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RESEARCH ARTICLE

Microbial communities biostimulated by ethanol during uranium (VI) bioremediation in contaminated sediment as shown by stable isotope probing

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Abstract Stable isotope probing (SIP) was used to identify microbes stimulated by ethanol addition in microcosms containing two sediments collected from the bioremediation test zone at the US Department of Energy Oak Ridge site, TN, USA. One sample was highly bioreduced with ethanol while another was less reduced. Microcosms with the respective sediments were amended with ^{13}C labeled ethanol and incubated for 7 days for SIP. Ethanol was rapidly converted to acetate within 24 h accompanied with the reduction of nitrate and sulfate. The accumulation of acetate persisted beyond the 7 d period. Aqueous U did not decline in the microcosm with the reduced sediment due to desorption of U but continuously declined in the less reduced sample. Microbial growth and concomitant ^{13}C-DNA production was detected when ethanol was exhausted and abundant acetate had accumulated in both microcosms. This coincided with U(VI) reduction in the less reduced sample. ^{13}C originating from ethanol was ultimately utilized for growth, either directly or indirectly, by the dominant microbial community members within 7 days of incubation. The microbial community was comprised predominantly of known denitrifiers, sulfate-reducing bacteria and iron (III) reducing bacteria including *Desulfovibrio*, *Sphingomonas, Ferribacterium, Rhodanobacter*, *Geothrix*, *Thiobacillus* and others, including the known U(VI)-reducing bacteria A*cidovorax, Anaeromyxobacter*, *Desulfovibrio, Geobacter* and *Desulfosporosinus.* The findings suggest that ethanol biostimulates the U(VI)-reducing microbial community by first serving as an electron donor for nitrate, sulfate, iron (III) and U(VI) reduction, and acetate which then functions as electron donor for U(VI) reduction and carbon source for microbial growth.

Keywords Stable isotope probing (SIP), ethanol, acetate, uranium reduction, sediment, bioremediation

1 Introduction

Uranium contamination of groundwater has been observed at many sites around the world. Uranium is listed as a groundwater contaminant at US Department of Energy (DOE) sites, and poses risks for liver damage and cancer. In early 1990s, bioreduction of mobile U(VI) to sparingly soluble and immobile U(IV) was proposed to be a promising approach to remediate U in situ [1]. Field tests for bioreduction and immobilization of uranium in contaminated aquifers and sediments have been performed [2–4]. During February 2004 to December 2008, a pilot scale in situ bioremediation system was implemented at the Field Research Center (FRC), Oak Ridge, Tennessee, USA, which established hydraulic control, conditioned groundwater and biostimulated indigenous microorganisms to reduce U(VI) to U(IV), rendering it immobile [5,6]

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as

$$UO_2^{2+} + 2e^- \rightarrow UO_{2(s)} (\Delta G^{\circ\circ} = -43.2\ kJ \cdot eq^{-1}). \quad (1)$$

Ethanol was used as an electron donor source throughout the test period [5,7,8]. The tests successfully reduced U concentrations in water from levels ranging from 50 to 60 $mg \cdot L^{-1}$ to concentrations below US EPA maximum contaminant levels for drinking water of 0.03 $mg \cdot L^{-1}$[7].

To better understand the bioremediation mechanism, we sought to identify the microorganisms that are responsible for U(VI) reduction and are responsive to biostimulation. Analyses of microbial communities at the site using 16S rRNA gene clone libraries, functional genes based on Geochip microarray analyses and high throughout pyrosequencing methods have documented a major shift in community composition and structure in sediments [9] and groundwater [10] following implementation of the bioremediation treatment system. Microbial communities increased in overall diversity and abundance of bacterial taxa known to reduce U(VI), Fe(III), sulfate and nitrate following treatment and biostimulation [9]. Under anaerobic condition, ethanol is degraded via nitrate or sulfate reduction or syntrophically with acetate as an intermediate

$$1/4CH_3CH_2OH + 1/4H_2O \rightarrow$$

$$1/4CH_3COO^- + 5/4H^+ + e^-$$

$$(\Delta G^{\circ\circ} = -37.46\ kJ \cdot eq^{-1}). \quad (2)$$

Acetate is then degraded further as

$$1/8CH_3COO^- + 3/8H_2O$$

$$\rightarrow 1/8CO_2 + 1/8HCO_3^- + H^+ + e^-$$

$$(\Delta G^{\circ\circ} = -26.36\ kJ \cdot eq^{-1}). \quad (3)$$

The electron acceptor can be nitrate, sulfate or even CO_2 (in case of methanogenesis). Microbial growth has been observed during in situ U(VI) reduction. However, the nature of the biostimulatory process remains largely unknown, including the role of ethanol as a carbon source for microbial growth and/or as an electron donor for reductive processes.

Stable isotope probing (SIP) enables the identification of microbes that derive carbon from a specific ^{13}C-labeled compound by tracing incorporation of ^{13}C into phylogenetic markers such as DNA [11], RNA [12] or fatty acids [13] during biodegradation and biotransformation as described by Uhlik et al. [14]. If ethanol served as a carbon source directly, DNA-SIP would identify sediment-associated bacteria that derive ^{13}C from ^{13}C-ethanol in the presence of ethanol. If the intermediate acetate served as a carbon source, ^{13}C-DNA would be only detected in the presence of ^{13}C-acetate produced from ^{13}C-ethanol degradation. To date, SIP with ethanol has not been applied to studies of biostimulation of U(VI) and other metal reduction. In this study, we performed SIP in two anaerobic microcosms containing contaminated sediments and groundwater collected from the wells within the treatment zone during the pilot U(VI) reduction tests at Oak Ridge, TN. By identifying organisms that are responsive to ethanol biostimulation and monitoring geochemical reactions in the microcosms, we gained insight into the process of biostimulation with ethanol in an actively uranium (VI)-reducing community. The results also suggested that acetate, which was generated from ethanol degradation, rather than ethanol itself was incorporated into bacterial biomass.

