Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid–uranium waste

Matthew W. Fields [a,b,*,1], Tingfen Yan [b], Sung-Keun Rhee [b,2], Susan L. Carroll [b], Phil M. Jardine [b], David B. Watson [b], Craig S. Criddle [c], Jizhong Zhou [b,1]

[a] Department of Microbiology, Miami University, Pearson Hall, Room 32, Oxford, OH 45056, USA
[b] Oak Ridge National Laboratory, Environmental Sciences Division, Oak Ridge, TN 37831, USA
[c] Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305-4020, USA

Abstract

Microbial communities were characterized at contaminated sites that had elevated levels of nitrate, nickel, aluminum, and uranium (up to 690 mM, 310 µM, 42 mM, and 30 µM, respectively). The bacterial community structure based upon clonal libraries of the SSU rRNA genes (screened clones = 876) was diverse at the background site, but the three acidic samples had decreased diversity and the majority of clones were closely related to *Azoarcus* and *Pseudomonas* species. *Arthrobacter* and *Novosphingobium* sequences were recovered from the background samples but not the acidic sites, and similar pseudomonad populations were present at the background and acidic sites albeit at different relative abundances. Heterologous sequence coverage analyses indicated the microbial communities at the contaminated sites were very similar ($p = 0.001$) but different from the background site. Bacterial isolates ($n = 67$) classified as β-or γ-*Proteobacteria*, high G+C Gram-positive or low G+C Gram-positive were obtained from the background and one contaminated sample, and some of the isolates had less than 95% sequence identity with previously observed microorganisms. Despite variations in nitrate and heavy metal levels and different proximities to the source ponds, the three acidic samples had similar microbial populations. However, the least contaminated site (lowest nitrate and aluminum) had increased diversity compared to the other acidic samples. The results suggested that the combined contamination has decreased the microbial diversity, and *Azoarcus* populations were observed at a drastically increased frequency compared to the background site that had a more even distribution of multiple taxa.

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1. Introduction

The remediation of mixed wastes and radionuclides is a significant challenge, and bioremediation with microorganisms remains potentially the most cost-effective cleanup technology. However, biodegradation or biotransformation of pollutants in the natural environment is a complex process that depends upon the quantitative and qualitative aspects of the contaminant present, the

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* Corresponding author.
  E-mail address: fieldsmw@muohio.edu (M.W. Fields).
[1] Virtual Institute of Microbial Stress and Survival (http://vimss.lbl.gov).
[2] Present address: Biodiversity Information Laboratory, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea.

structure and dynamics of the indigenous microbial community, and the geological and chemical conditions at the contaminated sites [1]. In addition, many disturbed sites contain multiple contaminants that can complicate treatment and/or maintenance. The elucidation of individual and community-wide microbial responses to the combination of mixed wastes and varied geochemical conditions is a crucial aspect for the improvement and implementation of bioremediation strategies.

The majority of studies dealing with radionuclide-associated environments have focused on cultivable, activity-based, or RFLP-based assessments of waste piles or repositories of nuclear fuel [2–5]. One study utilized clonal libraries of SSU rRNA gene sequences to characterize microbial diversity associated with naturally radioactive subsurface environments in Gabon, Africa [6]. The biogeochemical processes that occur in groundwater and sediments contaminated with heavy metals and radionuclides can impact the microbial community [7,8], yet little is known about the composition of the indigenous subsurface microbial communities at different sites contaminated with radionuclides. Moreover, even less is known regarding the relationship between the microbial community and the geochemical characteristics of contaminated sites.

The Field Research Center (FRC) is located within the Y-12 Security Complex near Oak Ridge, TN in the Bear Creek Valley, and the site includes 243-acres of a previously disturbed contaminated area. The subsurface at the FRC contains one of the highest concentration plumes of mobile uranium located in the United States, and contains various levels of nitrate, heavy metal, and organic contamination (http://www.esd.ornl.gov/nabirfrc/). Groundwater samples from wells that surround the S-3 ponds were collected, and the ponds were four unlined surface impoundments that received nitric acid/uranium bearing wastes. In order to determine how varying levels of multiple contaminants (e.g., nitrate, metals, radionuclides, low pH) have affected the microbial community structure, three contaminated wells in close proximity to the source ponds as well as a background site were used. In addition, the isolation of microorganisms in relation to nitrate reduction was attempted for the four tested sites.

2. Materials and methods

2.1. Sampling sites

The FRC site includes three areas of contaminated soil and groundwater and an uncontaminated background area that contains soils similar to those found in the contaminated areas. The site is contaminated with nitrate, uranium, heavy metals, and a variety of organic contaminants, and a full description can be located at

Table 1
The pH, nitrate, uranium, aluminum and nickel levels in the groundwater samples from four characterized FRC groundwater samples

Well	pH	Nitrate (mM)	Uranium (μM)	Nickel (μM)	Aluminum[a] (mM)
FW-300	5.8 ± 0.4	0.04 ± 0.05	ND	0.45 ± 0.5	0.01 ± 0.01
FW-005	4.0 ± 0.2	4.6 ± 2.5	30 ± 4.8	84 ± 1.0	1.74
FW-010	3.5 ± 0.1	694 ± 28	0.8 ± 0.1	310 ± 17	41.5
FW-015	3.5 ± 0.2	154 ± 27	31 ± 2.7	148 ± 2.5	22.9

FW-300 represents the background area. ND, not detected.
[a] Some samples measured once.

the FRC website (http://www.esd.ornl.gov/nabirfrc/). Groundwater samples were collected from four wells at the FRC (Table 1). The wells FW-005 and FW-010, located in Area 3, are 32.5 m apart, and are ≈20 m from the former waste pond. Well FW-015 is 27 m from the waste pond embankment in Area 1, and is ≈130 m from the wells in Area 3.

The background area is ≈2 km from the contaminated area in the same valley on the Y-12 security complex. The background area lies directly along the geologic strike of the contaminated area and is, therefore, underlain by nearly identical geology, mineralogy, and structure. Contaminants have not been previously or currently disposed at the background area throughout the history of Department of Energy operations (http://www.esd.ornl.gov/nabirfrc/index.html). However, it should be noted, that due to the contamination, the background area is not directly next to the contaminated areas.

Different background wells have very similar geochemical characteristics, and one background site was analyzed with increased coverage. The water table was ≈6 m from the surface, and water was collected from a screened interval below the water table at each of the four wells on the same day. Microbial cell numbers were quantified by direct cell counting using epifluorescence microscopy of acridine-orange stained samples and statistical analysis as described previously [9].

