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Linking bacterial diversity and geochemistry of uranium-contaminated groundwater

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Linking bacterial diversity and geochemistry of uranium-contaminated groundwater

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To understand the link between bacterial diversity and geochemistry in uranium-contaminated groundwater, microbial communities were assessed based on clone libraries of 16S rDNA genes from the USDOE Oak Ridge Field Research Centre (FRC) site. Four groundwater wells (GW835, GW836, FW113-47 and FW215-49) with a wide range of pH (3 to 7), nitrate (44 to 23,400 mg L^{-1}), uranium (0.73 to 60.36 mg L^{-1}) and other metal contamination, were investigated. Results indicated that bacterial diversity correlated with the geochemistry of the groundwater. Microbial diversity decreased in relation to the contamination levels of the wells. The highly contaminated well (FW113-47) had lower gene diversity than less contaminated wells (FW215-49, GW835 and GW836). The high concentrations of contaminants present in well FW113-47 stimulated the growth of organisms capable of reducing uranium (*Shewanella* and *Pseudomonas*), nitrate (*Pseudomonas*, *Rhodanobacter* and *Xanthomonas*) and iron (*Stenotrophomonas*), and which were unique to this well. The clone libraries consisted primarily of sequences closely related to the phylum *Proteobacteria*, with FW-113-47 almost exclusively containing this phylum. Metal-reducing bacteria were present in all four wells, which may suggest that there is potential for successful bioremediation of the groundwater at the Oak Ridge FRC. The microbial community information gained from this study and previous studies at the site can be used to develop predictive multivariate and geographical information system (GIS) based models for microbial populations at the Oak Ridge FRC. This will allow for a better understanding of what organisms are likely to occur where and when, based on geochemistry, and how these organisms relate to bioremediation processes at the site.

Keywords: uranium; microbial diversity; metal reduction; nitrate; LIBSHUFF

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1. Introduction

The fate of biologically reactive compounds in groundwater has been an area of intense study in recent decades. Contamination of groundwater with uranium, heavy metals and radionuclides is of concern not only to the environment, but also to human health. Metals and radionuclides occur in more than 50% of the facilities and waste sites of the US Department of Energy [1]. A promising approach to immobilize uranium is to catalyse the reduction of soluble U(VI) to the insoluble U(IV) [2–4]. The reduced form of uranium, U(IV), is of far less environmental concern [5,6]. Some microorganisms that have been reported to reduce U(VI) to U(IV) include fermenters such as *Clostridium* sp. [7], Fe(III) reducers such as *Shewanella* and *Geobacter* spp. [2,8], sulphate reducers such as *Desulfovibrio*, *Desulfosporosinus* and *Desulfotomaculum* spp. [9–11], and denitrifying bacteria such as *Acidovorax* and *Pseudomonas* spp. [8,12]. A mixture of these organisms has also been reported to reduce U(VI) to U(IV) [8].

Investigations within the previous Natural and Accelerated Bioremediation (NABIR) Program and at the Oak Ridge Field Research Centre (FRC) have primarily focused on actively manipulated and stimulated microbial communities under engineered bioremediation conditions. Investigations of native, unstimulated microbial communities and populations that would have relevance to natural attenuation processes have primarily been conducted on limited samples, to serve as comparisons between pre- and post-stimulated communities [13–15]. Because natural attenuation is mediated by indigenous microbial communities, a thorough evaluation of contaminant remediation in groundwater requires an approach that integrates qualitative and quantitative geochemical measurements of contaminant transformations and the identity, abundance and function of the key microorganisms involved in the transformation process. Although geochemical data can help researchers understand environmental conditions and the fate and transformation rates of contaminants, few investigators combine these observations with direct knowledge of the members of the indigenous community and the factors that may limit their *in situ* activity.

Mixed wastes are difficult and expensive to remediate effectively with current physical and chemical technologies. Although the indigenous microorganisms have great potential for cost-effective *in situ* remediation, successful and efficient applications of this technology remain a significant challenge. For example, at mixed waste sites where the concentrations of metal contaminants reach toxic

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levels, the reductive precipitation of radionuclides may be limited depending on the resistance of the endogenous microbial populations to metals. While a number of microbes have been shown to carry out reductive precipitation of radionuclides, the sensitivity of these microorganisms to non-reducible heavy metals could possibly limit their *in situ* activities [16]. Thus, understanding the microbial responses to combination of mixed wastes and varied geochemical conditions is crucial for the improvement and implementation of bioremediation strategies.

While it has become increasingly clear that certain microbial populations may be responsible for reducing contaminant loads under conditions where electron donors are added (biostimulation), it remains unclear what microbial populations and mechanisms are present and responsible for such processes under existing *in situ* conditions relevant to natural attenuation. A thorough knowledge of microbial community structure, the capabilities of the populations present, and how these populations affect their environment and vice versa should aid in the development of tools for predicting and monitoring natural attenuation. We utilized four newly installed multilevel sampling wells, across the Bear Creek watershed at the Oak Ridge FRC, which will allow for visualization of the interactions between geochemical parameters and microbial communities. Each sampling included geochemical parameters (e.g. pH, O_2 concentration, cation and anion composition, conductivity and dissolved organic carbon) and contaminant loads (uranium, technetium, nitrate and organic contaminants), as well as microbial community analysis (16S rDNA clone libraries). Four clone libraries were constructed to obtain more detailed information about the composition of the microbial communities. The results demonstrate how geochemistry affects the structure of the microbial dynamics within U(VI)-reducing communities.

2. Materials and methods

2.1. Sample collection

Groundwater samples for microbial community analysis were collected from groundwater wells FW113-47, FW215-49, GW835 and GW836. For each well, 500 mL groundwater samples were initially collected for geochemical analysis. An additional 5 L was then filtered through sterile Anodics filters (47 mm in diameter with 0.2 μm pore size; Whatman International Ltd, Maidstone, UK) in the field to collect cells for microbial community analysis. To collect water samples, a pump was set up near the well head with an inline stop valve between the well and pump. Two inline filters (8.0 and 0.2 μm) were connected in series downstream from the pump, and an outlet host was emptied into a carboy to collect the effluent water for subsequent disposal. Initially, the bleed valves were opened on the filter capsules and the pump was run at a very low speed to allow the capsules to fill with water. The bleed valves were then closed to commence filtering. After filtration, the filters were removed using tweezers and placed into sterilized 50 mL conical-bottom tubes and frozen in liquid nitrogen. The tubes were then stored on dry ice for transport to the laboratory. At the lab, the filters were stored at −80 °C until DNA could be extracted.