2 Materials and methods

2.1 Site and sediment source

The pilot scale bioremediation system was established in Area 3 of the DOE FRC at the Y-12 National Security Complex, Oak Ridge, TN and involved an outer groundwater recirculation loop protecting an inner loop test area as previously reported [5–7]. Ethanol was added into the injection well (FW104) of the inner loop two days per week along with recycled groundwater pulled from the extraction well (FW026) [5]. Multilevel sampling (MLS) wells within the treatment zone were used to monitor hydrogeology and remediation performance. To minimize entry of ambient groundwater, additional clean water was injected to FW024 at 0.7–0.9 $L \cdot min^{-1}$ [6,15]. This clean water was a mixture of tap water and groundwater treated by an aboveground system to remove nitrate via a bioreactor [5,16]. The remediation test was initiated on August 23, 2003 (day 1) by flushing with the clean water in order to remove most of the nitrate and aluminum from the treatment zone [5]. Beginning on January 7, 2004 (day 147), ethanol was added as an electron donor to stimulate bioactivity for U (VI) reduction [5]. Ethanol solution (industrial grade, 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 $COD \cdot L^{-1}$, and then was injected into FW104 over a 48-h period each week to result in a COD of 120–150 $mg \cdot L^{-1}$ at FW104.

Sediment samples for SIP experiments were collected from FW104 and FW026 on June 23, 2005 using a surging block technique as previously described [9,17]. Samples taken by this method are a mixture of the sediment along the well from 13.6 to 11.6 m below ground. The composition of groundwater and sediment samples is presented in Table 1. The sample from FW104 was most reduced, contained significant amount of Fe with black

Table 1 Chemical composition of groundwater and sediment samples used for microcosm test

microcosm	FW104	FW026
groundwater		
pH	5.88	5.86
HCO_3^-, mmol·L^{-1}	1.3	1.1
SO_4^{2-}, mmol·L^{-1}	1.38	1.95
S^{2-}, mmol·L^{-1}	0.18	0.023
NO_3^-, mmol·L^{-1}	0	0.001
Cl^-, mmol·L^{-1}	2.57	2.57
U, μg·L^{-1}	129	132
Fe, mmol·L^{-1}	0.028	0.025
Na^+, mmol·L^{-1}	1.71	1.90
K^+, mmol·L^{-1}	0.59	0.63
Ca^{2+}, mmol·L^{-1}	0.62	0.64
Mg^{2+}, mmol·L^{-1}	0.33	0.36
Mn^{2+}, mmol·L^{-1}	0.063	0.064
Be, mmol·L^{-1}	0.014	0.014
COD, mg·L^{-1}	146	9
sediment		
U content, m·kg^{-1}	6.0	1.20
U(VI) in total U, %	60	< 10
Fe content, g·kg^{-1}	199	47
Fe(II) in total Fe, %	53%	10%
Sulfide content, mg·kg^{-1}	23	0

color (FeS); while that from FW026 was also Fe-rich but less reduced, and was yellow in color.

2.2 SIP microcosm construction and incubation

Microcosms were made using sterile 1 L glass bottles sealed with rubber stoppers and secured with open-centered screw caps. Sediment samples mixed with groundwater (1 L volume) containing approximately 45 g and 125 g dry weight of sediment from FW104 and FW026, respectively. Microcosms were constructed in an anaerobic glove box, headspaces were purged with N_2, then they were spiked with solutions of $NaNO_3$ and Na_2SO_4 to achieve initial concentrations of 0.576 mmol·L^{-1} nitrate and 2 mmol·L^{-1} sulfate. Ethanol labeled with 99 atom % ^{13}C at the 2 position ($^{13}CH_3CH_2OH$) (Isotec, Miamisburg, OH, USA) was added to the microcosms to achieve an initial concentration of 2.5 mmol·L^{-1}. Concentrations of nitrate, sulfate and ethanol added were to simulate those observed in field tests [5]. Microcosms were incubated in an inverted position on a rotary platform shaker at 200 rpm at 25°C. Slurry samples were collected from the microcosms over a time course using an N_2-washed syringe with needle pierced through the rubber stopper, and then headspace was again purged with N_2. Samples were collected at the initiation of the experiment (0 h), and following incubation for 5, 10, 24, 29, 34, 46, 55, 70, 79 and 168 h (7 days). Following centrifugation, the supernatant was used for chemical analyses and sediment pellets were frozen at −80°C for later DNA extraction.

2.3 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 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 the ratio of U(IV)/total U were determined with XANES as described previously [5,7]. Ethanol and acetate concentrations were determined using 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) with Helium as carrier gas.

2.4 DNA extraction and isopycnic separation

DNA was extracted from sediment pellets with the Bio101 FastDNA Spin Kit for Soil (QBiogene, Irvine, CA). Density gradients were constructed using CsTFA solution (Amersham Biosciences, Piscataway, NJ) and fractionated as previously described [18]. DNA extracted following 0, 5, 10, 24 and 168 h (FW026) and 0, 24 and 168 h (for FW104) was analyzed. Buoyant densities (BD, g·Ml^{-1}) of fractions were determined gravimetrically based on control gradients (no DNA) run in parallel. Quantitative, real time PCR targeting 16S rRNA genes was performed on fractions to infer which fractions contained ^{13}C-DNA [18]. For each gradient, the series of fractions containing ^{13}C-DNA were compiled into one heavy DNA sample, and fractions containing unlabeled DNA were compiled into one light DNA sample for further analyses. To detect background contamination of DNA that can be present throughout density gradients, equivalent heavy fractions from a control unlabeled sample (DNA from 0 h incubation) were compiled and subjected to the same downstream analyses as ^{13}C-DNA.

