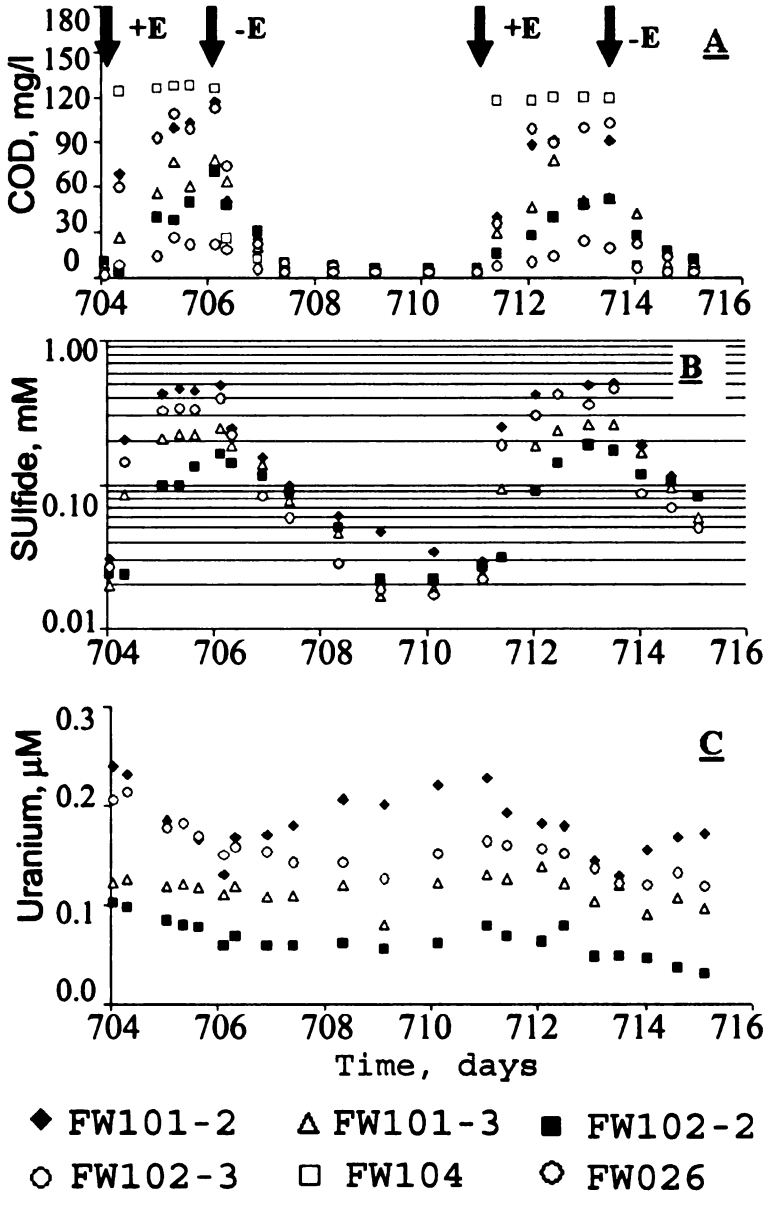

Figure 2.2. Typical biostimulation of U(VI) reduction by injecting ethanol to subsurface (day 704 to 714). (A) COD concentrations. (B) Sulfide concentrations. (C) Uranium concentration changes in MLS. U concentration in the injection well FW104 was 0.5 µM during this test period and is not shown due to scale. +E indicates start and –E indicates stop of ethanol biostimulation.

The COD in FW104 was mainly attributed to ethanol (>80%) and a small amount of acetate (<30 μM) while in MLS and FW026 wells, the COD came mainly from acetate (data not shown). No ethanol was detected in the MLS wells and FW026 well and methanol concentrations were below detection limit (<50 μM) for all the cases. On day 706, ethanol injection was stopped and the COD concentrations in MLS wells decreased rapidly. The same pattern was observed again on day 711 to 713 when ethanol was reinjected. Sulfide concentrations in all MLS wells increased after ethanol was injected and decreased after injection stopped (Figure 2.2 B) but remained at detectable levels (>20 μM). Sulfate concentrations in MLS wells decreased when ethanol was injected and rebounded when the injection stopped (data not shown) indicating the presence of SRB activity in the subsurface. Uranium concentration in all MLS wells decreased after ethanol was delivered and slightly rebounded when ethanol was not injected (Figure 2.2C). However, the uranium levels after the rebound were lower than those before ethanol injection. This rebound was likely due to uranium being carried in the recirculated water (~0.5 μm) and to the lack of electron donor for U(VI) reduction when ethanol injection stopped. During the ten-day test in figure 2.2, uranium concentration in the injection well FW104 was around 0.5 μM (data not shown in figure 2.2C) which was much higher than those in the MLS wells. Uranium levels in FW101-3 and FW102-2 were near or below EPA MCL (0.126 μM) throughout the 10 day-test period while U levels in FW101-2 and FW102-3 dropped even below 0.126 μM after ethanol injection. The relatively higher U levels in the latter two wells are likely due to the lack of enough electron donor after ethanol injection stopped. Lower U concentrations in the MLS wells

were achieved later as shown in Table 2.1. Aqueous Fe(II) concentrations were 10-20 μM in the MLS wells before ethanol injection, dropped below 5 μM during ethanol injection as sulfide concentration increased and then slowly rebounded after ethanol injection stopped and sulfide concentration decreased (data not shown). The decrease in Fe(II) concentration during ethanol injection is likely due to formation of more FeS precipitates by reaction with the produced sulfide. The FeS precipitates likely accumulated in sediments.

The reduced sediment samples showed black color (FW104) or dark green color (MLS). Reduced U(IV) was detected by XANES in the sediments samples from injection well and the MLS (Table 2.1). In the FW026 well, the U(IV) content was below the detection limit of XANES (<10% of total U). The highest content of reduced uranium was found in the injection well FW104 (50).

Groundwater flow pathway. The injection of the $NaBr^-$/ethanol solution to FW104 lasted for 50.75 h. Bromide concentration increased in the MLS and extraction (FW026) wells with different recovery ratio and mean travel time (Figure 2.3). These results indicate that all MLS wells were hydraulically connected to FW104 (Figure 2.3A). The Br^- recovery percentages were: FW101-2, 93%; FW101-3, 60%; FW102-2, 94%; FW102-3, 93%; and FW026, 50% (Figure 2.3A). FW101-2, FW102-2 and FW102-3 received more than 93% of water injected to FW104, while FW101-3 and FW026 received 50% and 60% of water from FW104, respectively. The rest water was from surrounding areas. Mean travel times from FW104 to the different wells were: FW101-2,

2.84 h; FW101-3, 18.4 h; FW102-2, 11.6 h; FW102-3, 3.7 h; and FW026, 46 h (23). Therefore, groundwater injected to FW104 reached FW101-2 and FW102-3 more rapidly than other two wells. The fraction of groundwater flow from injection well to each MLS well and the mean travel time may significantly influence microbial community in the MLS wells as discussed later. During the tracer test, COD concentrations in MLS and FW026 wells increased after ethanol injection (Figure 2.3B). The measured COD concentrations in the MLS wells were significantly lower than those calculated based on the Br^- concentration and the COD/Br^- ratio in the NaBr-ethanol tracer solution used. The difference was likely due to biodegradation of ethanol and acetate in the subsurface. Ethanol was observed only in the FW104 well during the tracer test. The COD in MLS and FW026 was mainly from acetate (>80%) based on acetate vs. COD measured. During the tracer test, sulfide concentrations increased in all MLS wells and U (VI) concentrations decreased in the same trend as shown in figure 2.2 (data not shown). Another separate tracer test by injecting bromide to outerloop FW024 indicated that innerloop extraction well FW026 received 17% of water injected to FW024 (23). The infiltration of water from outerloop may also influence the microbial community.

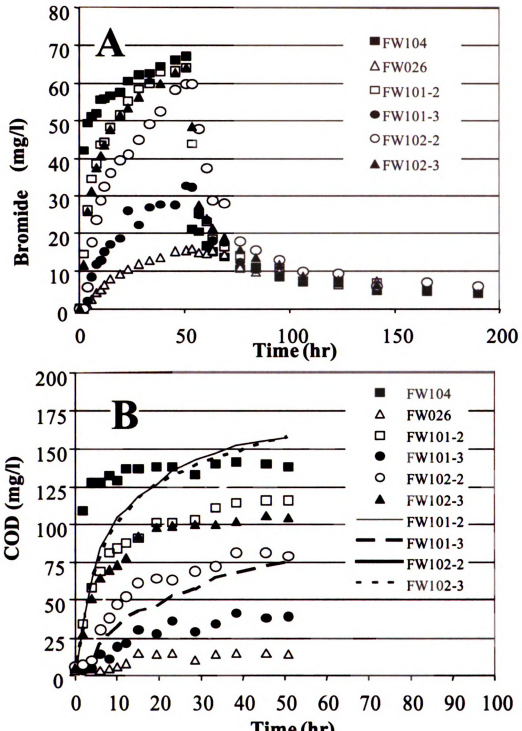

Figure 2.3. Tracer test performed with Bromide on day 801-803 shows the hydraulic connection between injection well FW104, extraction well FW026 and MLS and biodegradation of ethanol. A. Changes in bromide concentrations (23) B. Comparison of measured COD (marks) and calculated COD (lines) concentrations based on COD vs Br^- ratio (2.46 g/g).

Microbial enumeration. MPN results indicated that after biostimulation for nearly two years, increased levels of denitrifiers, SRB and FeRB were present in the sediments in the bioreduced area in comparison with control well FW106 (Table 2.2) that did not undergo stimulation and shares similar geochemical composition with the active area wells (Table 2.1). After biostimulation, the microbial concentrations in the treatment area were (in cells/g sediment): denitrifiers, 10^7-10^8; SRB, 10^6-10^8; and FeRB, 10^5-10^7. he highest levels for all three trophic groups were found in inner loop injection well FW104 where ethanol was injected. Relatively low levels were found in extraction well FW026. Consistent with the tracer study, FW101-2 (more connected to the injection well FW104 based on the tracer study) showed higher bacterial counts than FW102-2 (less connected) (Table 2.2). MPN counts from FW106 (groundwater) showed levels of denitrifier as low as 3.3 cells/ml groundwater (49). Neither SRB nor FeRB were found in FW106. Comparison with sediment MPN at day 453 showed a decrease in SRB and denitrifiers and no change for FeRB in FW104. MPN count at day 774 for all three groups remained around 10^8 cell/g sediment in FW104 (49). Integration of MPN at day 774 and three earlier timepoints (days 278, 354, 453) for FW101-2 showed an increase in the counts overtime with microbial levels at 774 around 10^7 cell/g sediment, one order lower than FW104. Conclusions from the MPN analysis must be qualified because we contrast sediments with groundwater samples. However, prior MPN studies using samples from the same well show a one log difference in counts in sediment versus groundwater samples (data not shown).

Table 2.2. MPN for three major trophic groups in the sediments of the treatment zone (day 774) in comparison with groundwater from control well FW106.

	Cells / g sediment		
Well	Denitrifiers	FeRB	SRB
FW104	7.23×10^8	9.40×10^7	1.53×10^8
FW101-2	1.54×10^8	2.05×10^7	2.06×10^8
FW102-2	2.39×10^7	5.48×10^5	1.87×10^7
FW026	1.10×10^7	1.93×10^6	1.07×10^6
FW106*	3.3 cell/ml	ND	ND

FeRB: Iron reducing bacteria; SRB: Sulfate reducing bacteria. ND: None detected. Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies. *Data on day 278 (49).

Sequence analyses. After discarding putative chimeric sequences, an average of 155 sequences per sample were used for each library. Rarefaction analysis at 97% similarity levels (Figure 2.4) showed that the estimated coverage (rarified number of OTUs divided by rarified Chao1 estimator) ranged from 36 to 58% in the stimulated area and was 93% in the background area (FW106). Even with low coverage in the biostimulated zone, clear differences in diversity were observed. Highest diversity was found in all the wells of the biostimulated area; working at 97% similarity for OTUs the number of OTUs ranged from 41.2 to 91.4 (rarified values). Only 6.6 OTUs were found in the background area FW106 (rarified value).

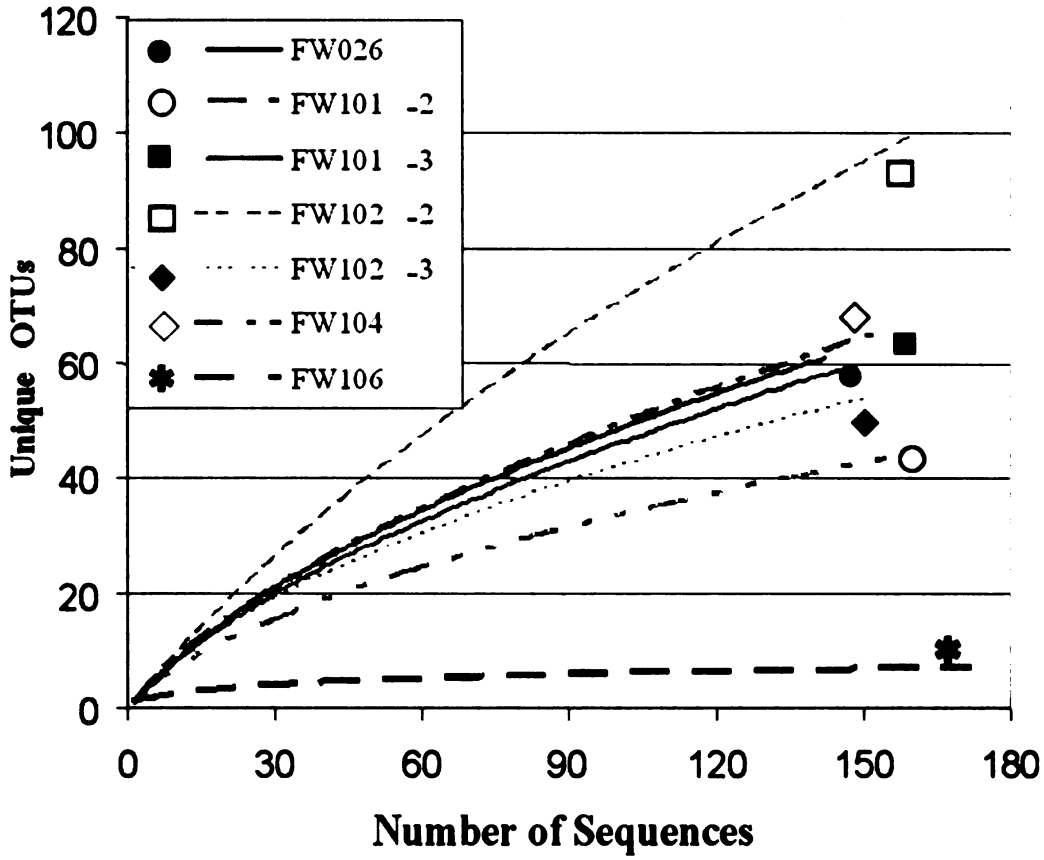

Figure 2.4. Rarefaction curves of the 16S rRNA gene libraries constructed. OTUs were defined at 97% sequence identity. The library from the untreated control (FW106) was close to complete sampling (93% coverage).

Microbial community structure and major groups detected. All libraries were dominated by members of the phylum proteobacteria, with *Betaproteobacteria* and *Deltaproteobacteria* being the most dominant proteobacterial classes in the biostimulated zone (Figure 2.5). In contrast, the background area was dominated by *Gammaproteobacteria.* Bacteria belonging to groups known to include U(VI)-, nitrate-, sulfate- and Fe(III)-reducers were detected in the active area but not in the background area library.

Sequences from the genera *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*,

Acidovorax and *Desulfosporosinus* were detected in the libraries. These genera are known to include U(VI) reducers and additionally can contribute with one or more of the following activities: iron (III)-, sulfate- and nitrate reduction (Figure 2.6). The contribution of the putative species responsible for the uranium reduction and their richness (measured as number of OTUs in a genus) was highly variable (Table 2.3). *Geobacter* and *Desulfovibrio* were detected in all the libraries of the active area. In five of the six samples from the active area, the relative contribution of *Desulfovibrio* was higher than that from *Geobacter* (Table 2.3). *Anaeromyxobacter*, *Acidovorax* and *Desulfosporosinus* sequences were not present in all the libraries.

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

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

Other commonly found soil bacteria like *Acidobacteria, Actinobacteria*,

Planctomycetes and *Verrucomicrobia* were also present in the samples. These bacteria were present in the active area and not in the untreated area.

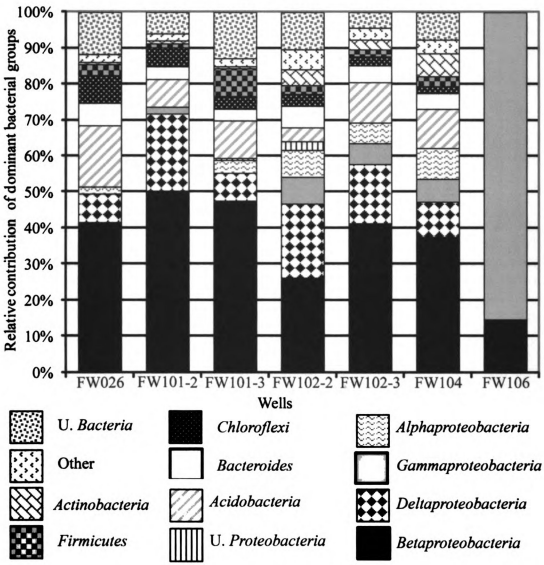

Figure 2.5. Microbial composition of the clone libraries based on the RDP Classifier. The "other" group category includes the phyla *Spirochaetes, Gemmatimonadetes, Verrucomicrobia, Chlamydia, Planctomycetes, Nitrospira, Cyanobacteria* and the proposed phyla, OP11, OP10, BRC1 and TM7. Bacteria that could not be assigned with the 80% confidence bootstrap value were included in an artificial "Unclassified *Bacteria*" (U. *Bacteria*) taxon. Wells are arranged according to their descending hydraulic connection to the injection well (FW104) based on the tracer studies.

Figure 2.6. Neighbor joining tree showing the relationship of selected representatives (shown in bold) from the groups similar to known U-reducing bacteria. Metabolic abilities of the clones' closest cultivated relatives are indicated. Non-model positions from the 16S rRNA were masked and Jukes-Cantor distance correction used. Type strains have a "(T)" label. Bootstrap values (10000 repetitions) are displayed if larger than 50%. (+) Present in closest relatives, (-) absent in closest relatives, * activity found some species of the genus, ? = unknown. The range of relative contribution to the different samples is also shown.

Figure 2.6. Neighbor-joining tree showing the relationship of selected representatives (shown in bold) from groups similar to known U-reducing bacteria

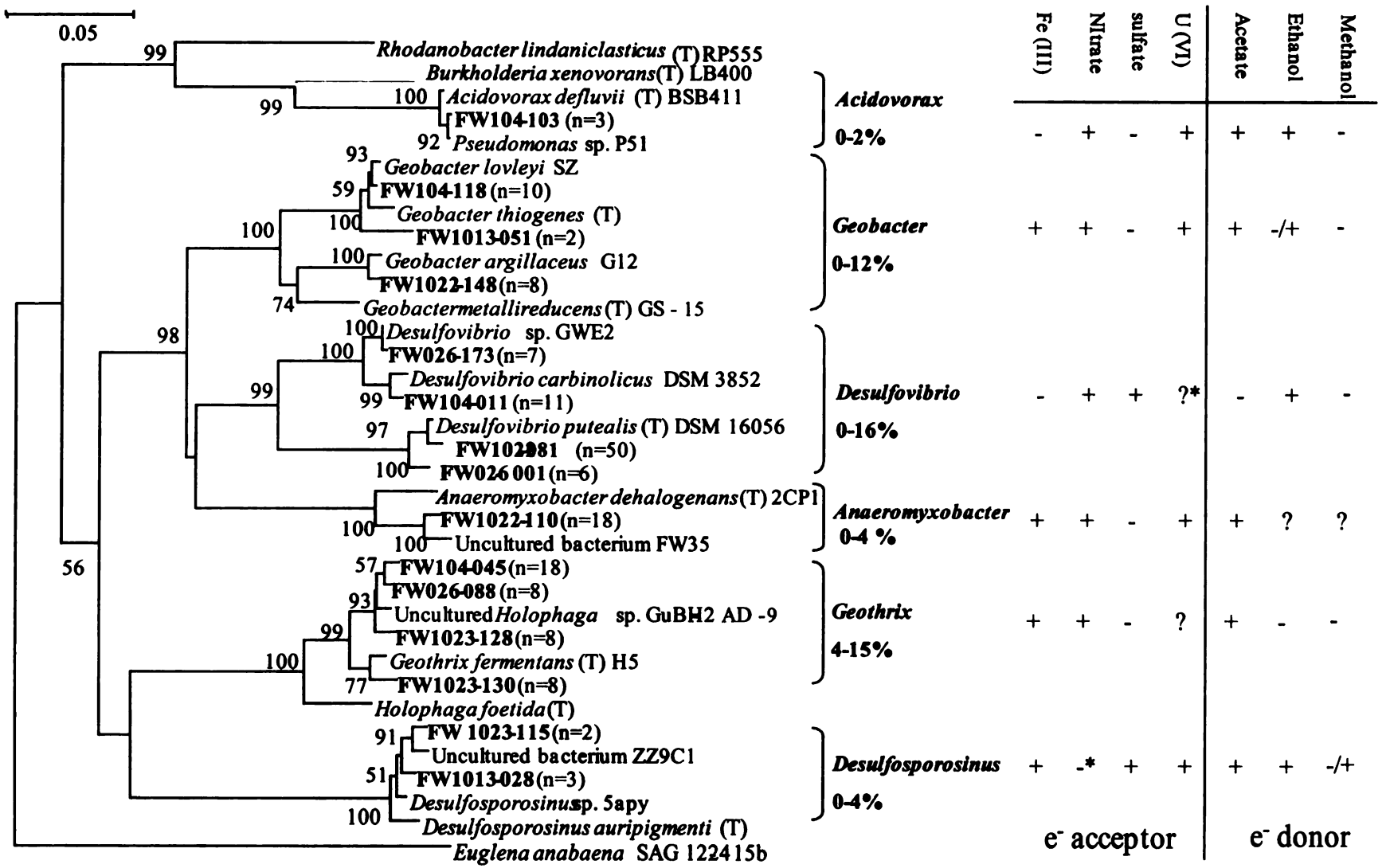

Table 2.3. Relative contribution(%) of known uranium-reducer genera in clone libraries from sediment samples in the Area 3 wells. The number of unique OTUs (97% similarity) and closest isolate (Seqmatch @ RDP) are reported.

