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U(VI) Reduction in Sulfate-Reducing Subsurface Sediments Amended with Ethanol or Acetate

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An experiment was conducted with subsurface sediments from Oak Ridge National Laboratory to determine the potential for reduction of U(VI) under sulfate-reducing conditions with either ethanol or acetate as the electron donor. The results showed extensive U(VI) reduction in sediments supplied with either electron donor, where geochemical and microbiological analyses demonstrated active sulfate reduction.

Contamination of groundwater with soluble U(VI) at sites involved in assembly of nuclear weapons during the Cold War remains a significant environmental problem. Microbial (enzymatic) reduction of U(VI) to the less soluble U(IV) has been well studied in pure-culture systems and bench-scale sediment incubation experiments. Microbial U(VI) reduction has also been shown to effectively immobilize uranium in field-scale bioremediation trials; however, questions remain regarding the organisms involved in this process under various biogeochemical conditions during *in situ* biostimulation (1). A variety of bacteria, including dissimilatory iron-reducing bacteria (DIRB), sulfate-reducing bacteria (SRB), and various others, are known to enzymatically reduce U(VI) (1, 2). No uranium-specific reductases have been identified, but a number of periplasmic and outer membrane-associated cytochromes from different organisms are thought to catalyze U(VI) reduction (1–5). Both laboratory- and field-scale bioremediation trials have implicated DIRB from the family *Geobacteraceae* in U(VI) reduction under Fe(III)-reducing conditions in response to ethanol or acetate stimulation (6–14).

There are conflicting studies on the role of SRB in U(VI) reduction *in situ*. Some studies have indicated that their role ranges from inhibitory to unclear, where U(VI) levels increased during sulfate reduction, or sulfate concentrations were too low for SRB to be involved in U(VI) reduction (6, 7, 13, 15, 16). Other studies, primarily conducted at the Oak Ridge National Laboratory Field Research Center (FRC) in Oak Ridge, TN, have suggested that SRB may enhance and possibly participate in U(VI) reduction with DIRB (10, 14, 17–20).

Although SRB were present at sites or in experiments involving acetate stimulation (6, 7), the organisms identified in these systems (e.g., *Desulfobacter* sp.) are not known to be capable of U(VI) reduction (21). To date, only studies in which ethanol amendment was used to stimulate anaerobic metabolism have implicated SRB in *in situ* U(VI) reduction (9, 14, 19, 20). In this report, we present findings on the proliferation of SRB and the simultaneous reduction of U(VI) in response to both acetate and ethanol metabolism in slurries of subsurface sediment that were previously driven into sulfate-reducing conditions via ethanol stimulation.

A single, large-volume (500-ml) slurry of dried and ground sediment (250 g liter^{-1}) from Area 2 at the FRC was prepared with piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-buffered artificial groundwater designed to match the groundwater geochemistry at Area 2 (10). A detailed description of the site geochemistry can be found in reference 22. The slurry was contained in a glass bottle sealed with a thick rubber stopper, flushed with N_2, and inoculated with a small quantity (2% [vol/vol]) of undried sediment. Previous studies have found it difficult to accurately document microbial reduction of endogenous U(VI) in long-term U-contaminated sediments (10); hence, our experiments were conducted with sediments from below the zone of major U(VI) contamination at Area 2 (22) to which exogenous U(VI) (ca. 150 μM; added from a 10 mM stock of uranyl acetate–30 mM $NaHCO_3$ buffer) was added prior to biostimulation. The slurry was initially amended with 5 mM ethanol, with periodic amendments of 1.5 mM ethanol. Concentrations of ethanol, nitrate, sulfate, 0.5 M HCl-extractable Fe(II), aqueous U(VI), and $NaHCO_3$-extractable U(VI) were measured over time as previously described (10). All slurry incubations were conducted at room temperature.

Nitrate was consumed in parallel with ethanol during the first 5 days of the experiment (Fig. 1A and B). Slight but detectable Fe(III) reduction [combination of Fe(III) oxides and Fe(III)-bearing phyllosilicates (10)] and sulfate consumption took place between day 5 and day 25, after which Fe(III) and sulfate reduction occurred in parallel (Fig. 1B). Fe(III) reduction ceased after ca. day 60, at which point the slurries were dominated by sulfate reduction, as evidenced by ongoing consumption of added sulfate and blackening of the sediment solids indicative of iron sulfide mineral formation. Significant quantities (0.2 to 0.84 mM) of acetate accumulated transiently during the period of Fe(III) and sulfate reduction (Fig. 1A), suggesting that ethanol metabolism proceeded through partial oxidation to acetate followed by acetate oxidation, as observed previously in ethanol-amended FRC Area 2 sediments (10). U(VI) reduction commenced during the period of Fe(III) and sulfate reduction (Fig. 1C), leading to removal of ca. 85% of aqueous and solid-associated ($NaHCO_3$-extractable) U(VI).