2.2. Water chemistry

Ground water pH was measured at the field site from multi-level wells with a pre-calibrated Yellow Springs Instruments YSI XL6000M multi-parameter probe (Yellow Springs Instruments, CO). Nitrate was analyzed with a Dionex 500 ion chromatograph (Dionex, Sunnyvale, CA) following EPA method 300.0, and TOC was measured by a Shimadzu total organic carbon analyzer (TOC-5000A, Tokyo, Japan) (EPA method 415.1). Nickel and aluminum were analyzed in acidified samples with a Thermo Jarrell Ash inductively coupled plasma PolyScan Iris Spectrometer (EPA reference SW846 ch 3.3), and uranium levels were analyzed with a Perkin–Elmer Elan 6000 ICP-MS (EPA reference 600/R-94-111).

2.3. Bacterial isolation, cultivation, and differentiation

Aliquots were removed from groundwater samples and used to inoculate different media. Denitrifying medium (DM) contained nutrient broth ($8 \, g \, l^{-1}$) and potassium nitrate ($1 \, g \, l^{-1}$), and MR2A medium has been described previously [10]. The pH values (4.0, 5.5, or 6.5) for both media were adjusted with nitric acid. Anaerobic media was prepared by boiling under oxygen-free N_2 gas and sealing the media in roll tubes with butyl stoppers and crimp seals. Samples were incubated in the dark at room temperature. Anaerobic plates were prepared in an anaerobic glove bag (Coy Laboratory Inc.) with a N_2:H_2 atmosphere (95%:5%). Colonies were picked based on differences in morphology, color, and margins.

Isolates were streaked 3–5 times to ensure purity, and then tested for nitrate reduction with inverted Durham tubes and/or Szechrome reagent (Polysciences, Inc.) according to the manufacture's instructions. Bacterial isolates were differentiated with BOX-PCR genomic fingerprinting. Each isolate was transferred to 0.04 M NaOH (50 μl) with a small inoculating loop, and then frozen at −80 °C. The samples were then quickly thawed at 65 °C, and subsequently heated at 96 °C for 10 min. The solution of disrupted cells served as the genomic DNA for BOX-PCR. BOX-PCR was done according to the protocol of Rademaker and de Bruijn [11]. PCR volumes were 25 and 1 μl of template DNA was used. The PCR parameters were as follows: 95 °C for 7 min – 1 cycle; 94 °C for 1 min; 53 °C for 1 min; 65 °C for 8 min – 30 cycles; 65 °C for 16 min – 1 cycle. Samples were observed on 1.5% TAE agarose gels. Gels were analyzed and compared with Molecular Analyst software 1.6 (BioRad, Inc.).

2.4. DNA extraction and purification

Groundwater samples (1–2 l) were collected, and transported to the laboratory in glass, amber bottles. Bacteria were harvested by centrifugation ($10,000g$ force, 4 °C for 30 min), and the pellets were stored at −80 °C until used for DNA extraction. The cell pellet was resuspended in a lysis buffer and the cells disrupted with a previously described grinding method [12]. DNA was extracted as previously described [12,13], and the precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up system, Promega) [12].

2.5. PCR amplification and cloning

The nearly complete SSU rRNA genes were amplified in a 9700 Thermal Cycler (Perkin–Elmer) with the primer pair FD1 F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1540R (5′ AAG GAG GTG ATC CAG CC 3′) (position 1 and 1540 *Escherichia coli* designations) [14]. The PCR reactions (20 μl) contained 50 mM KCl, 10 mM Tris–HCl pH 9.0 and 0.1% Triton X-100), 1.8 mM $MgCl_2$, 80 ng bovine serum albumin (Boehringer Mannheim, Indianapolis, IN), 0.25 mM $4\times$ dNTPs (USB Corporation, Cleveland, OH), 10 pmol each primer, 2.5 U Taq polymerase, and 1μl purified DNA (5–10 ng). To minimize PCR-induced artifacts, the optimal number of cycles was determined and five PCR reactions were combined prior to cloning as described previously [15]. The PCR parameters were as follows: 80 °C for 30 s; 94 °C for 2 min; 94 °C for 30 s; 58 °C for 1 min; 72 °C for 1 min, 26 cycles; 72 °C for 7 min. PCR products were analyzed on 1.5% (w/v) TAE agarose gels, and the insert size confirmed.

The combined PCR products were separated by electrophoresis in a low-melting point agarose gel (0.8%), the appropriate band excised, and the DNA extracted with a Wizard Prep Kit (Promega, Madison, WI) according to manufacturer's instructions. Recovered DNA was resuspended in 6 μl ddH$_2$O, 2 μl was ligated with pCR2.1 vector from a TA-cloning kit, and competent *E. coli* cells were transformed according to the provided protocol (Invitrogen, San Diego, CA).

2.6. Sequence and phylogenetic analysis

PCR products (100 μl) amplified with vector-specific primers via colony PCR were purified with the ArrayIt™ PCR Purification Kit (TeleChem International, Inc., Sunnyvale, CA) or treated with ExoSAP-IT™ (USB Corporation, Cleveland, OH) according to manufacturer's instructions. DNA sequences were determined with a BigDye Terminator kit (Applied Biosystem, Foster City, CA) using a 3700 DNA analyzer (Perkin–Elmer, Wellesley, MA) according to the manufacturer's instructions. DNA sequences were edited with the Sequencher™ software (version 4.0, Gene Codes Corporation, Ann Arbor, MI).

Partial sequences were determined from the purified inserts with the SSU rRNA gene primer 529r, 5′ CGC GGC TGC TGG CAC 3′ (*E. coli* numbering). Unique SSU rDNA clones ($\leqslant 97\%$ sequence identity) from each site were identified by direct sequence comparisons, and were designated as OTUs (operational taxonomic unit). The number of sequences determined for each site is listed in Table 3. The unique OTUs were compared to the closest related sequences from GenBank and the RDP, and nearly complete SSU rDNA sequences ($n = 82$) were determined for representative clones (Figs. 2–5).