Genus	OTUs	Closest isolated relatives	Relative contribution per well (%)					
			FW104	FW101-2	FW102-3	FW102-2	FW101-3	FW026
Desulfovibrio	7	*D. putealis* *D. carbinolicus*	6	16	12	4	5	6
Geobacter	7	*G. argillaceus* *G. lovleyi* *G. thiogenes* *G. humireducens* *G .psycrophilus*	3	1	1	12	1	1
Anaeromyxobacter	2	*A. dehalogenans*	1	4	3	3	2	0
Geothrix	9	*G. fermentans*	11	8	10	4	10	15
Desulfosporosinus	7	*D. auripigment,* *D. orientis.*	1	0	0	2	4	0
Acidovorax	3	*Pseudomonas P51*	2	1	0	0	0	1

Diversity analyses. All libraries from the stimulated zone showed greater diversity than the untreated zone based on both evenness and richness values as indicated by the diversity indices used (Table 2.4). One single OTU dominated the untreated area sample (FW106). This member of the *Xanthomonadaceae* family was also detected in a metagenome experiment for well FW106, where it was shown to carry a variety of metal resistance genes (13).

Table 2.4. Diversity indices for the different wells in the treated and untreated area (FW106).

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

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

Diversity-based clustering revealed that the FW106 community formed a cluster separated from the treatment zone wells (Figure 2.7). The two different clustering methods used resulted in congruent topologies. High similarity was observed between communities from FW102-3 and FW104, which were 53% similar according to a calculated Bray-Curtis index. ∫-LIBSHUFF comparison indicated that these two libraries were not significantly different (p=0.05). All the other pairwise comparison were non-

significant (libraries were significantly different, p=0.05).. Additional comparison of FW104 and FW102-3 communities with the LIBCOMPARE function of the RDP showed non-significant differences at (p=0.01) at all the different levels of taxonomy from phylum to genus. No clear relation was observed between the alpha diversity indices and the hydrology.

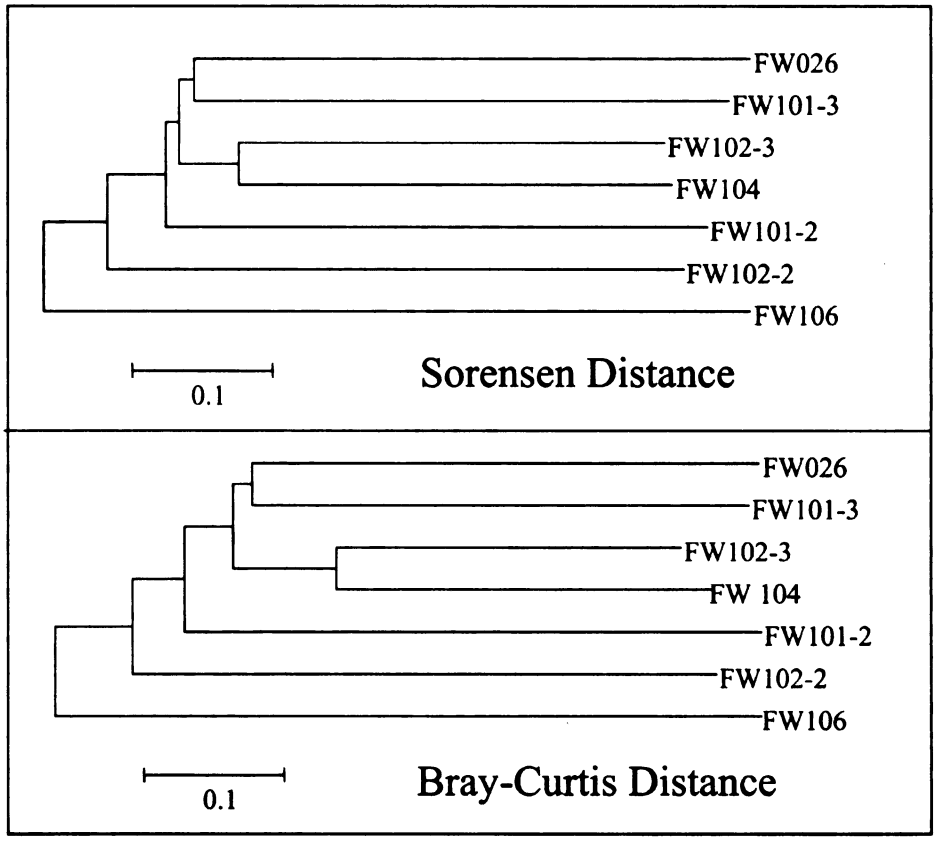

Figure 2.7. Clustering of the samples according to diversity patterns. The topology was similar when using the Sørensen index (Presence/Absence) and the Bray-Curtis index (Presence/Absence and abundance). The indices were normalized to account for differences in total number of sequences per library.

DISCUSSION

1. Remediation of uranium and metals in the subsurface

Microbial reduction of metals including uranium has been the proposed alternative to control the mobility of the contaminants in the groundwater (45). In this field experiment, ethanol injection successfully created a reducing environment capable of achieving uranium remediation and immobilization to levels below US EPA drinking water MCL (49). The biostimulation process changed the structure of the microbial communities from a small and low-diversity community to a more abundant and diverse community with bacteria capable of reducing the contaminants nitrate, sulfate and U(VI) *in situ*.

2. Microbial communities

2.a Major trophic groups detected

Microbial enumeration together with clone libraries revealed that after biostimulation viable FeRB, SRB and denitrifiers had grown in the treated area where uranium, nitrate, sulfate, ferric compounds and perhaps other compounds served as electron acceptors. At least three major microbial trophic groups appear to be involved in the bioremediation of the area. The first group, denitrifiers, can remove nitrate and provide a favorable low redox environment for U(VI) reducers, FeRB and SRB. These latter two groups may contribute to the remediation process by having members capable of reducing not only Fe(III) and sulfate but also U(VI). Microbial enumeration indicated

that these three trophic groups were present in the sediments at high levels after biostimulation. In contrast, the untreated area sample (FW106) showed the presence of low numbers of denitrifiers (3.3 cells/ml) in groundwater but neither SRB nor FeRB. Previous reports on groundwater MPN for this stimulated area (same wells, earlier timepoints) also show these three groups but the microbial counts were lower that our timepoint (49). The cell count in the untreated sediments is unknown but it could be significantly low since DNA extraction was unsuccesful. Clone libraries permitted detailed speciation of these trophic groups.

2.b. Carbon source as selective agent and the community of ethanol degraders

The use of ethanol as a carbon source likely increased the diversity of taxa and physiologies. Injected ethanol provided both the carbon source and the electron donor in the forms of ethanol, acetate (metabolic intermediate) and methanol (an impurity in industrial ethanol). Electron donor consumption and metabolism were likely performed by denitrifiers, FeRB and SRB (Figure 2.6). Based on groundwater analysis during tracer study, we hypothesize that the SRB were mainly responsible for ethanol consumption and acetate release between the injection well and MLS wells (where no nitrate was detected and sediments showed black or dark green color), while FeRB and denitrifiers utilized mainly acetate between MLS wells and the extraction well. At this well there was evidence of groundwater infiltration from the outer loop and the sediments were green-yellow in color. Close relatives of the SRB detected (*Desulfovibrio* spp. and *Desulfosporosinus* spp.) utilize ethanol and not acetate when doing sulfate reduction

though few strains of both groups can also use acetate (19, 33). The release of metabolic acetate can be used later by other species, including *Geobacter*. This hypothesis is supported by sulfide accumulation during ethanol injection (SRB activity) (Figure 2.2) and the detection of mostly acetate (and not ethanol) as carbon source in less connected wells. The sequential utilization of ethanol and release of acetate likely create a niche for acetate-consuming FeRB and acetate-consuming denitrifiers. The first group was represented in our libraries by *Geobacter* spp., *Geothrix* spp. and *Anaeromyxobacter* spp. These bacteria are capable of acetate and ethanol degradation mainly by Fe(III)-reduction as well as by denitrification (2, 6, 34). The second group is represented by denitrifiers such as *Acidovorax* spp., and *Thauera* spp. These *Betaproteobacteria* members utilize acetate and ethanol as electron donors and have been previously found in the aboveground denitrification reactor used to pre-treat groundwater at this site (16).

Methanol utilization may play a minor role because its small contribution (5% in the ethanol solution used), nevertheless it can be used for denitrification by almost all denitrifiers and also for sulfate reduction by some *Desulfosporosinus* spp. (33) and *Desulfovibrio carbinolicus* (25).

2.c. Other C sources and contaminants

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

Aromatic (phenols) and chlorinated compounds (TCE) were detected in the

groundwater prior to biostimulation but mainly removed during site preconditioning (48). These compounds are still present in the groundwater outside the treatment area and could have infiltrated to the inner loop (based on tracer test results) and provided additional carbon sources, electron donor and acceptors to the underground communities. Several detected groups of different phylogenetic affiliation show some potential for dechlorination or aromatic compounds degradation. In the case of dechlorination, at least 50% of the *Geobacteraceae* sequences were related to *G. lovleyi*, an isolate known to use chlorinated compounds such as TCE and PCE as electron acceptors (39). Close relatives of the chloro-phenol respirer *Anaeromyxobacter dehalogenans* 2CP-1 (34) and PCE respirer *Desulfosporosinus meridiei* were also detected (33). Aromatic compounds can also be used as carbon sources by some bacteria of the *Acidovorax* genus which was detected in the sediments. The *Betaproteobacteria Pseudomonas* sp. strain P51 can degrade chlorinated benzenes (43). Aromatic compounds and solvents present at the beginning of the bioremediation operation could have influenced the initial communities before the biostimulation began though their concentration is generally low and it is not clear if they can support growth at levels present at the site.

Humic substances can be used as electron acceptors (21). Sequences related to the humic-acid reducers *Geothrix* sp. (6) and *G. humireducens* (21) were detected in the libraries. Reduced humic substances can potentially abiotically reduce U(VI) far from the bacteria and they have been shown effective in increasing Fe(III)-reduction in subsurface environments (27).

In addition, other bacterial groups not directly related to bioremediation, as far as we know, were found in the sediments e.g. *Planctomycetes*, *Chloroflexi, Actinobacteria*,

etc. They are likely involved in the degradation or digestion of dead cells, soil humics, and extracellular substances produced during biostimulation in the subsurface. Chloroflexi members are known to grow in filaments, produce hydrogen and are proposed to be involved in dead cell recycling (52). This group can potentially contribute to the stability to the bacterial community by promoting the formation of biofilms and can facilitate bioremediation by transferring electrons in the form of hydrogen to other groups more likely involved in bioremediation such as *Desulfovibrio* spp. The Chloroflexi group was the only group not belonging to the FeRB, SRB or denitrifiers present in all the libraries from the active zone.

2.d. Putative genera involved in U(VI) reduction

Analyses of uranium oxidation state in sediments by XANES confirmed that the decrease in groundwater U concentration was due to reduction of U(VI) to U(IV). Elevated total U and U(IV) percentages of total U (18, 49, 50) were found in the inner loop injection well FW104 consistently with effective bioremediation (Table 2.1). In this study, we found a variety of previously reported U(VI)-reducing bacteria present in the reduced sediments including FeRB *Geobacter* spp. and *Anaeromyxobacter* spp., SRB *Desulfovibrio* spp. and *Desulfosporosinus* spp. as well as the denitrifier *Acidovorax* spp. Our results suggest that uranium reduction cannot be attributed to a single group and it is very likely that this role is taken by several different bacteria.

The contribution to the uranium reduction based solely on the relative frequency in our libraries and the reported literature ranks the contributor groups as following:

Desulfovibrio > *Geobacter* > *Anaeromyxobacter* > *Desulfosporosinus* > *Acidovorax*.

Desulfovibrio spp. were detected in all the samples of the active area and their contribution peaked in wells with high percentage of reduced uranium. This group was the most abundant group with reported uranium reducing abilities and was found in the sediment samples. Even though there are no reports of U(VI)-reduction by the closest relatives of the cloned sequence, the high frequencies of these bacteria in the sediment clones (up to 16%) suggest a substantial role in the groundwater ecosystem.

Geobacter spp. were found in all sediment sample of the active area. Our sequences were closely related to the uranium reducer *G. lovleyi* (39) and to the humic-acid reducer *G. humireducens* (21). Additionally, *Geobacter* spp. was found to be associated with U(VI) reduction at sites contaminated with uranium and U ore where acetate was added (2, 28, 44).

Anaeromyxobacter sequences were detected in all sediment samples from the active area except for FW026. These sequences were related to the known U(VI) reducer *A. dehalogenans* (47) and to a clone from a uranium mine sediment were uranium reduction was demonstrated (40).

Desulfosporosinus sequences were present in half the libraries of the active area and the closest isolate to most of the sequences was *D. orientis*, a SRB also known for reducing Fe(III), nitrate and U(VI) (26, 40). Despite their relative low contribution to the total community, *Desulfosporosinus* spp. may play a minor role in U(VI) reduction and a bigger role in the long term stability of the reduced uranium as they can form spores and survive under starvation conditions.

Acidovorax sequences were found in half the libraries of the active area. An *Acidovorax* sp. was shown capable of U(VI) reduction in microcosms tests with sediments from the FRC (29). Nitrate and nitrite has shown being able to re-oxidize and remobilize Fe(III) (37) and the presence of this denitrifier could contribute to the removal of these competing electron acceptors and ensure the stability of the reduced uranium.

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

Abiotic U(VI) reduction may also play a role under our operational conditions (i.e. pH 5.8-6.6 and HCO_3^- < 5 mM). Sulfide, the end product of SRB; and green rusts, products of FeRB can both reduce U(VI) to U(IV) (15, 30). Indirect U(VI) reduction by reduced humic substances can also be contributing an important piece given the constant presence of FeRB humic-reducing *Geothrix spp.* in all the tested sediments from the active area. Therefore, the activity of SRB and FeRB could also indirectly contribute to the reduction of U(VI) and maintenance of a stable and low level of uranium.

3. Dynamics of the community

3.a. Patterns of diversity detected

Microbial diversity varied in the sediments of the treated zone (Table 2.4). The utilization of standardized techniques in all of our community analyses allows us to conclude that the observed differences were due to real differences in the community, and not the consequence of PCR bias. The irregular pattern observed is likely due to the heterogeneity of groundwater flow and the distribution of contaminants as shown by the tracer studies. Microbial enumeration analyses for the three trophic groups studied (denitrifiers, FeRB and SRB) showed a decline in cell counts going from the injection well and its more connected wells, to the less connected wells (Table 2.2). Even though replication did not allow for statistical conclusions, some apparent trends were observed.

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

homogenous response to the bioestimulation.

When analyzing the carbon and electron acceptors, more ethanol-consuming organisms were detected in the more connected wells. Consistent with the expected sequential electron acceptor utilization (49), more denitrifiers where present in highly connected wells and FeRB were more abundant in the wells with lower connectivity. The exception to this pattern was *Desulfovibrio*, a SRB that showed high relative abundance in more connected wells, possibly due to its ability to utilize the injected ethanol. *Desulfovibrio* spp. were more abundant than *Desulfosporosinus* spp., also a SRB capable of using ethanol. The presence of *Desulfovibrio* in the aboveground bioreactor (16) could have given *Desulfovibrio* spp. an initial competitive advantage over *Desulfosporosinus* spp. by a continuous inoculation of *Desulfovibrio* spp. carried in the treated water from the bioreactor. On the other hand the ability of *Desulfosporosinus* to sporulate and degrade methanol may account for its survival. Overall the microbial methods were in agreement with the hydrological studies. However, more study is needed to understand the relation quantitatively.

3.b. Emergence of previously undetected populations

After preconditioning by pH adjustment and removal of inhibitors, the ethanol injection increased the microbial diversity of the subsurface as shown by diversity indices that consider richness and abundance (Table 2.4). The activity of the new complex bacterial community created favorable conditions where FeRB, SRB and denitrifiers thrive in contrast with the untreated area where they were either absent or in very low

levels.

It is very unlikely that the microbial community observed was fully derived from indigenous species that survived the extreme conditions of the area. The most likely source of new colonists was the upper soil where nitrate is low and pH neutral. These bacteria could have been transported by either natural groundwater infiltration or forced recirculation during the treatment. *Geobacter* and *Anaeromyxobacter* spp. have been found in FRC Areas 1 and 2 using iron-reducer enrichments with acetate and lactate (31). *Geobacter* spp. and *Geothrix* spp. have been also detected in the FRC Area 2, using high density 16S rRNA gene arrays (5). *Pseudomonas* spp. and *Azoarcus* spp. have been detected in the FRC Area 3 with 16S rRNA clone libraries prior to biostimulation (9). Therefore, these microorganisms are present in pH neutral soils at the site.

A second likely microbial source could be the aboveground bioreactor system. This reactor worked for 400 days to remove nitrate from the groundwater and the treated nitrate-free water was reinjected into the subsurface (48, 49). The reinjected water likely carried some bacteria to the treated area although it was filtered. Sequences related to *Desulfovibrio*, *Thauera*, *Azoarcus*, *Ferribacterium* and *Acidovorax* have been previously reported for the bioreactor (16) and were detected in the collected sediments of the active area in this study.

Our results suggest that biostimulation efforts successfully fostered communities comprised of a variety of bacterial groups (*Geobacter*, *Desulfovibrio*, *Geothrix*, *Anaromyxobacter*, *Desulfosporosinus* and *Acidovorax*) involved in the groundwater remediation process. This study provides a detailed view of the differences and

similarities among the microbial communities throughout the active area that correlated with the path of groundwater flow depicted by tracer studies. The results demonstrated that microbial communities can be established by *in situ* biostimulation with an electron donor to achieve successful reduction of U(VI) concentration below US EPA MCL. These findings contribute to an improved understanding of the composition, variability and controls on microbial communities in the subsurface associated with a successful bioremediation process and provide a foundation for future implementation and monitoring efforts applied to this and other contaminated sites.

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CHAPTER THREE

Massive parallel sequencing of microbial communities in a uranium remediation site

INTRODUCTION

Metals and radionuclides are two major contaminants at Department of Energy (DOE) facilities and waste sites (45). One of these sites, the former Y-12 National Security Facility at Oak Ridge, Tennessee, stored its waste from uranium enrichment operation in four unlined ponds of 9.5 million liter capacity each for over 31years. The ponds received acidic uranium nitrate (~30% uranium nitrate) from its uranium enrichment operations as well as waste from other DOE sites. After 31 years of operations the ponds were neutralized, subject to nitrate removal, and its water treated for nitrate removal before the ponds were capped (the contaminated sludges remained). The contamination, however, was not contained within the ponds but expanded to nearby areas through groundwater movement. The nearby Area 3 contains uranium in concentrations as high as 250 μM in groundwater and 800 mg/kg in sediments (8, 58).

