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Biogeochemical Processes In Ethanol Stimulated Uranium-contaminated Subsurface Sediments

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A laboratory incubation experiment was conducted with uranium-contaminated subsurface sediment to assess the geochemical and microbial community response to ethanol amendment. A classical sequence of terminal electron-accepting processes (TEAPs) was observed in ethanol-amended slurries, with NO_3^- reduction, Fe(III) reduction, SO_4^{2-} reduction, and CH_4 production proceeding in sequence until all of the added ^{13}C-ethanol (9 mM) was consumed. Approximately 60% of the U(VI) content of the sediment was reduced during the period of Fe(III) reduction. No additional U(VI) reduction took place during the sulfate-reducing and methanogenic phases of the experiment. Only gradual reduction of NO_3^-, and no reduction of U(VI), took place in ethanol-free slurries. Stimulation of additional Fe(III) or SO_4^{2-} reduction in the ethanol-amended slurries failed to promote further U(VI) reduction. Reverse transcribed 16S rRNA clone libraries revealed major increases in the abundance of organisms related to *Dechloromonas*, *Geobacter*, and *Herbaspirillum* in the ethanol-amended slurries. Phospholipid fatty acids (PLFAs) indicative of *Geobacter* showed a distinct increase in the amended slurries, and analysis of PLFA $^{13}C/^{12}C$ ratios confirmed the incorporation of ethanol into these PLFAs. A increase in the abundance of ^{13}C-labeled PLFAs indicative of *Desulfobacter*, *Desulfotomaculum*, and *Desulfovibrio* took place during the brief period of sulfate reduction that followed the Fe(III) reduction phase. Our results show that major redox processes in ethanol-amended sediments can be reliably interpreted in terms of standard conceptual models of TEAPs in sediments. However, the redox speciation of uranium is complex and cannot be explained based on simplified thermodynamic considerations.

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Introduction

The oxidized form of uranium [U(VI)] is relatively soluble and mobile as U(VI)-carbonate complexes in most oxic circumneutral pH sedimentary environments (*1*). U(VI) can be enzymatically reduced by dissimilatory iron- and sulfate-reducing bacteria, leading to removal of uranium from solution through precipitation of the insoluble U(IV) mineral phase uraninite [UO_2(s)] (*2, 3*). Recent studies indicate that stimulation of microbial U(VI) reduction activity, through addition of acetate or ethanol as electron donor, can be used to precipitate UO_2(s) in U(VI)-containing aquifers, thus providing a mechanism for remediation of U(VI)-contaminated groundwaters, (*4–7*) analogous to the natural process of roll-front UO_2(s) deposition (*2*).

Assessing the linkage between aqueous/solid-phase geochemical conditions, microbial community development, and patterns of U(VI) reduction activity represents a continuing challenge for in situ uranium bioremediation research (*8*). We examined these linkages in suspensions of ethanol-amended, uranium-contaminated subsurface sediment from the Area 2 site at the U.S. Department of Energy Field Research Center (FRC) located at Oak Ridge National Laboratory (ORNL) in Oak Ridge, TN. Microbial communities associated with shifts in terminal electron-accepting processes (TEAPs) were assessed by 16S rRNA (clone library analysis of 16S rDNA or reverse-transcribed 16S rRNA) and phospholipid fatty acid (PLFA) techniques, including stable isotope probing (^{13}C incorporation) of PLFAs. The results verify existing conceptual models of the temporal segregation of TEAPs in sediments, and provide a data set for the development of microbial physiology-based reaction models suitable for incorporation into field-scale reactive transport simulations of ethanol-driven redox metabolism.

Experimental Section

Site Description. Sediment for the slurry incubation experiment was obtained from Area 2 at the ORNL FRC (see http://www.esd.ornl.gov/orifc). The Area 2 site is a shallow pathway for migration of contaminated groundwater to seeps in the upper reach of Bear Creek at ORNL. Detailed descriptions of the stratigraphy and sediment/groundwater characteristics of Area 2 are available elsewhere (*9*).