The sequences were aligned with ClustalW, and alignments compared with reference sequences from the database [16]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [17], and phylogenetic trees were constructed with distance

matrices and the minimum evolution method with a Jukes–Cantor distance model. The initial trees were obtained with the neighbor-joining method and interior branch tests were determined. The presumptive phylogenetic relationships between the clonal sequences are being used as a relative comparison between the observed sequences and not as definitive designation for phylogeny. The nucleic acid sequence accession numbers in GenBank for the bacterial isolates and the unique clonal sequences described in this study are: AY661909–AY662049. The diversity (Shannon–Wiener and Simpson's index) and evenness (equitability) indices were based upon the distribution of unique OTUs obtained from the clonal libraries using equations from Krebs [18].

LIBSHUFF (version 1.2) analysis computes the homologous and heterologous coverage within and between clonal libraries [19]. The analysis estimates the similarity between clonal libraries from two different samples based upon evolutionary distances of all sequences. Thus, the sampled diversity of a community can be directly compared to another community. The predicted coverage of a sampled library is denoted by the homologous coverage, and the heterologous coverage is the observance of a similar sequence in a separate library. The values are reported over a sequence similarity range or evolutionary distance (D) based upon a distance matrix. Analyses were performed according to specified directions given at the LIBSHUFF website (http://www.arches.uga.edu/~whitman/libshuff.html).

3. Results

3.1. Site characteristics

The chemical characteristics were significantly different among the four samples (Table 1), and the wells except background had nitrate levels over the drinking water standards of 10 ppm or 0.16 mM. The TOC (total organic carbon) levels were similar for FW-005 and FW-015 (5.8 mM vs. 5.4 mM, respectively), and were ≈2-fold above background (2.5 mM). Site FW-010 had a TOC value that was ≈6-fold increased compared to background (14.6 mM). The substratum of the sampling sites was similar, and consisted of shale with inner-embedded limestone that has been weathered to clay, silty saprolite. The groundwater flow was in a south–southwest direction from the source ponds towards FW-005, FW-010, and FW-015.

3.2. Bacterial isolates

Bacteria were isolated from FW-300 ($n = 59$) and FW-005 ($n = 9$) with either MR2A medium [10] or nutrient broth amended with nitrate. Isolates were not ob-

tained from FW-010 or FW-015. Microbial isolates with significant sequence identity to predominant clones were not obtained from any of the samples, except for isolate DM-E3 (FW-300) which had 97% sequence identity with clone 005Aa-H03 that accounted for 7% of the sampled diversity from FW-005 (Fig. 3). All isolates from FW-005 could be classified as β-*Proteobacteria*, except 005-J which was related to *Microbacterium*, and could grow at pH values between 4.0 and 7.0 in MR2A medium. All isolates could reduce nitrate in MR2A medium and the closest phylogenetic associations are listed in Table 2.

3.3. Community structure based on partial SSU rRNA sequence comparisons

The collected biomass from the groundwater samples yielded PCR products, and 876 clones were screened via comparison of partial sequences of the V2–V6 region of the SSU rRNA gene sequence (≈400–500 nucleotides at the 5′ end). Between 100 and 320 clones were screened for each sample (Table 3), and rarefaction analyses indicated that the majority of recovered diversity was sampled within 85 analyzed clones for FW-010, ≈135 analyzed clones for FW-005 and FW-015, and 245 clones for FW-300 (Fig. 1). The background, FW-300, displayed the highest diversity compared to the acidic, contaminated sites (Table 3). At the time of sampling, bacterial numbers in FW-300 groundwater were estimated to be ≈10^6 cells ml^{-1} with acridine orange and ≈10^5 cells ml^{-1} in the acidic groundwater.

A significant proportion of the FW-005 clones were *Azoarcus*-like (005C-F11) and comprised almost 40% of the FW-005 library (Table 4). Two different *Pseudomonas*-like sequences (005C-G12 and 005Aa-H03) were also relatively abundant and were 20% and 7% of the library, respectively. Ecological indices indicated that FW-005 had the highest diversity and evenness values of the contaminated sites and 27 OTUs were estimated based on ≤97% similarity of SSU rRNA gene sequences (Table 3).

The well, FW-010, also had an increased occurrence of *Azoarcus* and *Pseudomonas*-like sequences (010B-F11, 010D-B08, and 010B-E10), and these genera comprised almost 90% of the FW-010 library (Table 4). The nitrate and aluminum levels of FW-010 groundwater were the highest of the sampled sites, and FW-010 had the lowest number of estimated OTUs. An increased proportion (40%) of the FW-010 library appeared to be *Pseudomonas*-like compared to the other acidic samples, and 12 OTUs were less than 2% of the library. The majority of recovered sequences for FW-015 were also closely related to *Azoarcus* and *Pseudomonas*. The *Azoarcus* group accounted for 49% of the library and pseudomonads accounted for ≈25% (Table 4).

Table 2
Bacterial isolates obtained from FW-300 (background) or FW-005

Sample	Percent identity to closest match (%)	Closest match
FW-005		
005-L	99	*Delftia acidovorans* (AF526915)
005-M	97	*Acidovorax* 3DHB1 (AF458096)
005-3	95	*Burkholderia phenazinium* (AB021394)
005-2	94	*Burkholderia phenazinium* (AB021394)
005-D	96	*Pandoraea* G5084 (AF247693)
005-4	96	*Chromobacterium violaceum* 52 (AY117554)
005-J	94	*Microbacterium* VKM (AB042074)
FW-300		
M-A24	90	*Variovorax paradoxus* (AJ420329)
AM-Z2	95	*Burkholderia phenazinium* (AB021394)
DM-R2	88	*Chromobacterium violaceum* 52 (AY117554)
DM-L2	90	*Chromobacterium violaceum* 52 (AY117554)
DM-P3	97	*Chromobacterium violaceum* 52 (AY117554)
AnM-A3	98	*Stenotrophomonas detusculanense* (AF280434)
M-A21a	99	*Stenotrophomonas rhizophila* (AJ293463)
DM-P2	96	Biofilm clone IAGBH3 (AF286178)
DM-N2	95	Biofilm clone IAGBH3 (AF286178)
AnM-E3	96	Biofilm clone IAGBH3 (AF286178)
DM-A3	90	*Serratia proteamaculans* (AJ508694)
DM-D3	92	*Serratia proteamaculans* (AJ508694)
M-12	95	*Acinetobacter johnsonii* (AB099655)
M-3	94	*Acinetobacter johnsonii* (AB099655)
DM-E3	95	*Pseudomonas syringae* (AB001439)
DM-V2	96	*Pseudomonas aeruginosa* (AY162139)
M-A12	93	*Rhizobium galegae* (Z79620)
M-11	93	Clone R10 (AF407686)
M-1	99	Clone ph5Lac302-37 (AY527741)
M-4	96	Clone ph5Lac302-37 (AY527741)

All isolates from FW-005 were isolated with MR2A medium. Isolates from FW-300 designated with a DM were isolated with nitrate amended nutrient broth, and all other isolates from FW-300 were obtained with MR2A.