Since metals cannot be degraded, other strategies are needed to reduce their bioavailability. One of these alternatives is microbial dissimilatory reduction, an anaerobic process in which microbes use metals such as Fe(III), Mn(IV), Cr(VI) , Se(VI) and radionuclides such as U(VI), Tc(VII) as final electron acceptors to support their growth. Metal reduction involves changes in oxidation state than can trigger changes in water solubility making some metals more soluble and easier to remove from the system (e.g. Mn), and others less soluble, hence less mobile and less bioavailable, like in the

case of uranium. Since uranium reduction was first discovered it has been proposed as an alternative to control U bioavailability (2, 3, 23, 29, 34, 58).

The ability to reduce U(VI) to U(IV) was first shown in the Fe(II) reducing bacteria (FRB) *Geobacter metallireducens* (34) and later in the sulfate- reducing bacteria (SRB) *Desulfovibrio desulfuricans* (33). Since then, U(VI) reducers have been found in a variety of phylogenetic groups such as Delta-, Beta- and Gamma-*Proteobacteria*, *Firmicutes, Deinococci, Actinobacteria* among others groups (55). Beside the ability to reduce uranium, there is no common theme among uranium reducing bacteria as they represent metabolically different groups such as FRB, SRB, fermenters, and denitrifiers.

Uranium reduction for bioremediation purposes has been tested successfully with pure cultures of Fe(III)-reducing bacteria such as *Geobacter* spp.; sulfate-reducing bacteria such as *Desulfovibrio*, *Desulfoporosinus, Shewanella*, and *Desulfotomaculum* spp.; fermenters such as *Clostridium*; heterotrophs such as *Deinococcus* spp. and denitrifying bacteria such as *Acidovorax* spp., among others (55). More recently, batch serum bottles (18, 25, 61), microcosms (43), and sediments columns (20, 56) have been used to study this approach in controlled conditions. Field scale studies have recently started at a former uranium ore processing facility (Old Rifle site, now part of the Uranium Mill Tailings Remedial Action program of the DOE) at Rifle, Colorado (54), and at the Field Research Center (FRC) at Oak Ridge, Tennessee, the site containing residue from the former Y-12 uranium enrichment facility (3, 29, 58).

Studies of uranium reduction have shown response of FRB and/or SRB, though the stimulatory conditions were different. *Geobacter* was shown to be abundant at the

Old Rifle site, where uranium reduction was promoted through acetate injection into the subsurface (3, 25, 42, 54). A different study also at the Old Rifle Site found enrichments of FRB (*Geobacter* spp.) and also SRB (*Desulfobacterales*) (54). A recent publication from this site has shown that uranium removal from groundwater continued even after acetate injection stopped due to uranium absorption into biomass of *Firmicutes*-related bacteria (40). Though not expected to be uranium reducers but dead-cell recyclers, their effect in controlling uranium bioavailability is real and promising. In field studies from the FRC Area 3 where ethanol was used as stimulatory electron donor, *Desulfovibrio* spp. and *Geobacter* spp. have been associated to the uranium (10, 27); and *Geobacter* and *Geothrix* have been found in enrichments using Area 2 samples with lactate as electron donor (6). In anaerobic enrichments of contaminated sediments of a uranium mine, sequences related to the SRB *Desulfosporosinus*, and fermentative *Clostridium* (53) appeared to be major contributors to U(VI) reduction.

This present work studies the approach taken at the FRC Area 3 for uranium and nitrate bioremediation. The bioremediation strategy was based on a hydraulic control system that creates an area, isolated from groundwater intrusion and which allows electron donors to be injected into the system to promote growth of local communities of uranium reducers. The system was created using a series of wells that pump and recirculate the groundwater. Over a two year period, the treatment was successful in removing nitrate and conditioning the subsurface for the establishment of uranium reducing communities (58). After conditioning, ethanol was injected and uranium levels in groundwater dropped. X-ray Absorption Near Edge Structure (XANES) confirmed that uranium was being reduced, discarding the possibility that uranium removal from water

was due to sorption or complexation with inorganic compounds (59). Microbial surveys of sediments from six wells from Area 3 detected genera known to harbor U(VI)-reducing members such as *Geobacter*, *Desulfovibrio*, *Aneromyxobacter*, *Desulfosporosinus,* and *Acidovorax* after uranium reduction was established in the zone (10) . Microbial counts, measured as Most Probable Number, correlated with the hydraulic path; however, the proportion of U present as U(IV) did not follow a linear relationship with the hydraulic connection (10). This suggests that factors other than electron donor availability are influencing the presence and/or activity of the uranium reducing populations. A more recent study tracked the microbial communities of FRC Area 3 groundwater over a 1.5 year period and found that environmental factors such as nitrate, uranium, sulfide, and ethanol were correlated with particular bacterial populations (27). More interestingly, the engineering adjustments to control dissolved oxygen and deliver nutrients to the subsurface were also found to be significant in explaining the biological variability (27).

In this study we explored the role of environmental conditions, including hydrology, on the structure of the sediment microbial communities of FRC Area 3 in a period of high uranium reduction. The use of sediments allows for a more precise spatial characterization than using groundwater samples where filtering large volumes of water is required because of low biomass. Additionally, groundwater samples may not reflect the attached communities present in sediments, especially in oligotrophic aquifers such as the FRC (22).

For this purpose we thoroughly surveyed the microbial communities present in the area to include a wider range of environmental conditions. We used massive parallel

16S rRNA gene sequencing to obtain a deeper survey of the microbial communities, and higher confidence in the detection indicator species. We were able to detect groups associated with uranium reduction, to explain differences in community structure with environmental factors, and to manage with high confidence the massive number of sequences to provide candidate groups likely important for the bioremediation of the Area 3.

MATERIAL AND METHODS

Site description and sampling. Samples were retrieved from Area 3 of the DOE FRC at the Y-12 National Security Complex, Oak Ridge, Tennessee, USA. In this area, a hydraulic control system was used to promote *in situ* bioremediation in a controlled fashion. The system consisted of an outer groundwater recirculation loop (injection well FW024 and extraction well FW103) that isolated an inner groundwater loop (injection well FW104 and extraction well FW026) preventing penetration by highly contaminated groundwater from the source zone (Figure 3.1). Well FW105 was used to feed water to the above ground treatment system and provided additional hydraulic control to the outer loop (58). The outer loop was expected to respond to changes in groundwater movement, while the inner loop's shape and flow was expected to remain stable (58). The remediation started on August 23th, 2003 (day 0). During the first 137 days, groundwater was pumped and treated *ex situ* to adjust pH, remove Al, Ca and nitrate (via a denitrification bioreactor). This process removed nitrate competition as a terminal electron acceptor, avoided formation of stable Ca-U-CO_3 products and clogging by gas and biomass due to denitrification. Reduction of U(VI) to U(IV) was accomplished

through ethanol injection to the inner loop, starting on day 137. Multilevel sampling (MLS) wells, FW100, FW101 and FW102 were used to monitor hydrogeology and remediation performance over seven levels (-1 to -7). Except for the multisampling wells, the wells were 14.6 m depth with screens between 11.28 m and 13.77 m. Multisampling wells retrieved sediments from 15.2 , 13.7, 12.2, and, 10. 7 m for levels -1, -2, -3, and -4, respectively. Water and sediments samples were retrieved on October 5th, 2005(day 775) as previously described (10).

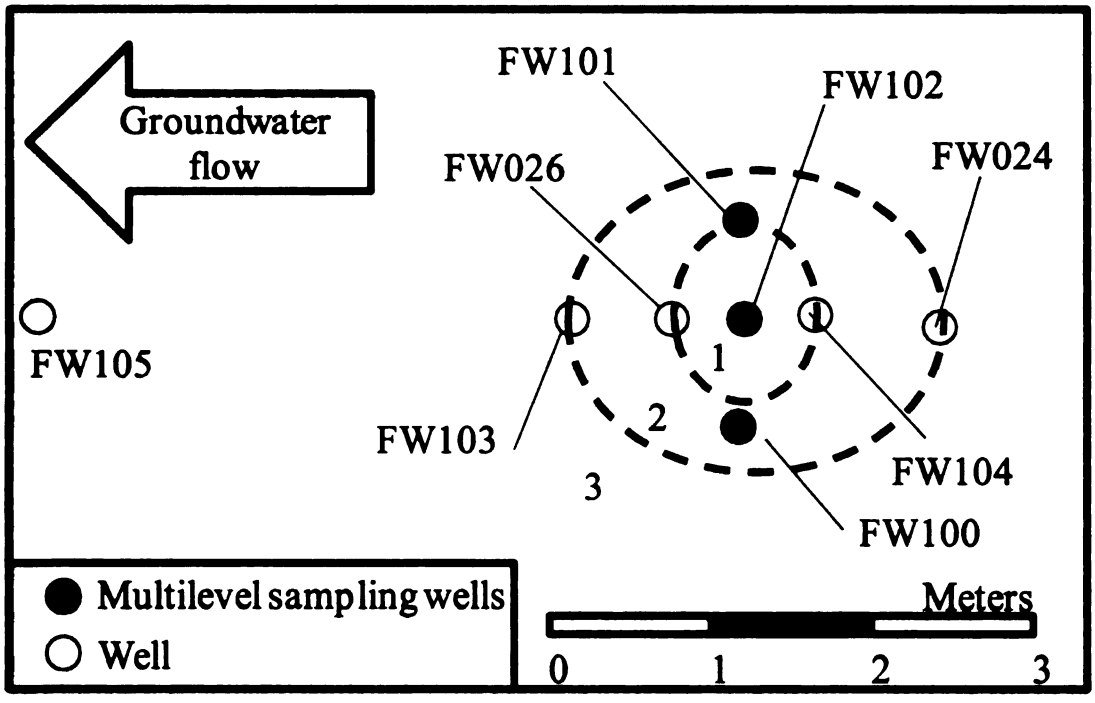

Figure 3.1. Scheme of the DOE Field Research Center Area 3 treatment system. Wells FW104 and FW024 are injection wells, FW026,FW105 and FW103 are extraction wells. Wells FW100, FW101, and FW102 are multilevel sampling wells. The controlled hydraulic scheme creates an outer cell (2) that protects an inner cell (1) from intrusion of contaminated groundwater from the background (3). The stimulatory electron donor, ethanol, is injected through FW104 into the inner cell. The terms inner/outer loop and inner/outer cells are used interchangeably throughout this article.

Chemicals and analytical methods. Chemical oxygen demand (COD), sulfide

and Fe(II) were determined using a Hach DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Anions (including NO_3^-, Br^-, Cl^-, SO_4^{2-} and PO_4^{3-}) were analyzed with an ion chromatograph as previously described (10). Metals (Al, Ca, Fe, Mn, Mg, U and K etc.) were determined using an inductively coupled plasma mass spectrometer (ICPMS) (Perkin Elmer ELAN 6100), and U reduction state was determined with XANES as described elsewhere (59, 60). The proportion of Fe(II) to the total Fe was measured as HCl (10%) extractable. Two hydrology parameters that described the connectivity of the different locations to the injection well were measured with a tracer study that introduced a conservative tracer (bromide) at the injection well (FW104) were previously determined (35). Tracer recovery (%) was considered a proxy for the amount of the stimulatory electron donor received at each location. Similarly, mean travel time (from FW104) was used as proxy for the composition of the electron donor as ethanol is converted to acetate by the microbial communities.

DNA extraction and direct sequencing. DNA was extracted from 0.5 g of sediments with the Fast soil prep kit (MoBio Inc., San Diego, CA, USA) following the manufacturer's instructions. Ribosomal RNA genes were amplified using a primer set that flanked the v4 hypervariable region of the 16S rRNA gene at corresponding *Escherichia coli* positions 563 and 802. Primers 563F (5'-GCCTCCCTCGCGCCATCAG(*barcode*)**AYTGGGYDTAAAGVG**-3') and 802R (5'-GCCTTGCCAGCCCGCTCAG**TACNVGGGTATCTAATCC**-3') were a combination of universal eubacterial primers and sequence adaptors (**in bold**) used in the amplification

step of the 454 Roche pyrosequencing process. The forward primer additionally contained a short run of nucleotides used as bar codes. The bar codes were designed to be distinct from each other by at least two nucleotide changes.

Primers were dual HPLC-purified (Integrated DNA Technologies, Coralville, IA). Each PCR mixture contained 1 μM of each primer (dual HPLC-purified, Integrated DNA Technologies, Coralville, IA), 1.8 mM $MgCl_2$, 0.2 mM for each dNTP, 3 μg BSA (New England Biolabs, Beverly, MA), 1 unit of FastStart High Fidelity PCR System enzyme blend (Roche Applied Science, Indianapolis, IN), and 10 ng of DNA template. Each sample was run in triplicate. The PCR cycling conditions were: 95°C for 3 min; 95 °C for 45s, 57 °C for 45s, 72°C for 1min, for 30 cycles; 72 °C for 4 min. PCR products were run in a 1% (wt/vol) agarose Tris-acetate-EDTA gel and bands between 270-300 bp were excised. Bands for each triplicate were pooled together for gel purification with the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). After gel extraction, products were cleaned for a second time with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA) and eluted with 20 μl of EB buffer (10 mM Tris-Cl, pH 8.5). Clean products were quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and mixed in equal amounts, 20 ng each for direct sequencing by Genome Sequencer FLX System (454 Life Sciences).

Pyrosequencing analysis. Sequences were processed with the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (14). Sequences were first trimmed to remove the pyrosequencing adaptor sequences, and then sorted according to their bar

codes (used in the PCR amplification). Two quality filters were applied to remove sequences with aberrant lengths and to discard sequences with more than two changes in the primer portion. The RDP also provided a complete linkage clustering tool and secondary-structure-based alignment tool based on INFERNAL version 8.1 (http://infernal.janelia.org/) and a 16S rRNA secondary structure model (9).

Community analysis. Sequences from different samples were classified using RDP classifier with a 80% bootstrap confidence (57). To rapidly separate sequences into taxonomic groups, RDP's Classifier was used at a 50% confidence. e.g. to obtain all *Firmicutes* from sample A. Sequences from that particular taxon from different samples were later aligned and clustered (complete linkage) in order to track the distribution of a specific group among samples.

Operational taxonomic units (OTUs) were defined from 0% to 20% sequence dissimilarity (distance) to calculate richness of individual samples at different levels of taxonomy. The results were rarified to the lowest number of sequences per sample from the group of samples being compared. When an individual phylum or class was studied, OTUs were defined at 5% distance (~genus level). When an individual genus was studied, OTUs were defined at 1% distance (species level).

To compare individual samples according to their diversity patterns (beta diversity analysis), a modified Sorensen index was used. This index has been corrected by using abundance information to account for unseen species in the survey (11). Clustering of samples was done using the average neighbor clustering algorithm on the

modified Sorensen index matrix. To find groups that represent a specific branch of the clustering tree, indicator species analysis was used on the resulting topology (16). This method was preferred because it provides a consistent approach to analyze the high number of sequences per sample. The indicator value algorithm considers both frequency of occurrence, and relative abundance, and its higher when a species is present in all the samples and highly abundant in one specific group (16), this avoids considering indicator species groups that appear only on one location. Since we test each OTU for significance in the indicator species analysis, and multiple tests could increase the chance of finding significant results by chance, we used a false discovery rate (FRD) approach (5). The FDR estimates the chance of reporting a false positive in all the significant results (q-value) instead of using excessively conservative corrected p-values. Q values were generated from p-values using the freely available software package QVALUE Ver. 1.0 (51, 52) using the bootstrap method.

Multivariate analysis. Environmental variables were standardized (z-score) and tested for correlation using a principal component analysis (PCA) on their correlation matrix. This result was used to find variables correlated to uranium reduction, sulfate reduction and iron reduction, to create a model on how the treatment changes the geochemical characteristics, and to select environmental for direct gradient analysis. For direct gradient testing, environmental variates were normalized using a log(x+1) or arcsin($\sqrt{x}$) transformations, pH was left unchanged. Hellinger transformation was used in some cases on OTUs abundance data to make it suitable even if when many zeros were present (32). Principal coordinates analysis (PCoA) and non-dimensional scaling, was performed on untransformed species data.

Statistical analyses were conducted using the R environment v2.80 (http://www.R-project.org.) with packages Vegan v1.8-8 (50), BiodiversityR (13), Labdsv v1.3-1 (46) (indicator species analysis), and QVALUE v1.1 (available at http://genomics.princeton.edu/storeylab/qvalue/) (51, 52).

RESULTS

Chemical and hydrological characterization. Analysis of DOE FRC Area 3 hydrology (Table 3.1), groundwater (Table 3.2) and sediments (Table 3.3) showed differences in geochemical and hydrological composition due to the creation of the three zones (inner loop, outer loop and background) by the treatment system. One of the hydrology parameters, percentage of tracer recovery, an indication of how much water from the injection well FW104 reached to a particular well, was significantly higher in the inner loop ($p<0.01$) than in the outer loop. Mean travel time, the second hydrology parameter and an indication on how long does it takes groundwater to move from the injection well FW104 was not significantly different in the two loops. When grouping the two multilevel sampling (MLS) wells from the inner loop (FW101 and FW102) according to the depth they sampled, wells at level -1 and -4 received significantly less water from FW104 ($p<0.05$), and were less connected ($p<0.001$) than wells from levels -2 and -3. This is likely because the injection at FW104 occurred at depths between levels -2 and -3.

When compared to the outer loop, inner loop groundwater was richer in sulfate ($p<0.01$), Fe ($p<0.01$), K ($p<0.01$), and chloride ($p<0.05$); and more depleted in sulfide ($p<0.05$). This trend remained when compared to the background area (FW105) though

no statistical comparison was possible (only one sample from background well existed). Samples from depths 2 and 3 of the inner loop were significantly higher in K ($p<0.01$), and Fe (<0.05) and lower in chloride ($p<0.05$) than samples from levels 1 and 4.

Sediment analysis showed reduced U(IV) only in wells from the inner loop, and a higher proportion of reduced Fe(II) in the inner loop ($p=0.08$). Sulfide levels of sediments were also higher in the inner loop, although not significantly. These three trends remained when adding the background well FW105, though no statistical test was possible.

Overall, the bioremediation system seems to have influenced the composition of the groundwater by creating gradients in electron donor availability (due to differences in connectivity) and maintaining the isolation of the inner loop from contaminated background groundwater. The differences found can be related to the activity of SRB and FRB as observed in changes in sulfate/sulfide and total Fe/reduced Fe(II).

Table 3.1. Hydrological characterization of the DOE FRC Area 3 wells.

Well*	Tracer studies results	
	Mean travel time (hr)	Tracer recovery (%)
FW104	0	100
FW101-1	387	18
FW101-2	2.9	93
FW101-3	17.9	60
FW101-4	298	18
FW102-1	222	3
FW102-2	11.6	94
FW102-3	3.7	94
FW102-4	790	6
FW026	8.1	50
FW024	7.8	4.5
FW100-1	211	9
FW100-2	223	12
FW100-3	43	18
FW100-4	211	8
FW103	7.8	10
FW105	710	6
Inner loop (mean± SE.)	174.1 ± 82.3	53.6 ± 12.6
Outer loop (mean± SE)	117.3 ± 44.1	10.3 ± 1.9
Background	710	6
P-value**	0.622	0.008

Results from hydrology studies using bromide (conservative tracer) on days 801 to 802 (November 1st to 2nd, 2005). Tracer was injected at well FW104, tracer recovery and travel time at this well are 100% and 0 h by definition. The treatment system created three different areas: inner loop (FW104, FW026 and multilevel wells FW101 and FW102), outer loop (FW024, FW103 and multilevel well FW100), and a local background (represented by FW105).*For multilevel sampling wells the well location is followed by the depth where the sample was taken. **Comparison of inner and outer loops was done with a two-tailed t-Test, alpha=0.05.

Table 3.2. Chemical composition of groundwater from wells from DOE FRC Area 3.