Slurry Preparation. Core material from the zone of maximum total U(VI) concentration (ca. 0.25 μmol g^{-1} of bicarbonate-extractable U(VI) at ca. 5.5 m depth) was air-dried, ground with a mortar and pestle, and passed through a 0.5 mm sieve. Dry sediment (125 g) was suspended in 500 mL of a Pipes-buffered artificial groundwater (PBAGW835) designed to mimic the groundwater in well 835 at Area 2 ($CaCl_2 \cdot 2H_2O$, 1.85 mM; $CaSO_4 \cdot 2H_2O$, 1.0 mM; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM, $MgCl_2 \cdot 6H_2O$ 1.1 mM, KCl 0.16 mM, $Na_{1.5}$-Pipes 10 mM; initial pH ca. 6.5). The slurry was prepared in a 1 L glass bottle fitted with a cap that incorporated a glass pressure tube with a thick rubber stopper through which samples could be collected by needle and syringe. The slurries were inoculated with a small quantity (2% vol/vol) of a suspension of undried sediment (10 g in 20 mL of PBAGW835) from the same depth interval. Two slurries were amended with 9 mM of ^{13}C-ethanol, and two slurries were left unamended. The slurries were incubated in the dark at 20 °C, and were periodically sampled by syringe and needle.

Analytical Techniques. Techniques used for determination of various dissolved (Fe(II), U(VI), anions), solid-phase (Fe(II), Fe(III), U(VI)), and gas-phase (CO_2 and CH_4) compounds in the slurries are described elsewhere (*10–12*). The

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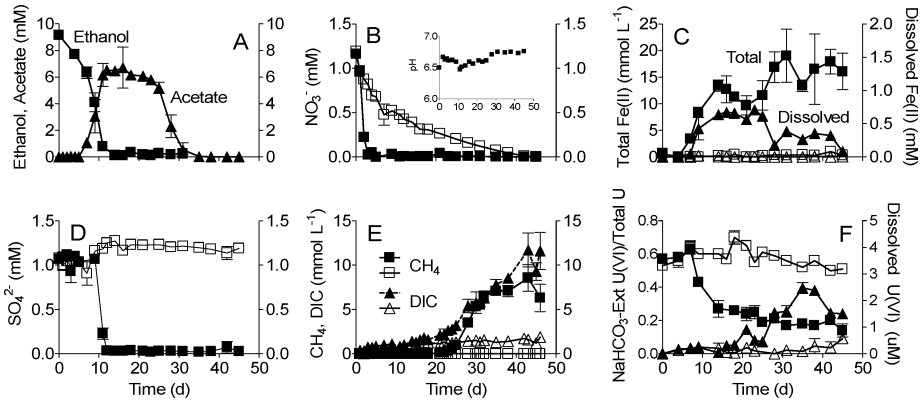

FIGURE 1. Redox metabolism in the ethanol-amended (closed symbols) and unamended (open symbols) slurries. Each data point represents the mean ± range of duplicate slurries. The high temporal variability in HCl-extractable Fe(II) content (panel C) is attributed to difficulty in obtaining subsamples (via needle and syringe) with uniform particle density from the large (1 L) incubation vessels. Squares in panel F indicate the ratio of $NaHCO_3$-extractable U(VI) to total ($NaHCO_3$ + HNO_3 extractable) U; triangles indicate dissolved U(VI) concentrations.

procedures used for bacterial PLFA (including $^{13}C/^{12}C$ ratios), respiratory quinone, and 16S rRNA clone library analyses are described in the Supporting Information.

^{57}Fe Mössbauer spectroscopy. Mössbauer analysis of the pristine sediment was performed on an air-dried sample, whereas those of biostimulated sediments (including a 0.5 M HCl-extracted sample) were performed on samples dried in an anaerobic chamber. Only spectra obtained at 12 K, where Fe-oxides and most Fe(II)-phases (e.g., siderite) magnetically order, are reported in this study. Details of the Mössbauer instrumentation, sample preparation procedure, and guidelines for modeling are available elsewhere (*13, 14*).