2.5 Microbial community analysis

Bacterial community profiling was performed using terminal restriction fragment length polymorphism (T-RFLP) analyses of the 16S rRNA gene as previously described [18]. To analyze community composition, 16S

rRNA gene clone libraries were generated and sequenced. Clone libraries were constructed from total community DNA extracts of sediments collected from FW104 and FW026, and 192 clones were picked and sequenced for each sample. T-RFLP profiles from these samples shared the same T-RF peaks as those generated from total community DNA and heavy DNA obtained in this SIP experiment (data not shown). Sequences with anomalies such as chimeras were detected using MALLARD [19]. 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 [20]. Sequences confirmed as anomalous with Pintail were excluded from the analyses. To associate terminal restriction fragments (T-RFs) with specific bacterial taxa, 16S rRNA gene sequences from clone libraries were subjected to *in silico* digestion with *Hha*I to predict the associated T-RF size for each sequence as previously described [18]. *In silico* digestions were performed using a Python script that locates *Hha*I restriction sites nearest the 5′ end of the 16S rRNA gene sequence and calculates the size of the resultant fragment between this site and the 27F primer. Because the first approximately 50 bp of a sequence read are generally of low quality, the first 50 bp following sequencing primer site 27F could not be reliably obtained with sequencing. The script accounted for this section of DNA by aligning the sequence to nearest matching 16S rRNA gene sequences in the Ribosomal Database Project (RDP) [21] to predict the number of base pairs missing and compute an inferred T-RF size.

2.6 Most probable number (MPN) analysis

Within 24 h following sample collection, denitrifying bacteria, iron- reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) were enumerated using the Most Probable Number (MPN) technique with five tubes for each dilution using 10 mL receptive medium in 22 mL pressure tubes sealed with butyl rubber stopper and aluminum cap at ambient temperature condition (21°C–23°C). MPNs were performed separately for groundwater and sediments collected from FW104 and FW026 following methods previously described [9].

3 Results and discussion

3.1 Biostimulation performance in situ

Sediment samples for the SIP experiment were taken from inner loop injection well FW104 and extraction well FW026. Prior to sampling, ethanol was injected to the subsurface for one week. The geochemical characterization of groundwater is presented in Table 1. During ethanol injection, COD concentrations increased in injection well FW104 and MLS wells FW101-3 and FW102-2 as well as extraction well FW026, indicating that the injected electron donor penetrated through the treatment zone. Uranium concentrations in FW104 and FW026 were approximately 130 $\mu g \cdot L^{-1}$, and originated from the aquifer surrounding the treatment zone. In MLS wells, uranium concentrations were approximately 20 $\mu g \cdot L^{-1}$ or lower, which was much lower than FW104 because of bioreduction of mobile U(VI) to U(IV) [2,6]. Sulfide concentrations increased during the ethanol injection period in all wells. Fe(II) concentrations also increased during ethanol injection (data not shown). These results indicated that sulfate and metal reducing activities were present in the treatment zone when the samples were collected for the SIP experiment.

3.2 Microbial community composition

MPN of total bacterial communities indicated the presence of denitrifiers, SRB and FeRB in the groundwater and sediment samples of both FW104 and FW026 (Table 2). These three microbial trophic groups are major components of microbial community and involved in the metabolism of ethanol and its intermediate acetate using nitrate, sulfate and iron(III) as electron acceptors. In general, the population levels in injection well FW104 were much higher than those in extraction well FW026 by almost 10-fold. In FW104, the level of SRB was much higher than FeRB and denitrifying bacteria. This is probably due to consumption of bioavailable iron (III) and low nitrate concentration in the injection well. In FW026, the levels of FeRB were similar to that of SRB. Microbial U(VI) reduction and immobilization has been known to mainly be related to the activities of FeRB and SRB [1,22–24].

Microbial analyses based on 16S rRNA sequencing was conducted using clone libraries (Table 3). Of the 192 clones sequenced for each sample, 173 quality sequences were obtained from FW104, and 170 sequences from FW026. 16S rRNA gene clone libraries detected five genera of known U(VI) reducers associated with sediments, *Desulfovibrio*, *Anaeromyxobacter, Geobacter, Acidovorax* and *Desulfosporosinus* [1,23,25–27]. *Geothrix* and *Ferribacterium* are known to reduce iron and nitrate [4,28], and were among the most frequently detected genera in libraries from both FW104 and FW026. *Thiobacillus* spp. were found in both sediments (Table 3, Fig. 1). The genus *Thiobacillus* includes strictly aerobic bacteria that oxidize Fe(II), sulfur and sulfide with O_2 and facultative anaerobes such as *T. denitrificans* which can use nitrate as an electron acceptor to oxidize H_2S and FeS [29]. The presence of *Thiobacillus* spp. in the sediments is likely due to the presence of nitrate in groundwater and infiltration of oxygen from above ground [8,30].

Frequently-detected 16S rRNA gene sequences in clone libraries were subjected to *in silico* digestion (Table 3), and then were matched to peaks in T-RFLPs generated from the

Table 2 Most probable numbers (MPN) of denitrifiers, sulfate reducing bacteria (SRB) and iron (III) reducing bacteria (FeRB) in groundwater and sediment samples used for microcosm test.

well	sample type	MPN		
		denitrifiers	SRB	FeRB
	groundwater (cells·mL^{-1})			
FW104 (injection)	groundwater	9.4×10^5	5.4×10^6	1.4×10^4
FW026 (extraction)	groundwater	2.8×10^5	5.5×10^5	5.1×10^5
	sediment (cells·g^{-1} dry weight)			
FW104 (injection)	sediment	7.2×10^8	1.53×10^8	9.4×10^7
FW026 (extraction)	sediment	1.1×10^7	1.1×10^6	1.9×10^6

Table 3 Dominant microorganisms detected in the sediment samples and predicated terminal restriction fragments (T-RF) length.