	Chemical Composition of Groundwater (mM)												
Well*	pH	Sulfate	Sulfide	Nitrate	Cl-	U	Na	K	Mg	Al	Fe	Mn	Ca
FW104	5.75	1.18	0.30	0.000	2.34	0.569	2.10	0.85	0.32	0.017	0.031	0.072	0.65
FW101-1	6.78	0.83	0.00	0.520	2.42	0.559	1.92	0.49	0.48	0.012	0.004	0.065	2.50
FW101-2	6.23	1.07	0.43	0.000	2.32	0.150	2.02	1.40	0.30	0.012	0.034	0.074	0.67
FW101-3	6.10	1.20	0.11	0.003	2.30	0.111	2.20	0.87	0.35	0.012	0.005	0.063	0.67
FW101-4	5.56	1.41	0.00	0.290	2.49	0.634	2.51	0.49	0.28	0.103	0.005	0.047	0.69
FW102-1	6.38	0.29	0.00	34.419	2.65	0.739	5.78	0.15	2.82	0.016	0.014	0.269	13.15
FW102-2	6.45	1.01	0.16	0.011	2.32	0.083	1.97	1.63	0.31	0.011	0.039	0.077	0.80
FW102-3	6.23	1.10	0.36	0.001	2.31	0.061	2.02	1.33	0.31	0.012	0.045	0.083	0.62
FW102-4	4.43	1.23	0.00	0.018	2.44	5.427	1.47	0.76	0.21	0.103	0.002	0.036	0.29
FW026	5.74	1.20	0.04	0.007	2.36	0.525	2.05	0.80	0.32	0.016	0.006	0.071	0.64
FW024	5.87	1.58	0.00	0.011	2.32	0.092	2.49	0.15	0.45	0.013	0.003	0.014	0.79
FW100-1	6.67	1.23	0.00	1.903	2.23	0.923	1.65	0.29	0.63	0.012	0.005	0.048	3.19
FW100-2	5.50	1.54	0.00	0.017	2.38	0.979	2.05	0.48	0.33	0.018	0.003	0.047	0.78
FW100-3	5.87	1.45	0.00	0.023	2.27	0.326	2.43	0.21	0.43	0.014	0.003	0.027	0.77
FW100-4	5.80	1.44	0.00	1.597	2.26	1.505	2.27	0.32	0.47	0.093	0.003	0.082	1.60
FW103	5.92	1.51	0.01	0.000	2.31	0.196	2.53	0.29	0.41	0.012	0.004	0.022	0.78
FW105	4.66	1.73	0.00	2.108	2.27	5.239	1.29	0.55	0.35	0.187	0.000	0.102	1.14

Table 3.2. (cont'd)

Well*	Chemical Composition of Groundwater (mM)												
	pH	Sulfate	Sulfide	Nitrate	Cl-	U	Na	K	Mg	Al	Fe	Mn	Ca
Inner loop (mean± SE)	5.97 ± 0.21	1.05 ± 0.1	0.14 ± 0.05	3.527 ± 3.433	2.39 ± 0.03	0.886 ± 0.511	2.4 ± 0.38	0.88 ± 0.14	0.57 ± 0.25	0.031 ± 0.012	0.019 ± 0.005	0.086 ± 0.021	2.07 ± 1.25
Outer loop (mean± SE)	5.94 ± 0.16	1.46 ± 0.05	0.00 ± 0.00	0.592 ± 0.368	2.29 ± 0.02	0.67 ± 0.226	2.24 ± 0.14	0.29 ± 0.05	0.45 ± 0.04	0.027 ± 0.013	0.003 ± 0	0.04 ± 0.01	1.32 ± 0.4
Background	4.66	1.73	0	2.1081	2.2694	5.2387	1.291	0.55	0.35	0.1872	0.000	0.1023	1.145
P- value**	0.926	0.003	0.027	0.417	0.053	0.706	0.690	0.003	0.657	0.821	0.018	0.070	0.578

Samples were retrieved on day 774 (October 5th, 2005). Concentrations are in mM (except pH). Treatment system created three different areas: inner loop (FW104, FW026 and multilevel wells FW100 and FW200), outer loop (FW024, FW103 and multilevel well FW100), and background (represented by FW105). *For multilevel sampling wells the well location is followed by the depth where the sample was taken. **Comparison of inner and outer loops was done using a two- tailed t-Test, alpha=0.05.

Table 3.3. Chemical composition of sediments from DOE FRC Area 3.

Well*	Chemical Characteristics of Sediments						
	Bioactivity	U (mg/kg)	U(IV)/ Total U (%)	Total Fe (g/kg)	Fe(II)/ Total Fe (%)	COD (g/kg)	Sulfide (g/kg)
FW104	High	10.3	61	199.1	0.53000	337.9	23.000
FW101-1	Low	0.051	0	48.3	0.02640	8.6	0.000
FW101-2	High	1.249	54	43.3	0.27400	23.9	0.667
FW101-3	High	1.825	51	29.4	0.29400	24.2	0.265
FW101-4	Low	0.619	0	42.6	0.04660	27.1	0.000
FW102-1	Low	0.041	0	39.4	0.00560	22.6	0.000
FW102-2	High	0.517	17	33.7	0.30900	20	0.557
FW102-3	High	0.875	30	36.4	0.28700	22.5	0.431
FW102-4	Low	0.481	0	37.1	0.00660	12.7	0.000
FW026	Medium	1.22	0	47.1	0.14800	22.7	0.034
FW024	Low	0.371	0	50.8	0.21900	33.2	0.070
FW100-1	Low	0.215	0	55.6	0.00142	10.5	0.000
FW100-2	Medium	0.98	0	32.4	0.12640	7.5	0.000
FW100-3	Medium	1.098	0	36.2	0.06360	13.4	0.000
FW100-4	Low	1.501	0	34.4	0.05450	23.8	0.000
FW103	Low	0.658	0	44.9	0.00510	32.1	0.000
FW105	Low	0.978	0	47.8	0.00480	30.7	0.000
Inner loop (mean ± SE)	-	1.718 ± 0.97	21.3 ± 8.1	55.6 ± 16	0.19272 ± 0.05515	52.2 ± 31.8	2.495 ± 2.28
Outer loop (mean ± SE)	-	0.8 ± 0.197	0	42.4 ± 3.9	0.07834 ± 0.03374	20.1 ± 4.6	0.012 ± 0.012
Background	-	0.978	0	47.8	0.0048	30.7	0
P-value**	-	0.377	ND	0.441	0.157	0.343	0.304

Samples retrieved on day 774 (October 5th, 2005). Treatment system created three different areas: inner loop (FW104, FW026 and MLS FW100 and FW200), outer loop (FW024, FW103 and MLS FW100), and a local background (represented by FW105).

*For multilevel sampling wells the well location is followed by the depth where the sample was taken. **Comparison of inner and outer loops with a two tailed t-Test, alpha=0.05.ND, mathematically not defined. Bioactivity is a qualitative descriptor based on Most Probable Number (MPN) results for iron reducing bacteria, sulfate reducing bacteria and denitrifiers. This variable is used more as a general guide rather than a strictly quantitative indicator. The MPN methodology is described elsewhere (10).

Correlation of environmental variables. Correlation analysis showed that the environmental variables measured were highly correlated. PCA on the correlation matrix (Figure 3.2) showed that most of the variables were collinear with at least one other variable as shown in the correlation matrix (Table 3.4).

Hydrology factors (mean travel time and tracer recovery) seemed to influence key variables such as pH. Mean travel time was correlated with pH, Al and U in groundwater. It is not clear if U levels in groundwater were influenced by pH directly or controlled by microbial activity. The percentage of tracer recovery was correlated with sulfide levels in groundwater, potassium and iron in groundwater.

Reduced uranium in sediments (U(VI)/Total U) was strongly correlated with bioactivity and reduced iron and moderately correlated with sulfide levels in sediments, uranium levels in sediments, iron in sediments and chemical oxygen demand (COD). Nitrate was highly correlated ($r>0.9$) with Na, chloride, Mg, Mn, and Ca. Nitrate was also correlated with chloride and negatively with sulfate but with smaller strength ($r= 0.72$ and -0.72 respectively). The correlation of reduced U(IV) with variables related to FRB and SRB and not with nitrate reduction suggests that FRB and SRB are more important for U(VI) reduction at this location than nitrate reducing populations.

Based on the correlation analysis, the following variables were kept: pH, uranium in sediment, tracer travel time, nitrate, and COD in sediments.

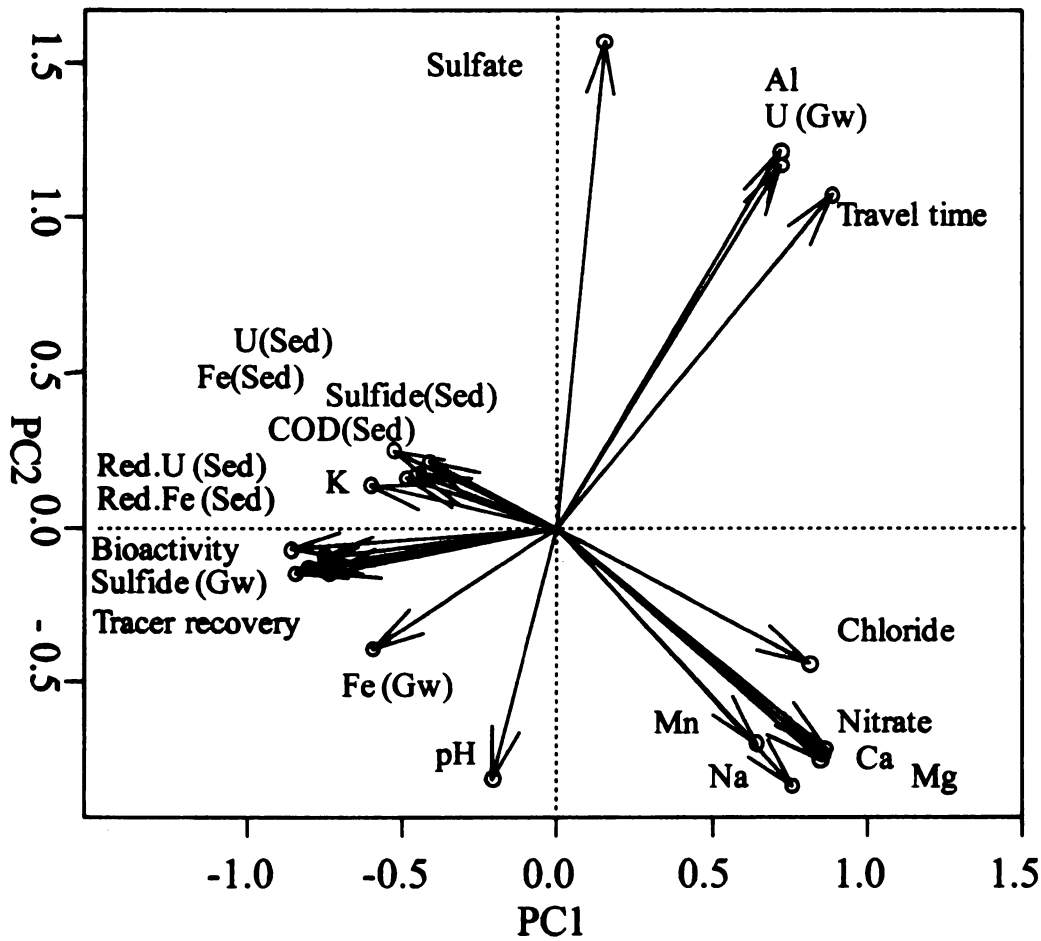

Figure 3.2. Correlation plot of environmental variables measured for 15 locations at DOE FRC Area 3. Variables were z-score transformed prior to correlation analysis. Bioactivity was converted as following, High=3, Medium=2 and Low=1.

Table 3.4 Correlation of chemical characteristics of groundwater, sediments and hydrology of the DOE FRC Area 3 wells.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. pH	1.00																					
2. Sulfate	-0.55	1.00																				
3. Sulfide	0.25	-0.23	1.00																			
4. Nitrate	0.20	-0.70	-0.18	1.00																		
5. Chloride	-0.02	-0.65	-0.18	0.72	1.00																	
6. U (gw)	-0.81	0.27	-0.32	-0.01	0.04	1.00																
7. Na	0.31	-0.64	-0.15	0.91	0.72	-0.32	1.00															
8. K	0.11	-0.20	0.78	-0.31	-0.16	-0.11	-0.35	1.00														
9. Mg	0.30	-0.70	-0.21	0.99	0.68	-0.10	0.93	-0.37	1.00													
10. Al	-0.76	0.43	-0.31	-0.07	-0.01	0.83	-0.27	-0.14	-0.16	1.00												
11. Fe (gw)	0.37	-0.41	0.89	0.00	-0.02	-0.37	0.02	0.81	-0.02	-0.36	1.00											
12. Mn	0.19	-0.74	0.05	0.92	0.63	0.04	0.77	-0.03	0.88	0.04	0.22	1.00										
13. Ca	0.33	-0.73	-0.22	0.98	0.68	-0.06	0.88	-0.36	0.99	-0.13	-0.03	0.89	1.00									
14. Bioactivity	0.17	-0.10	0.31	-0.25	0.12	-0.33	-0.11	0.19	-0.25	-0.20	0.06	-0.16	-0.23	1.00								
15. U (sed)	-0.08	0.01	0.43	-0.15	-0.08	-0.07	-0.12	0.12	-0.16	-0.07	0.31	-0.04	-0.17	0.17	1.00							
16. Reduced U* (sed)	0.24	-0.25	0.49	-0.16	0.12	-0.23	-0.11	0.26	-0.16	-0.15	0.29	-0.04	-0.12	0.13	0.17	1.00						
17. Fe (sed)	-0.02	-0.06	0.33	-0.02	-0.03	-0.07	-0.02	0.04	-0.02	-0.11	0.29	0.04	-0.02	0.13	0.17	0.13	1.00					
18. Reduced Fe** (sed)	0.13	-0.06	0.41	-0.22	0.07	-0.09	-0.07	0.16	-0.22	-0.23	0.18	-0.02	-0.14	0.13	0.17	0.13	0.13	1.00				
19. COD (sed)	-0.06	-0.01	0.41	-0.12	-0.07	-0.08	-0.08	0.13	-0.12	-0.09	0.34	-0.09	-0.10	0.13	0.17	0.13	0.13	0.13	1.00			
20. Sulfide (sed)	-0.04	-0.05	0.40	-0.08	-0.02	-0.09	-0.05	0.12	-0.09	-0.11	0.33	0.00	-0.10	0.13	0.17	0.13	0.13	0.13	0.13	1.00		
21. Mean travel time	-0.64	0.12	-0.45	0.08	0.24	0.92	-0.21	-0.22	0.01	0.78	-0.46	0.09	0.07	0.13	0.17	0.13	0.13	0.13	0.13	0.13	1.00	
22. Tracer recovery	0.30	-0.25	0.90	-0.26	-0.20	-0.40	-0.20	0.88	-0.29	-0.38	0.88	0.00	-0.30	0.13	0.17	0.13	0.13	0.13	0.13	0.13	0.13	1.00

Table 3.4 (cont'd)

Variable	1. pH	2. Sulfate	3. Sulfide	4. Nitrate	5. Chloride	6. U (gw)	7. Na	8. K	9. Mg	10. Al	11. Fe (gw)	12. Mn	13. Ca	14. Bioactivity	15. U (sed)	16. Reduced U* (sed)	17. Fe (sed)	18. Reduced Fe** (sed)	19. COD (sed)	20. Sulfide (sed)	21. Mean travel time	22. Tracer recovery
14														1.00	0.45	0.83	0.25	0.85	0.35	0.38	-0.20	0.36
15															1.00	0.64	0.94	0.70	0.98	0.98	-0.19	0.45
16																1.00	0.50	0.85	0.58	0.59	-0.11	0.44
17																	1.00	0.59	0.98	0.98	-0.18	0.38
18																		1.00	0.66	0.68	-0.31	0.44
19																			1.00	0.99	-0.21	0.46
20																				1.00	-0.19	0.45
21																					1.00	-0.54
22																						1.00

*Percentage of U(IV) to total U. **Percentage of Fe(II) to total Fe. Variables 1 to 13 were measured in groundwater (gw) samples, variables 14 to 20 in sediments (sed), and variables 21 and 22 in tracer studies. Pearson correlation is significant if greater than 0.482 (two tailed, alpha=0.05)

Pyrosequencing results

The 16S rRNA gene surveys produced a total of 97, 610 sequences for 17 samples that covered a wide range of conditions in the FRC Area 3 including wells from the inner and outer loops, three MLS wells at four different depths and one well (FW105) outside the treatment area. The high throughput provided a wide view of the microbial diversity present at the site (Table 3.5). The surveys were powerful enough to detect phyla poorly represented in the databases (Table 3.6). In some of these cases there were more sequences in the libraries for a given phyla than in the complete Ribosomal Database Project dataset, though RDP Classifier tools is better trained for well studied groups.

Two samples yielded a low sequence count and were discarded from the analysis (FW100-1 and FW101-1). Without considering those samples there was no significant difference in microbial richness recovered between inner and outer loops. Rarefaction curves (Figure 3.3) suggests that even with thousands of sequences per sample the sampling would be incomplete. This was not surprising given the high microbial diversity expected for non-extreme environments (17, 26). Estimates of coverage (data not shown) were similar to previous experiments that used ~100 clones per library (10). Given that we obtained 20 to 80 times more sequences per sample, it is clear that rarefaction curves are only informative when a plateau has been reached, otherwise the coverage estimation is deemed to fail, especially if using non-parametric models such as Chao1.

Table 3.5. Sequences per sample and number of groups detected at different levels of taxonomy.

Well	Sequences	Phylum	Class	Order	Family	Genus
FW104	6671	18	20	34	66	99
FW101-1*	52	7	10	11	16	15
FW101-2	6769	15	19	34	67	94
FW101-3	6458	18	20	34	34	107
FW101-4	5450	17	21	40	91	146
FW102-1	8566	18	21	41	36	165
FW102-2	8881	18	20	39	92	170
FW102-3	5954	16	20	37	75	118
FW102-4	7118	20	25	43	91	143
FW026	6188	15	22	36	74	106
FW024	8121	15	18	36	68	92
FW100-1*	83	13	16	13	16	17
FW100-2	2050	17	21	40	79	95
FW100-3	5564	18	24	41	82	121
FW100-4	5451	20	34	40	89	169
FW103	7345	17	20	37	80	128
FW105	6889	17	21	39	77	122
Whole set	97610	26	29	57	151	251

Classification based on RDP Classifier at 80% bootstrap confidence. *Discarded from later analyses.

Table 3.6. Detection of phyla poorly represented in 16S rRNA databases in libraries from FRC area 3 samples

Phyla	Found in library	Present in RDP*	Average identity to closest isolated relative
WS3	7	203	84.5%
TM7	185	883	77.1%
Tenericutes	7	2570	91.8%
Op10	2	369	82.0%
OD1	545	195	74.6%
Nitrospira	6	2045	95.6%
Lentisphaerae	16	271	92.7%
Gemmatimonas	2099	1572	89.9%
Fusobacteria	23	1929	86.6%
Fibrobacter	23	364	95.6%
Deinococcus- Thermus	13	1305	95.0%
Chlamydiae	4076	654	91.3%
BRC1	121	86	82.2%

*RDP Release 10, Update 6 of 12/3/2008 containing 715,637 aligned 16S rRNA gene sequences. Comparison is against the complete RDP dataset (Both partial and near-full length sequences, from clones or isolates, of good or suspicious quality. Sequences were assigned to a particular phylum using RDP Classifier with 80% bootstrap confidence.

Richness (number of different OTUs at 95% similarity) correlated with the number of sequences per site (r^2=0.6) as expected when coverage is not complete. However, richness in some samples was high albeit low sequence counts. This is probably due to a more even community structure that makes it easier to capture a wider range of richness (Figure 3.4), as well as incomplete sampling. Nevertheless, the communities were generally dominated by few OTUs, in 10 most abundant OTUs in any sample accounted for 40% of the sequences for that particular sample. Singletons, sequences with abundance equal to one in one library, represented on average 50.1% of the richness for any given site.

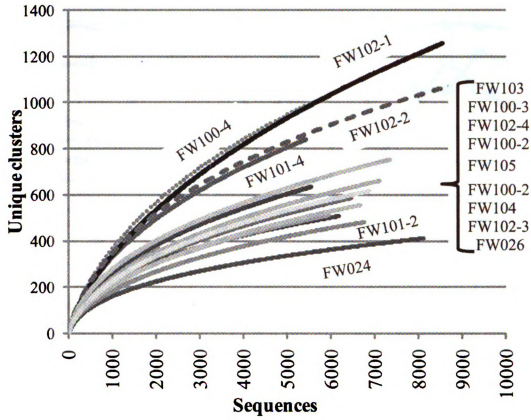

Figure 3.3. Rarefaction curves for FRC Area 3 samples. The shape of the curves suggests that a plateau has not been reach and incomplete sampling may occurred.

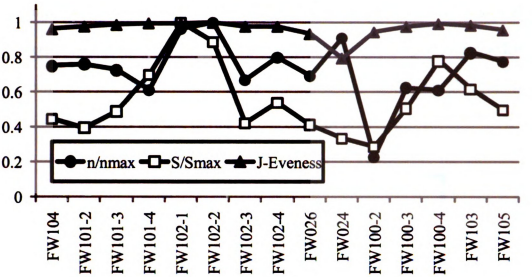

Figure 3.4. Richness estimations for samples from DOE FRC Area 3 as influenced by evenness and sampling effort. S = Richness (number of different groups), n = number of sequences sampled, J-eveness = Eveness indicator (range 0 to 1), n_{max} and S_{max} refer to the maximum number of sequences and richness the complete set of samples, respectively. Changes in sampling correlated with changes in richness. In some wells, e.g.FW100-2, richness was still high even when sampling was low due to a more even community.