Results

Microbial Redox Metabolism. Ethanol was completely consumed within 2 weeks in the ethanol-amended slurries (Figure 1A). Substantial accumulation of acetate (up to ca. 7 mM) took place in conjunction with ethanol metabolism. A clearly defined temporal pattern of TEAPs was observed in the ethanol-amended slurries, with NO_3^- reduction, Fe(III) reduction, SO_4^{2-} reduction, and CH_4 production proceeding in sequence (Figure 1, panels B–E, solid symbols) until all of the electron donor was consumed. Production of CH_4 (and DIC) was coupled to consumption of acetate after ca. 20 d incubation. Only a slow consumption of NO_3^- took place in the nonamended slurries; no reduction of Fe(III) or SO_4^{2-} or production of CH_4 was observed (Figure 1, panels B–E, open symbols). Although measurements of Mn(II) were not conducted in this study, subsequent slurry experiments with similar FRC Area 2 materials indicated the presence of ca. 2 mmol L^{-1} of microbially reducible Mn(IV) oxide. Reduction of this Mn(IV) likely took place during the first week of incubation, prior to the onset of significant Fe(II) accumulation (*15, 16*), and could have accounted for a small quantity (<0.4 mM) of ethanol consumption.

Approximately 60% of $NaHCO_3$-extractable U(VI) was reduced during the Fe(III) reduction phase between 4 and 12 d in the ethanol-amended slurries (Figure 1F). No further U(VI) reduction took place during the ensuing periods of sulfate reduction and methanogenesis. No significant reduction of $NaHCO_3$-extractable U(VI) took place in the unamended slurries. Dissolved U(VI) concentrations remained less than 1 μM in the nonamended slurries (Figure 1F, open triangles). In contrast, dissolved U(VI) concentrations increased to 2–3 μM during the period of CH_4 production in the ethanol-amended slurries (Figure 1F, closed triangles).

Phospholipid Fatty Acid (PLFA) and Quinone Analysis. The bulk abundance of PLFAs was significantly higher in ethanol-amended slurries compared to the unamended

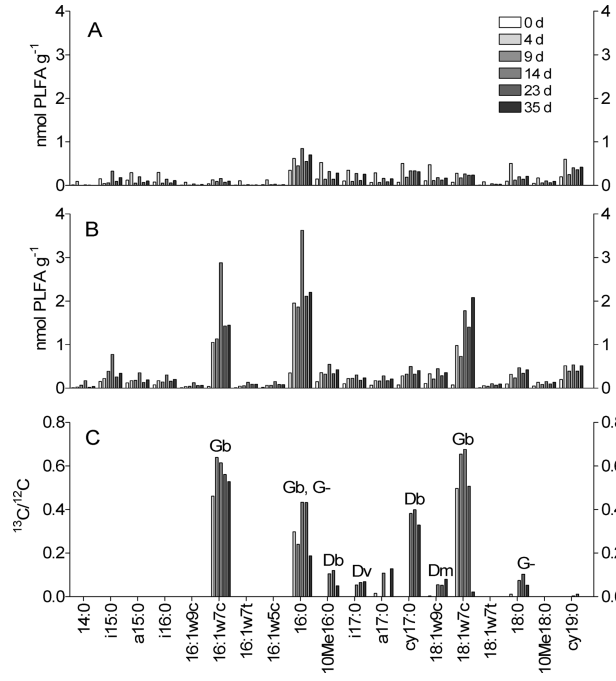

FIGURE 2. Specific PLFA abundances in unamended (A) and ethanol-amended slurries (B) and ^{13}C incorporation into PLFAs in the ethanol-amended slurries (C), expressed as the ratio of ^{13}C to ^{12}C in the PLFA. Each bar represents the mean of duplicate slurries. The designations shown in panel C are provisional, based on the known PLFA contents of relevant groups of microorganisms, including various gram negative bacteria (G–), and organisms associated with the genera *Geobacter* (Gb), *Desulfobacter* (Db), *Desulfovibrio* (Dv), and *Desulfotomaculum* (Dm).

controls (Supporting Information Table S1). The ratio of ubiquinones to menaquinones was lower in the ethanol-amended slurries (Table S1). The latter result makes sense because both ubiquinones and menaquinones are utilized in aerobic and denitrifying respiratory metabolism, whereas menaquinones are required for anaerobic respiratory pathways (*17*), which were obviously stimulated by ethanol addition.