Table 3
Characteristics and diversity estimates for SSU rRNA gene clones from four FRC groundwater samples

Well	Number of clones[a]	OTU[b]	H′[c]	1/D[d]	Evenness[e]
FW-300	321	79	5.17	22.7	0.97
FW-005	211	27	3.18	4.91	0.82
FW-010	113	19	2.62	3.81	0.77
FW-015	231	34	2.84	3.43	0.73

FW-300 represents the background area.
[a] Number of clones analyzed from each library.
[b] Operational taxonomic units based on unique, partial SSU rRNA gene sequences ($\leqslant$97%).
[c] Shannon–Weiner index, higher number represents more diversity.
[d] Reciprocal of Simpson's index, higher number represents more diversity.
[e] As Evenness approaches 1, the population is more evenly distributed.

Table 4
The distribution of observed SSU rRNA gene sequences for FW-005, FW-010, FW-015, and FW-300 based on partial sequence determination

Sample	Clone	Number of clones	Closest match
FW-005	005C-F11	82/211	*Azoarcus* FL05 (AF011330)
	005C-G12	42/211	*Pseudomonas stutzeri* (U65012)
	005Aa-H03	14/211	*Pseudomonas marginalis* (AF364098)
	005Aa-B11	13/211	*Rhizobium giardinii* (U86344)
	005C-G01	8/211	*Wautersia eutropha* (M32021)
	005Aa-C05	6/211	*Stenotrophomonas rhizophila* (AJ293463)
	005Aa-F09	6/211	Unidentified clone (Z93955)
FW-010	010B-F11	51/113	*Azoarcus* FL05 (AF011330)
	010D-B08	37/113	*Pseudomonas stutzeri* (AJ270451)
	010B-E10	14/113	*Pseudomonas marginalis* (AF364098)
	010A-E08	2/113	*Ralstonia metallidurans* CH34 (Y10824)
FW-015	015d-A03	113/231	*Azoarcus* FL05 (AF011330)
	015B-H08	39/231	*Pseudomonas stutzeri* (U65012)
	015C-D08	18/231	*Pseudomonas marginalis* (AF364098)
	015C-F06	7/231	Uncultivated clone (AF072924)
	015E-E03	5/231	*Ralstonia metallidurans* (Y10824)
	015C-F09	5/231	*Acidovorax delafiedii* (AF332187)
FW-300	300I-E04	51/321	*Arthrobacter polychromogenes* (Y80741)
	300A-A08	29/321	*Pseudomonas marginalis* (AF364098)
	300C-C3	25/321	*Novosphingobium subterraneum* (AB025014)
	300I-A04	22/321	*Acidovorax delafiedii* (AF332187)
	300I-E02	19/321	Uncultured clone (AY037565)
	300E2-D10	10/321	*Pseudomonas stutzeri* (U65012)
	300I-F2	6/321	*Propionivibrio limicola* (AJ307983)
	300E2-F01	6/321	*Sphingobium yanoikuyae* (AF509480)
	300A-H04	6/321	*Bdellovibrio bacteriovorus* (AF084850)
	300A-C09	6/321	Unidentified clone (Z93955)

Unique OTUs are at the 97% sequence identity level. Each predominant clone is listed with the number of times a similar sequence was detected in the respective library as well as the closest match from the GenBank database (accession numbers in parentheses).

The background, FW-300, displayed the highest diversity and evenness, and 79 unique OTUs were observed from a clone library of 321 analyzed clones compared to between 19 and 34 unique OTUs at the acidic sites (Table 3). Two *Pseudomonas*-like OTUs were observed at all tested sites, but the occurrence in the background library was reduced compared to the acidic samples (9% vs. 17–33% or 3% vs. 7–9%, respectively). The majority of the observed OTUs from the FW-300 clonal library were unique with respect to the acidic sites.
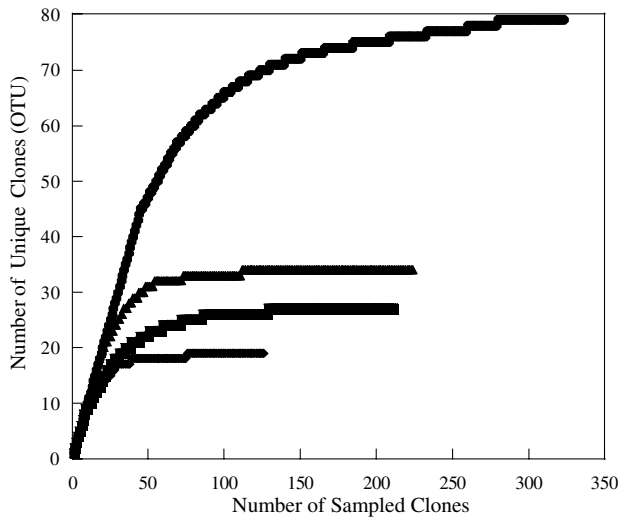
Fig. 1. Number of unique clones versus the number of sampled clones from groundwater samples FW-300 (●), FW-005 (■), FW-010 (◆), and FW-015 (▲).

3.4. LIBSHUFF analyses

The SSU rRNA gene clonal libraries were compared between the sites with differences between coverage curves (LIBSHUFF analysis, version 1.2) as previously described [19]. The acidic samples had the lowest ΔC_{xy} values when compared to each other, and FW-010 and FW-015 were the most similar (Table 5). The majority of difference between the acidic samples was observed at decreased evolutionary distances (>95%). The sites, FW-005 and FW-300, appeared to be the most dissimilar, and the FW-010 and FW-015 also displayed significantly increased dissimilarity with the FW-300 library (3.5 and 5.2, respectively).