2.2. Geochemistry of the groundwater wells

The pH and conductivity values were determined with an Orion multimeter; dissolved oxygen (DO) was measured using a flow cell during purging; dissolved organic carbon (DOC) was filtered through 47 mm diameter Whatman GF/C filters and acidified to pH 3. Samples were kept in the dark at 4 °C until analysed on a Shimadzu TOC5000A with platinum-catalysed high-temperature combustion to CO_2 and infrared detection; U(VI) concentrations were determined by kinetic phosphorescence analysis using a kPa-11 analyser (ChemCheck Instruments, Richland, WA, USA). Anions (NO_3^-, Cl^-, SO_4^{2-}) were analysed with an ion chromatograph equipped with an IonPac AS 14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale CA, USA). Cations (Al, Ca, Fe, Mn, Mg and K) were determined using an inductively coupled plasma–atomic emission spectrophotometer (Thermo Jarrell Ash PolyScan Iris Spectrometer).

2.3. Molecular analysis

2.3.1. DNA extraction, PCR amplification and DNA sequencing

Prior to DNA extraction, the biomass collected on the filters was washed in 1 × PBS (phosphate buffered saline), vortexed and then centrifuged. Cell pellets were suspended in 1 × buffer and DNAs were isolated using a Power Soil™ DNA isolation kit (Mobio Laboratories, Inc. Carlsbad, CA, USA). Community 16S rRNA genes were amplified in mixtures containing 50 ng μL^{-1} DNA, 1 × High Fidelity PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each of the four deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.2 μm each of the forward (FD1; 5′ AGA GTT TGA TCC TGG CTG AG ′3) and reverse (1540R; 5′ AAG GAG GTG ATC CAG CC 3′) primers, and one unit of High Fidelity Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) per 20 μL. The following PCR conditions were applied: initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 sec, annealing for 58 °C for 1 min, extension for 1 min, and completion with a final extension period of 72 °C for 7 min. Negative PCR controls without DNA template were run concurrently for each sample. The PCR products were visualized in a 1.5% (wt/vol) agarose Tris-acetate-EDTA gel to confirm the size of the product. Five replicate PCR runs were performed for each DNA extract. The replicate PCR products were combined (100 μL) and loaded into a 0.8% agarose gel, excised,

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extracted with Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA) and eluted in 30 μL of EB buffer (10 mM Tris-Cl, pH 5.0).

2.3.2. Clone library construction

To construct clone libraries, purified PCR products were cloned using a TOPO TA PCR 2.1 cloning kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Transformants for each clone library were checked for inserts by PCR amplification using M13 primers. The applied PCR conditions were similar to the conditions described above except that 30 cycles were used with an annealing temperature of 60 °C and a final extension time of 10 min. The amplification products were analysed by gel electrophoresis. The PCR products from each clone were purified with a Montage PCR plate (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Purified PCR products of the 16S rRNA inserts were sequenced using a Prism Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) with 100 ng of template DNA. The sequences were obtained with an internal sequencing primer 529r (5′ CGC GCC TGC TGG CAC ′3). The DNA sequences were determined on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3.3. DNA sequence analysis

The DNA sequences were analysed using Chromas Pro version 1.5 (Digital River Inc., Eden Prairie, MN, USA) and vector sequences were removed. Chimeric sequences were checked by the Check_Chimera program available at the Ribosomal Database Project (RDP II) [17]. Sequences that appeared chimeric were excluded from further analysis. The closest known relative of the groundwater microorganisms represented by the recovered sequences were identified with the Sequence Match Program of RDP II. Unique 16S RNA sequences were parsed into operational units (OTUs) based on 97% sequence identity. Sequences were aligned with Clustal W [18] and the resulting alignments were used to construct phylogenetic trees. The maximum parsimony, maximum likelihood and neighbour-joining algorithms were each used to generate tree topologies. Trees generated from these three methods were congruent, with only minor rearrangements in branching order. Phylogenetic trees and evolutionary distance calculations were generated using the Jukes–Cantor distance model (MEGA version 5.0) [19]. Ten thousand replicates were used to obtain confidence estimates for the phylogenetic trees within Mega version 5.0 [19]. RDP Classifier [20] was used to provide rapid taxonomic placement of the 16S rRNA sequence data. The data provides taxonomic assignments from domain to genes, with confidence estimates for each assignment.

2.3.4. Diversity estimates

Diversity matrices (Shannon–Weiner index [H′], Simpson's index [1/D] and Chao1 index) rarefaction curves and the sample coverage for each clone library were generated using the DOTUR (Distance-based Operational Taxonomic Unit and Richness Determination) program [21]. The evenness index was calculated within Krebs software as previously described [22]. The H′ index considers the equitability of the OTU distribution, I/D also considers both richness and abundance, and Chao1 is a non-parametric estimation of OTU richness.

2.4. Statistical analyses

LIBSHUFF analysis was used to construct a pairwise comparison of the 16S rRNA gene libraries [23,24]. To determine the significance of differences between two clone libraries (e.g. X and Y), differences (ΔC_{xy}) between homologous $C_X(D)$ and heterologous coverage curves $C_{XY}(D)$ were calculated using the LIBSHUFF software (http://libshuff.mib.uga.edu/) [24]. The homologous coverage of clone library X is calculated using the equation $C_X = 1 - (N_X/n)$, where N_X is the number of unique sequences in the sample and n is the total number of sequences. In a similar way, the heterologous coverage of clone library X by a second clone library Y is defined as: $C_{XY} = 1 - (N_{XY}/n)$, where N_{XY} is the number of sequences in clone library X that are not found in the second clone library Y, and n is the number of sequences in X. Both N_X and N_{XY} can be defined at different levels of evolutionary distance (D), e.g. homology of the sequenced 16S rDNA fragments, to generate a coverage curve. If clone libraries are similar, then the coverage curves $C_X(D)$ and $C_{XY}(D)$ are also expected to be similar. The significance of ΔC_{xy} is described by P, which is calculated by randomly shuffling sequences (e.g. 999 times) and estimating ΔC_{xy} after each shuffling. The randomized values plus the empirical value of ΔC are ranked from largest to smallest, and then the P value is estimated to be $r/(N + 1)$, where r denotes the rank of the empirical value of ΔC_{xy} [24]. This provides a quantitative comparison of 16S rDNA gene clone libraries from environmental samples [24]. UPGMA and the neighbour-joining method were used to construct phylogenetic trees with distance matrices based upon correlation values of ΔC_{xy} with MEGA version 5.0 [19]. Pearson's product-moment correlation was used to calculate general correlation between diversity indices (H′, 1/D and Chao1) and geochemical parameters. Correlation values were estimated using the SYSTAT statistical computing package (SYSTAT Version 9.0, SPSS Inc., Chicago, IL, USA).