All of the 19 individual PLFAs detected by GC/MS were more abundant in the ethanol-amended slurries (compare Figure 2, panels A and B). Three specific PLFAs (16:1w7c, 16:0, and 18:1w7C) showed a pronounced response to biostimulation. These same PLFAs, and well as several others

TABLE 1. Abundance of 16S rDNA (0 d) or Reverse-transcribed 16S rRNA Sequences in Clone Libraries from the Ethanol-amended Slurries[a]

	day of sampling					
taxa	0 d (90)	4 d (114)	9 d (87)	14 d (76)	23 d (104)	35 d (136)
Anaeromyxobacter	2	1	1	2	0	3
Burkholderaceae	2	10	1	0	2	0
Clostrideaceae	40	0	8	2	2	0
Dechloromonas	3	15	9	11	28	40
Desulfotomaculum	4	0	1	0	0	0
Desulfosporomusa	2	0	0	1	0	0
Desulfosporosinus	1	1	0	1	0	1
Flavobacterium	3	0	0	0	0	0
Geobacteraceae	5	35	36	38	43	67
Herbaspirillum	0	25	19	15	8	12
Rhodocyclaceae	3	4	2	0	8	1
Uncultured	4	1	4	0	2	0
Others	21	22	6	6	11	12

[a] The libraries were constructed with nucleic acids extracted from pooled 10 mL samples from duplicate slurries. A sequence similarity of 95% was used as cutoff value for genus (or family) level identification. Number in the parenthesis represents total number of clones analyzed.

(10Me16:0, i17:0, a17:0, cy17:0, 18:1w9c, 18:0) showed significant incorporation of ^{13}C from the ^{13}C-ethanol (Figure 2C).

16S rRNA Clone Libraries. Samples obtained from the prestimulation (0 d) time point did not provide sufficient rRNA for reverse transcription; hence, a library was constructed with extracted 16S rDNA that was sufficient to provide a template for PCR. Most of these clones belonged to the family *Clostrideaceae* (Table 1). Upon incubation with ethanol, reverse-transcribed 16S rRNA sequences related to the genera *Dechloromonas*, *Geobacter*, *Herbaspirillum* became predominant in the 16S rRNA libraries, accounting for 66−88% of total clones. The frequency of *Dechloromonas* and *Geobacter* clones increased during methanogenic phase (day 23 and 35 samples), whereas the abundance of *Herbaspirillum* declined. A list of the clones included in the "Other" category in Table 1 is provided in the Supporting Information (Table S2).

Discussion

Fe(III) Reduction. Mössbauer measurements revealed that the nonstimulated sediment contained significant quantities of small-particle, Al-substituted goethite (ca. 70% of total Fe), phyllosilicate Fe (Illite, vermiculite; ca. 25% of total Fe), and a small amount of hematite (<5% of total Fe) (Figure 3A). Approximately 25% of the phyllosilicate Fe was in the Fe(II) redox state in the unreduced material. Biostimulation partially reduced both goethite and phyllosilicate Fe(III) (Figure 3B). Decreases in the goethite and phyllosilicate Fe(III) contents of the sediment, estimated by Voigt-based (*18*) simulation of Mössbauer spectra, suggested that approximately equal amounts of 0.5 M HCl-extractable Fe(II) were produced by goethite and phyllosilicate Fe(III) reduction. Mössbauer analysis of 0.5 M HCl-extracted reduced sediment (Figure 3C) verified that 0.5 M HCl extraction liberated most (>75%) of the reduced phyllosilicate domains, as indicated by the similar phyllosilicate Fe(II)/Fe(III) ratios in the pristine (ca. 0.25) and 0.5 M HCl-extracted biostimulated sediment (ca. 0.33; Figure 3C). In general, our results are in agreement with other recent work on ORNL saprolite materials that indicate that both Fe(III) oxide (goethite) and phyllosilicate Fe(III) (e.g., Illite) are quantitatively important electron acceptors for microbial Fe(III) reduction (*14, 19*)