Received 7 February 2013 **Accepted** 21 April 2013
Published ahead of print 26 April 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00420-13.

doi:10.1128/AEM.00420-13

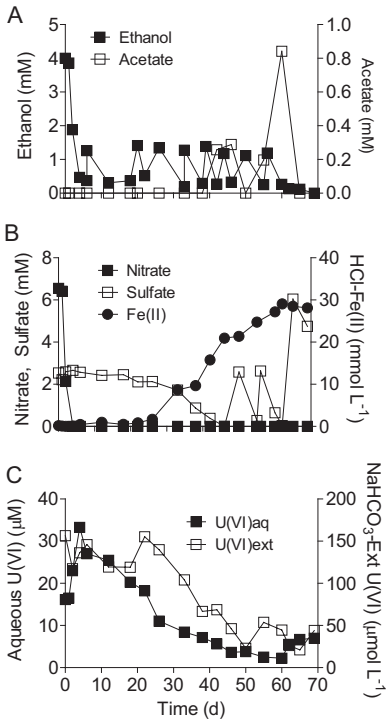

FIG 1 Concentrations of ethanol, acetate, nitrate, sulfate, 0.5 M HCl-extractable Fe(II), aqueous U(VI), and $NaHCO_3$-extractable U(VI) during the initial phase of the sediment slurry experiment designed to drive the slurries into sulfate-reducing conditions.

The sulfate-reducing sediments generated during the initial phase of the experiment were used to assess the potential for reduction of U(VI) with either ethanol or acetate as the electron donor in comparison to a no-electron donor control. Three 100-ml portions of slurry were transferred to sterile, N_2-flushed bottles. Approximately 100 μM exogenous U(VI) was added to each bottle, together with (in the case of the organic-amended slurries only) either 1.5 mM ^{13}C-labeled ethanol or 1.5 mM ^{13}C-labeled acetate to fuel sulfate reduction. The slurries contained ca. 4 mM sulfate at the time of electron donor addition. Virtually all of the aqueous U(VI) (mean of 98% ± 1% across the last three time points), and 83% ± 3% of $NaHCO_3$-extractable U(VI), was consumed in conjunction with sulfate reduction in the ethanol- and acetate-amended slurries compared to only 84% ± 0.4% and 47% ± 11% consumption in the unamended (control) slurry (Fig. 2). U(VI) reduction in the unamended slurry was probably linked to residual sulfate reduction activity, as indicated by the slight consumption of sulfate (Fig. 2A and B). The absence of any change in HCl-extractable Fe(II) content suggested that Fe(III) reduction did not take place in any of the slurries. As observed in first phase of the experiment, ethanol metabolism appeared to proceed via partial oxidation to acetate (Fig. 1A).

It should be acknowledged that abiotic U(VI) reduction via reaction with reduced Fe or S phases would confound assignment of a biological basis (e.g., linked to sulfate reduction) for U(VI) reduction in the second phase of our experiments, which did not include a sterile, microbially reduced control. However, the stimulation of U(VI) reduction by addition of electron donors, relative to the unamended slurry, strongly suggests a biological basis for U(VI) reduction. Although it is conceivable that U(VI) reduction was driven by production of additional biogenic reduced Fe or S phases in response to organic substrate addition, numerous prior studies have demonstrated that U(VI) does not react extensively with reduced Fe and S phases in sediments (23–25). This is particularly true in circumneutral-pH systems containing high levels of dissolved inorganic carbon (DIC), presumably because of the formation of thermodynamically stable aqueous U(VI)-carbonate complexes (26). Our slurries initially contained ca. 5 mM DIC, and more than 20 mM additional DIC was likely generated as a result of periodic ethanol and acetate amendment (total of ca. 24