3.5. Phylogenetic analysis

The most frequently recovered OTU (005C-F11) for all three acidic samples had 99% sequence identity with *Azoarcus* FL05, and the clonal sequences had 99% sequence identity with one another (Fig. 2). *Azoarcus*-like or *Ralstonia*-like sequences were not observed at the background site, nor were any microbial isolates obtained that had high sequence similarity with these groups. Interestingly, the bacterial isolate, 005-L, from FW-005 had 99% sequence identity with the clone, 005Aa-B06, and 98% sequence identity with *Delftia acidovorans* (Fig. 2).

The majority of pseudomonad sequences grouped with *Pseudomonas stutzeri* and was similar for all sites (although the relative abundances were markedly different). However, the second cluster of pseudomonad sequences grouped with *Pseudomonas marginalis* and *P. rhodesiae*, and was observed at similar frequencies (8–12%) for all four samples. In addition, sequences closely

related to the *Enterobacteriaceae* were not identified from any of the clonal libraries, but six bacterial isolates were obtained from FW-300 that could be classified in the *Enterobacteriaceae* (Fig. 3).

Indicative of the increased diversity indices for FW-300, sequences that represented the α-,β-,γ-,δ-*Proteobacteria*, *Acidobacterium*, high G+C, and *Verrucomicrobia* were observed, many were not observed at the acidic samples, and some of the clones were not closely related to previously cultivated microorganisms. Clonal sequences unique to FW-300 had 99% or 96% sequence identity with *Novosphingobium subterraneum* or *Sphingobium yanoikuyae*, respectively, and accounted for a total of 10% of the sampled diversity from the background (Fig. 4). A majority of the novel sequences could be classified as δ-*Proteobacteria*, low G+C, *Acidobacteria* or *Verrucomicrobiales* (Fig. 5).

A high G+C OTU represented by 300I-E04 constituted 16% of the sampled diversity from FW-300 and had 98% sequence identity with *Arthrobacter polychromogenes* (Fig. 5). Few clonal sequences that could be classified as low G+C were obtained from any of the samples, but unique microorganisms were isolated from FW-300 with MR2A medium. Isolates M-1-300 and M-4-300 were most closely related to FRC sediment clone ph5Lac302-37 (GenBank, AY527741) and had 98% and 96% sequence identity, respectively.

4. Discussion

PCR-based cloning approaches can be used to assess diversity and community structure, and can be a valuable tool for microbial community analyses [20]. However, the abundance, dominance, or diversity may not be completely epitomized by clone distribution; therefore, the measurements and indices are used for relative comparisons [20]. The formation of chimeras, heteroduplexes, and mutations can be intrinsic to PCR amplification [15], and protocols were followed to minimize PCR amplification-induced artifacts as described in Section 2. Multiple studies exist in the literature that employ SSU rRNA gene clonal libraries, and for the present study 97% sequence identity was used for the designation of unique OTUs at the species level. It should also be noted that the cloning and sequencing of SSU rRNA sequences could vastly underestimate the number of bacterial species, but can provide information on some of the more significant species present in a given environment [21,22].

Previous studies have shown that mixed microbial communities can differ significantly between attached and unattached cells with respect to biochemical capacity, cell division, and exopolymer production [23]. Microbial populations and communities within groundwater and sediment from the same sampling site could
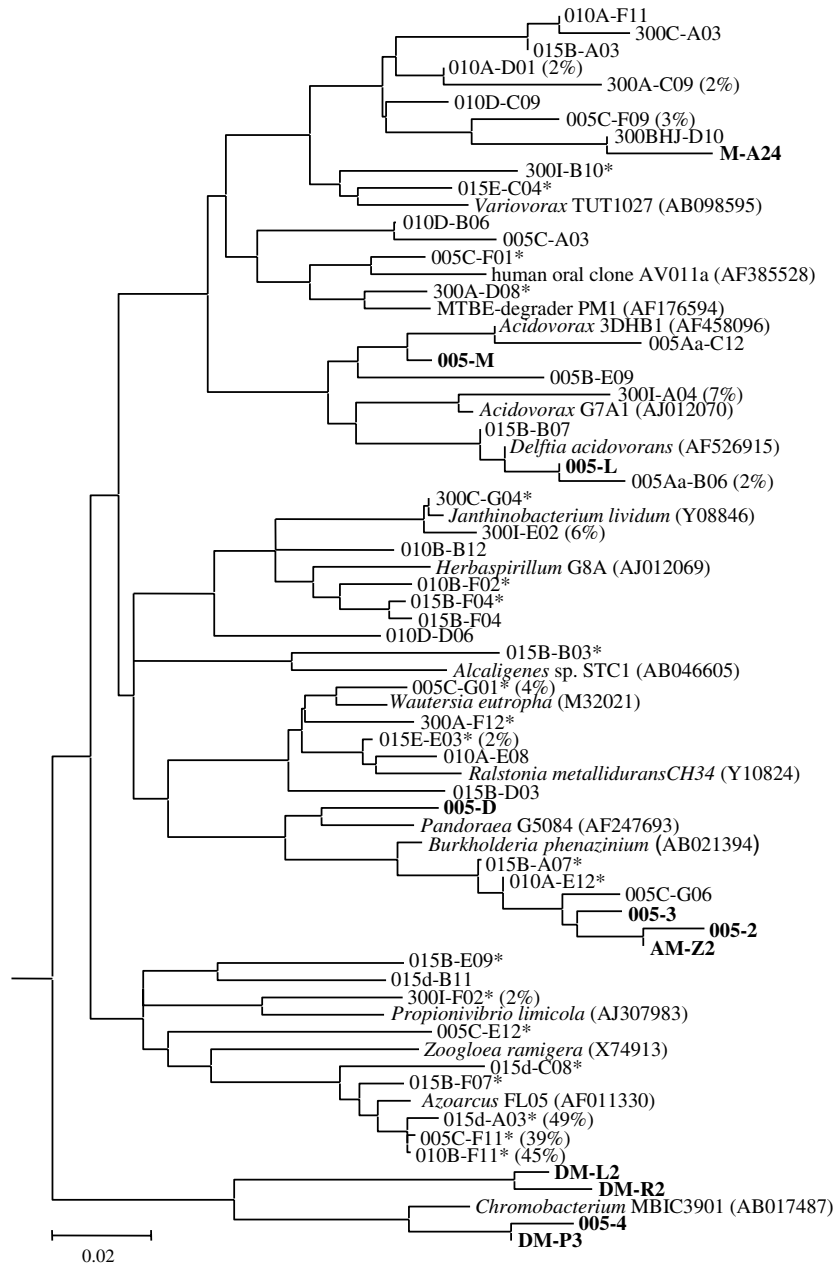
Fig. 2. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the β-*Proteobacteria*. Minimum evolution phylogenies were constructed in MEGA (version 2.1; http://megasoftware.net) with Jukes–Cantor distance model. The sub-tree for the β-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. The percentage (%) represents the abundance of predominant clones from the sampled diversity. Sequences identified as numbers and letters are FRC clones (i.e., 015C-F06, a clone from FW-015), and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for reference sequence are listed in the figure (e.g., *Azoarcus* FL05 (AF011330)), and nomenclature was based upon most recent release of taxonomic outline [31].

