Biogeochemical Processes and Microbial Community Structure in Ethanol-Amended FRC Area 2 Sediments

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Abstract
A laboratory incubation experiment was conducted with uranium-contaminated subsurface sediment from Oak Ridge National Laboratory in order to evaluate the potential for U(VI) reduction by native microorganisms in the sediment, and to generate a dataset for use in development of a reaction-based model of terminal electron-accepting processes (TEAPs) for incorporation into field-scale simulations of in situ biostimulation. Sediment from the zone of maximum uranium contamination was suspended in an anoxic Pipes-buffered artificial groundwater medium (250 g dry sediment per L) whose composition was designed to mimic the chemistry of groundwater at the field site. Duplicate slurries were amended with 10 mM ethanol, and changes in aqueous and solid-phase geochemical conditions were monitored over a 52-d incubation period in comparison to duplicate unamended slurries. Nitrate was consumed within a few days in ethanol-amended slurries. Parallel Fe(III) and U(VI) reduction commenced upon depletion of NO_3^-. Approximately 60% of the 100 mM $NaHCO_3$-extractable U(VI) content of the sediment was reduced during the ca. 7-d period of Fe(III) reduction. No further U(VI) reduction took place during the ensuing periods of SO_4^{2-} reduction and methanogenesis. Nitrate was consumed slowly in the nonethanol-amended slurries, with concentrations falling to below 10 µM only after 45 d of incubation. No reduction of Fe(III), U(VI), or SO_4^{2-}, or production of CH_4, took place in the non-amended slurries. Dissolved U(VI) remained below 1 µM in the non-amended slurries. In contrast, dissolved U(VI) increased to ca. 2 µM toward the end of the incubation period in ethanol-amended slurries. This increase in dissolved U(VI) can likely be explained by complexation of U(VI) by dissolved inorganic carbon produced during methanogenic oxidation of acetate that was generated earlier in the experiment via incomplete oxidation of ethanol. These results suggest that (1) spatial and temporal variations in organic substrate metabolism and associated TEAPs is likely to have an important influence on aqueous/solid-phase U(VI) partitioning during in situ biostimulation.

Total and metabolically active microbial community composition associated with U(VI) bioreduction during the Fe(III) and SO_4^{2-} reduction stages was investigated with 16S rDNA and rRNA gene clone libraries. A good correspondence between the two types of libraries was observed for the Fe(III)-reducing phase. *Geobacter* and *Oxalobacter* were predominant, representating 20% and 33% in rDNA and 41% and 21% in rRNA gene clone libraries. For the SO_4^{2-} reduction stage, both clone libraries consisted of a broader community and were not well correlated with each other. Specific PLFAs indicative of Gram-negative bacteria and the major PLFAs in *Geobacter* (Rooney-Varga et al., 1999) increased in the amended slurries. However, PLFAs known to be specific to sulfate reducing bacteria did not increase in abundance.

Purpose
The purpose of the sediment slurry incubation experiment was to (1) evaluate the potential for U(VI) reduction by native microorganisms in Area 2 sediment and (2) determine the predominant and metabolically active organisms via 16S rDNA and rRNA gene clone library and membrane phospholipids fatty acid (PLFA) analysis at different stages of TEAPs.

Experimental Set-up
Core material from the zone of maximum U contamination (ca. 18 ft depth; see Fig. 1) in ORNL Area 2 sediments was dried and ground with a mortar and pestle. The sediment was suspended in Pipes-buffered artificial groundwater (composition listed in Table 1) and bubbled with N_2. The slurries (initial volume 500 mL; see Fig. 2) were then inoculated with a small quantity (2% vol/vol) of an anoxic slurry of undried sediment from the same depth interval. Two slurries were then amended with 10 mM of ^{13}C-ethanol, and two slurries were left unamended. The slurries were incubated at 28C and sampled periodically by syringe and needle for various aqueous and solid-phase geochemical, molecular parameters, which are summarized in Table 2.