2.5. Nucleotide sequence accession numbers

Nucleotide sequences have been submitted to the GenBank database under accession numbers JN420322, JN420323,

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JN420324, JN420325, JN420326, JN420327, JN420328, JN420329, JN420330, JN420331, JN420332, JN420333, JN420334, and JN420335.

3. Results

3.1. Groundwater characteristics

The geochemical characteristics of the four groundwater samples were significantly different (Table 1). Groundwater from well FW113-47, being the most contaminated sample, had the highest concentrations of DOC, nitrate, chloride, cations (Al, Ca, Fe, Mn, Mg and K) and uranium, and the lowest pH. The uranium and nitrate concentrations of the groundwater from FW113-47 were 60 times and >100 times higher, respectively, than those of FW215-49, GW835 and GW836. Sulphate concentration was highest in the groundwater sample from FW215-49 (3798 mg L^{-1}) and lowest in GW836 (78 mg L^{-1}). Groundwater from GW836 was the least contaminated. It had the highest pH (6.96) and lowest concentrations of nitrate (44 mg L^{-1}), uranium (0.73 mg L^{-1}), sulphate (78 mg L^{-1}), Fe (0.015 mg L^{-1}), Mn (0.03 mg L^{-1}), Mg (8.67 mg L^{-1}) and K (3.70 mg L^{-1}). The DOC and DO concentrations of groundwater from FW113-47 and FW215-49 were similar but were different from those of GW835 and GW836 (Table 1).

3.2. Clone libraries and LIBSHUFF analyses

Between 60 and 180 clones were screened for each sample. The clones were screened via comparison of partial sequences of the V2–V6 region of the 16S rDNA sequence (~400–500 nucleotides at the 5′ end). After discarding putative chimeric sequences, 59 to 173 sequences per sample were used for each clone library (Table 2). Rarefaction analysis at 97% similarity levels (Figure 1) indicated that the majority of the recovered diversity was sampled within 41 analysed clones for FW113-47, 108 for FW215-49, 81 for GW835 and 165 for GW836. The least contaminated sample (GW836) displayed the highest diversity (richness) and evenness (frequency), whereas the most contaminated sample (FW113-47) showed the lowest (Table 2).

In an attempt to determine the significance of differences between clone libraries based on available sequence data, LIBSHUFF analysis was used (Table 3). A comparison of all libraries revealed that bacterial community composition based on 16S rDNA sequence differed significantly between the four groundwater wells ($P = 0.001$ for each combination) (Table 3). More detailed information on the differences between clone libraries was obtained by examining the distribution of ΔC_{xy} as a function of evolutionary distance (D). The coverage curves for representative pairs of clone libraries clearly show major differences also at low levels of genetic distance ($D > 0.2$)

Table 1. Physicochemical properties of the groundwater in different wells.

	Groundwater wells			
Chemical properties	FW113-47	FW215-49	GW835	GW836
pH	3.47	6.65	6.38	6.96
Conductivity (mS cm^{-1})	6.52	0.39	0.58	0.55
DO (mg L^{-1})	0.17	0.21	0.32	0.35
DOC (mg L^{-1})	41.34	43.36	33.96	23.66
NO_3^- (mg L^{-1})	23409	50	180	44
SO_4^{2-} (mg L^{-1})	109	3798	116	78
Cl^- (mg L^{-1})	619	11	14	244
Al (mg L^{-1})	420.822	0.003	0.0001	0.008
Ca (mg L^{-1})	748.94	105.46	100.18	120.23
Fe (mg L^{-1})	0.423	0.038	0.018	0.015
Mn (mg L^{-1})	59.97	0.89	1.58	0.03
Mg (mg L^{-1})	98.57	16.68	17.14	8.67
K (mg L^{-1})	73.39	4.83	4.96	3.70
U (mg L^{-1})	60.36	1.23	1.03	0.73

Table 2. Diversity estimates for 16S rRNA gene clones from different groundwater wells.

Groundwater wells	Number of clones[a]	OTU[b]	H′[c]	1/D[d]	Chao1[e]	Evenness[f]
FW113-47	59	18	2.40	0.115	0.0027	0.83
FW215-49	128	98	4.42	0.008	0.0438	0.97
GW835	95	62	3.89	0.017	0.0230	0.94
GW836	173	139	4.86	0.003	0.0404	0.99

[a]Number of clones sequenced from each library.
[b]Operational taxonomic unit; based on partial 16S rRNA gene sequences (≥97% nucleotide sequence similarity).
[c]Shannon–Weiner index; higher number represents higher diversity.
[d]Reciprocal of Simpson's index; higher number represents lower diversity.
[e]Chao diversity index; higher number represents higher diversity.
[f]Evenness; higher number represents higher diversity.

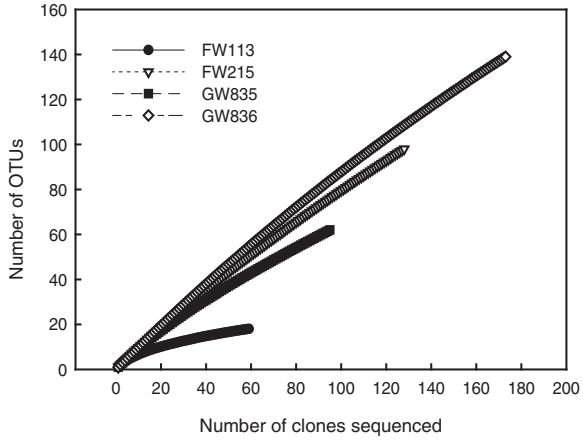

Figure 1. Rarefaction curves of 16S rDNA clone library illustrating the relationship between the number of OTUs and the number of clones sequenced (OTUs were defined at ≤97% nucleotide sequence identity).

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Table 3. LIBSHUFF analyses of the groundwater communities based on 16S rDNA clone libraries.

Sample comparison	Clones (N_x)	Clones (N_y)	ΔC_{xy}	P value
FW113-47 versus FW215-49	18	98	0.80	0.001
FW113-47 versus GW835	18	62	0.45	0.001
FW113-47 versus GW836	18	139	0.60	0.001
FW215-49 versus GW835	98	62	0.25	0.001
FW215-49 versus GW836	98	139	0.30	0.001
GW835 versus GW836	62	139	0.20	0.001

Note: the number of clones for each clone library is given by N_x and N_y. ΔC_{xy} represents the difference in coverage between two clone libraries. Higher ΔC_{xy} represents greater dissimilarity between two clone libraries.