Hierarchical clustering of samples

Clustering of the samples based on their microbial community yielded four clusters (Figure 3.5). The clustering was consistent with differences in bioactivity and connectivity. Cluster A contained only samples with low bioactivity and poor connectivity to the injection well. Some samples from this group came from the inner loop but at depths (levels -1 and -4) that make them less likely to have received the electron donor (injected between levels -2 and -3). Cluster B contained all the samples with the highest bioactivity and U(VI) reduction ability. Three subgroups were present in cluster B, with subgrouping consistent with pH levels suggesting that even when the communities respond to the treatment in a similar fashion, there is variation that can be

attributed to differences in pH, non homogenous starting populations or other variables. Clusters C and D contained wells with medium to low bioactivity and intermediate connectivity. Samples present in clusters B, C and D showed similar pH levels within clusters but cluster A did not, having a wide range of pH.

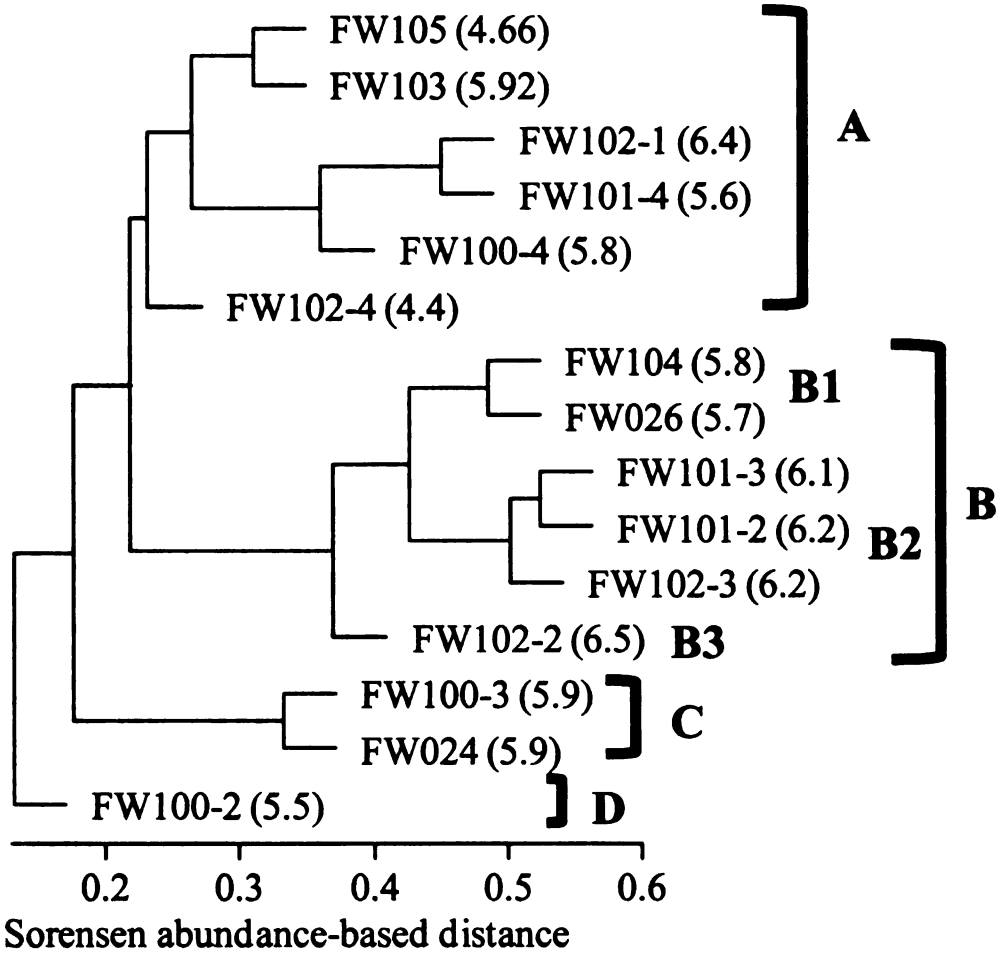

Figure 3.5. Clustering of samples by abundance-based Sorensen index. Well name and (pH) shown. Four clusters were defined (A to D). Cluster A was comprised of wells with low activity and connectivity to the carbon injection well. Cluster B has the most active communities and the highest connectivity. Clusters C and D contained wells with medium activity. In clusters B and C-D, the subgroups seemed to be influenced by pH. Hierarchical clustering used an average neighbor algorithm.

Microbial community structure

The microbial communities were dominated by *Proteobacteria* and *Acidobacteria*, this agrees with previous surveys of the site (10, 27). On average *Proteobacteria* and *Acidobacteria* together contributed 52% of the sequences, with combined contributions ranging from 24% at FW100-2 to 74% at FW024. *Proteobacteria* contributed 39% on average, with a maximum of 71% at FW024 and minimum of 18% at FW100-2. At FW024, 46% of the sequences were related to *Sulfuricurvum*, an anaerobic sulfide oxidizer that can use nitrate and hydrogen as electron donor (30).

Acidobacteria contributed 13% of the sequences with a maximum of 33% at FW026 and minimum of 3% at FW026.The *Actinobacteria* contribution was around 6% with the exception of one well (FW100-2) where its contribution was 36%. The *Proteobacteria* contribution varied the most, followed by *Actinobacteria, Acidobacteria* and *Firmicutes* (Figure 3.6).

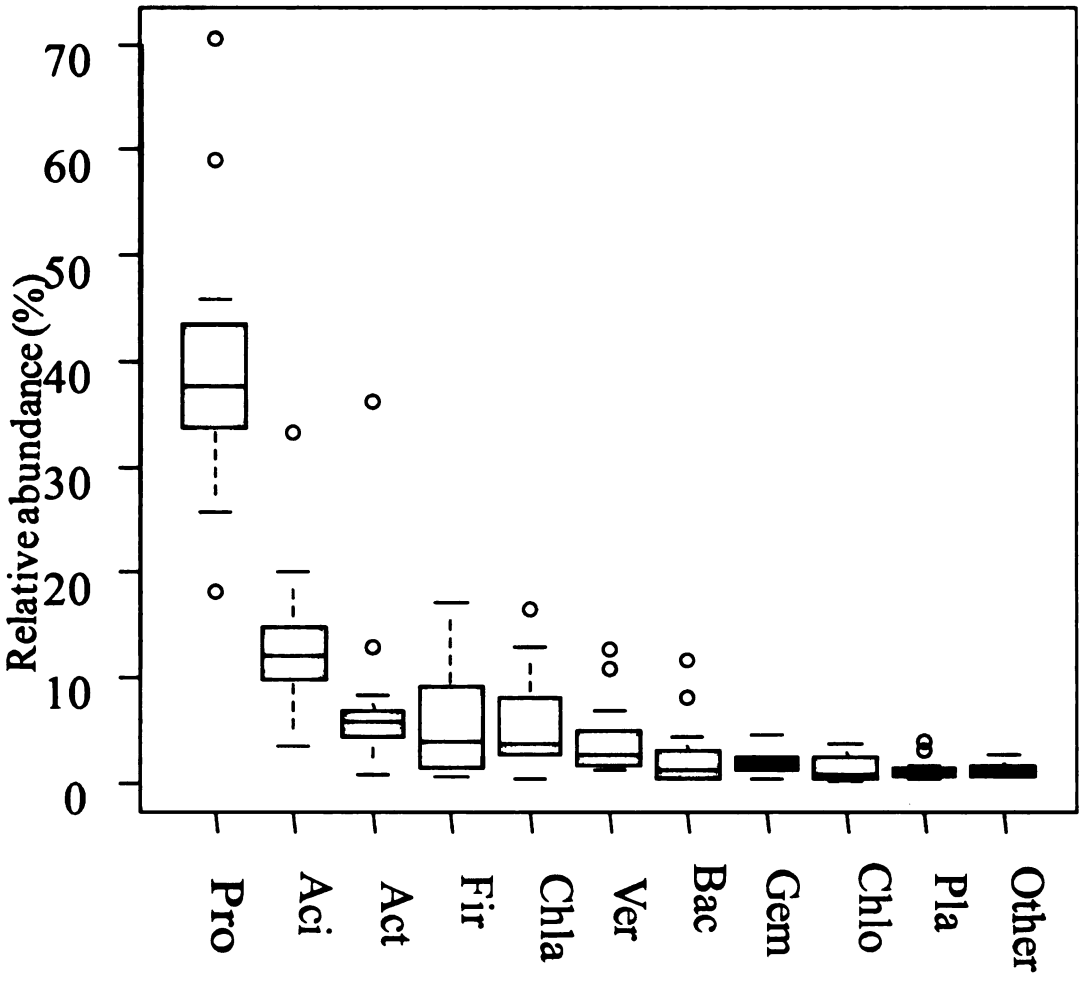

Figure 3.6. Relative contribution of most abundant phyla to the microbial community from 15 samples from DOE FRC Area 3. Boxplots show medians, lower and upper quartile (box limits), range of observations (whiskers) and outliers if any (circles). Pro = *Proteobacteria*, Aci = *Acidobacteria*, Act = *Actinobacteria*, Fir = *Firmicutes*, Chla = *Chlamydiae*, Ver = *Verrucomicrobia*, Bac= *Bacteroidetes*, Gem = *Gemmatimonadetes*, Chlo = *Chloroflexi*, Pla = *Planctomycetes*, Other = other phyla. Unclassified bacteria are not shown.

When comparing the structure of samples in the inner and outer loops, *Acidobacteria* ($p<0.01$), *Chloroflexi* ($p<0.05$) *and Chlamydiae*($p<0.05$) were significantly higher in the inner loop, while *Fibrobacter* was significantly lower ($p<0.05$) in this zone. *Fibrobacter* is a rare phylum originally found in the gut of herbivores where it plays a role in the anaerobic degradation of cellulose. It has been detected recently outside the gut in landfills (37). Our putative *Fibrobacter* sequences were on average 95.6% identical with its closest isolated relative in the RDP.

When comparing the four sample clusters produced in the clustering analysis, cluster D was dominated by *Actinobacteria* and cluster C by *Proteobacteria* (Figure 3.7). Clusters A and B shared a similar profile, however cluster B had a significantly higher proportion of *Chloroflexi* ($p<0.01$), *Chlamydiae* ($p<0.01$), and *BRC1* ($p<0.01$), and a significantly lower proportion of *Verrucomicrobia* ($p<0.05$), *Actinobacteria* ($p<0.05$), *TM7*($p<0.05$), and *Bacteroidetes* ($p<0.05$) than cluster A. *Chloroflexi* are a group of facultative aerobes known to subsist on carbohydrates and yeast extract and thought to have a role in cellular matter degradation (62), therefore, its abundance in wells with higher bacterial biomass could be due not to its ability to utilize the stimulatory carbon source but to recycle dead cells. *Verrucomicrobia* are ubiquitous in soil, with tolerance to acidic conditions and oligotrophy and thus a good competitor under these limiting conditions at the FRC.

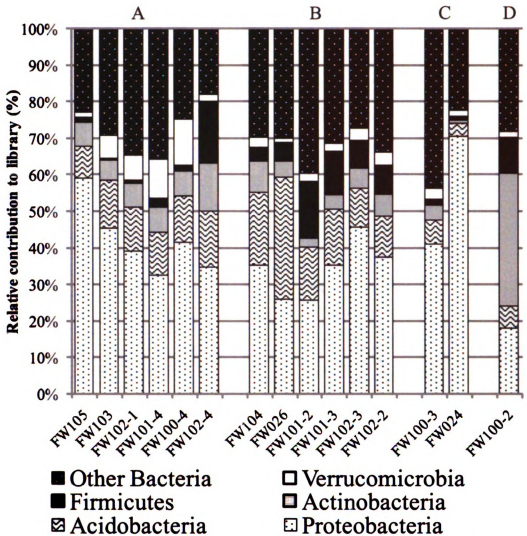

Figure 3.7. Relative contribution of the five most abundant phyla present at samples from the FRC Area 3. Samples are ordered into four clusters (A, B, C and D) according to the clustering analysis described earlier. Other *Bacteria* include *Chlamydiae, Chloroflexi, Bacteroidetes, Gemmatimonadetes, Planctomycetes, Tenericutes, Nitrospira, Fusobacteria, Deinococcus-Thermus, Deferribacteres, TM7, Spirochaetes, WS3, OD1, OP10, Fibrobacteres, BRC1, Cyanobacteria, Lentisphaerae,* and unclassified *Bacteria.* Not all the mentioned phyla were not present in all sites.

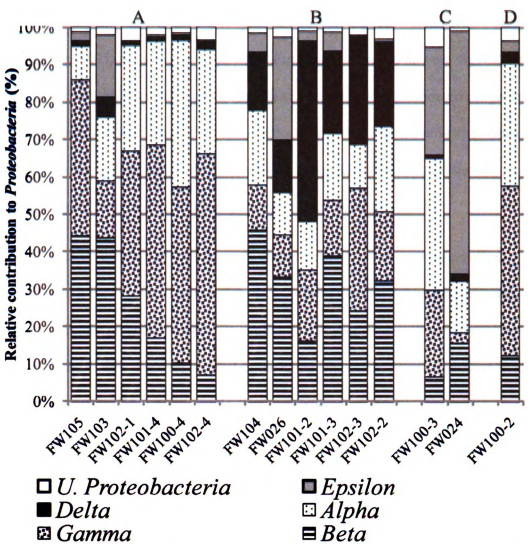

Figure 3.8. Class composition of the *Proteobacteria* from FRC Area 3 samples. U. *Proteobacteria* accounts for sequences classified as *Proteobacteria* but that could not be assigned with a 80% confidence to any of the classes of the *Proteobacteria* using RDP's Classifier. Samples are ordered into four clusters (A, B, C and D) according to the clustering analysis described earlier.

Taxonomic classes of the *Proteobacteria* phyla varied among the sites (Figure 3.8). When comparing sample clusters A and B from the clustering analysis (inactive vs active), cluster B was enriched in *Deltaproteobacteria* ($p<0.01$), while cluster A had a significantly higher proportion of *Gammaproteobacteria* and *Alphaproteobacteria*. Cluster D was similar to cluster A in terms of abundance of *Gammaproteobacteria*.

When comparing the inner and outer loops, we found *Deltaproteobacteria* in higher proportions ($p<0.05$) at the inner loop, while *Epsilonproteobacteria* were significantly lower ($p<0.05$) in this zone. The great variability observed in Proteobacteria is probably due to the range of conditions surveyed in terms of pH and carbon availability (measured as chemical oxygen demand due to connectivity differences. Also the proteobacteri are the most metabolically and ecologically diverse group of bacteria (19).

Given that the *Deltaproteobacteria* class has many of the known uranium reducers such as *Geobacter (Desulfomoronales)*, *Desulfovibrio (Desulfovibrionales),* and *Anaeromyxobacter (Myxococcales),* we evaluated if orders of this class were present in the samples and if they were distributed in the same abundance in samples from inactive wells versus samples from active wells. The results showed that the cluster B's *Deltaproteobacteria* were enriched in members of the orders *Myxococcales* ($p=0.01$), *Desulfobacterales* ($p<0.05$) and *Desulfovibrionales* ($p<0.01$) in contrast to low activity wells.

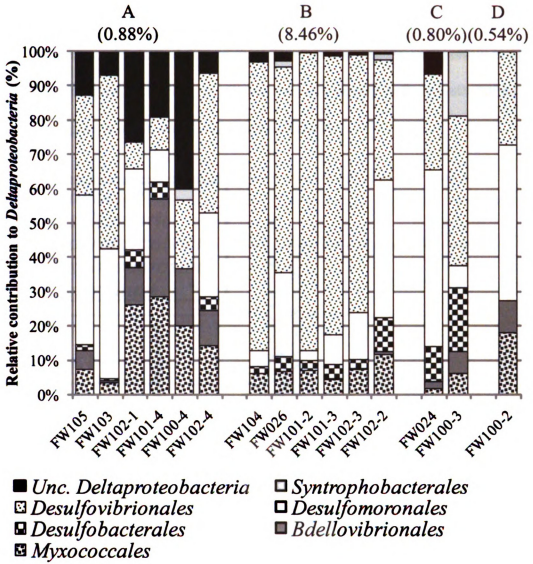

Figure 3.9. Relative abundance of *Deltaproteobacteria* orders in FRC Area 3 samples. Samples are ordered in the four clusters (A to D) that were defined previously in clustering analysis. The average contribution of the *Deltaproteobacteria* to the total community in that specific cluster of samples is given in parenthesis. U. *Deltaproteobacteria* are sequences that cannot be assigned with >80% confidence to one of the orders of the *Deltaproteobacteria* class by RDP classifier.

Presence of genera with members known to reduce uranium

Genera known to contain U(VI) reducers were detected in the samples. *Desulfovibrio* was on average the most abundant one followed by *Desulfosporosinus, Geobacter, Anaeromyxobacter* and *Clostridium.* The first four genera were present in all wells with high bioactivity (Figure 3.10), *Clostridium* was present in these wells but its abundance was higher at a low activity well from cluster A.

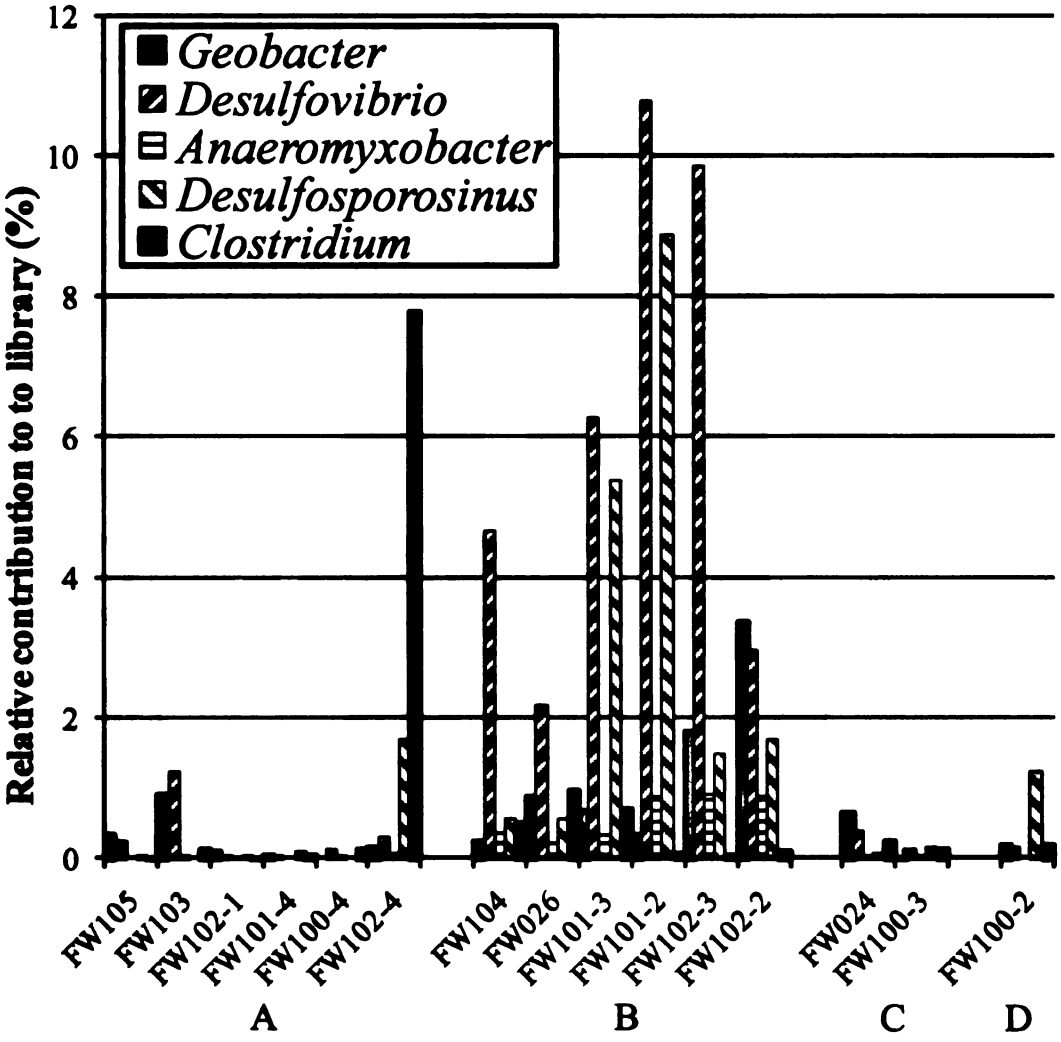

Figure 3.10. Relative contribution of most common uranium reducing genera found in the samples from DOE FRC Area 3. Samples are ordered in the four clusters (A to D) defined previously in clustering analysis. Cluster B contains samples with highest bioactivity.

Abundance of *Geobacter, Anaeromyxobacter ,Desulfosporosinus,* and *Anaeromyxobacter* sequences were higher at wells with higher connectivity, as measured by tracer recovery (Figure 3.11). The location where the population peaked in abundance differed for each group (order: *Desulfovibrio*> *Geobacter* > *Anaeromyxobacter* > *Desulfosporosinus*), suggesting that some niche specialization may be have occurred along the electron donor gradient. The location of the peak for *Geobacter* is consistent with a study that found elevated *Geobacter* abundance in clone libraries when organic carbon (measured as COD) was elevated (27).