Microbial Community Response to Ethanol Amendment. The three PLFAs (16:1w7c, 16:0, and 18:1w7C) that showed the most pronounced response to biostimulation in terms of total lipid biomass (Figure 2A,B) and ^{13}C incorporation (Figure 2C) are known to be abundant in the cell membranes of dissimilatory iron-reducing bacteria (DIRB) such as *Geobacter* and *Shewanella* (*20, 21*). The high frequency of *Geobacteraceae*-related clones in 16S rRNA libraries from the ethanol-amended slurries (Table 1) together with the extensive Fe(III) reduction activity observed in ethanol-

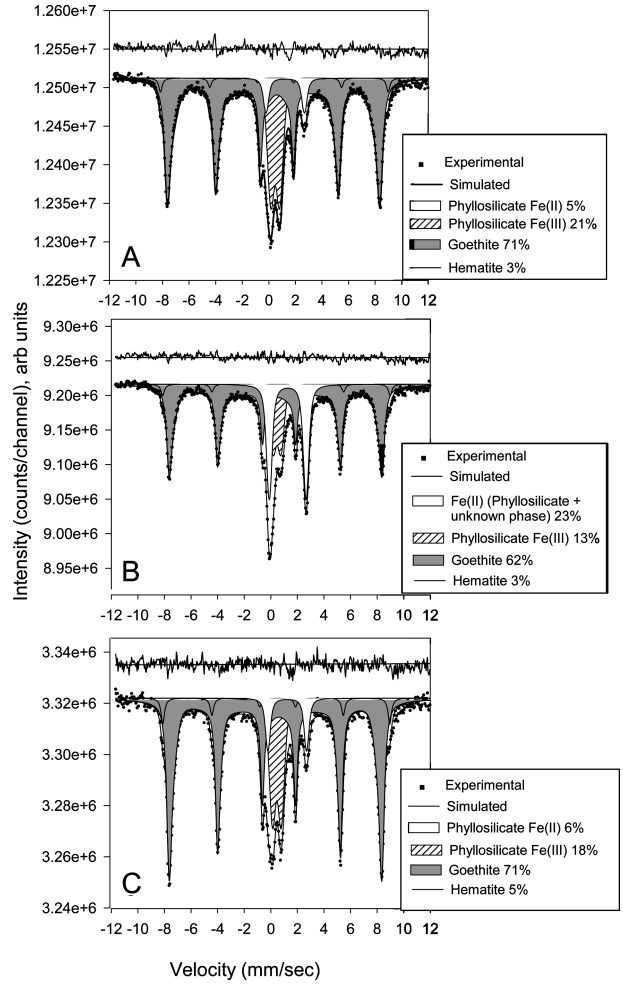

FIGURE 3. Mössbauer (12 K) spectra of (A) undreduced, (B) reduced (ethanol-amended), and (C) reduced, 0.5 M HCl-extracted sediment. Lines represent Voigt-based fits to the observed spectra.

amended slurries (Figure 1C) suggest that organisms from the *Geobacteraceae* (but not *Shewanella*) were stimulated by ethanol addition. The apparent stimulation of *Geobacteraceae* in conjunction with Fe(III) and U(VI) reduction is consistent with results from in situ and laboratory experiments with acetate-amended U(VI)-contaminated subsurface sediments (*5, 22–24*). The above PLFAs are, however, widely distributed in other types of gram-negative bacteria (*25*), and it is likely that some portion of the response to biostimulation could be attributed to proliferation of other bacterial groups, including those discussed below.

Organisms related to *Dechloromonas* and *Herbaspirillum* were present in relatively high frequency in the 16S rRNA libraries (Table 1). Both genera are denitrifying taxa widely distributed in soil and sedimentary environments (*26, 27*), including uncontaminated ORNL sediments (*23, 28*). Such organisms were clearly active during the initial nitrate reduction phase of the experiment (Figure 1B). The lack of significant ammonium production during nitrate reduction (data not shown) is consistent with denitrification as the main pathway for nitrate consumption. It is not clear, however, what role (if any) these organisms, or *Geobacter*, may have played in the latter stages of the incubation when methanogenesis was the predominant TEAP.