(Figure 2). These differences were even more obvious for clone libraries from FW113-47 and GW835 (Figure 2b), FW215-49 and GW835 (Figure 2d), FW215-49 and GW836 (Figure 2e), and GW835 and GW836 (Figure 2f). The ΔC_{xy} values indicated that the most contaminated well (FW113-47) was significantly different from the other three wells. These results coincided with what was observed between diversity indices (i.e. OTU, H′, I/D, Chao and evenness) and geochemical parameters (Tables 1 and 2). Clone libraries from GW835 and GW836 had more similarities than clone libraries from FW113-47 and FW215-49 (Table 3).

Diversity-based clustering showed that the microbial community of the most contaminated well (FW113-47) formed a cluster and separated from the microbial communities found in FW215-49, FW835 and GW826 (Figure 3), corroborating the result of LIBSHUFF clone library comparisons (Figure 2 and Table 3). High similarity was observed between communities from GW835 and GW836. The similarity was 80% according to the calculated Bray–Curtis index. An $\int$-LIBSHUFF comparison showed that these two libraries were not significantly different ($P = 0.05$). All other pairwise comparisons showed significant difference between each other.

3.3. *Microbial community structure*

All four bacterial 16S rDNA clone libraries included sequences affiliated with most classes or phyla previously detected in contaminated groundwater (Figure 4). Among the 317 unique sequences, 37% share 85% to 93% sequence

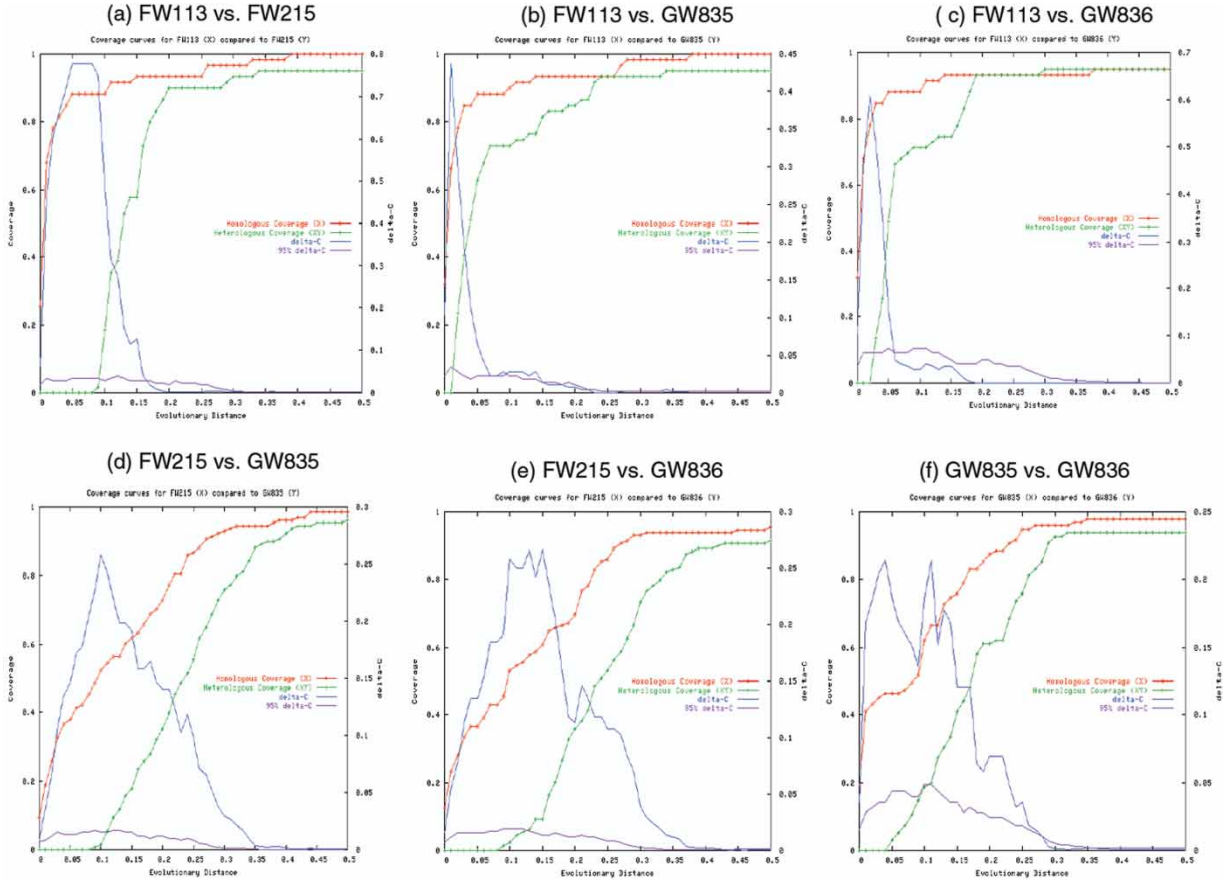

Figure 2. Results of LIBSHUFF comparisons of clone libraries from (a) FW113-47 and FW215-49; (b) FW113-47 and GW835; (c) FW113-47 and GW836; (d) FW215-49 and GW835; (e) FW215-49 and GW836; and (f) GW835 and GW836. Homologous (red lines) and heterologous (green lines) coverage curves for 16S rDNA. Blue lines indicate ΔC_{xy} for the original samples at each value of evolutionary distance (d). Violet lines indicate the 950th value (or $P = 0.05$) of ΔC_{xy} for the randomized samples.

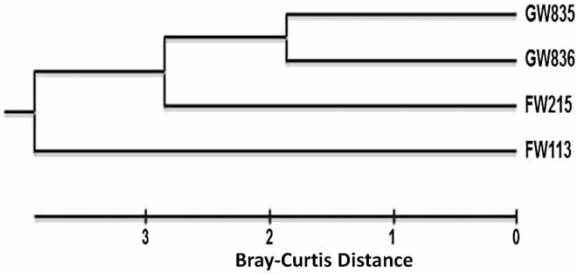

Figure 3. Clustering of samples according to the diversity patterns using the Bray–Curtis distance (presence/absence and abundance). The data were normalized to account for differences in the total number of sequences per library.