Four other genera with known uranium reducers were also detected: *Desulfitobacterium, Shewanella, Acidovorax, a*nd *Deinococcus.* They, however, were not present in all the wells where uranium reduction was established and their relative abundance was generally low (0.1 to 1%). Hence, their role in uranium reduction is difficult to establish and for that they were not considered in the analysis.

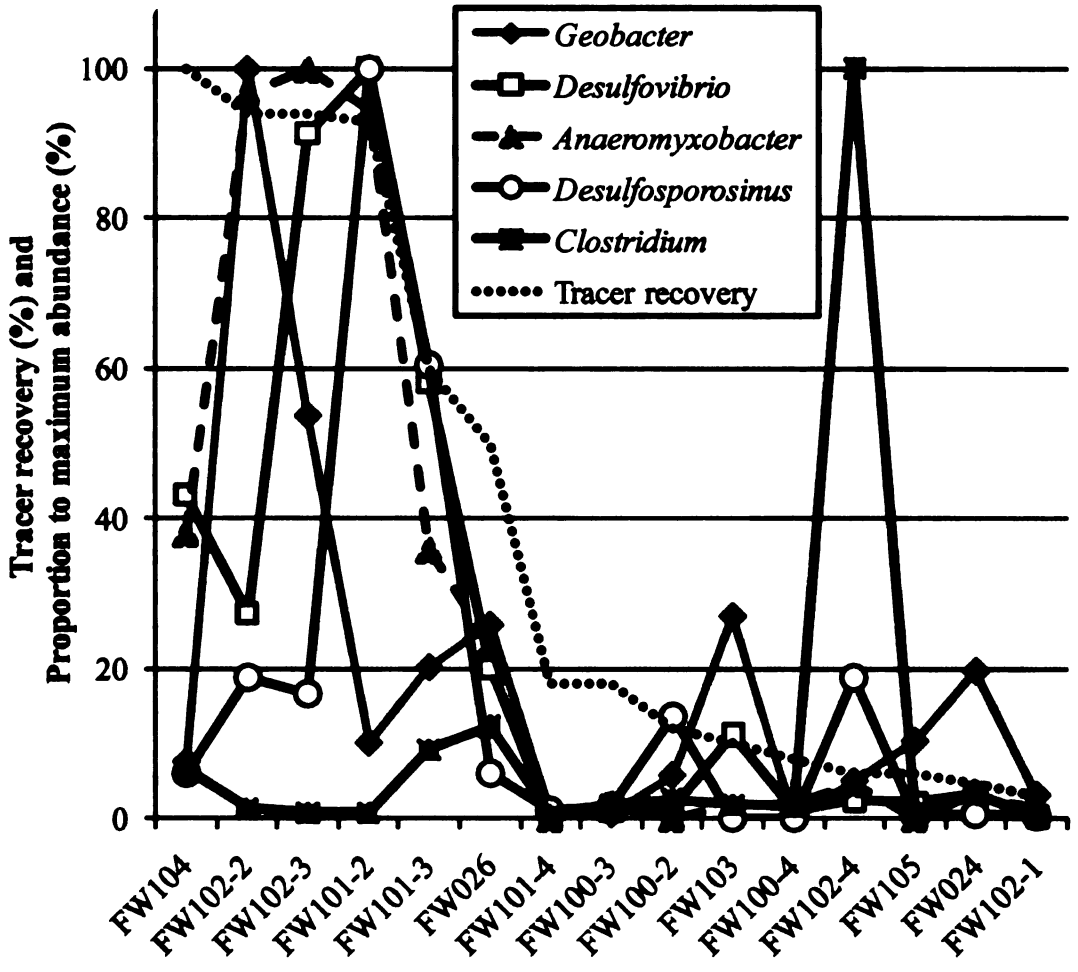

Figure 3.11. Location of maximum abundance for uranium reducing genera along the connectivity gradient at FRC Area 3. Plotted are the ratio of abundance to maximum abundance for selected groups and the proportion of tracer recovery, all in a 0 to 100% scale.

Indicator species

Indicator species analysis is an approach that can greatly reduce the complexity of the dataset by finding sequences that represent samples, or cluster of samples. Additionally, the indicator value is provided with an indication of statistical confidence. We used abundance information for 4719 OTUs over 15 samples to obtain indicator species.

The first level of analysis focused on the four sample clusters found by clustering analysis (Clusters A, B, C, and D). Cluster A (mostly inactive wells) was represented mostly by *Betaproteobacteria* and *Gammaproteobacteria* OTUs (Table 3.7), two classes known to have nitrate reducing organisms. The top 15 representatives (ranked according to descending indicator value) contained three *Rhodanobacter* and two *Castellinella* OTUs. *Rhodanobacter*, a *Gammaproteobacteria,* was previously detected in high abundance in FW106, a location in Area 3 meters away from the treatment zone (10). FW106 has not received any stimulatory electron donor and it is still acidic (pH 3.6) and highly contaminated. A metagenome analysis of a FW106 groundwater sample additionally revealed a *Rhodanobacter* sp. with a wide variety of metal resistance genes and indication that these genes were horizontally transmitted (24). *Castellinella* is a denitrifying *Betaproteobacteria* that has been found in biostimulated wells from the FRC Area 1 where it was the dominant *nirK*-containing denitrifier (49). Area 1 is also close to the main contamination source at the FRC, and has acidic conditions as well as high nitrate levels. The main differences between these areas 1 and 3 reside in the U levels, with Area 3 being higher, and the treatment system used. Area 1 experienced a pull-push

approach with ethanol while Area 3 used the hydraulic control system previously described. *Castellinella* isolates from FRC Area 1 were recovered under acidic (pH 4.5) and neutral (pH 7.5) conditions. It was suggested that this group may outcompete other denitrifiers under acidic conditions characteristic of the FRC contaminated areas (49). *Castellinella* has also been detected in microcosm enrichment from the FRC Area 2 (1). The rest of the top 15 representatives of cluster A did not include genera known to be involved in bioremediation of U or nitrate. A *Comamonas* sp. was also found in top 15 ranking at #2, this genus is known for their ability to grow on organic compounds such as phenols. These compounds were present in the contamination source (58) and may be still present in locations poorly connected to the injection well. The main physiological theme in cluster A representatives was their resistance to harsh conditions like those from the FRC.

The top 15 representative OTUs of cluster B contained seven OTUs related to U(VI)-reducing genera (Table 3.8). The wells in this cluster are the most active in microbial activity, and are also the only locations where U(VI) reduction has been detected. Out of the seven OTUs, four belonged to *Desulfosporosinus* (SRB *Firmicutes*), two to *Desulfovibrio* (SRB *Deltaproteobacteria*) and one to *Anaeromyxobacter* (FRB *Deltaproteobacteria*).

Two denitrifiers, *Vogesella* (#3) and *Thauera* (#12) were also present as top indicator species. *Thauera* spp. have been previously found in the denitrifying bioreactor used *ex situ* to precondition the site by removing nitrate (28). Since aboveground treatment system was connected to the subsurface, it is likely that *Thauera* spp, were injected with the treated water.

Bacteria related to iron reduction/oxidation had a high indicator value for this cluster of sequences: *Gallionella* (#15, Ind.value 0.93, p=0.001), *Thiobacillus* (#16, Ind.value=0.928, p=0.001), *Geothrix* (#22, Ind.value=0.883, p=0.001), and *Ferribacterium* (#28, Ind.value=0.883 p=0.115). *Geobacter*, a FRB also known to reduce U(VI), ranked slightly lower at position 53 (indicator value 0.73, p=0.065). A special case is that of *Thiobacillus,* a bacteria capable of reducing iron and nitrate but also able to oxidize iron and uranium (the later while reducing nitrate) (4). Its presence in the top of the indicator list suggest that it plays a role in the most active uranium reducing communities and may influence the mobility of uranium *in situ.*

Two amoeba endosymbiont relatives were found in high in the indicator species ranking. The putative endosymbiont come from two different phyla ,*Chlamydiae* and *Proteobacteria*, and share the same likely host, *Acanthamoeba*, a common free-living protozoa that feeds on bacteria, algae and yeast in the environment (36). Considering that the sediments with high activity in occasions clog the wells with biomass (10), it is quite possible that predatory populations of amoeba exist at these locations, though this remains to be established directly by microscopy, cultivation, or PCR based methods.

Clusters C and D did not contain known U(VI) reducers in their top 15 ranking taxa (Table 3.9 and Table 3.10, respectively). In cluster D, *Geobacter* was positioned #21 (p=0.065) and *Desulfovibrio* #99 (p=0.109). These results suggest that even when populations capable of reducing U(VI) exist in these locations, they are not good representatives of the main selection trends in these sites. In both C and D, the most frequent groups in the top of the list were related to *Alpha-* and *Gammaproteobacteria.*

Indicator species analysis was also performed on the cluster B. Subgroup B2 was formed by three samples, FW101-2, FW101-2 and FW102-3, and was consistently high in uranium reduction. In these three samples *Desulfovibrio* was a top indicator species where it ranked #1 (p=0.055) and #12 (p=0.03), respectively. *Desulfosporosinus* ranked #8 at B2 but the statistical confidence was low (p=0.317). In these samples *Desulfovibrio* spp. were always in the 10 most abundant species. Interestingly, *Geothrix*, an iron reducer *Acidobacteria* was also highly present in these samples, and always more abundant than *Desulfovibrio*.

It is also likely that there are taxa not previously described as U(VI) reducers present and actively reducing uranium at this site. Candidates for these roles are iron reducers such as *Geothrix* (*Acidobacteria*) and *Ferribacterium* (B*etaproteobacteria*). The indicator list for this group of samples could potentially be used to target specific groups in isolation efforts since the uranium reduction ability seems to be more widespread than originally described.

Table 3.7. Top 15 indicator species for sample cluster A, their classification and closest isolated relative.

Rank	Ind. Value	p-value	Classification[1]	Closest isolate in RDP[2]	Identity[3] (%)
01	1.000	0.002	*Treponema* (*Spirochaetes*)	*Parvibaculum* sp. psc10; EU930870	90.7
02	0.997	0.002	*Comamonas* (*Betaproteobacteria*)	Bacterium rJ9; AB021327	99.5
03	0.987	0.008	U. *Sphingobacteriales* (*Bacteroidetes*)	*Niastella* sp. RHYL-67; EU917053	94.1
04	**0.985**	**0.001**	***Castellaniella* (*Betaproteobacteria*)**	***Castellaniella defragrans*; 62Car; AJ005449**	**99.5**
05	0.973	0.005	*Rhodanobacter* (*Gammaproteobacteria*)	Cimanggu media isolate 88; Cimanggu media 88; B; AF229452	99.0
06	**0.941**	**0.001**	***Rhodanobacter* (*Gammaproteobacteria*)**	***Rhodanobacter ginsengisoli*; GR17-7; EF166075**	**99.0**
07	0.938	0.004	U.*Rhizobiaceae* (*Alphaproteobacteria*)	*Ensifer xinjiangensis*; Rx22; AF250353	99.0
08	0.904	0.064	*Opitutus* (*Verrucomicrobia*)	*Opitutus* sp. VeSm13; X99392	97.1
09	0.886	0.002	*Rhodanobacter* (*Gammaproteobacteria*)	Cimanggu media isolate 88; Cimanggu media 88; B; AF229452	99.0
10	0.872	0.004	Gp6 (*Acidobacteria*)	Unidentified eubacterium clone BSV07; AJ229181	83.9
11	0.866	0.002	*Castellaniella* (*Betaproteobacteria*)	*Castellaniella* sp. 7.5A2; EF175378	99.5
12	0.847	0.006	*Gp6* (*Acidobacteria*)	Marine gamma proteobacterium HTCC2246; AY386337	85.8
13	0.843	0.065	U. *Bacteria*	*Sphingomonas* sp.JQ1-3; DQ118953	82.3
14	0.833	0.071	U. *Verrucomicrobiales* *(Verrucomicrobia)*	Bacterium Ellin516; AY960779	88.7
15	0.833	0.007	U. Hypomicrobiaceae *(Alphaproteobacteria)*	*Devosia ginsengisoli*; Gsoil 326; AB271045	98.0

Ind.value = Indicator value, p-value = probability of obtaining as high an indicator values as observed over 1000 iterations. [1] Classification based on RDP Classifier with a 80% bootstrap confidence. [2] Closest relative was found with RDP SeqMatch function using an isolates-only dataset. [3] Sequence identity with the closest isolate from the RDP. Unclassified sequences (U) were not able to be assigned to a lower taxon with the 80% confidence. Wells in this cluster: FW100-4, FW101-4, FW102-1, FW102-4, FW103, and FW105. Groups in bold were significant (q-value <0.05) under the false discovery rate analysis.

Table 3.8. Top 15 indicator species for sample cluster B, their classification and closest isolated relative.

Rank	Ind. Value	p-value	Classification[1]	Closest isolate in RDP[2]	Identity[3] (%)
01	**1.000**	**0.001**	***Desulfosporosinus* (*Firmicutes*)**	***Desulfosporosinus* sp. 5apy; AF159120**	**99.0**
02	**0.997**	**0.001**	***Desulfosporosinus* (*Firmicutes*)**	***Desulfosporosinus* sp. 5apy; AF159120**	**99.0**
03	**0.979**	**0.001**	**U. *Burkholderiales* (*Betaproteobacteria*)**	***Vogesella indigofera*; ATCC 19706T; AB021385**	**95.6**
04	**0.977**	**0.001**	**U. *Peptococcaceae* (*Firmicutes*)**	**Anaerobic bacterium Prop2; AY756143**	**92.7**
05	0.974	0.002	U. *Chlamydiales* (*Chlamydiae*)	Endosymbiont of *Acanthamoeba* sp. UWC22; AF083616	91.7
06	**0.971**	**0.001**	***Desulfosporosinus* (*Firmicutes*)**	***Desulfosporosinus* sp. 5apy; AF159120**	**98.0**
07	**0.970**	**0.001**	***Desulfovibrio* (*Deltaproteobacteria*)**	***Desulfovibrio putealis* (T); DSM 16056; AY574979**	**99.5**
08	**0.968**	**0.001**	**U. *Firmicutes***	***Clostridium* sp. 9B4; AY554416**	**92.7**
09	**0.967**	**0.001**	***Desulfovibrio* (*Deltaproteobacteria*)**	**Sulfate-reducing bacterium F1-7b; AJ012594**	**99.5**
10	0.962	0.002	U. *Bacteria*	*Desulfomonile limimaris* (T); AF230531	86.3
11	**0.959**	**0.001**	**U. *BRC1***	**Bacterium Ellin371; AF498753**	**82.8**
12	0.957	0.006	U. *Betaproteobacteria*	*Thauera* sp. R-28312; AM084110	99.0
13	**0.948**	**0.001**	***Anaeromyxobacter* (*Deltaproteobacteria*)**	***Anaeromyxobacter dehalogenans* (T); 2CP-1; ATCC BAA-258; AF382396**	**96.1**
14	**0.943**	**0.001**	**U. *Alphaproteobacteria***	**Endosymbiont of *Acanthamoeba polyphaga*; AF132138**	**90.2**
15	**0.929**	**0.001**	**U. *Betaproteobacteria***	***Gallionella ferruginea* subsp. *capsiferriformans*; DQ386262**	**96.6**

Ind.value = Indicator value, p-value = probability of obtaining as high an indicator values as observed over 1000 iterations. [1] Classification based on RDP Classifier with a 80% bootstrap confidence. [2] Closest relative was found with RDP SeqMatch function using an isolates-only dataset. [3] Sequence identity with the closest isolate from the RDP. Unclassified sequences (U) were not able to be assigned to a lower taxon with the 80% confidence. Wells in this cluster: FW026, FW101-2, FW101-3, FW102-2, FW102-3, and FW104. Groups in bold were significant (q-value <0.05) under the false discovery rate analysis.

Table 3.9. Top 15 indicator species for sample cluster C, their classification and closest isolated relative.

Rank	Ind. Value	p-value	Classification[1]	Closest isolate in RDP[2]	Identity[3] (%)
01	1.000	0.009	U. *Chlamydiales* (*Chlamydia*)	*Neochlamydia hartmannellae* (T); A1Hsp; AF177275	84.9
02	1.000	0.013	*U. Gammaproteobacteria*	*Legionella* sp. II29; AB058910	90.7
03	1.000	0.010	U. *Bacteroidetes*	*Tuber borchii* symbiont b-Z43; b-Z431; AF233292	89.3
04	1.000	0.013	*Rhodocista (Alphaproteobacteria)*	*Azospirillum* sp. DA10-2; AY118225	93.2
05	1.000	0.008	GpV (*Cyanobacteria*)	*Pseudophormidium* sp. ANT.PROGRESS2.2; AY493583	95.6
06	1.000	0.012	U. *Bacteria*	*Chondromyces lanuginosus* (T); Sy t2; AJ233939	82.9
07	0.981	0.043	U. *Bacteria*	*Clostridium paradoxum;* DSM 7308T; para2; Z69941	79.0
08	**0.968**	**0.001**	**Subdivision 3 (*Verrucomicrobia*)**	**Bacterium Ellin514; AY960777**	**94.6**
09	0.964	0.003	U. *Bacteria*	*Neochlamydia hartmannellae* (T); A1Hsp; AF177275	85.2
10	**0.957**	**0.001**	***OD1***	***Geobacter sulfurreducens* PCA; AE017180**	**75.6**
11	0.947	0.008	*Rhabdochlamydia* (*Chlamydiae*)	*Rhabdochlamydia crassificans;* CRIB01; AY928092	93.2
12	0.946	0.010	U. *Bacteria*	Sulfate-reducing bacterium R-PropA1; AJ012591	82.0
13	0.943	0.010	Gp3 (*Acidobacteria*)	Bacterium Ellin371; AF498753	89.3
14	0.931	0.086	U. *Proteobacteria*	*Desulfohalobium utahense* (T); EtOH3; DQ067421	82.9
15	0.931	0.006	U. *Alphaproteobacteria*	*Azospirillum amazonense*; 21R; AY741146	89.3

Ind.value = Indicator value, p-value = probability of obtaining as high an indicator values as observed over 1000 iterations. [1] Classification based on RDP Classifier with a 80% bootstrap confidence. [2] Closest relative was found with RDP SeqMatch function using an isolates-only dataset. [3] Sequence identity with the closest isolate from the RDP. Unclassified sequences (U) were not able to be assigned to a lower taxon with the 80% confidence. Wells in this cluster: FW024 and FW100-3. Groups in bold were significant (q-value <0.05) under the false discovery rate analysis.

Table 3.10. Top 15 indicator species for sample cluster D, their classification and closest isolated relative.

Rank	Ind. Value	p-value	Classification[1]	Closest isolate in RDP[2]	Identity[3] (%)
1	1.000	0.070	U. *Bacteria*	Candidatus *Brocadia* sp. 40; AM285341	81.5
2	1.000	0.076	U. *Lachnospiraceae* (Firmicutes)	*Clostridium herbivorans* (T); 54408; L34418	95.6
3	1.000	0.064	U. *Bacteria*	*Verrucomicrobia bacterium* YM31-066; AB372850	87.8
4	1.000	0.069	U. *Bacteria*	Unidentified eubacterium clone BSV28; AJ229190	82.0
5	1.000	0.072	*Flavimonas* (*Gammaproteobacteria*)	*Pseudomonas* sp. SV4; AF251334	92.2
6	1.000	0.070	U. *Bacteria*	Candidatus *Brocadia* sp. 40; AM285341	80.5
7	1.000	0.053	U. *Bacteria*	*Bacillus* sp. BR (T); AM050346	82.0
8	1.000	0.060	*Roseburia* (*Verrucomicrobia*)	Butyrate-producing bacterium A2-194; AJ270473	95.6
9	1.000	0.064	U. *Bacteria*	*Treponema porcinum* (T); 14V28; AY518274	88.3
10	1.000	0.073	U. *Actinomycetales* (*Actinomycetes*)	*Actinobaculum* sp. P2P_19 P1; AY207066	94.6
11	1.000	0.062	Isosphaera (*Planctomycetes*)	*Isosphaera* -like str. C2-3; AF239692	99.5
12	1.000	0.066	*Desulforomonas* (*Deltaproteobacteria*)	Delta proteobacterium 112 (T); AY835388	95.6
13	1.000	0.079	U. *Bacteria*	*Sphaerobacter thermophilus*; DSM 20745T; AJ420142	78.4
14	1.000	0.063	U. *Clostridiales* (*Firmicutes*)	*Clostridium termitidis*; DSM 5396; X71854	91.7
15	1.000	0.064	Treponema (*Spirochaetes*)	*Treponema bryantii* (T); RUS-1; M57737	88.7

Ind.value = Indicator value, p-value = probability of obtaining as high an indicator values as observed over 1000 iterations. [1] Classification based on RDP Classifier with a 80% bootstrap confidence. [2] Closest relative was found with RDP SeqMatch function using an isolates-only dataset. [3] Sequence identity with the closest isolate from the RDP. Unclassified sequences (U) were not able to be assigned to a lower taxon with the 80% confidence. Cluster formed by well FW100-2. Groups in bold were significant (q-value <0.05) under the false discovery rate analysis.