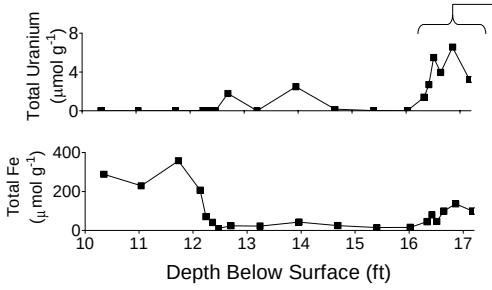

Fig. 1. Depth profile distribution of Total U and Fe in FRB sediment core. Sample used in this study, collected from maximum U contamination.

Table 1. Composition of artificial groundwater medium

Component	Concentration (mM)
Ca^{2+}	4.10
K^+	0.170
Mg^{2+}	1.10
Na^+	23.0
NH_4^+	0.100
Cl^-	10.7
$Pipes^-$	10.0
PO_4^{3-}	0.01
HCO_3^-	0.500
NO_3^-	1.00
SO_4^{2-}	1.00
Ionic strength	36.4

Table 2. Analytical, molecular parameters and methods

Parameter(s)	Method
Ethanol	Enzymatic kit (Boehringer)
NO_3^-, SO_4^{2-}, CH_3COO^-	Ion chromatography
Dissolved Fe(II)	Spectrophotometry with Ferrozine
Total Fe(II)	HCl (0.5M) extraction & Ferrozine
Dissolved U(VI)	Kinetic Phosphorescence Analyzer (KPA)
Total U(VI)	100 mM NaHCO extraction & KPA
CO_2(g), CH_4(g)	Gas chromatography
pH	Combination electrode
Dissolved inorganic carbon	Calculation from CO_2(g) and pH
DNA/RNA extraction	Hurt et al., 2001
RT-PCR (cDNA)	Retro-script (Ambion)
Primer 27F	5'-AGA GTT TGA TCC TGG CTC AG-3'
907R	5'-CCG TCA ATT CMT TTR AGT TT-3'
Cloning	pGEM-T easy vectorsystem (Promega)
PLFA extraction, GCMS	Blig and dyer, 1956

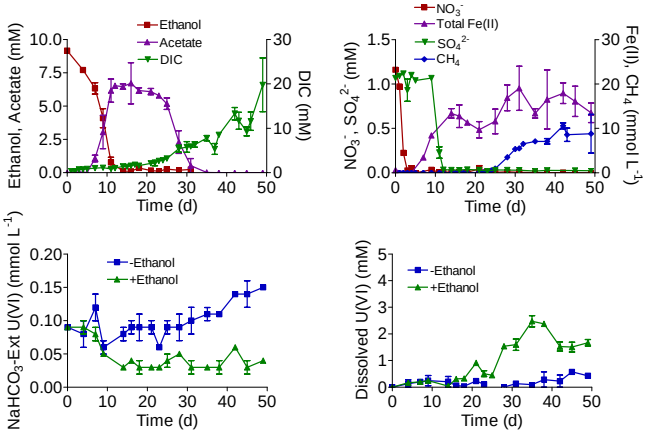

Fig. 3. Time course of microbial metabolism and uranium speciation in ethanol-amended slurries. Data points represent the means of duplicate slurries; error bars show the range of the duplicates.

Fig 2. Sediment slurries after ca. 2 months of incubation with or without ethanol amendment