be different, and the groundwater populations at the sampled FRC sites might be different from populations associated with the sediment. However, sequences most closely related to *P. marginalis* or *P. rhodesiae* were recently reported from acetate and iron enrichments from background sediments [24]. In addition, Petrie et al. [24] reported low G+C Gram-positive sequences from sediment enrichments that were closely related to our iso-

lates, M-1-300 and M-4-300. The iron-reducing enrichments had either glycerol or acetate and sediments from the background or a contaminated site. Future work includes the assessment of the microbial community structure for different FRC sediments.

The nitrate levels in different groundwater samples from a mill-tailings disposal site (Shiprock, NM) were between 0.002 and 126 mM, and a predominance of
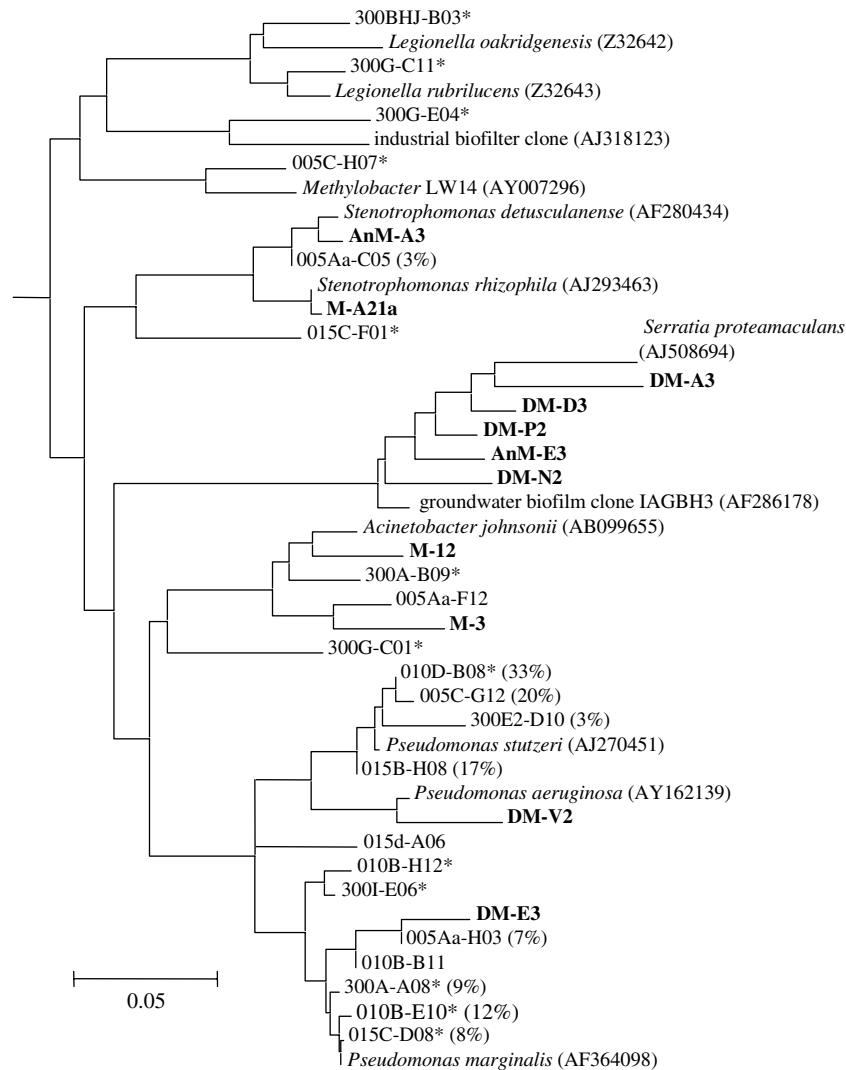
Fig. 3. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the γ-*Proteobacteria*. The tree was constructed as described in the legend of Fig. 2. The sub-tree for the γ-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The percentage (%) represents the abundance of the clone in the respective library. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

the ammonia-oxidizing bacterium, *Nitrosomonas*, was observed over *Nitrosospira* [25]. Based on SSU rRNA gene clonal libraries, *Nitrosomonas* or *Nitrosospira* were not detected as significant populations in the FRC groundwater, and this result may be due to the acidic nature of the contaminated samples. Recent results from *amoA* (ammonia monooxygenase) clonal libraries of the same samples suggested the presence of *amoA*-like sequences from *Nitrosomonas* and *Nitrosospira* (unpublished results); however, specific SSU rRNA primers or probes were not tested.

The three acidic sites tested at the FRC had increased levels of nitrate, heavy metals, and aluminum, and the clonal libraries indicated that all three were predominated by β-*Proteobacteria* related to *Azoarcus*. A pre-