similarity with cultured bacterial strains. The remaining clones (<85% sequence similarity) were also distinct from any cultured bacterial species. Pairwise comparisons of all sequences in the four clone libraries showed high heterogeneity in the libraries since only 41 sequences (13%) shared more than 93% similarity to any other sequences published in GenBank. The RDP classifier grouped the 16S rDNA sequences to different classes: FW113-47 consisted of 4 classes; FW215-49, 18 classes; GW835, 13 classes; and GW836, 19 classes (Figure 4). The number of classes observed for each library corresponded with the results of the various diversity indices obtained (Table 2). The greater the number of classes found in the libraries (Figure 4), the greater was the reported diversity (Table 2). All libraries were dominated by members of the phyla α-, β-, δ- and γ-*Proteobacteria* and *Clostridia*. In addition, a few clones (fewer than 10 per group) were affiliated with *Actinobacteria*, *Anaerolineae*, *Aquificae*, *Bacilli*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Cyanobacteria*, *Flavobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, *Planctomycetacia*, *Sphingobacteria*, *Thermomicrobia* and *Verrucomicrobiae*. The least diverse clone library (FW113-47) almost exclusively contained 16S rDNA sequences similar to the γ-*Proteobacteria* (93.22%). Sequences within the γ-*Proteobacteria* were closely related to *Rhodanobacter*, *Shewanella*, *Stenophonomas*, *Pseudomonas*, *Pseudoxanthomonas*, *Xanthomonas*, *Flavimonas* and *Lysobacter* spp. The other three clone libraries were dominated by α-*Proteobacteria*, β-*Proteobacteria*, δ-*Proteobacteria*, and *Clostridia*: with *Clostridia* being the most prevalent group in FW215-49 (27% of clones in library); α-*Proteobacteria*, in GW835 (21% of clones in library); and β-*Proteobacteria*, in GW836 (19% of clones in library).

Sequences affiliated with α-*Proteobacteria* accounted for 16% of the total clones, but varied widely among the libraries (Table 4). Most clones affiliated with α-*Proteobacteria* grouped into six families, including *Acetobacteraceae*, *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Rhodobacteraceae*, *Rhodospirillaceae* and *Sphingomonadaceae*. Members of the β-*Proteobacteria* were not found in FW113-47 but accounted for 12–27% of the clones in FW215-49, GW835 and GW836 (Figure 4). Sequences affiliated with β-*Proteobacteria* were most closely related to *Burkholderiaceae*, *Comamonadaceae*, *Hydrogenophilaceae*, *Methylophilaceae*, *Neisseriaceae*, *Oxalobacteraceae*, *Procabacteriaceae* and *Rhodocyclaceae* (Table 4). γ-*Proteobacteria* were frequently encountered in all four clone libraries, representing 93%, 6%, 13% and 5% of the clones from FW113-47, FW215-49, GW835 and GW836, respectively (Figure 4). *Enterobacteriaceae* were detected in all clone libraries whereas *Xanthomonadaceae* were found only in FW113-47, FW215-49 and GW836. *Gemmatimonadaceae* and *Hahellaceae* were found only in GW835 and GW836, and *Pseudomonadaceae* were found only in FW113-47 and GW835 (Table 4). Predominant δ-proteobacterial sequences were affiliated with *Cystobacterineae*, *Desulfobulbaceae*, *Desulfohalobiaceae*, and *Syntrophobacteraceae*. A few δ-proteobacterial sequences

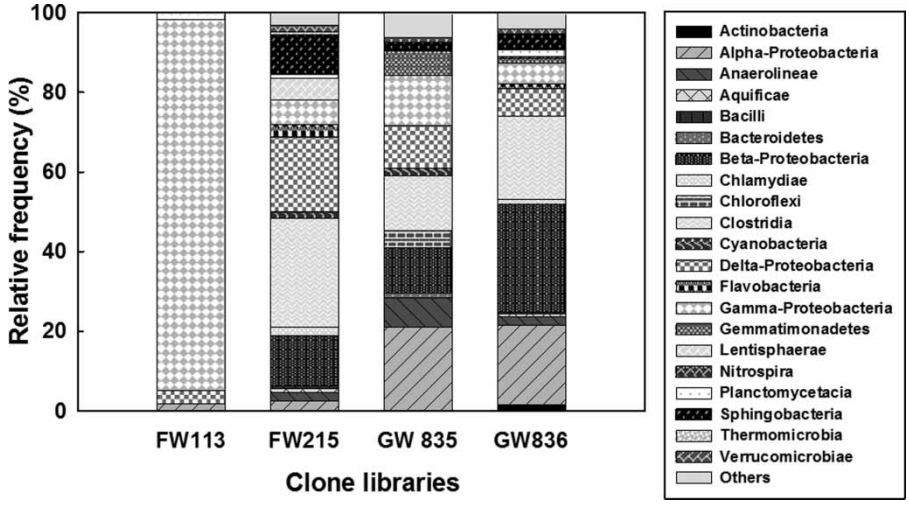

Figure 4. Microbial composition of the clone libraries based on the RDP Classifier. Bacteria that could not be assigned with the 80% confidence bootstrap value were included in an artificial 'nonaffiliated groups or others'.

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Table 4. Distribution (% of total) of clones similar to *Proteobacteria* from groundwater samples.

Taxonomic groups	Percentage of phylotypes in the clone libraries			
	FW113-47	FW215-49	GW835	GW836
		α-Proteobacteria		
Acetobacteraceae	0	33.33	0	6.06
Anaplasmataceae	0	0	0	3.03
Beijerinckiaceae	0	0	7.69	0
Bradyrhizobiaceae	100	33.33	0	15.15
Caulobacteraceae	0	0	0	9.09
Hyphomicrobiaceae	0	0	7.69	24.24
Phyllobacteriaceae	0	0	0	3.03
Rhizobiaceae	0	0	0	6.06
Rhodobacteraceae	0	0	23.08	12.12
Rhodospirillaceae	0	33.33	61.54	0
Sphingomonadaceae	0	0	0	15.15
		β-Proteobacteria		
Burkholderiaceae	0	0	0	10.52
Comamonadaceae	0	38.46	100.00	71.05
Hydrogenophilaceae	0	0	0	2.63
Methylophilaceae	0	15.38	0	0
Neisseriaceae	0	0	0	2.63
Oxalobacteraceae	0	7.69	0	5.263
Procabacteriaceae	0	0	0	2.63
Rhodocyclaceae	0	38.46	0	5.26
		γ-Proteobacteria		
Chromatiaceae	0	0	0	9.09
Coxiellaceae	0	0	11.11	0
Ectothiorhodospiraceae	0	0	5.56	0
Enterobacteriaceae	76.36	50.00	33.33	27.27
Gemmatimonadaceae	0	0	33.33	18.18
Hahellaceae	0	0	0	9.09
Legionellaceae	0	0	5.56	18.18
Methylococcaceae	0	12.50	5.56	0
Oceanospirillaceae	0	0	0	9.09
Pseudomonadaceae	7.27	0	5.56	0
Thiotrichaceae	0	25.00	0	0
Xanthomonadaceae	14.55	12.50	0	9.09
Shewanellaceae	1.82	0	0	0
		δ-Proteobacteria		
Bacteriovoracaceae	0	0	0	8.33
Coxiellaceae	0	0	9.09	0
Cystobacterineae	0	16.67	27.27	33.33
Desulfobulbaceae	0	0	9.09	41.67
Desulfohalobiaceae	100.00	45.83	27.27	0
Desulfurellaceae	0	0	9.09	0
Geobacteraceae	0	0	0	8.33
Sorangineae	0	0	9.09	0
Syntrophobacteraceae	0	37.50	9.09	8.33

were affiliated with *Bacteriovoracaceae*, *Coxiellaceae*, *Desulfurellaceae*, *Geobacteraceae* and *Sorangineae* (Table 4).