organism	relative abundance /%[a]		electron acceptor				electron donor		T-RF length (bp)	T-RF label on Fig.3
	FW104	FW026	Fe(III)	Nitrate	Sulfate	U(VI)	Ethanol	Acetate		
Acidobacteria										
Geothrix	11.0	15.5	+	+	−	?	−	+	96, 375	B, E
Chloroflexi										
Anaerolinea	1.9	7.7	−	−	−	?	−	−	213	C
Firmicutes										
Desulfosporosinus	1.3	0.0	+	−*	+	+	+	+	386	nd
Gemmatimoadetes										
Gemmatimonas	1.3	0.0	−	−	−	−	−	+	360	D
Proteobacteria										
Acidovorax	1.9	0.7	−	+	−	?*	+	+	204	C
Anaeromyxobacter	0.6	0.0	+	+	−	+	?	+	94, 224	B
Desulfovibrio	6.5	5.6	+	+	+	+	+	−	95	B
Duganella	9.0	0.7	?	−	?	?	+	+	67	A
Ferribacterium	11	14.1	+	+	−	?	−	+	67, 207	A, C
Geobacter	2.6	0.7	+	+	−	+	+	+	95	B
Thiobacillus	4.5	4.9	−*	+	−	?	−*	−	67, 451,570	A, F, G
Rhodoferax	0.0	3.5	+	+	−	−	−	+	206	C
Rhodanobacter	6.5	0.0	?	−	−	?	−	?	209	C
Sterolibacterium	0.0	1.4	?	+	?	?	−	−	67	A
Sphingomonas	5.2	0.7	?	+	?	?	?	−*	96	B

Note: [a] Relative abundance was calculated as a percentage of the total number of clones successfully sequenced (173 clones from FW104, 170 clones from FW026). Metabolic abilities are those in the literature for the closest isolates of organisms detected in clone libraries. ? = unknown or untested, * = activity not preset in closest related isolates but present in some members of the genus.Sizes are listed for most frequent T-RF for each genus produced by in silico digestions of clone libraries. T-RFs of other sizes may also have been produced in low abundance but are not listed for brevity.

starting sediment material (Fig. 1). Dominant T-RFs matched those predicted from sequences detected in clone libraries. FW104 and FW026 shared the same major T-RFs, although these peaks occurred in different proportions in the profiles (Fig. 1), which may reflect differences in relative abundance of community members, although PCR biases cannot be ruled out. *Gemmatimonas* was detected in low numbers in clone libraries, although matched a high peak on T-RFLPs (peak D). In general, T-RFLP peak heights may be more accurate indicators of the relative abundance of taxa than clone library detection frequency, since rarefaction curves indicated that our clone libraries did not provide complete community coverage (data not shown). A pairwise comparison of the two libraries using $\int$-LIBSHUFF indicated that the libraries were significantly different [9] ($p = 0.05$). The differences among the two libraries occurred within the phylum Actinobacteria, class γ-Proteobacteria, family Sphingobac-

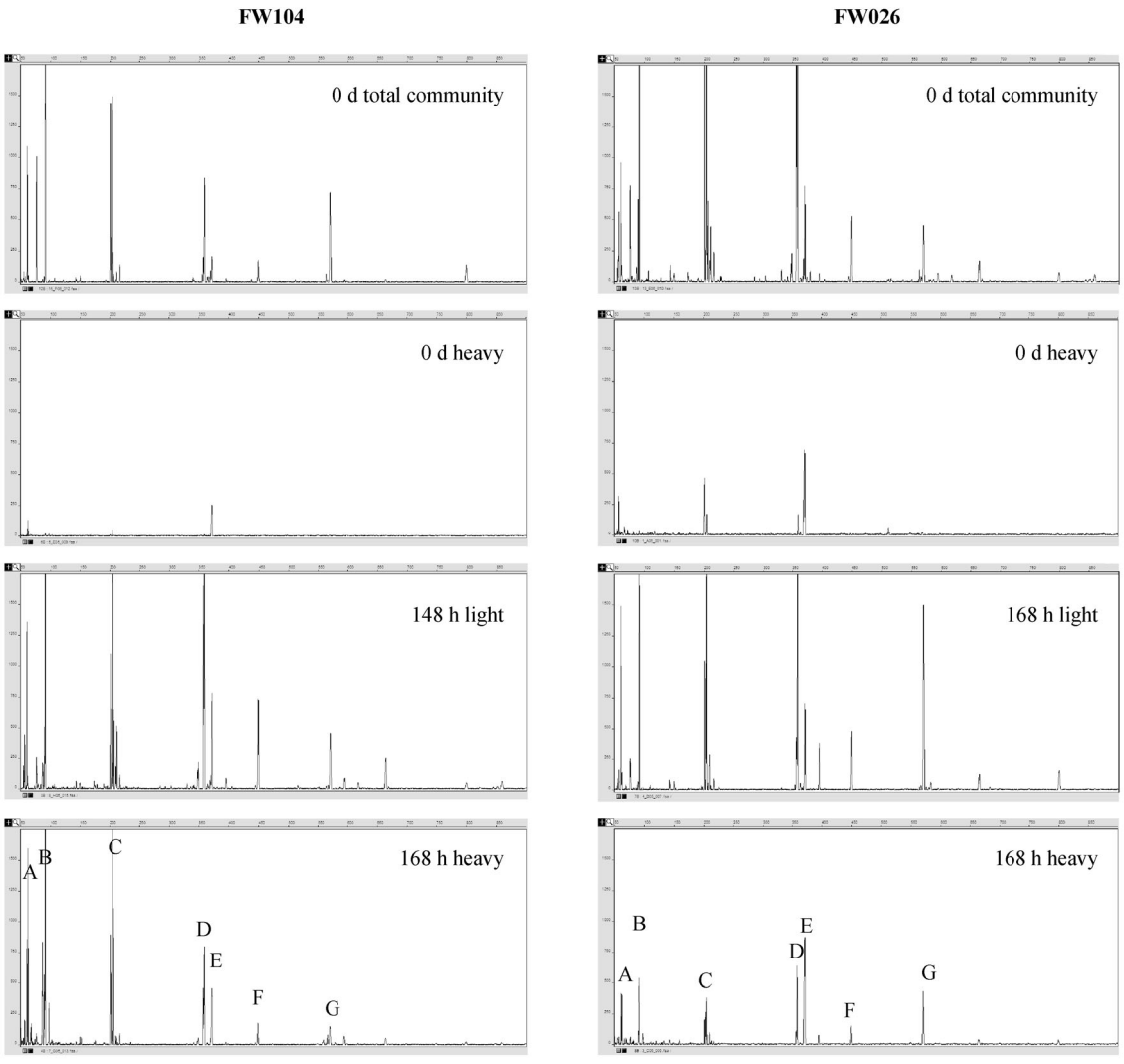

Fig. 1 T-RFLP profiles of total community DNA, heavy background control DNA (representing contamination) and heavy DNA following 7 d incubation with ^{13}C-ethanol for each microcosm.