dominant sequence type identified in a groundwater plume contaminated with coal-tar was closely related to the genus *Azoarcus* [26], however, the presumptive *Azoarcus* sequences from the FRC groundwater could represent different microorganisms. *Azoarcus* species were shown previously to be denitrifiers capable of anaerobic degradation of organic contaminants [12,27], and organic volatile concentrations (including toluene) are between $5 \mu g \, l^{-1}$ and $10 \, mg \, l^{-1}$ across the FRC. Anaerobic enrichments inoculated with acidic, FRC groundwater were positive for toluene degradation and nitrate removal, but isolates were not obtained (data not shown). The results indicated that denitrifying *Azoarcus* spp. capable of anaerobic degradation of organic pollutants may be present at the three acidic sites,
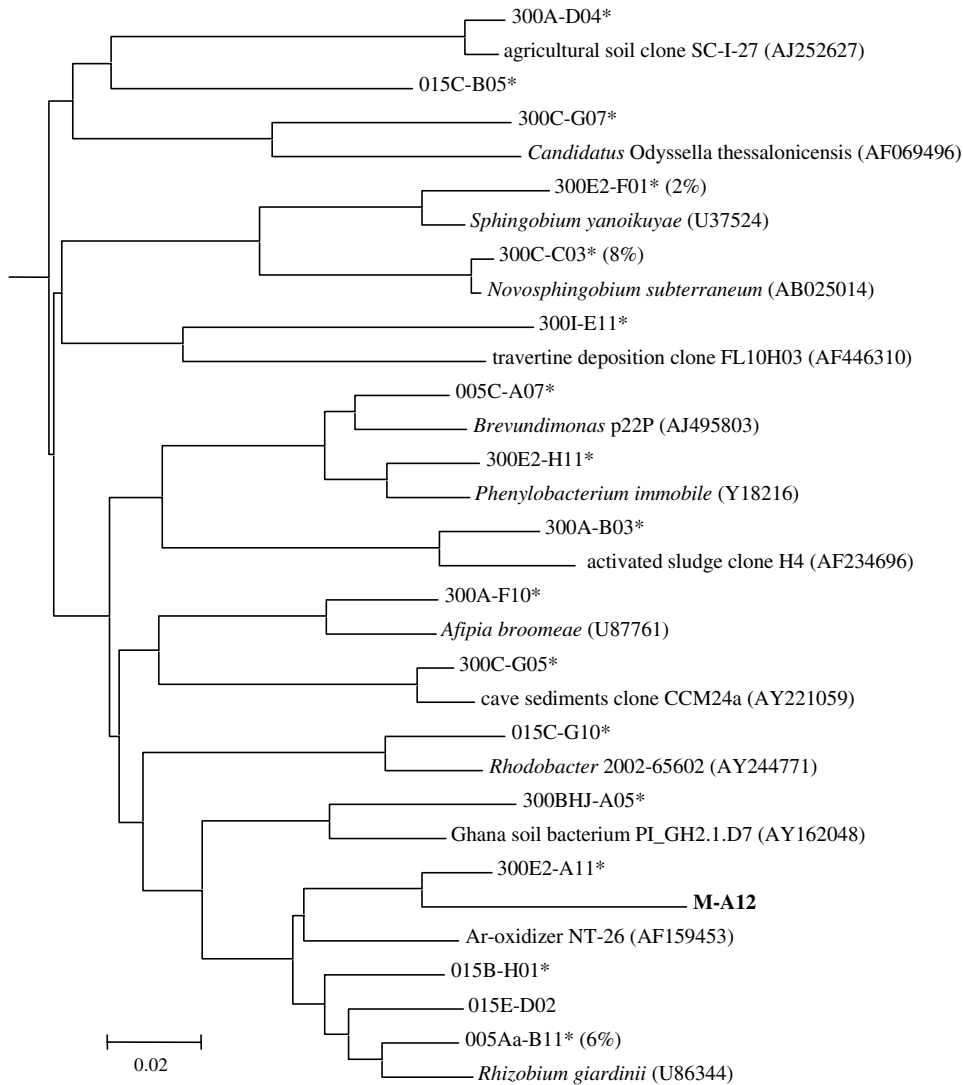
Fig. 4. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the α-*Proteobacteria*. The sub-tree for the α-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. The tree was constructed as described in the legend of Fig. 2. The percentage (%) represents the abundance of the clone in the respective library. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

but further work is needed to confirm the existence of these microorganisms as well as any biochemical activity. Current work includes the metagenomic sequence determination for the FW-010 groundwater community in order to obtain the nearly complete genomes from the predicted 20 phylotypes.

Similar *Pseudomonas* OTUs were observed at the background and acidic sites, but these populations were decreased 5–11-fold at the background site. The second *Pseudomonas* OTU (*P. marginalis*) was observed at similar frequencies for FW-300, FW-005, FW-010, and FW-015 (9%, 7%, 12%, and 8%, respectively). The results suggested that similar *Pseudomonas* populations were present at all sites but at different proportions of

the sampled diversity. The only other γ-*proteobacterial* clone sequence observed at a significant level was closely related to *Stenotrophomonas* DFK5, and accounted for 3% of the sampled FW-005 diversity. Interestingly, *Stenotrophomonas* DFK5 was reported to be isolated from heavy water (GenBank, AB045277). The data suggested that the conditions at the acidic sites have caused the pseudomonad populations to shift, but it is difficult to ascertain the relative contributions of decreased diversity and increased contaminant load for the relative distribution of similar populations.