Ordination of samples by principal coordinates analysis

Principal coordinates analysis showed an ordination that was consistent with previous clustering analysis as shown with the minimum spanning tree overlaying the ordination (Figure 3.12 left panel). When testing the ordination against gradients of environmental factors, chemical oxygen demand (COD) in sediments, sulfide in sediments, total iron in sediments, and uranium levels in sediments were significant in explaining the ordination ($p<0.05$). However, we previously established that colinearity was present in these variables. We hypothesize that COD is the independent variable of this group. COD represents the organic carbon content in sediments, and also probably an indication of electron donor availability in this electron-donor-limited environment. COD likely influenced sulfide, iron, and uranium levels through the activity of sulfate-, iron- and uranium-reducing microorganism that used the organic compounds. This results agrees with our hypothesis that the injection of the stimulatory carbon, ethanol, helped shape the community structure.

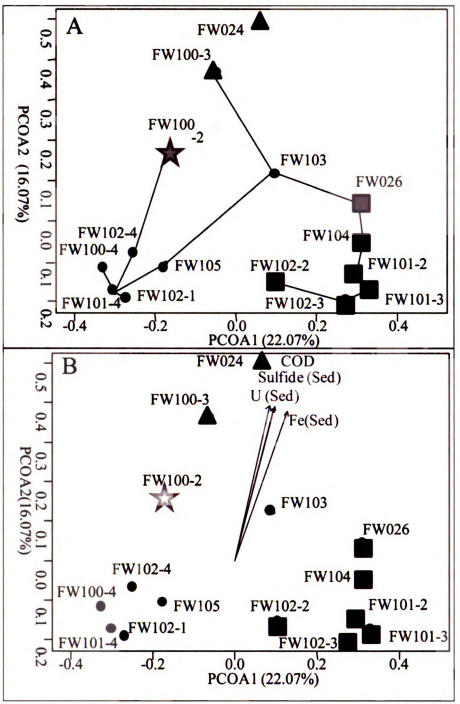

Figure 3.12. Principal coordinates ordination on abundance-based Sorensen distances for samples from the FRC Area 3. First two dimensions together explain 38% of the original variability. The grouping of samples is consistent with previous clustering results as shown with a minimum spanning tree on top of the ordination (panel A). Vectors that explained significantly the ordination ($p<0.05$) are shown (panel B), though vectors were collinear (correlated). Cluster A (●), cluster B (■), cluster C (▲), and D (★).

We also used non metric dimensional scaling (NMDS) to study the distribution of specific taxonomic groups likely related to the U(VI) reduction process. This model was preferred because it does not assume a specific distribution of species along a gradient. It is not clear if the measured environmental gradients were pronounced enough for our groups of interest to react to; thus, we cannot assume that our populations have a unimodal distribution (single peak along a gradient). In this case we tested only five main groups of variables, they were called pH, nitrate, chemical oxygen demand (COD), mean travel time and U in sediments. Mean travel time is a hydrology terms that refers to the time water from the injection wells takes to travel to a given location, the more connected the location the shorter the travel time. Because the variables were correlated, these names actually represent several collinear variables. The names used are to our best estimation of what is the independent variable for the group, though it is possible that a collinear variable is responsible for the response. Four of the five groups of variables were significant for at least one group of organisms (Table 3.11).

COD and Uranium levels in sediment were the most common factor to be found significant in explaining the variability of the groups of interest. COD explained significantly ($p<0.05$) the distribution of *Bacteria, Anaeromyxobacter*, and *Desulfovibrio* along the samples. Uranium levels in sediment were highly significant in explaining the distribution of *Deltaproteobacteria, Desulfovibrio and Desulfosporosinus* (Table 3.11). However, this variable is correlated with sulfide and iron levels in sediments, so part of the response could be due to changing levels of sulfate and/or iron. Nitrate was weakly significant ($p<0.1$) in explaining the distribution of *Geobacter* OTUs. Nitrate was highly

correlated with sulfate, na, mg, Mn, chloride and Ca. The results suggest that SRB *Desulfovibrio* and *Desulfosporosinus*, as well as FRB *Anaeromyxobacter* are responsible for the uranium levels, and that they responded to the injection of the stimulatory electron donor.

Table 3.11 Environmental factor that explain distribution of groups along the samples.

Group	Nitrate	U(sed)	COD (sed)	Travel time
Acidobacteria	-		•	-
Gp8	-	*	•	-
Betaproteobacteria	-	•	•	-
Deltaproteobacteria	-	-	-	•
Anaeromyxobacter	-	**	*	*
Desulfovibrio	-	**	*	*
Geobacter	•	-	-	-
Firmicutes	-	*	*	•
Desulfosporosinus	-	**	•	-
Verrucomicrobia	-	-	-	-
Bacteria	-	•	*	-

Environmental factor that significantly explain the distribution of clusters in NMDS ordination space. •p<0.1,*P<0.05, **p<0.01. Similarity threshold: Genus level analysis=99%, Phyla and class level = 95%. pH was not significant in explaining the distribution of any of the groups studied.

DISCUSSION

Effect of hydraulic control on microbial communities of the treatment area

The establishment of a controlled hydraulic system created an opportunity to study the bioremediation of a contaminated aquifer that went through different stages from

denitrification to iron reduction to sulfate reduction (27, 58, 59). We observed differences in the spatial scale that are a consequence of the hydraulic flow regime and the injection of ethanol as the stimulatory electron donor. The geochemical gradients shaped the microbial communities to resist toxicity, denitrify, reduce iron and reduce uranium in different zones of the study area, creating differences in the spatial patterns. Other work that tracked the succession of communities in FRC Area 3 since the stimulation started determined the establishment of these different types of communities (toxicity resistant/denitrifiers/metal reducers) but in a temporal scale (27). In biological terms, the establishment of spatial gradients increased the diversity of metabolism for the site creating a biological transition zone. This zone potentially works as buffer zone, protecting the inner loop from intrusion of contaminated water if the hydraulic system is perturbed and could be important in the long term bioremediation of the site.

Besides the hydraulic control and ethanol injection, an additional factor in shaping the microbial communities of the site is the conditioning phase that used a fluidized bed bioreactor to remove nitrate (competing electron acceptor). This bioreactor worked for over 4 months treating water *ex situ* and reinjected treated water as well as microbial cells into the study area (28). This bioreactor was initially inoculated with biomass from the Y-12 wastewater treatment plant (300 m away from Area 3, also at the FRC) (28), and biomass from a pilot study FBR that showed denitrification as well as U(VI) reduction (61) . The pilot scale reactor was also inoculated with site water (TBP-16 well the FRC). In both reactors, pilot and full scale, ethanol was used as electron donor and populations of SRB such as *Desulfovibrio* spp. were detected (28, 61) . In the pilot scale bioreactor, uranium reduction was also present and additions of molybdate (a SRB inhibitor)

decreased sulfate reduction as well as U(VI) reduction, suggesting that SRB were partly responsible for the uranium reduction (61). Other groups detected in the bioreactors that were also detected in the sediment samples include *Acidovorax* (denitrifier, also U(VI) reducer), *Thauera* (denitrifier *Betaproteobacteria*), *Sporomusa* (acetogen *Firmicutes*), *Stenotrophomonas*, *Pseudomonas*, *Decholormonas* (denitrifier *Betaproteobacteria*), *Hydrogenophaga* (hydrogen oxidizing *Betaproteobacteria*), and *Clostridium* among others (28, 61). No FRB known to reduce uranium where detected in either bioreactor. However, *Geobacter* and *Anaeromyxobacter,* both U(VI)-reducing FRB, were detected in enrichments of sediments of Area 1 of the FRC (42). The bioreactor is likely a source inoculum in the subsurface ecosystem, helping U(VI)-reducing SRB to establish in the aquifer and compete with local FRB populations that are more likely in lower numbers. The FBR inoculums is probably a reason why SRB are predominant in this location, and U(VI) reduction works for this site. Since not all SRB reduce U(VI) and many SRB can utilize ethanol as electron donor, the success formula of this site may not occur in other sites. In a different site also contaminated with uranium, injection of acetate stimulated an early response of FRB (*Geobacter* spp.) that reduced uranium but later succession with SRB *Desulfobacterales* decreased uranium reduction (25).

Fate of electron donor injected and populations that responded to the treatment

Several studies have shown that ethanol is the best electron donor for uranium reduction at the FRC (1, 59). Ethanol, however, is rapidly converted into acetate at the injection point, acetate accumulates and it is later consumed (1, 10, 39). The oxidation of ethanol

to acetate and its release to the environment is consistent with the activity of SRB such as *Desulfovibrio,* and *Desulfosporosinus* (31, 47). Additionally when ethanol has been used as electron donor in studies at the FRC, sulfate and uranium reduction occur simultaneously (1, 38, 59). This suggests that at the FRC, SRB are more important in uranium reduction than FRB. In our analysis, SRB *Desulfovibrio* and *Desulfosporosinus* correlated with uranium reduction in ordination analysis and are characteristic of sites with high U(VI) reducing abilities in the indicator species.

This however does not discount that FRB partially contributed to the reduction even though acetate provides less energy than ethanol. Akob and colleagues found that FRB *Geobacter* spp. reacted to ethanol amendments in microcosm enrichment from Area 2 that showed U(VI) reduction (59). In our case FRB *Geothrix*, *Ferribacterium*, and *Geobacter* were also abundant in sites with high U(VI) reducing activity. The three of them are known to utilize acetate to reduce iron but U(VI) reduction has been demonstrated for *Geobacter* only (34). In all the active wells, the FRB *Geothrix* was the more abundant than any SRB. At the injection well, FW104, where ethanol is the main electron donor, *Geothrix* and *Ferribacterium* where the most dominant FRB, being more abundant than *Geobacter*. In this well, reduced iron was at its maximum, so competition for oxidized iron, or unknown abilities of the specific *Ferribacterium* and *Geothrix* spp. could have excluded *Geobacter*.

In later sample wells when acetate is the main electron donor, *Geobacter* peaked in abundance. After *Geobacter, Anaeromyxobacter* (also FRB) and *Desulfovibrio* (SRB) peaked in abundance. The fact that the SRB were less abundant in these wells could be due to sulfate reduction being less favorable than iron reduction when acetate is the

electron donor. Another possible explanation is that since acetate oxidation is not a frequent characteristic of *Desulfovibrio*, this SRB could be growing as the hydrogen-consuming partner in a syntrophic association with an acetate-consuming partner. This type of association has been demonstrated for pure cultures of *Geobacter* and *Desulfovibrio* (15).

In general, it seems that SRB dominate these samples because of their ability to oxidize ethanol, the time of sampling in the succession event, and the initial advantage given by the FBR. FRB likely use mainly acetate to reduce iron and benefit from the presence of iron oxidizers (aerobic and anaerobic) at the site. This dynamic seems to be present in our site since many iron oxidizing genera such as *Gallionella* and *Thiobacillus* were detected in the most active wells. This dynamic could also be applied to sulfur since *Sulfuricurvum*, an anaerobic sulfide oxidizer was present in high abundance but only in one site (FW024).

Uranium reduction and its correlation with microbial groups

Comparison of the levels of uranium reduction in different wells with the locations where the putative iron reducers peaked, suggests that multiple populations are responsible for uranium reduction. The highest uranium reduction (61%) was at the injection site FW104, where ethanol was rapidly converted to acetate (likely by SRB) and FRB *Geothrix* and *Ferribacterium* dominated. In slightly less connected wells, FW102-2, and FW102-3, *Geobacter* peaked in abundance. In these wells the average uranium reduction was 24%. Finally at wells FW101-2 and FW101-3, uranium-reducing

candidates with sulfate-reducing activities peaked in abundance. Those wells had 54% and 51% of reduced uranium, respectively. We hypothesize that FRB are responsible in the more connected wells for uranium reduction, and SRB are responsible for uranium reduction at the injection site and less connected points. This agrees with previous findings of *Geobacter* and *Geothrix* being associated with high levels of COD and no dissolved oxygen control (27). In our case more connected wells are the equivalent of that because they have more carbon available and a higher chance of having dissolved oxygen (due to injection process). *Desulfovibrio* spp. were associated with dissolved oxygen control in previous studies (27), this agrees with our findings of *Desulfovibrio* peaking in less connected wells where oxygen is less likely to be present due to its consumption in earlier wells.

It remains unclear if the FRB *Ferribacterium* and *Geothrix* are involved directly in uranium reduction or if they are only benefitting from the added electron donor while reducing iron or humic acids. Since reduced iron compounds (from FRB)(44) as well as reduced humic acids (from a humic reducer such as *Geothrix*) (12, 41) can potentially reduce uranium abiotically, *Ferribacterium* and *Geothrix* activities could be linked to uranium reduction. *Geothrix* has been associated with uranium reduction in other studies of the FRC, though no direct evidence of uranium reduction exists to date (7, 10, 27). The role of humic acids as electron shuttles for uranium reduction also remains unknown (39).

Clustering, direct gradient ordination, and indicator species analysis suggests that in more active communities, *Deltaproteobacteria,* and more specifically its SRB members seemed to be playing a bigger role in the immobilization of uranium at the time of the sampling. In less active communities, the lack of electron donors has selected for species

capable of life under acidic and toxic conditions. Our indicator species analysis suggests that the groups more likely to reflect the uranium-reducers are related to *Desulfovibrio, Desulfosporosinus, Anaeromyxobacter,* and *Geobacter.* Differences in uranium reducing populations at the active locations can be attributed mainly to differences in connectivity to the injection well that likely influenced that carbon available for the microbes.

There are populations that were not related to uranium reduction but were a consequence of the establishment of high number of bacteria in the active zone, e.g. populations likely involved in recycling dead cell material such as Chloroflexi were significantly higher in active sediments.

Patterns of diversity

The communities coming from samples with reduced uranium were not the richest communities. This is likely due to a combination of incomplete sampling and differences in evenness. Uneven communities reflect on one extreme a thriving community with highly enriched groups (e.g. SRB in FW104) and on the other extreme a community struggling to survive toxic conditions (e.g. FW105). Clustering by diversity patterns was more informative, and showed that communities responsible for uranium reduction were similar in composition suggesting that the same main groups in these communities were reacting to the treatment. The treatment changed the subsurface by creating better conditions for microbes in terms of pH, heavy metals, and electron donor availability. Electron donor availability was probably the most important change since energy source is commonly the limiting factor in uranium reduction in groundwater (59). The impact of

the treatment can be tracked to groups that increased in abundance such as *Chloroflexi, Acidobacteria, Deltaproteobacteria, Desulfovibronales, Myxococcales* and *Desulfobacterales.* In general the treatment shaped the community to provide specific functional groups for nitrate, iron, sulfate, and uranium reduction.

When those databases are compared to our previous clone libraries results, some differences in abundance are noted. Since we used two different systems in terms of primers, product size, and amplification conditions; some differences in abundance can be due to primer coverage (primer bias), sampling, and regions flanking the template region (21). Additionally, the lack of a cloning step could potentially avoid cloning bias. Cloning bias has been reported for *Firmicutes* rRNA genes cloned into a *Gammaproteobacteria* host (48). In our case, *Desulfosporosinus* contribution comprised 1% of clone libraries and 8% of the pyrosequencing produced, using the same DNA extract. This difference is more likely attributable to difference in bias and sampling.

Beside avoiding cloning bias, our methodology also allowed us to explore more deeply the microbial diversity of the site, and to track specific populations that occur at low frequency (e.g. 0.5% for *Anaeromyxobacter*) and whose trends were previously difficult to follow.

CONCLUSIONS

The results from a much larger number of 16S rRNA gene sequences allowed us to more comprehensively study the microbial communities in response to treatment than was previously feasible, and to compare their patterns of diversity with a statistical

confidence.

Clustering analysis showed differences in community structures that can be related to connectivity and pH among wells as well as organic carbon. In contrast with other sites where *Geobacter* populations are more abundant, our results suggest that SRB such as *Desulfovibrio* were more involved in uranium reduction at this particular location at the time of sampling, this is likely due to the choice of electron donor, its metabolism by SRB and FRB, and the use of a bioreactor that likely injected SRB into the aquifer.

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CHAPTER FOUR

Cardenas, E., and J. M. Tiedje. 2008. New tools for discovering and characterizing microbial diversity. Curr Opin Biotechnol **19:**544-9.

New tools for discovering and characterizing microbial diversity

Summary

To discover and characterize microbial diversity, approaches based on new sequencing technologies, novel isolation techniques, microfluidics and metagenomics among others are being used. These approaches have contributed to discovery of novel genes from environmental samples, to massive characterization of functional and phylogenetic genes and to isolation of members of formerly uncultured yet ubiquitous groups like *Verrucomicrobia, Acidobacteria*, OP10 and methanogenic *Archaea.* Cheaper sequencing is key in this process by making available applications that were previously restricted to big research centers, complementing previously available methodologies and potentially replacing some of them. The new tools are reshaping the way we study the environment, increasing the resolution at which microbial communities, their complexities and dynamics, can be studied to reveal their genetic potential and their functional diversity.

Introduction

Discovering new microbes and characterizing their functions are major goals in the study of microbial diversity. Historically, this was achieved through cultivation and subsequent characterization of strains. Decades later, culture-independent methods have provided new tools to study the microbial world. Recently, metagenomics is providing a gene-based exploration of the community as a whole while isolation and cultivation

continue as the main-stay for testing metabolic abilities and more detailed genomic studies of individual members of this community. As more and more environments were sampled, it became evident that the majority of the microbes have yet to be cultured (30), driving the development of new methods to unveil the unknown (Figure 4.1). These methods benefit from advances in sequencing, isolation and microfluidics and allow researchers to study microbial communities in their natural environments and with higher resolution.

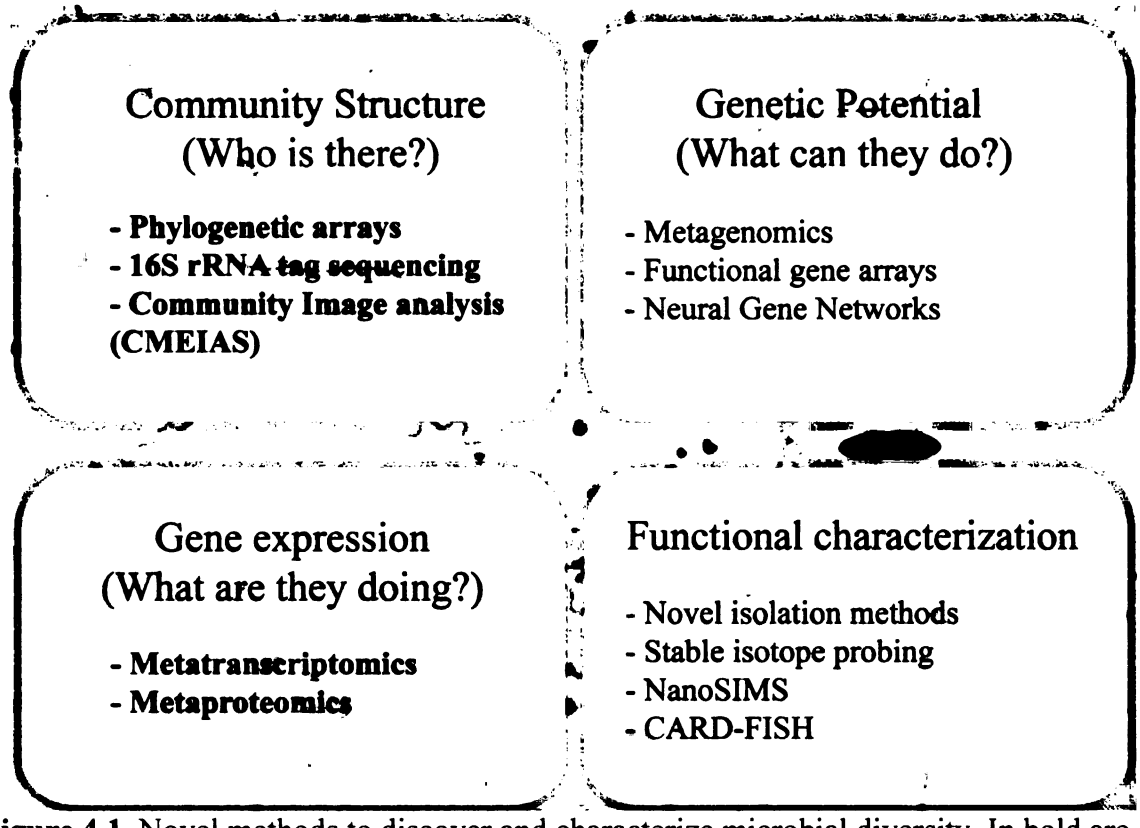

Figure 4.1. Novel methods to discover and characterize microbial diversity. In bold are the methods reviewed in this article. The image showing the morphological diversity from an anaerobic bioreactor community is courtesy of Frank Dazzo.