Except for FW113-47, all clone libraries contained sequences affiliated with *Clostridia* (Table 5). This group contains members of *Acidaminococcaceae* (*Allisonella*, *Dendrosporobacter* and *Quinella* spp.),

Table 5. Distribution (% of total) of clones similar to *Clostridia*, *Sphingobacteria*, *Anaerolineae* and *Planctomycetes* from groundwater samples.

Taxonomic groups	Percentage of phylotypes in the clone libraries			
	FW113-47	FW215-49	GW835	GW836
		Clostridia		
Acidaminococcaceae	0	8.57	23.08	8.33
Clostridiaceae	0	0	7.69	2.78
Halobacteroidaceae	0	5.71	0	0
Peptostreptococcaceae	0	54.29	46.15	86.11
Syntrophomonadaceae	0	28.57	15.38	0
Thermodesulfobiaceae	0	2.857	7.69	2.78
Acidaminococcaceae	0	8.57	23.08	8.33
		Sphingobacteria		
Crenotrichaceae	0	53.85	50	42.85714
Flexibacteraceae	0	46.15	0	7.142857
Saprospiraceae	0	0	50	7.142857
Sphingomonadaceae	0	0	0	42.85714
		Anaerolineae		
Anaerolinaeceea	100.00	100.00	100.00	100.00
		Planctomycetes		
Planctomycetaceae	100.00	100.00	100.00	100.00

Clostridiaceae (*Acetivibrio* and *Faecalibacterium* spp.), *Halobacteroidaceae* (*Selenihalanaerobacter* spp.), *Peptostreptococcaceae* (*Sporanaerobacter* spp.), *Syntrophomonadaceae* (*Anaerobaculum* spp.), and *Thermodesulfobiaceae* (*Thermodesulfobium* spp.). *Peptostreptococcaceae* populations dominated FW215-49 (54% of the *Clostridia* clones present in the library), GW835 (46% of the *Clostridia* clones present in the library) and GW836 (86% of the *Clostridia* clones present in the library). Several members of the class *Shingobacteria* were also found in the clone libraries FW215-49, GW835 and GW836. All libraries contained sequences affiliated with *Anaerolinaeceea* and *Planctomycetaceae*.

Bacterial groups known to reduce U(VI), nitrate, sulphate, Fe(III) as well as fermenters were detected in the clone libraries (Figure 5). Sequences from the genera *Acidovorax*, *Polyangium*, *Pseudomonas* and *Shewanella* were detected in the clone libraries. All four genera are known to include U(VI) reducers and can also contribute with one or more of the following activities: iron(III) reduction, sulphate reduction and nitrate reduction. *Acidovorax* was detected in GW835, whereas *Polyangium* was detected in GW836, and *Pseudomonas* and *Shewanella* were detected in the FW113-47 clone library. Most of the nitrate reducers were members of the phylum *Proteobacteria*, including *Pseudomonas* and *Ferribacterium*, *Acidovorax*, *Rhodanobacter*, *Xanthomonas and Herbaspirillum*. Iron(III) reducers were represented by *Polyangium* (*δ-Proteobacteria*), *Ferribacterium* (*β-Proteobacteria*), *Rhodoferax* (*β-Proteobacteria*) and *Stenotrophomonas* (*γ-Proteobacteria*).

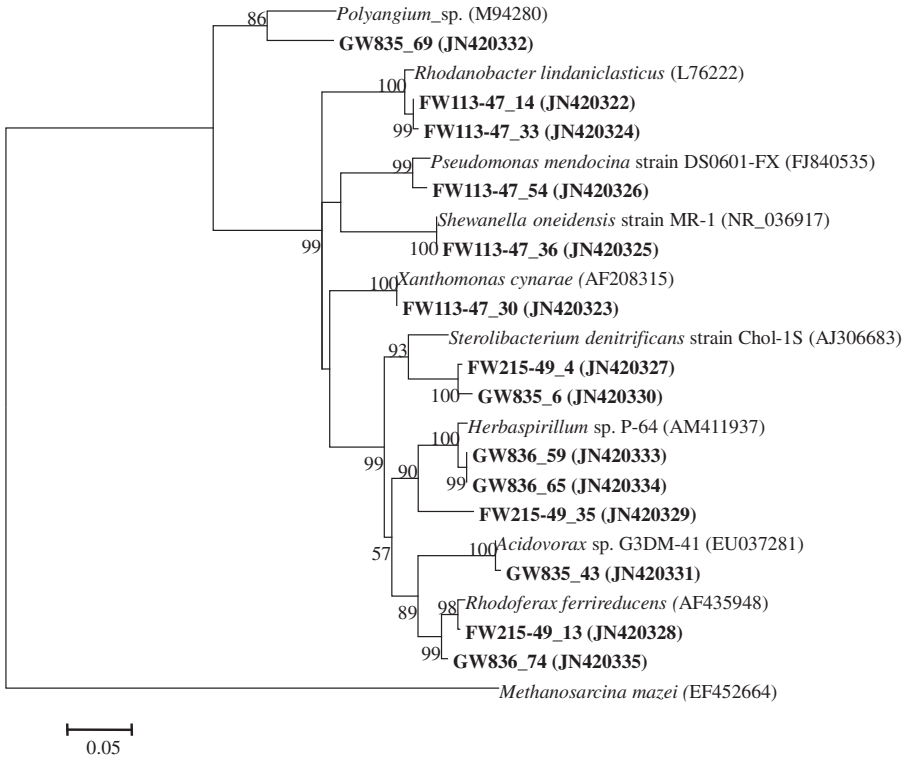

Figure 5. Phylogenetic dendogram showing the relationship of selected representatives from groups similar to known U(VI)-, iron-, nitrate- and sulphate-reducing bacteria. Nodal values represent bootstrap probabilities bases on 10,000 replicates.

Table 6. Pearson's product-moment correlation coefficients between diversity indices and geochemical properties of the groundwater retrieved from four different monitoring wells.