Neither the bulk PLFA measurements nor the 16S rRNA libraries provided strong evidence for proliferation of sulfate-reducing bacteria (SRB) tied to the brief period of sulfate reduction activity in the ethanol-amended slurries. However, there was a distinct upturn in ^{13}C incorporation into PLFAs, indicative of SRBs from the genera *Desulfobacter* (10Me16:0, cy17:0), *Desulfotomaculum* (18:1w9c), and *Desulfovibrio* (i17:0) (*29–31*), between day 9 and 14 (Figure 2C), just at the time when sulfate reduction took place (see Figure 1D). These results illustrate how stable isotope probing can reveal relatively subtle shifts in microbial community structure that are not discernible through bulk lipid biomarker analysis. The modest but significant apparent stimulation of *Desulfovibrio* is significant in terms of U(VI) reduction potential since *Desulfovibrio*, but not *Desulfobacter* or *Desulfotomaculum*, is capable of enzymatic U(VI) reduction (*32*).

Terminal Electron-accepting Processes in Ethanol-amended Sediments. The sequence of TEAPs observed in the ethanol-amended slurries conformed to classical thermodynamic expectations, with more energetically favorable reactions preceding less favorable ones (*33*). Although the segregation of different TEAPs in space and time in sediments is actually determined by the physiological properties of the organisms that catalyze those TEAPs (see refs *34* and *35* for review), the temporal/spatial sequence of redox reactions first outlined in thermodynamic terms by Ponnamperuma for hydromophic soils (*36*), and Froelich and colleagues for marine sediments (*37*), are observed in most natural soil, sediment, and groundwater systems (*33, 38*). The results of the subsurface sediment slurry experiments described here (Figure 1) provide an explicit demonstration of this basic principle.

A redox titration simulation was conducted with the geochemical modeling program PHREEQC (*39*) in order to illustrate how thermodynamic principles can be used to interpret the sequence of TEAPs in reaction systems that arise during biostimulation with ethanol. A summary of the simulation is available in the Supporting Information. The simulation reproduced the pattern of TEAPs in the ethanol-amended slurries (Figure 4), with a few important exceptions. First, the measured amount of electron donating equivalents remaining in the system, when plotted against the total number of electron equivalents accounted for by the sum of NO_3^- consumption (5 e^- equiv mol^{-1}), SO_4^{2-} consumption (8 e^- equiv mol^{-1}), Fe(II) production (1 e^- equiv mol^{-1}), and CH_4 production (8 e^- equiv mol^{-1}), did not match the linear

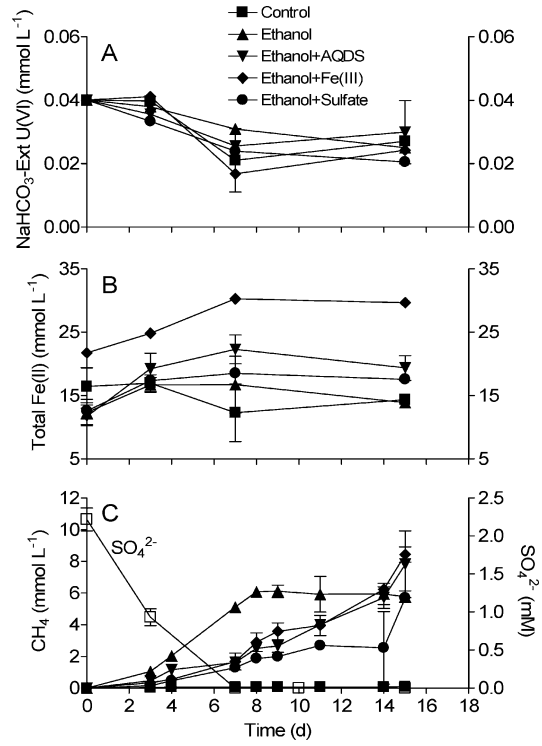

FIGURE 5. Results of the follow-up experiment to assess possible metabolic limitations on residual U(VI) reduction. All slurries except the control were amended with 5 mM ethanol and either 10 mmol L^{-1} of synthetic amorphous Fe(III) oxide, 2 mM sulfate, or 0.1 mM AQDS. Data points represent the means of duplicate slurries; error bars show ± range of the duplicates.