Novosphingobium- and *Arthrobacter*-like sequences accounted for a majority of the recovered diversity at the background site and not the contaminated sites.
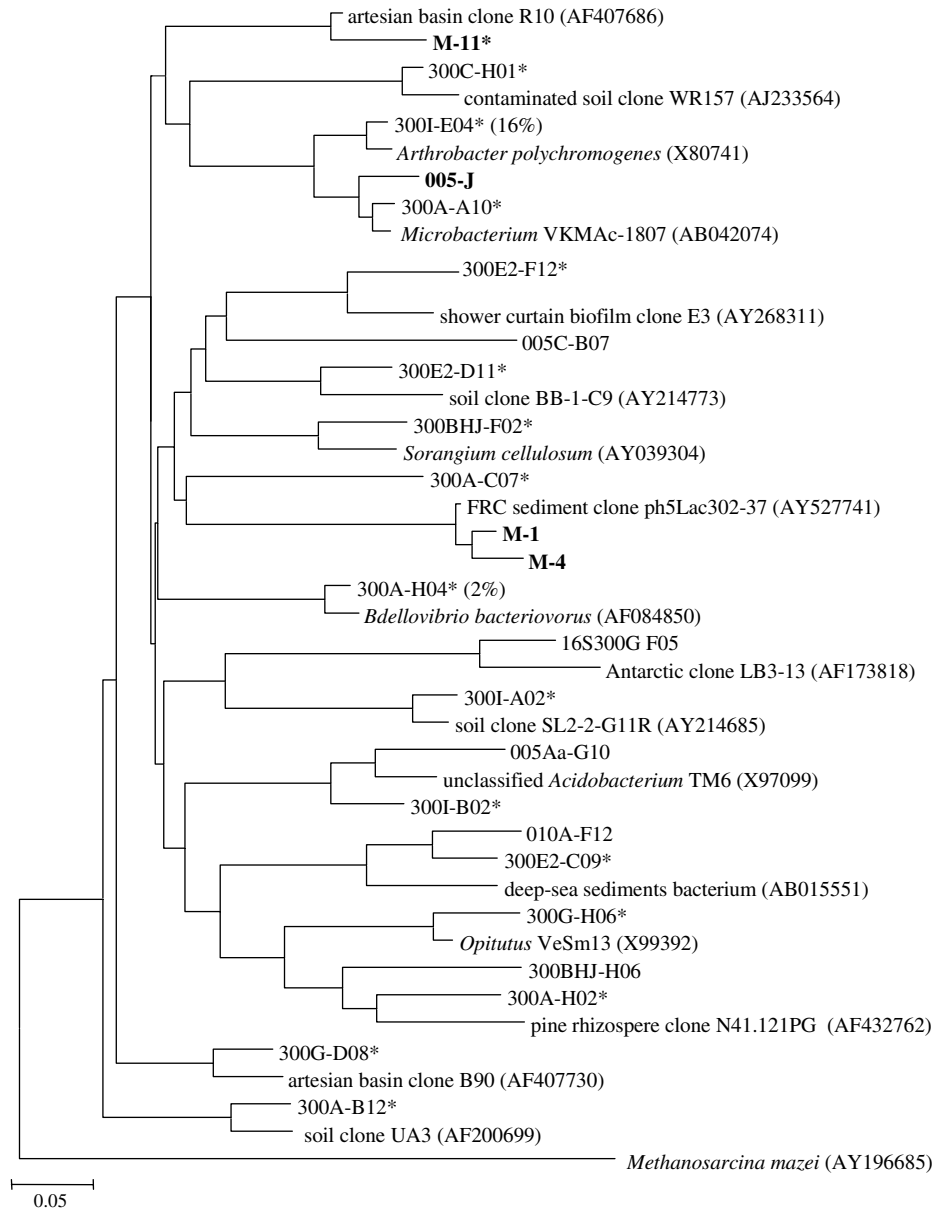
Fig. 5. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the δ-*Proteobacteria*, *Acidobacteria*, high G+C, and low G+C microorganisms. *Methanosarcina mazei* was used as the outgroup. The tree was constructed as described in the legend of Fig. 2. The percentage (%) represents the abundance of the clone in the respective library. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

The data suggested that *Novosphingobium* and *Arthrobacter* species might not tolerate increased nitrate, increased metals, and/or acidic pH. However, isolates closely related to *Novosphingobium* and *Arthrobacter* were not obtained and could not be tested for sensitivity. FW-005 isolates were evaluated for the ability to grow at different pH values in MR2A medium, and some of the tested isolates (e.g., 005-2, 005-3, 005-L) could grow at pH values down to 4.0 and tolerate nickel concentrations up to 10 mg l^{-1} (data not shown).

Few of the isolates had greater than 97% sequence identity with observed clones from the same sample, and previous studies have reported differences between cultivable and molecular assessments of the same community [28,29]. A previous study by Pedersen et al. [6] described microbial populations from groundwater at a naturally radioactive subsurface. The pH values ranged from ≈5.0 to 7.0, but metal and anion levels were not reported. Samples were analyzed from five wells and β-*Proteobacteria* were a predominant group in most

Table 5
LIBSHUFF analyses of the groundwater communities based on SSU rRNA clonal libraries

Sample comparison	Clones (n_x)	Clones (n_y)	ΔC_{xy}	P value
FW-005 vs. FW-010	211	113	0.63	0.001
FW-005 vs. FW-015	211	231	0.61	0.001
FW-005 vs. FW-300	211	321	8.61	0.001
FW-010 vs. FW-015	113	231	0.44	0.001
FW-010 vs. FW-300	113	321	3.52	0.001
FW-015 vs. FW-300	231	321	5.18	0.001

The respective clone number for each sample is given by n_x and n_y, and ΔC_{xy} represents the difference in coverage of the two sequence libraries (an increased ΔC_{xy} represents greater dissimilarity between the given communities). The software for the analysis was used according to specified directions (http://www.arches.uga.edu/~whitman/libshuff.html).

of the tested wells [6]. The occurrence of similar sequences might be more of an attribute of groundwater itself and not the contamination. Extensive geochemical data was not reported for the Oklo site [6], but the FRC groundwater is most likely very different due to the combination of low pH and multiple contaminants.

Direct comparison of the entire sequence libraries between the sites (LIBSHUFF analysis) indicated that the contaminated samples were significantly different from the background site, and that the acidic sites were significantly similar. In addition, bacterial isolates were only obtained from one acidic site, and FW-005 had the lowest measured aluminum and nitrate levels. However, linear relationships were not observed between the diversity indices (i.e., OTUs, $1/D$, ΔC_{xy}) and the tested geochemical parameters. Further work is needed to identify the key factors that affect microbial community structure and relative abundances at the FRC, as well as major metabolic activities in situ.

Our previous analysis of the geochemical data for the different sites indicated that nitrate was a major factor, but similar *nir*K and *nir*S gene sequences accounted for a majority of the diversity at the background and acidic samples [30]. Based upon SSU rRNA gene sequences, the only similar, significant OTUs between background and the acidic samples were the pseudomonads. However, it is possible that different genera at the FRC have similar *nir*K and/or *nir*S genes. Preliminary results with some of the isolates indicate that the microorganisms can denitrify FRC groundwater amended with electron donors after the pH has been adjusted. Further work is underway to characterize the denitrification capacity of these organisms in the presence of heavy metals, and evaluate the effects of pH on indigenous microorganisms.

In summary, the results showed that: (i) groundwater community structure was affected in a similar fashion for the contaminated samples compared to the background site (diversity decreased with increasing nitrate and aluminum); (ii) *Arthrobacter* and *Novosphingobium*

spp. predominated the background sample but were not detected at the acidic sites; (iii) novel sequences (genus and species level) from isolates and clones were identified in contaminated, subsurface groundwater (high nitrate, acidic pH); (iv) similar pseudomonad populations were present at the background and acidic sites, but at different relative abundances; (v) similar *Azoarcus* spp. predominated the acidic samples. Despite variations in nitrate and heavy metal levels and different proximities to the source ponds (different flow paths), the three acidic samples had similar microbial communities. However, the acidic sample with the reduced nitrate and aluminum levels had an increased diversity index compared to the other acidic samples. Future work is needed to characterize the associations between environmental conditions and the microbial communities, to elucidate the response of microorganisms and communities to relevant stresses, and better understand biotic and abiotic factors on functional dynamics.

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