Chemical properties	H′	1/D	Chao1	Evenness
pH	0.965^{***}	-0.999^{***}	0.920^{**}	0.989^{***}
Conductivity (μS)	-0.920^{**}	0.995^{***}	-0.885^{*}	-0.959^{***}
DO ($mg\,L^{-1}$)	0.689^{ns}	-0.715^{ns}	0.473^{ns}	0.703^{ns}
DOC ($mg\,L^{-1}$)	-0.555^{ns}	0.465^{ns}	0.322^{ns}	-0.552^{ns}
NO_3^- ($mg\,L^{-1}$)	-0.919^{**}	0.995^{***}	-0.878^{*}	-0.959^{***}
SO_4^{2-} ($mg\,L^{-1}$)	0.333^{ns}	-0.344^{ns}	0.572^{ns}	0.345^{ns}
Cl^- ($mg\,L^{-1}$)	-0.725^{ns}	0.887^{*}	-0.745^{ns}	-0.798^{ns}
Al ($mg\,L^{-1}$)	-0.918^{**}	0.994^{***}	-0.876^{*}	-0.958^{***}
Ca ($mg\,L^{-1}$)	-0.907^{*}	0.991^{***}	-0.868^{ns}	-0.950^{**}
Fe ($mg\,L^{-1}$)	-0.917^{**}	0.993^{***}	-0.861^{ns}	-0.956^{**}
Mn ($mg\,L^{-1}$)	0.926^{**}	0.996^{***}	-0.883^{*}	-0.963^{***}
Mg ($mg\,L^{-1}$)	-0.944^{**}	0.998^{***}	-0.890^{*}	-0.976^{***}
K ($mg\,L^{-1}$)	-0.923^{**}	0.995^{***}	-0.879^{*}	-0.962^{***}
U ($mg\,L^{-1}$)	−0.919	0.994	−0.875	−0.959

Single, double, and triple asterisks indicate *P*-values less than 0.05, 0.01 and 0.001, respectively; ns = not significant.

3.4. Relationship between microbial diversity and geochemistry

The relationships between diversity indices (Shannon-Weiner [H′] index, Simpson's index [1/D], and Chao1 index) and geochemical properties (pH, conductivity, DO, DOC, NO_3^-, SO_4^{2-}, Cl^-, Al, Ca, Fe, Mn, Mg, K and U) in groundwater were explored (Table 6). Correlations were derived from all water data collected at all four wells (FW113-47, FW215-49, GW835 and GW836). Shannon diversity index and evenness were positively correlated with pH but were negatively correlated with conductivity, NO_3^-, Al, Ca, Fe, Mn, Mg, K and U. A reverse pattern was observed for the reciprocal of Simpson's index (1/D), where it correlated negatively with pH and positively correlated with conductivity, NO_3^-, Al, Ca, Fe, Mn, Mg, K and U (Table 6).

4. Discussion

The present study characterized geochemical parameters within a contaminated aquifer and identified abundant bacterial groups associated with each groundwater well. The data indicate that: (1) the diversity of the microbial communities are affected by geochemical properties including, pH, conductivity, NO_3^-, SO_4^+, Fe, Mn, Al, Mg and U (Table 6); (2) the bacterial communities include genera related to known U(VI), nitrate, sulphate and Fe(III) reducers, and fermenters (Figure 5); (3) distinct bacterial groups were observed on monitoring wells with varying geochemical properties; and (4) the most common groups at monitoring wells belong to α-, γ- and δ-*Proteobacteria*.

High levels of NO_3^-, Cl^-, Al, Ca, Fe, Mn, Mg, K and U had a significant impact on the level of bacterial diversity present in the groundwater. The presence of large quantities

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of the aforementioned contaminants seemed to inhibit the growth of a wide variety of microbes including *Actinobacteria*, *Anaerolineae*, *Aquificae*, *Bacilli*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Clostridia*, *Cyanobacteria* and other bacteria that are normally found in groundwater under less severe levels of contamination [15,25–27]. The high levels of contamination found in FW113-47 seemed to select few groups of bacteria to grow including α-, γ- and δ-*Proteobacteria* along with *Planctomycetaceae*. This limited diversity suggests that the bacteria that were found to survive in highly contaminated groundwater either were able to utilize the contaminants present for their cellular processes or possessed effective defences against the contaminants, which allowed them to survive or tolerate high levels of contamination. Bacterial diversity present in wells FW215-49, GW835 and GW836 was much higher than that of FW113-47. The contamination in these three wells was orders of magnitude less than that found in well FW113-47 (Table 2). In well FW215-49, the majority of the bacterial community present belongs to *Clostridia*, δ-*Proteobacteria* and β-*Proteobacteria*. Wells GW835 and GW836 also hosted large populations of *Clostridia* and β-*Proteobacteria* and had similar geochemical properties to well FW215-49, except for a lack of a large abundance of SO_4^{2-} and smaller populations of δ-*Proteobacteria*. In well FW113-47, the majority of the bacterial populations (93%) belong to α-*Proteobacteria*, whereas δ-*Proteobacteria* and β-*Proteobacteria* constitute a small fraction of the community (Figure 4). In other studies, the low pH and the presence of heavy metals, as seen in well FW113-47, were thought to cause selection pressure that favours species boasting acid tolerance and the capability to utilize multiple electron acceptors, therefore limiting diversity [27–29]. In this case, it would appear that the low pH and high concentration of inorganic ions (such as NO_3^-, SO_4^{2-} and Cl^-) and heavy metals (U, Al, Fe, Mn and Mg) have created a selection pressure that has limited the bacterial diversity in well FW113-47 when compared with wells FW215-49, GW835 and GW836. The high concentration of nitrate (23,409 mg L^{-1}) in well FW113-47 may have also been a factor that limited the growth of metal-reducing bacteria in the well, despite the ample concentrations of uranium and iron, because Fe(III) and U(VI) will only be used as electron acceptors after the nitrate in the environment has been exhausted [15]. The addition of electron donors such as acetate or ethanol to well FW113-47 could possibly determine whether the high amount of nitrate is what hinders the growth of metal-reducing bacteria and limits the microbial diversity [30]. Another study, by Fields *et al.* [25], conducted using groundwater collected from the Oak Ridge FRC found that nutrient availability may be more likely to affect diversity than are high concentrations of U(VI), as uranium is not the only toxic contaminant present in most of the groundwater wells. Transitory trends in nutrient usage by groundwater bacterial communities at Oak Ridge FRC were also suggested by a shift from nitrate-reducing to iron-reducing and finally sulphate-reducing populations in response to geochemical changes [26]. Such studies suggest that nitrate is utilized first by the microbial populations, which provides a low redox environment that causes the reductions of Fe(III), U(VI) and SO_4^{2-} to become more favourable and allows the microbes that undergo those reductions to flourish [27]. Pearson's product-moment correlation coefficients between diversity indices and geochemical properties demonstrated that increases in pH correlated positively with bacterial diversity, whereas increases in NO_3^-, Al, Ca, Fe, Mn, Mg, K and U concentrations correlated negatively with bacterial diversity. Elevated SO_4^{2-} concentrations, as observed in well FW215-49, did not significantly affect the bacterial diversity, as seen in Table 6. Although this data does support the role of nitrate as the geochemical parameter that affects diversity the most at the FRC, our limited sample size of four wells prevents such generalizations from being made without relying on previous studies for confirmation.