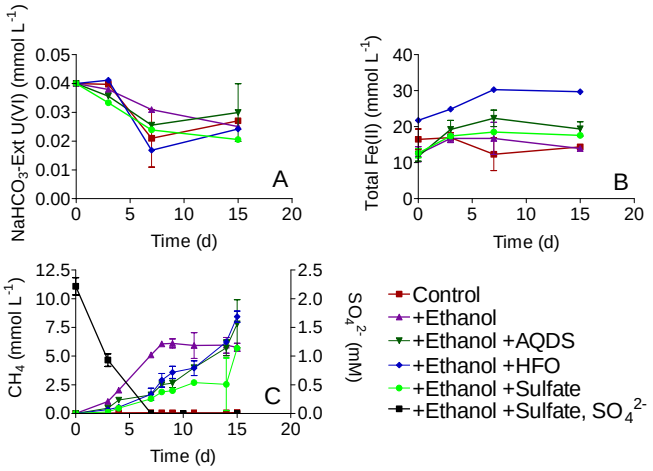

Fig 4. Results of follow-up experiment to assess controls on residual U(VI) reduction. Data points represent the means of duplicate slurries; error bars show the range of the duplicates

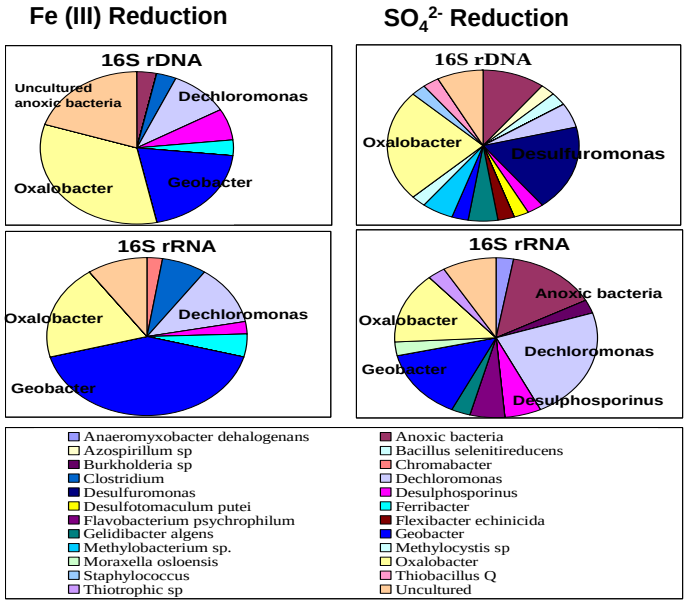

Fig.5. 16SrDNA/rRNA clone library at Fe and SO_4^{2-} reduction phase in FRB sediment amended with ethanol

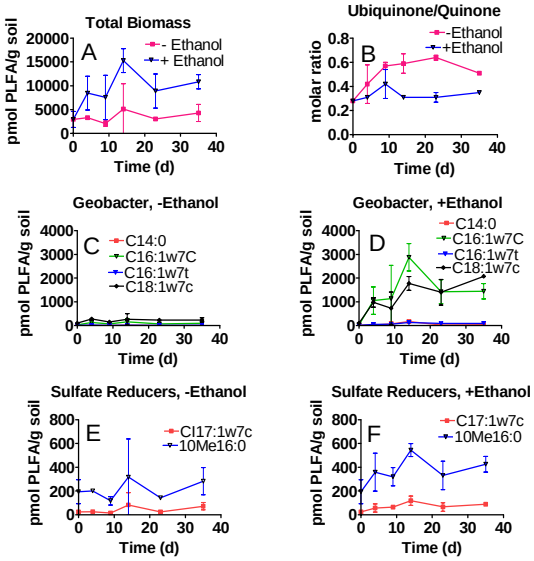

Fig. 6. Total PLFA (A), ubiqionone/quinone molar ratio (B), *Geobacter* (C–without ethanol, D–with ethanol) and sulfate reducer (E–without ethanol, F-with ethanol) specific PLFAs in the sediment slurries

RESULTS AND DISCUSSION

Microbial metabolism. Ethanol was consumed within two weeks in the amended slurries (Fig. 3A), resulting in the transient accumulation of significant quantities of acetate. A classical sequence of terminal electron-accepting processes (TEAPs) took place in the amended slurries: nitrate reduction was followed by Fe(III) reduction (Fe(II) production), which was followed by sulfate reduction, which in turn was followed by methanogenesis (Fig. 3B). Only a slow consumption of nitrate occurred and no reduction of Fe(III), sulfate, or production of methane, occurred in the nonamended slurries (data not shown).