Refer to Table 3 for T-RF sizes. T-RFs matched to clone sequences based on *in silico* digestions are labeled as follows A. *Duganella**, *Ferribacterium*, *Sterolibacterium*, *Thiobacillus*; B. *Desulfovibrio***, *Sphingomonas***, *Geobacter, Geothrix, Anaeromyxobacter;* C. *Ferribacterium***, *Rhodanobacter***, *Rhodoferax**, *Anaerolinea***, *Acidovorax** ; D. *Gemmatimonas*, E. *Geothrix***, F. *Thiobacillus**, G. *Thiobacillus*.* = Sequence associate with T-RF abundant in clone libraries. ** = Abundant in clone libraries (> 5% of clones, Table 3).

teriaceae and genus *Duganella*, which were more frequently detected in the FW104 than in the FW026 clone library.

3.3 Reductive processes

Observations of the two sediment samples indicated that they differed in their geochemical properties. FW104 sediments appeared highly reduced, based on their black color and higher concentration of sulfide and less or no ferric (hydro) oxides, whereas FW026 sediments were light brown in coloration with lower sulfide concentration and presence of ferric (hydro) oxides (Table 1). Since FW104 was the site of ethanol injection and entry of contaminated groundwater, reduced products should be present in greater abundance.

Nitrate was rapidly reduced in the microcosms. Measured initial concentrations of nitrate were 0.347 and 0.323 mmol·L^{-1} for FW026 and FW104, respectively. In both microcosms, nitrate dropped to below detection limits (< 0.001 mmol·L^{-1}) before the first sample was collected at the 5 h incubation time (Fig. 2(c)).

Sulfate was also actively reduced in both microcosms (Fig. 2(d)). Following addition of 2 mmol·L^{-1} sulfate to microcosms, initial measured sulfate concentrations were 3.03 and 1.98 mmol·L^{-1} sulfate in FW026 and FW104, respectively, with the higher sulfate concentration in FW026 attributed to the presence of sulfate in ground-

water. Sulfate levels declined rapidly within the first 24–29 h of incubation, which was correlated with ethanol degradation, and then continued to decline slowly (Fig. 2 (d)), suggesting that the initial sulfate removal was performed using ethanol as electron donor. Sulfide accumulated from 0.13 to 0.62 mmol·L^{-1} within 24 h in FW104 (Fig. 2(e)). In FW026, sulfide accumulation was minimal, increasing from 0.0003 to 0.0079 mmol·L^{-1} within 5 h and then declining to levels ranging from 0.0006 to 0.002 mmol·L^{-1} throughout the remainder of the incubation. The non-stoichiometric relationship between sulfate disappearance and sulfide accumulation in both microcosms is likely due to the formation of FeS precipitates, effectively removing sulfide from the aqueous solution being measured.

The initial U(VI) concentrations in microcosms FW026 and FW104 were 2,010 and 343 μg·L^{-1}, respectively. The low U(VI) concentration in the injection well (FW104) microcosm is attributable to the more extensive U reduction in situ. During the 168 h of incubation, aqueous U(VI) concentrations in FW026 declined from 2,010 to 241 μg·L^{-1} (Fig. 2(f)). A slight increase in U(VI) concentrations observed in the FW026 microcosm during the initial 5 h incubation period may be accounted for by reoxidation of U(IV) by nitrate [31] added at the start of the incubation. In FW104, initial concentrations of U(VI) were considerably lower at 343 μM and increased to 551 μM after 168 h incubation. The change in U(VI) concentration was not continuously monitored afterwards but the U(VI) concentrations in both microcosm samples eventually dropped to less than 30 μg·L^{-1} after one month, indicating that U(VI) reduction was achieved (data not shown).

Differences in apparent U(VI) reduction rates between FW026 and FW104 may be due to several factors. Kinetic analysis indicates that U(VI) reduction can be expressed as a first order reaction [32] in which the rate of U(VI) reduction is related to U(VI) concentration such that a higher U(VI) reduction rate occurs at higher U(VI) concentration. Differences in U(VI) desorption rates in FW026 versus FW104 may also have caused apparent differences in U(VI) disappearance rates. The desorption of U(VI) from sediments is dependent on pH, with increased carbonate concentrations resulting in increased U(VI) desorption [5]. As sulfate reduction occurred in the first 24 h, pH decreased in the microcosms from 6.90 to 6.72 (FW026) and 6.62 to 6.47 (FW104), perhaps due to increased bicarbonate concentration, which may have released slightly more U(VI) to the aqueous phase as a uranyl-carbonate complex [24]. The groundwater of FW026 contained more FeRB but less SRB than those in FW104 (Table 2). The U(VI) reduction rates in the two microcosms may be regulated by their U(VI)-reducing functional species in the presence of different Fe(III) and sulfate. The extensive and rapid disappearance of U(VI) from FW026 may have been due to a more rapid rate of U (VI) reduction relative to U(VI) desorption from sediment in the presence of relatively high U(VI) concentrations. In the FW104 microcosm, the initial U(VI) concentration of U(VI) was much lower than that in FW026 microcosm. The rate of U(VI) reduction during the 168 h test period may have been slower than the rate of U(VI) desorption from the sediment causing an apparent net increase in U (VI) concentration. Due to extensive reduction of U(VI) in situ in the injection well, less total U(VI) was likely present sorbed to sediments in the extraction well.