The largest portion of the bacterial community found in well FW113-47 belongs to the group γ-*Proteobacteria*, whose members are capable of heavy metal reduction, including the reduction of U(VI) to U(IV) (*Shewanella and Pseudomonas*) and Fe(III) reduction (*Stenotrophomonas*), and also the reduction of nitrate (*Rhodanobacter*, *Xanthomonas* and *Pseudomonas*). In a recent study, focusing on uranium bioremediation in high salinity subsurface sediments, it was found that the family *Geobacteraceae* thrived during metal reduction in lower salinity subsurface sediments, whereas when induced by the addition of acetate in higher salinity environments *Pseudomonas* species populations increased [31]. *Geobacteraceae*, a known metal reducer, was found in well GW836 along with another member of δ-*Proteobacteria*, *Cystobacterineae*, which is also known to reduce metals including U(VI) [8]. Most bacteria known to reduce U(VI) are part of a group of dissimilatory metal-reducing bacteria (DMRB) which are capable of reducing Fe(III), SO_4^-, NO_3^- and other metals [8]. In well FW113-47, 85% of the γ-*Proteobacteria* present, including *Enterobacteriaceae*, *Pseudomonadaceae and Shewanellaceae*, are known to contain species capable of U(VI) and other metal reduction [8]. Members of β-*Proteobacteria*, γ-*Proteobacteria* and δ-*Proteobacteria* capable of metal reduction were found in all of the wells tested, with the largest percentage of γ-*Proteobacteria* metal reducers in well FW113-47, β-*Proteobacteria* in well FW215-49 and δ-*Proteobacteria* in well GW836.

In wells GW836, GW835 and FW215-49 some of the clones recovered were very similar to *Sporanaerobacte*r (17.4%, 6.38% and 14.1%, respectively) and *Desulfonauticus* (1.70%, 3.15% and 7.62%, respectively), which have been observed to reduce SO_4^{2-} [32]. Nitrate reducers were also detected in GW835 (*Acidovorax* and *Sterolibacterium*) and FW215-49 (*Sterolibacterium*), as well as in GW836 (*Herbaspirillum*). Wells GW836 and FW215-49 included clones related to the Fe(III)-reducing organism *Rhodoferax*, while GW835 included clones related to *Acidovorax*. The

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U(VI) reducers *Acidovorax* and *Polyangium* were detected in well GW835. The diversity and abundance of metal-reducing organisms found in all four wells suggest that there is potentialfor successful bioremediation of the groundwater at the FRC.

Unlike other studies utilizing groundwater at Oak Ridge FRC, this study did not include the injection of ethanol or acetate to promote a reducing environment in order to stimulate the growth of organisms capable of reducing U(VI), Fe(III) and sulphate. Therefore, the discrepancy between the results presented in this study, with respect to the nascent populations of uranium-reducing bacteria present in groundwater, and the results presented by others [27,31] can be attributed to the stimulation of bacteria such as *Shewanella*-like and *Pseudomonas*-like species present at low levels in well FW113-47. Well FW113-47 also contained very small populations of known metal reducers (7% of clones in the library), which may not have a significant impact on the bioremediation process unless an electron donor such as ethanol is added to promote an iron-reducing environment. Given that the dominant family in well FW113-47 is γ-*Proteobacteria*, whose members are known to possess pathways that utilize higher energy substrates such as nitrate, and that this well has a high nitrate concentration (23,409 mg L^{-1}), it is likely that the nitrate would have to be exhausted before the community could be involved in the natural attenuation of U(VI). Since wells FW215-49, GW835 and GW836 contain significant populations of δ-*Proteobacteria* capable of reducing U(VI) to U(IV) (*Acidovorax* and *Pseudomonas*), and their nitrate concentrations are lower (50–180 mg L^{-1}), it is possible that those microbial communities could affect the process of natural attenuation of U(VI).

All monitoring wells contained bacterial populations that belong to α-, γ-, and δ-*Proteobacteria*. These bacterial populations have also been observed as principal constituents of the microbial community in other studies conducted at Oak Ridge FRC [15,16,33–35] The abundance of α-, β-, γ-, and δ-*Proteobacteria* observed in wells FW215-49, GW835 and GW836 is similar to previous studies on groundwater at the Oak Ridge FRC [26,27]. Moreover, the large populations of γ-*Proteobacteria* and absence of β-*Proteobacteria* in well FW113-47 are similar to results observed at wells with similar geochemical characteristics reported in other studies [26,27]. Similar populations of *Anaerolineae*, *Clostridia*, *Sphingobacteria and Verrucomicrobiae* were all found in wells FW215-49, GW835 and GW836, whose geochemical variables were less extreme than well FW113-47. Hwang *et al*. [26] suggested that groundwater sampling alone may not fully encompass the microbial diversity of any given site. This study observed that different populations could be generated from groundwater and a surrogate solid media contained in a porous receptacle or biofilm coupon. These biofilm coupons can be colonized by microbes in the environment and promote the association of these microbes into communities. Some research has also proposed that attached microbes make up the majority of an environment's biomass and activity [36–40]. Seeing that this study was focused on sampling groundwater, the low abundance of metal-reducing microbes may be due to differences in populations between planktonic microbes and those that are attached to mineral matrices.

Our results suggest that the low pH and high concentration of ions and heavy metals like those found in well FW113-47 limited the diversity of the microbial community and created a pressure that would select for bacteria able to withstand or even utilize the contaminants present. The high concentrations of contaminants present in well FW113-47 limited the bacterial diversity, but stimulated the growth of uranium- (*Shewanella* and *Pseudomonas*), nitrate- (*Pseudomonas*, *Rhodanobacter* and *Xanthomonas*) and iron- (*Stenotrophomonas*) reducing organisms unique to this well. Groundwater geochemistry influenced the bacterial populations capable of surviving and thriving in an environment; bioremediation efforts may benefit from the knowledge of groundwater geochemistry and how it affects microbial diversity. The presence of genes from a variety of metal-resistant microbes and genes for metal- and nitrate-reduction indicate great potential for successful bioremediation of the contaminated groundwaters through biostimulation.

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