Solid-phase U speciation. Partial (ca. 60%) reduction of $NaHCO_3$- extractable U(VI) took place in conjunction with Fe(III) reduction in the amended slurries (Figs. 3C,D). No further U(VI) reduction took place during the ensuing periods of sulfate reduction and methanogenesis. A follow-up experiment was conducted with reduced sediment slurry to assess microbial metabolic factors that might have limited U(VI) reduction (Fig. 4). None of the treatments (ethanol alone or ethanol plus 0.1 mM AQDS, 10 mmol/L HFO, or 2 mM sulfate) stimulated significant additional U(VI) reduction (Fig. 4A), despite the presence of active microbial metabolism (Fig. 4B,C).

Dissolved U(VI). Virtually all U(VI) remained associated with the solid-phase in the nonamended slurries (Fig. 3C,D). In contrast, a significant increase in dissolved U(VI) occurred toward the end of the amended slurry incubations. This increase in dissolved U(VI) can likely be explained by complexation of U(VI) by dissolved inorganic carbon (> 10 mM) produced during methanogenic oxidation of acetate that was generated earlier in the experiment via incomplete oxidation of ethanol (see Fig. 3A). These results indicate that spatial and temporal variations in organic substrate metabolism and associated TEAPs is likely to have an important influence on aqueous/solid-phase U(VI) partitioning during in situ biostimulation at ORNL.

Microbial community structure. 16S rRNA and rDNA gene clone libraries were constructed from samples obtained from the Fe(III) (day 9) and SO_4^{2-} (day 14) reduction stages of the slurry experiment (Fig. 5). A good correspondence between the two types of libraries was observed for the Fe(III)-reducing phase (Fig. 5), and significant percentage of clones in the DNA and cDNA libraries (ca. 20% and 40%, respectively) were 99% similar to the known Fe(III)-reducing bacterium *Geobacter humireducens*. In contrast, the two libraries obtained from the SO_4^{2-}-reducing phase were less similar to each other, and only a small number sequences (<10% of total) similar to known SO_4^{2-} reducers (*Desulphosporosinus*) were obtained. We are currently constructing additional 16S rDNA/RNA clone libraries for each of the major TEAP phases of the experiment (Table 2), and investigating the potential physiological role of *Oxalobacter* in ethanol-amended sediments.

Phospholipd fatty acid (PLFA) and Quinone analysis. Biomass as measured by total PLFA per gram of soil increased in the ethanol amended treatment (Fig. 6A). Microbial respiratory quinone ratios (Ubiquinone/Total Quinone) showed an increase in anaerobic activity in the ethanol amended treatment (Fig. 6B). Specific PLFA indicative of Gram-negative bacteria and the major PLFA in Geobacter (Rooney-Varga et al., 1999) also increased in the amended treatment (Fig. 6C,D), however PLFA known to be specific to sulfate reducing bacteria (Macalady et al., 2000) did not increase over time (Fig. 6E,F). Preliminary results of C^{13} PLFA analysis showed uptake of donor by the microbial community with the highest incorporation in Gram-negative PLFA (data not shown). More detailed analysis of the PLFA, quinone and C^{13} incorporation are undertaken.

Table 2. Ongoing 16S rDNA/RNA clone library from FRC 2 sediment amended with ethanol at different TEAPs.

Time	Nominal TEAP condition	Actual or Planned[a] No. of Clones rDNA	cDNA
0		32[a]	
3	NO_3^- reduction		100[a]
9	Fe(III) reduction	29	40,100[a]
14	SO_4^{2-} reduction	38	35,100[a]
23	CH_4 production		100[a]
35	CH_4 production		100[a,b]

[a] Libraries prepared, sequencing in process
[b] Bacterial Primers only

References

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