Impact of the new sequencing technologies in microbial ecology

As we write this report, new sequencing technologies are changing the way we study microbial communities. Traditionally, the study of genes from natural environments included cloning DNA into a vector, inserting that vector into a host, screening, and Sanger sequencing. Sequence-by-synthesis methods provide faster, cheaper and simpler methods for (meta)genome sequence that bypass the cloning bias and labor-intensive Sanger method. New sequencing technologies differ in type of output, read length, speed, and error rate distribution (Table 4.1); for a more detailed review see ref. (34). Single molecule sequencing, expected soon by Helicos and Pacific Biosciences, will provide another leap in sequence capacity with the latter company

projecting reads as long as 10 kilobases. The different sequencers are being used for different applications: longer reads being preferred for genome sequencing and gene surveys while shorter reads being the choice for transcriptomes, small RNAs analysis and comparison of closely related strains or strain variants. These methods compensate their shortcomings in read length and error bias with massive output (and thus coverage), speed and simplicity. The new methodologies create a situation where the limitation is not the ability to produce sequence data but the ability to store and analyze it in new revealing ways.

Table 4.1. Currently available sequencing technologies and their features. Not included in the table are technologies under development by Visigen Biotechnologies, Polonator, Pacific Biosciences, Intelligent Biosystems, Complete Genomics. N/A, not available.

Method	Output per run (per day)	Read length (bases)	System described
Sanger	52-86 kB (0.69-2.1MB)	550-900	ABI 3730xl DNA Analyzer. 96 Well
Pyrosequencing	400-600 MB	400	Roche 454 Life Sciences GS FLX Titanium Series Reagents
Helicos	N/A (600-2160 MB)	30	The HeliScope Single Molecule Sequencer
SOLiD	6 GB (600MB)	35	SOLiD system
Illumina (Solexa)	3GB (600MB)	36	Illumina Genome Analyzer

Currently pyrosequencing is favored for microbial genome sequencing and metagenomic studies. It is valuable for sequencing regions that are technically difficult (e.g. due to strong secondary structure or high GC content) and for covering regions recalcitrant to cloning in *Escherichia coli* (36). In one example, a Sanger/pyrosequencing hybrid approach completely eliminated gaps in two genomes and reduced gaps by 86% in

four other genomes (12). In the large sequencing centers, the Sanger method is being replaced as the main sequencing method for bacterial genomes though it remains an important tool for closing them. See also (32) for a more historical perspective of 454 sequencing.

A major advantage of the new sequencing technologies is the substantial reduction in cost, democratizing genomics and making massive gene surveys feasible as a way to discover new microbes and genes. This is especially useful for the study of microbial communities by 16S rRNA gene analysis, the most commonly used species proxy. By combining primers that target hypervariable regions of the 16S rRNA gene with nucleotide barcodes, it is now more feasible to survey environments with thousands of sequences at the time. The hypervariable regions targeted are short enough (100-350 bases) to be covered by some of the new sequencing technologies yet long enough to be informative for classification by the current rRNA databases (e.g. Ribosomal database project (RDP), Greengenes, Silva), though they are poor for phylogeny in comparison to the full length 16S rRNA gene (~1500 bases). The RDP has reported accuracy in classification of partial sequences to the genus level for 400-base reads and to the family level for 200-base reads (44). The first in the new wave of massive 16S rRNA gene studies was of deep sea sediments using the V6 hypervariable region (35). Since then, other regions have been used to study temperate and tropical soils (31). Highlighting the magnitude of the species diversity question, in one case 750,000 sequences were not enough to recover all the microbial diversity present in deep sea communities near hydrothermal vents (15). Longer reads will allow targeting of regions that provide a

better classification (44) and to use the sequences for more accurate phylogenetic studies. A complementary approach to massive sequencing is the adoption of nucleotide barcodes in the amplification primers. In this method, samples from different origins can be mixed in one run and after sequencing their data can be separated according to their barcode (22, 25). This approach decreases the cost per sample since more samples can be pooled in the sequencing run rather than sequencing fewer samples to greater depth. For example, using 454 FLX pyrosequencing 400,000 reads are usually generated; by using 80 barcodes, 5000 reads per sample can be obtained. This number of sequences is at least one order of magnitude higher than those from traditional clone libraries.

Transcriptomics is also being transformed by the new sequencing technologies. Gene prediction algorithms, probe design, cloning, and hybridization steps involved in traditional transcriptomic analyses can all be bypassed by massively parallel direct sequencing of cDNA and subsequently mapping the reads to a reference genome to quantify known transcripts and even detect putative new genes. For example, Illumina (Solexa) sequencing has been used to detect genes, intron usage and alternative initiation codons in yeast (23). In a more recent article, 512 new genes were predicted for the nitrogen-fixing plant symbiont *Sinorhizobium meliloti* after a transcriptome analysis that used pyrosequencing (19).

A new look at metagenomics

Metagenomics, the study of microbial communities based on its genetic material (6), has paved the way for the discovery of new genes, proteins and biochemical

pathways. Genes involved in anaerobic methane oxidation were discovered with a metagenomics effort that targeted an association of *Archaea* and sulfate-reducing bacteria (13). Metagenomics has proved more valuable in recovering complete genomes in systems with low diversity, such as the acid drainage from an abandoned mine (43) or in highly enriched cultures such as the first genome of the annamox (anaerobic ammonia oxidation) group, *Kuenenia stuttgartiensis* (41). When applied to more diverse environments, such as soils, the yield of assembled genomes has been poor (38). However, assembly is not necessary to make some important inferences. One particularly valuable use of a metagenome can be as the basis for metranscriptomics (9) and metaproteomics (28).

Array-based approaches for studying microbial diversity

The study of the microbial community structure and its functional diversity has benefited from the use of microarrays that target phylogenetic markers and functional genes. Arrays provide high throughput, specificity and relative quantification free of cloning bias. A functional gene array, Geochip (14), has been helpful in the study of nitrogen and carbon cycles in Antarctic soils (47) and PCB contaminated soils (18). Arrays targeting the 16S rRNA gene have been used to study microbial communities involved in uranium reduction and reoxidation (4). Phylogenetic arrays easily detect more microbial diversity than traditional clone libraries (7). Nevertheless, probe design limits detection to what is already known, or what can be placed on a chip, and hence microarrays are tools better suited to generally characterize ecosystems rather than to discover new genes and functions.

Microfluidics, manipulation at micro(be) scale

Microfluidics, the manipulation of small volumes, is becoming a powerful tool in microbial ecology. By working at dimensions relevant to microbes it has been possible to study specific microbes via isolation, gene amplification or genome reconstruction. Microfluidics allowed for individual members of termite hindgut flora to be sorted and screened by multiplex PCR (24). By targeting the 16S rRNA gene and functional genes it was possible to unambiguously link phylogeny and function (no cultivation required), and to detect horizontal gene transfer events (24). This approach has been coupled with whole-genome amplification steps that target more genes and to sequence much of an organism (37). A genome from the candidate phylum TM7 has been reconstructed from human mouth samples with the help of a microfluidic device that selected for rod-like morphology, amplified individual genomes and screened for TM7 signatures (20).

The formerly unculturables – New approaches for isolation

Renewed efforts in isolation are being taken to capture more of the microbial functional diversity. These novel methods demonstrate that culturing is often possible if niche conditions are mimicked. In contrast with traditional cultivation, the novel isolation conditions try to better reflect natural environments in terms of nutrients (composition and concentration), oxygen levels, pH and natural associations with other microorganisms. These novel isolation methods also benefit from advances in microfluidics.

Novel lineages of *Actinobacteria, Acidobacteria, Proteobacteria* and *Verrucomicrobia* were isolated using diluted nutrient broth solidified with gellan (16). Gellan is a good alternative solidifying reagent producing gels that are clearer (easier screening) and more stable than agar under a wider range of temperatures and pHs. Gellan also worked as a carbon source when isolating the first cultured member of the OP10 division from soils (40). In one study, colony counts increased up to 21.3 times when replacing agar with gellan in culture medium (42). In terms of oxygen concentration, microaerophillic conditions have led to increased recovery of termite gut bacteria (46) and soil *Acidobacteria* (8).

In situ and trap cultivation introduce sterile media to natural environments to use local conditions for enrichment. Using *in situ* cultivation with diffusion chambers, the diversity of isolates recovered from freshwater sediments increased and isolates from some rarely cultivated groups, such as *Acidobacteria* and *Verrucomicrobia*, were recovered (3). Trap cultivation has been used to capture novel soil *Actinobacteria*, known secondary metabolite producers, whose filaments penetrate the medium when growing (10).

Because organisms rarely live in pure cultures, cocultivation is another strategy for isolation. This is well established for hydrogenotrophic methanogenic *Archaea* and their partners. In these associations *Archaea* consume hydrogen, a product of incomplete substrate oxidation in anaerobic environments and make the oxidation reaction thermodynamically feasible (17). Through cocultivation, a globally abundant methanogen

(with a propionate-oxidizing, H_2 producing partner) (33), and an anaerobic phenol degrader (and its hydrogen consuming Archaeal syntroph) (27) have been isolated. Carbon exchange occurring in natural associations can also be used for isolation purposes. Novel heterotrophic partners of algae have been isolated by adding algae-excreted organic material to culture media (21) or by creating defined media to simulate the exudates' composition (45).

Another novel isolation approach uses of a combination of high throughput screening, microfluidics and dilution-to-extinction techniques originally used by Casida (5). In a current version of this approach(39), small volumes of inocula, sometimes picoliters, are added to sterile medium, typically in a 96-well plate format. By having few cells per well, the competition with fast growers is greatly reduced. After incubation different preparations can be screened for growth using fluorescent staining or group-specific probes. The most dominant group in marine ecosystems (SAR11 clade) (29) and novel isolates of *Proteobacteria, Actinobacteria, Firmicutes and Flavobacteria-Cytophaga* (11) have been obtained using this approach. There appears to be no reason why most of the formerly unculturable microbes cannot be made culturable with sufficient study, cleverness and patience. Study of cultured isolates remains the most straightforward method to characterize an organism's functions and evaluate its biotechnological potential.

Metatranscriptomics and metaproteomics, fields in their infancy

The study of gene expression and translation into proteins within natural environments are two emerging fields in microbial ecology which hold special promise for studying function. Recovering mRNA and proteins from natural environments is challenging, yet several groups have pace-setting examples. The first metatranscriptome study recovered transcripts encoding *Archaea* and *Bacteria* genes involved in carbon, sulfur and nitrogen cycling from marine and freshwater bacterioplankton communities (26). A marine metatranscriptome study found that hypothetical genes were among the most highly expressed in *Prochlorococcus* spp. and that a large fraction of the transcripts recovered were novel, indicating the immense metabolic diversity hidden in the oceans (9). In a soil transcriptome, even though most of the transcripts coded for housekeeping proteins, genes linked to soil processes were also found (1). In addition, 32% of eukaryote transcripts recovered carried novel genes (1), again highlighting the vast unknown within hypothetical genes. In the case of metaproteomics, proteins identified as chlorocatechol dioxygenases were recovered from a chlorobenzene-contaminated aquifer, a finding that agrees with the predicted degradation pathway of chlorobenzene (2).

Many challenges remain in metagenomic, metatranscriptomic and metaproteomic approaches, including DNA, RNA and protein extraction, mRNA instability, low abundance, and low proportion of mRNA in total RNA. As these are still novel approaches, new solutions to these technical problems are expected to arise, which would allow them to reach their full potential of characterizing functional diversity. Databases and software tools (Table 4.2) are essential and their further development is needed to

deal with the growing bottleneck in the analysis of metagenomic, metatranscriptomic and metaproteomic data.

Table 4.2. Selected resources available for metagenomics, metatranscriptomics and metaproteomics.

Resource	Features	Website
CAMERA	Data repository and bioinformatics tools resource	http://camera.calit2.net
Integrated Microbial Genomes with Microbiome samples (IMG/M)	Comparative metagenome analysis and annotation tools	http://img.jgi.doe.gov/cgi-bin/m/main.cgi
Human Oral Microbiome Database	Database on bacteria from the human oral cavity	http://www.homd.org/
SEED	Comparative genomics environment	http://www.theseed.org
MEGAN	Metagenome Analysis Software	http://www-ab.informatik.uni-tuebingen.de/software/megan/
Metagenomics RAST server	Automated metagenome analysis using SEED environment	http://metagenomics.nmpdr.org/
PFAM	Collection of protein families using multiple sequence alignments and hidden Markov models	http://pfam.janelia.org/
Phylofacts	Structural phylogenomic encyclopedia for protein functional and structural classification	http://phylogenomics.berkeley.edu/phylofacts/index.php
Uniprot	Protein sequence and functional information	http://www.uniprot.org/
Mascot	Search engine that uses mass spectrometry data to identify proteins from primary sequence databases	http://www.matrixscience.com/home.html

Future challenges and directions

Massively parallel sequencing, metagenomics, metatranscriptomics, metaproteomics, and isolation will allow us to study deeper and deeper layers of the microbial diversity, including genes behind novel or valued functions. These methods promise to broaden the use of (meta)genomics and offer the chance to study specific groups that were previously unknown and uncharacterized. We can now ask more complex questions such as what is the role of low abundant organisms?, can we estimate the total diversity of the system and assess its value?, how important is it to know in what organism particular genes reside?, and more importantly, how can we use this information to better characterize, manage and/or harvest even greater functional diversity. The ball is in our court.

Reference annotations

• of special interest •• of outstanding interest

Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, Arrieta JM, Herndl GJ: **Microbial diversity in the deep sea and the underexplored.** *Proc Natl Acad Sci U S A* 2006, **103**:12115-12120.

••First paper on massive parallel 16S rRNA gene surveys. Deep sea samples were studied by targeting the V6 hypervariable region. By comparing against a custom V6 database, it

was found that 80% of the sequences were divergent by 10% or more, suggesting an immense diversity present in rare members of the community.

Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M, Snyder M: **The transcriptional landscape of the yeast genome defined by RNA sequencing.** *Science* 2008, **320**:1344-1349.

••Transcriptomic study using massive parallel sequence of cDNA. By comparing the transcripts against the yeast genome it was possible to detect alternative splicing, initiation sites and delimit the boundaries of genes.

Mao C, Evans C, Jensen RV, Sobral BW: **Identification of new genes in Sinorhizobium meliloti using the Genome Sequencer FLX system.** *BMC Microbiol* 2008, **8**:72.

• Massive sequencing of cDNA using pyrosequencing. Putative new genes were found, these genes were likely missed by prediction algorithm because they were short or had low scores against known databases.

Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW, DeLong EF: **Microbial community gene expression in ocean surface waters.** *Proc Natl Acad Sci U S A* 2008, **105**:3805-3810.

••A study of DNA and cDNA from the same marine community. Proof that gene expression in complex systems if possible by mapping the transcripts to the metagenome. Interestingly, some of the more highly expressed genes were hypothetical and a large

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Brodie EL, Desantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, Hazen TC, Richardson PM, Herman DJ, Tokunaga TK, et al.: **Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation**. *Appl Environ Microbiol* 2006, **72**:6288-6298.

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•An approach that combines flow cytometry, whole genome amplification and screening to allow single cells to be separated and their genome amplified. The method allows targeting metabolic and phylogenetic markers in the same uncultured microbe.

Marcy Y, Ouverney C, Bik EM, Losekann T, Ivanova N, Martin HG, Szeto E, Platt D, Hugenholtz P, Relman DA, et al.: **Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth**. *Proc Natl Acad Sci U S A* 2007, **104**:11889-11894.

••A microfluidic device that allows selection of specific uncultured bacterium and amplification and recovery of their genome.

Stott MB, Crowe MA, Mountain BW, Smirnova AV, Hou S, Alam M, Dunfield PF: **Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand**. *Environ Microbiol* 2008.

• A fine example of novel isolation methods that combine low nutrient conditions, and alternative gelling agents to recover novel bacteria from soils.

Bollmann A, Lewis K, Epstein SS: **Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates**. *Appl Environ Microbiol* 2007, **73**:6386-6390.

• By using a diffusion chamber incubated in situ, rarely recovered groups from Deltaproteobacteria, Verrucomicrobia, Spirochaetes, and Acidobacteria were isolated.

Stingl U, Tripp HJ, Giovannoni SJ: **Improvements of high-throughput culturing yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast and the Bermuda Atlantic Time Series study site**. *ISME J* 2007, **1**:361-371.

•• Excellent example of high-throughput dilution-to-extinction culturing yielding novel isolates from the globally abundant SAR11 group.

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CHAPTER FIVE

CONCLUSIONS

Comparative 16S rRNA gene analyses have allowed us to explore the diversity of the microbial world present in the Field Research Center Area 3 where uranium remediation is being applied. The results from both studies have suggested that bacteria related to sulfate-reducing bacteria, *Desulfovibrio* and *Desulfosporosinus* and iron reducing bacteria are directly involved in uranium reduction in situ. *Desulfovibrio* seems to be the most important group given its relative abundance in wells where uranium reduction occurs, and also because the rapid conversion of ethanol to acetate observed in this site.

In addition to the uranium reducing groups, other bacteria seems to contribute important functions to the community such as the ability to eliminate nitrate, a competing electron (e.g. *Thauera*); create resistance structure such as biolfilms (e.g. Chloroflexi), recycle nutrients (e.g. *Chloroflexi, Acanthoamoeba*) and provide uranium reduction indirectly through reduced iron (FRB, e.g. *Ferribacterium*), sulfur (SRB, e.g. *Desulfosporosinus*) and nitrogen compound (Denitrifiers e.g *Acidovorax*) as well as reduced humic acids (*Geothrix*).

One surprising find was the abundance of iron-oxidizing populations such as *Thiobacillus* and *Gallionella. Thiobacillus* can oxidize uranium while reducing nitrate, *Gallionella* can oxidize iron under microaerophillic conditions, and iron oxides can directly reoxidize uranium. The effect of iron oxidizers on uranium stability needs further attention.

The ability of *Acidobacteria* iron reducers such as *Geothrix*, to reduce uranium also needs further study. In our experiments, *Geothrix*-related sequences were associated with the wells of high uranium reducing activity. These bacteria can reduced humic acids and through them potentially reduced uranium, yet the ability to reduce uranium directly is unknown. The use of *Acidobacteria* in uranium reduction would be a useful application given their ability to grow under a wide range of pH, resist environmental stresses and grow on oligotrophic conditions.

In terms of diversity, what started as a low diversity community of a heavily contaminated site has changed to a diverse community that can perform the task we stimulated it for, uranium reduction. Even when the response was heterogeneous i.e. different uranium reducing population responded in different sites, the diverse response was partially explained by hydrology parameters. Using the hydrology information helped us to understand the gradients present in the site and better understand how the communities respond to the stimulation. We have demonstrated that hydrology information is no longer relevant only for engineering purposes but can also be used to explain the natural variability in the microbial communities.

Method wise, massive parallel sequencing has shown to be powerful in detecting minor members of the community that seemed to respond to stimulation such as *Anaeromyxobacter* and *Desulfosporosinus,* it also potentially avoided cloning bias. Additionally, massive sequencing provides the opportunity to perform multivariate analyses that are powered by high sequence counts. It is expected that in the future this technology will be widely adopted for the analysis of microbial communities, this works uses for the first time a combination of massive sequencing and indicator species

approach to provide a consistent and statistically sound method to detect candidate species for a given groups. We expect this approach to be more adopted to deal with the high complexity of massive parallel sequencing databases.

Overall this work has answered some questions regarding the identity of the populations involved in uranium reduction but has also raised new question regarding the role of non-uranium reducers on the stability of reduced uranium, and their role in the community. The massive aspect has potentially shown areas of the microbial diversity where isolation efforts should be made in order to obtain microorganism relevant to the cleanup of this site e.g. in the isolation of new *Acidobacteria* associated with uranium reduction.

APPENDIX

My research has greatly benefited from a year of work in the Ribosomal Database Project where I developed and produced video tutorials to increase the adoption of the Functional Gene Database / Repository as part of the NIEHS Research translation core of the Michigan State University Superfund group. Additionally I led in the development of a new feature of the Ribosomal Database Project, Assignment Generator. This application provides instructors the chance to create a realistic assignment on comparative 16S rRNA gene analysis.

These two projecta, the tutorials and the Assignment Generator, have been published as part of the Ribosomal Database Project's papers in the journal *Nucleic acid research* in 2007(1) and 2009 (2), respectively.

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