3.4 Fate of ethanol

^{13}C-Ethanol disappeared from both microcosms within 24 h of addition (Fig. 2(a)). The specific ethanol degradation rates during the initial 10 h were 0.066 and 0.035 mmol·g dry $weight^{-1}$·day^{-1} for the sediments from FW104 and FW026, respectively. The higher rates measured in FW104 sediments are consistent with its higher MPN values relative to FW026 (Table 2). Concomitant with ethanol disappearance, acetate accumulated as an apparent product of ethanol degradation and remained at high levels in microcosms throughout the incubation (Fig. 2(b). In sediments derived from FW026, acetate accumulated in a stoichiometric ratio of near 1:1 mol/mol to the amount of ethanol degraded. The transformation of ethanol to acetate, and subsequent persistence of acetate observed, is consistent with activities of non-acetate utilizing SRB such as *Desulfovibrio* spp [33]. Acetate accumulation in conjunction with ethanol disappearance has recently been reported in another study of U(VI) contaminated sediments near this site [34].

In microcosm FW104, a higher initial acetate concentration of 1.73 mmol·L^{-1} was present due to in situ injection of ethanol to FW104 when the sample was collected. Acetate accumulated in a ratio of approximately 1:1 mol/mol during ethanol disappearance, however as the incubation continued, more acetate accumulated than could be accounted for by ethanol conversion by a factor of approximately twofold in FW104. The additional acetate may be a product of acetogenesis [35]. Acetogens are broadly distributed phylogenetically, and can oxidize a variety of substrates (aromatic acrylate groups, fumarate, etc.) to acetate or oxidize H_2 to generate reductant for the conversion of CO_2 to acetate [35]. Although the microcosm headspace was initially purged with N_2, CO_2 may have been generated metabolically. *Desulfosporosinus*, a genus of SRB known to include acetogens [35], was detected in clone libraries from FW104 (approximately 1% of clones) and in lower abundance in FW026. Several taxa present are also known to possess carbon monoxide dehydrogenase (CODH) orthologs, including *Desulfovibrio* [36], *Anaeromyxobacter, Geobacter, Desulfitobacterium* [37], some Clostridia and *Desulfotomaculum* [39]. Low pH, low temperature and carbon limited conditions favor acetogenesis [35], and may account for the presence of acetogens in this aquifer. The more extensive acetogen-

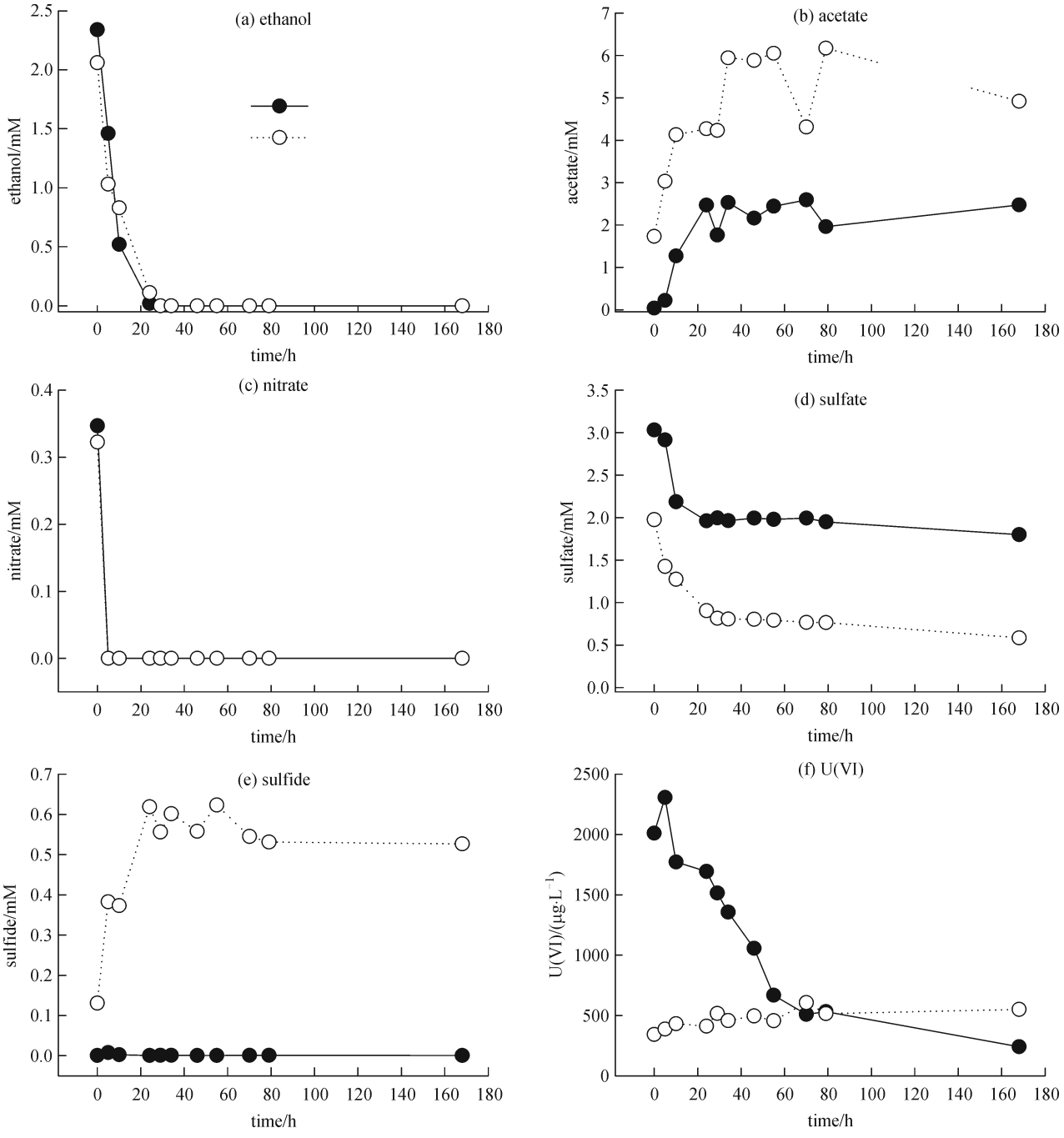

Fig. 2 Concentrations of (a) ethanol; (b) acetate ; (c) nitrate; (d) sulfate; (e) sulfide; and (f) U(VI) in microcosms during the SIP incubation period.

esis observed in FW104 than FW026 may be due to higher cell numbers (S1) and/or greater relative abundance of acetogenic bacteria in the injection well. Alternatively, acetate may have accumulated in FW104 due to decomposition of microbial biomass in this sample.