relationship that was implicit in the simulation (Figure 4A). Incorporation of carbon into microbial biomass and (more importantly) transient accumulation of acetate (see Figure 1A) can account for this result, as well as the lower observed accumulation of DIC compared to the simulation (Figure 4E). A thermodynamics-based approach that takes into account the formation of microbial biomass as part of the redox reaction network has been applied to this data set to depict the growth of various groups of fermentative and respiratory organisms (*40*). However, only kinetic models (discussed below) can reproduce the accumulation of acetate during partial oxidation of ethanol observed here and in other experiments (*41*) with ORNL sediments.

A key disconnect between the experimental data and the simulation results is that the simulation predicted complete reduction of U(VI) at the outset the Fe(III) reduction phase, whereas U(VI) reduction did not proceed to completion (Figure 4F). Other studies have documented incomplete reduction of solid-associated U(VI) in reduced subsurface sediments that contain excess electron donor and abundant Fe(II) as a potential chemical reductant for U(VI) (*10, 42, 43*). The persistence of substantial solid-associated U(VI) during active Fe(III) reduction provides an explanation for the increase in dissolved U(VI) that took place later on during the methanogenic phase of the experiment: complexation of residual U(VI) by DIC (>10 mM) produced during methanogenic oxidation of acetate could have easily shifted the balance between aqueous and surface-associated U(VI) (*44*).

Limitations on U(VI) Reduction. A follow-up experiment was conducted with subsamples of the reduced slurries to assess possible metabolic (as opposed to geochemical) reasons for incomplete U(VI) reduction observed in the ethanol-amended slurries. The PLFA and 16S rRNA clone library data (see above) suggested that DIRB and SRB were present in the slurries during the latter stages of the

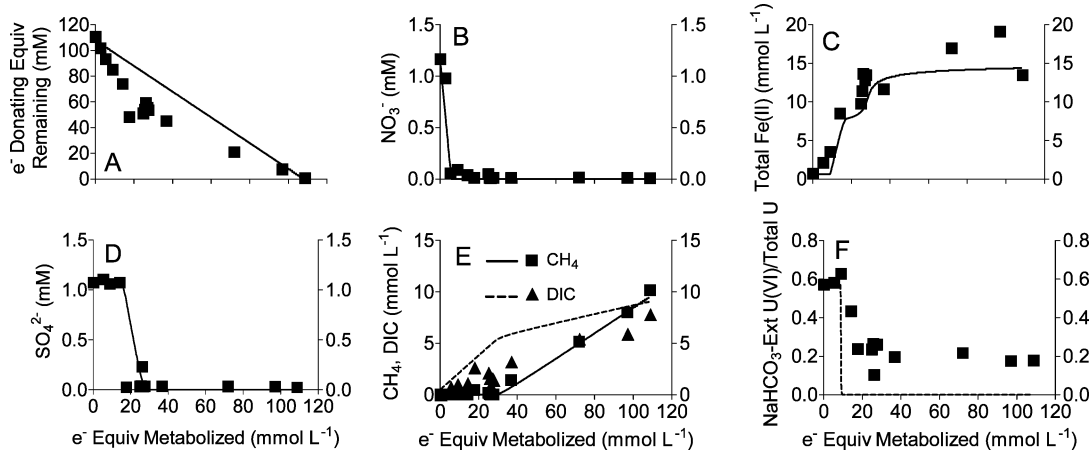

FIGURE 4. Equilibrium thermodynamic simulation (solid lines) of ethanol-amended slurry results using PHREEQC (see Supporting Information for details). Reactant or product concentrations (symbols) are plotted against the cumulative number of electron equivalents accounted for by electron acceptor consumption (NO_3^-, SO_4^{2-}) and end-product accumulation (Fe(II), CH_4). The amount of electron donating equivalents remaining in the slurries (panel A) was computed from the amount of ethanol (12 e^- equiv mol^{-1}) and acetate (8 e^- equiv mol^{-1}) measured in solution at a given time point. Each symbol represents the mean of duplicate slurries.