The incorporation of ^{13}C originating from ethanol into microbial DNA was assessed using SIP. The presence of ^{13}C-labeled DNA was assayed by screening density gradient fractions using QPCR targeting 16S rRNA genes of incubated samples relative to initial samples (Fig. 3). No ^{13}C-enriched DNA was detected following 5, 10 or 24 h incubation for FW026 or 24 h for FW104 (data not shown). Following 168 h incubation, ^{13}C-DNA was evident in heavy fractions in both samples (Fig. 3). The presence of ^{13}C-DNA was most pronounced in FW104, suggesting that more bacterial growth occurred in FW104 using carbon originating from ethanol than in FW026. This may be a result of the injection well (FW104) community

being more adapted to ethanol conversion and utilization. It is apparent from previous groundwater analyses that, in situ, FW026 receives acetate rather than ethanol as electron donor and carbon source due to the rapid ethanol oxidation upstream.

Based on the timing of ethanol disappearance, acetate accumulation and cell growth (^{13}C-DNA), it is unlikely that ethanol was utilized directly for microbial growth during the initial 24 h. Instead, bacteria apparently converted ethanol to acetate and following ethanol depletion switched to the utilization of acetate as an electron donor and carbon source. This metabolic pattern has been observed in different settings [30]. Cell growth, as determined by presence of ^{13}C-DNA, was not evident during the first 24 h of incubation (data not shown), when active reduction of nitrate, sulfate and U(VI) occurred in the microcosms and when acetate accumulated concomitantly with ethanol disappearance (Fig. 2). Acetate concentrations in the microcosms appeared to decrease slightly in FW104 between 70 and 168 h and in FW026 between 34 and 168 h, which may indicate the utilization of acetate for growth. The exact determination of when ethanol-derived acetate may have been utilized for growth is complicated by the simultaneous accumulation of acetate from acetogenesis and/or degradation of biomass in FW104. The ^{13}C derived from ethanol would have been diluted by acetate generated from other sources, reducing the strength of the SIP signal. However, ^{13}C-DNA was detected in FW104 and FW 026 (Fig. 3 and Fig. 1), indicating that some ^{13}C-acetate was directly assimilated and/or the acetate pool was actively turned over.

The majority of U(VI) reduction occurred after conversion of ethanol to acetate, suggesting that acetate functions as an electron donor and carbon source for U(VI) reducers. Alternatively, U(VI) reduction may have occurred via reduced iron or humic materials generated in the actively reducing phase during ethanol depletion [38,39]. Iron reducing bacteria (*Geobacter, Ferribacterium, Desulfovibrio*, *Geothrix,* and *Anaeromyxobacter*) and humic acid reducing bacteria (*Geobacter humireducens and Geothrix*) were present in clone libraries.

Nearly all peaks detected in the total community were also found in ^{13}C-DNA (Fig. 1), indicating that the carbon from ethanol flowed into the majority of microbial community members within 168 h. These abundant and active organisms included denitrifiers, SRB and FeRB

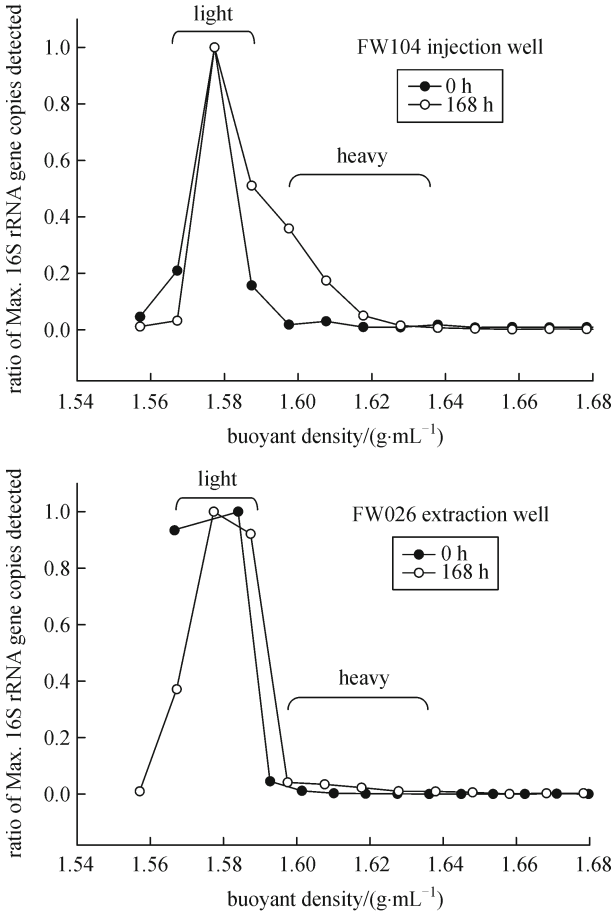

Fig. 3 Quantitation of 16S rRNA genes in density gradient fractions at initiation of experiment (0 h) and following 168 h incubation with ^{13}C-ethanol. *Y* axis shows abundance of 16S rRNA gene copies as a ratio of the maximum copies detected in each gradient.

such as *Duganella*, *Ferribacterium*, *Sterolibacterium*, *Thiobacillus*, *Desulfovibrio*, *Sphingomonas*, *Rhodanobacter*, *Rhodoferax*, *Anaerolinea*, *Gemmatimonas* and possibly *Geothrix*. T-RFLP profiles of ^{13}C-DNA did not always reflect those in the total community, likely indicating differences in growth rates under the conditions evaluated. Apparent differences in peak relative abundance should be interpreted cautiously, due to the potential for biases during PCR reactions.