incubation, and we speculated that depletion of their preferred electron acceptors may have limited their ability to reduce U(VI) reduction in the slurries. Duplicate slurry subsamples were amended with nothing (control), 5 mM ethanol, 5 mM ethanol + 10 mmol L^{-1} of synthetic amorphous Fe(III) oxide, 5 mM ethanol + 2 mM SO_4^{2-}, or 5 mM ethanol + 0.1 mM AQDS. Addition of Fe(III) oxide and sulfate were designed to stimulate DIRB and SRB activities. AQDS is a soluble electron shuttling compound that accelerates rates of microbial Fe(III) oxide reduction in sediments (*45*) and that may stimulate solid-associated U(VI) reduction by reacting with U(VI) associated with sediment surfaces that are inaccessible to direct microbial reduction (*10*).

None of the treatments stimulated significant additional U(VI) reduction (Figure 5A), despite the presence of active microbial metabolism as indicated by additional Fe(II) accumulation (Figure 5B), sulfate consumption (Figure 5C), and CH_4 production (Figure 5C). AQDS stimulated reduction of residual Fe(III) phases in the sediment (Figure 5B), but in contrast to previous studies (*10*) did not promote solid-associated U(VI) reduction. Together, these results indicate that the main limitation posed on residual U(VI) reduction was geochemical rather than microbiological in nature.

Practical Implications. There are two key practical implications of this study. First, the results suggest that standard conceptual models of TEAPs in sediments should be valid for predicting the response of ORNL FRC Area 2 (and other) subsurface sediments to in situ ethanol amendment, that is, in terms of the segregation of major TEAPs over space and time. A sequence of TEAPs analogous to that observed in the slurry incubation (up to the point of sulfate reduction) was recently documented in an situ ethanol biostimulation experiment conducted at the Area 2 research site (*46*). The conformation of the data to thermodynamic theory provides a sound basis for development of microbial physiology-based kinetic models, which can reproduce the zonation of TEAPs typically observed over space and time in sediment systems (cf., ref *47*). The detection by rRNA and PLFA methods of functional groups of microorganisms known to be associated with major TEAPs provided confirmation that such groups were in fact activated during ethanol biostimulation. The slurry data have been used as a basis for development of a kinetic microbial reaction model that accurately reproduces the consumption of ethanol, transient accumulation of acetate, and major TEAPs observed in the slurry experiment (*48*). A modified version of this model was incorporated into a general biogeochemical simulator (*49, 50*), linked to the reactive transport code HBGC123D (*51*), and used to design the in situ biostimulation experiment at Area 2.

A second key implication of our findings is that the redox behavior of uranium, unlike that of other major redox couples, could not be explained on the basis of standard thermodynamic considerations. The seemingly irreversible association of U(VI) with particle surfaces that are inaccessible to enzymatic (and abiotic) reduction observed here and in other subsurface sediments (*10, 42, 52*) is puzzling and cannot be rationalized in terms of existing models of aqueous/solid-phase U speciation (*1*). This phenomenon is of practical significance in that it may limit the overall effectiveness of in situ remediation of highly contaminated U(VI) source zones such as those present at ORNL. Our results highlight the need for studies on the physiochemical nature of such nonreducible U(VI) species.

Acknowledgments

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Supporting Information Available

Methods for bacterial PLFA/respiratory quinone analysis and 16S rRNA clone library construction; total PLFA abundance and ubiquinone:menaquinone ratio in ethanol-amended and unamended slurries (Table S1); list of 16S rDNA or 16S rRNA clones included in the "Other" category in Table 1 (Table S2); description of PHREEQC redox titration simulation; and input file for the simulation (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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