Iron reducers from the genera *Ferribacterium*, *Geothrix*, *Rhodoferax, Geobacter* and *Anaeromyxobacter* were detected in the clone libraries and most were associated with peaks on T-RFLP profiles (Table 3, Fig. 1). *Ferribacterium* and *Rhodoferax* are β-Proteobacteria that couple iron reduction with oxidation of acetate and other organic acids but not ethanol [28,40]. *Geothrix* was an abundant FeRB from the phylum, Acidobacteria, that grows on simple organic acids such as lactate and acetate [4]. *Geobacter* and *Anaeromyxobacter* are δ-Proteobacteria known to reduce U(VI) in pure cultures [25,41,42]. Some *Geobacter* strains can utilize ethanol.

SRB were represented by *Desulfovibrio* and *Desulfosporosinus* spp., which are members of the δ-Proteobacteria and Firmicutes, respectively. Both genera can reduce U (VI) in pure cultures [41]. *Desulfovibrio* releases acetate when using ethanol as electron donor during sulfate reduction. *Desulfosporosinus* can use both ethanol and acetate as electron donors [43].

The most abundant denitrifiers were β-Proteobacteria, with *Ferribacterium* being the most abundant denitrifier. Other denitrifiers detected in the libraries were *Geothrix, Sphingomonas, Acidovorax* and *Sterolibacterium* among others.

Genera known to include U(VI)-reducers detected in clone libraries were *Acidovorax, Anaeromyxobacter*, *Desulfovibrio, Geobacter* and *Desulfosporosinus* (Table 3). Because *Anaeromyxobacter* and *Geobacter* both produced T-RFs that overlapped with the abundant clone *Desulfovibrio*, their incorporation of ^{13}C from ethanol could not be ascertained with certainty. No peaks were detected at or near 586 bp, where *Desulfosporosinus* would appear. *Acidovorax* produced a TRF of 204 bp in size (peak C, Table 1, Fig. 1), overlapping with several other dominant organisms. The U(VI)-reducing activity observed at the site and in our microcosm FW026 may be attributable to these organisms and/or other SRB and FeRB present that have not yet explicitly been shown to reduce U(VI). A SIP study in sediments collected elsewhere in Area 2 indicated that *Geobacter, Desulfobacter, Desulfovibrio, and Desulfotomaculum* may have derived carbon from ethanol during a biostimulation microcosm experiment [34]. However these identifications were based on phospholipid fatty acids (PLFAs) that are widespread among Gram-negative bacteria making identifications somewhat uncertain.

Only three T-RFs present in heavy DNA were associated with background contamination. T-RFs sized 62 bp (peak A, FW104), 205 bp (peak C, FW026) and 372 bp (peak E, FW104 and FW026) were detected in background controls. Peak heights in the background were considerably smaller than in heavy DNA for T-RFs sized 62 bp and 205 bp, suggesting that although these organisms were present as contamination they may also have been enriched with ^{13}C in incubated samples. However the T-RF sized 372 bp (Fig. 1, peak B) was associated with *Geothrix* by *in silico* digestion, a genus detected in abundance (11%–15.5%) in libraries (Table 3). Whether *Geothrix* actually derived carbon from ethanol or simply appeared in heavy fractions as contamination from total community DNA cannot be determined with confidence.

Using SIP methods to investigate the role of ethanol and acetate in microbial U(VI) reduction, this study provides new insights into the biostimulation process in an active U (VI)-reducing community. Ethanol functioned as electron donor but not directly as a growth substrate during active reduction of nitrate and sulfate. Ethanol was converted to acetate during nitrate and sulfate reduction, and this ethanol-derived acetate was apparently supplemented by additional acetate from acetogenesis in FW104. This indicates that acetogenesis may occur in the treatment system, and the role of acetogens in providing additional carbon to the community via the reduction of CO_2 warrants further exploration. Bacterial growth using carbon originally derived from ethanol occurred after 24 h, following conversion of ethanol to acetate, completion of nitrate and sulfate reduction, and during the period of U(VI) reduction in FW026. Thus, based on temporal appearance of ^{13}C in bacterial DNA, acetate served as the primary electron donor and carbon source for bacterial growth that coincided with U(VI) reduction. Carbon derived from ethanol was dispersed throughout nearly the entire bacterial community, either via utilization of acetate for growth or via secondary carbon flow from metabolites or dead ^{13}C-biomass within 7 days of ethanol addition. This study confirms that ethanol addition ultimately biostimulated known the U(VI) reducers *Acidovorax, Anaeromyxobacter*, *Desulfovibrio, Geobacter* and *Desulfosporosinus*, as well as diverse SRB and FeRB that may also contribute to U(VI) reduction, within an actively U(VI)-reducing microbial community.

4 Conclusions

Field test results demonstrated that in situ bioactivity and microbial populations were stimulated by ethanol injection. Microcosm tests with contaminated sediments amended with ^{13}C-labeled ethanol indicated that ethanol was rapidly converted to acetate within 24 h accompanied with the reduction of nitrate and sulfate and the accumulation of acetate as an intermediate that persisted beyond the 7 d test period. Low initial aqueous U was

detected in the microcosm with the highly reduced sediment sample and the U concentration did not decline during initial 7 days, perhaps due to desorption of U from sediment, but a rapid decline of U concentration was observed in the less reduced sample with higher initial U concentration. ^{13}C-DNA production in microorganisms occurred when ethanol was exhausted and when abundant acetate had accumulated in both microcosms, and coincided with U(VI) reduction in the less reduced sample. ^{13}C originating from ethanol was ultimately utilized for growth, either directly or indirectly (via acetate), by the majority of dominant microbial community members within 7 days of incubation. The active microbial community was comprised predominantly of known denitrifiers, sulfate-reducing bacteria and iron (III) reducing bacteria including *Desulfovibrio*, *Sphingomonas, Ferribacterium, Rhodanobacter*, *Geothrix*, and others. They included the known U(VI)-reducing bacteria A*cidovorax, Anaeromyxobacter*, *Desulfovibrio, Geobacter* and *Desulfosporosinus.* The findings suggest that ethanol biostimulates the U(VI)-reducing microbial community indirectly by first serving as an electron donor for nitrate, sulfate, iron (III) and U(VI) reduction, and producing acetate which then functions as electron donor for U(VI) reduction and carbon source for microbial growth.

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