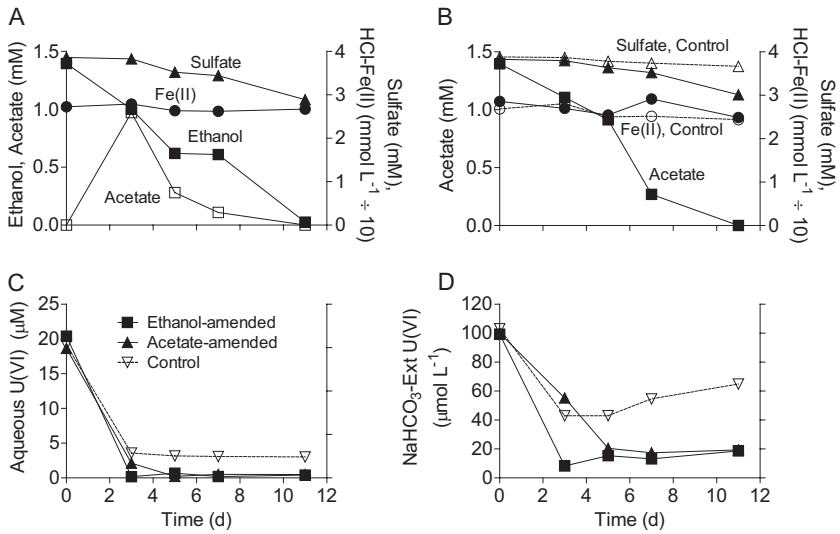

FIG 2 (A and B) Concentrations of ethanol (panel A only), acetate, sulfate, and 0.5 M HCl-extractable Fe(II) in ^{13}C-labeled ethanol (A)- or ^{13}C-labeled acetate (B)-amended slurries during the second (sulfate-reducing) phase of the sediment slurry experiment; panel B also shows sulfate and Fe(II) concentrations in unamended (control) slurries. (C and D) Concentrations of aqueous U(VI) and $NaHCO_3$-extractable U(VI) in ethanol- or acetate-amended or unamended (control) slurries during the second phase of the sediment slurry experiment.

mM added organic carbon). Prior experiments showed a complete absence of U(VI) (ca. 100 μM) reduction during addition of 10 mM $Na_2S \cdot 9H_2O$ to a slurry of Fe(III) oxide-rich sediment (similar to that found in FRC Area 2) in 30 mM $NaHCO_3$ buffer (E. Roden, unpublished data). Based on these considerations, we assume that U(VI) reduction was an enzymatically catalyzed process in our experiments and discuss below the likely microbiological basis for this process.

Quantitative PCR was conducted on DNA extracted from each slurry (MoBio UltraClean soil DNA isolation kit, used according to the manufacturer's protocols) using Bio-Rad iQ SYBR green Supermix with a Bio-Rad iQ5 multicolor real-time detection system. Previously described primers and cycling conditions were used to quantify the 16S rRNA gene of bacteria (27) and the *dsrA* gene of SRB (28) in order to estimate the relative abundances of putative SRB. The cycling conditions for the 16S rRNA primers were modified slightly for quantitative PCR (qPCR) as follows: initial denaturation at 92°C for 2 min and 30 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Standard, sample, and control DNAs were amplified in duplicate. Serial dilutions of full-length PCR amplicons generated from genomic DNA were used to construct standard curves based on the amount of DNA added. The full-length *Escherichia coli* DH5α 16S rRNA gene and the full-length *dsrA* gene from *Desulfovibrio vulgaris* ATCC 29579 were amplified using previously described primers (primers 27f and 1492r and primers DSR1F and DSR4R) and cycling conditions (29). No corrections for rRNA gene copy number were included. The analyses showed that, at the end of the ^{13}C-substrate experiments, *dsrA* gene copy numbers represented 75%, 65%, and 140% of the total 16S rRNA gene copy number in the control, ethanol-amended, and acetate-amended slurries, respectively (data not shown). These results are generally consistent with the presence of a bacterial community dominated by SRB.

At the end of the experiment, phospholipid fatty acids (PLFAs) were extracted and quantified by gas chromatography-flame ionization detection (GC-FID) (30), and their stable isotope composition ($\delta^{13}C$ values) was determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) (31). Bulk and ^{13}C-labeled PLFA profiles (Fig. 3A) were consistent with a mixed community of anaerobic Gram-negative bacteria (30). A challenge in comparing these data is the differences in resolution between the GC-FID and GC-IRMS PLFA profiles; the need for baseline resolution and peak spreading during IRMS leads to reporting groups of unresolved (by GC-IRMS) fatty acids as summed features. The PLFAs that were well resolved by both systems (14:0, 15:0, i17:0, and cy19:0) showed three different patterns of incorporation: fatty acids 14:0 and i17:0 showed high levels of incorporation ($\delta^{13}C$ = ~5,000 to 7,000), 15:0 showed moderate incorporation ($\delta^{13}C$ = ~1,000 to 2,000), and cy19:0 showed virtually no incorporation. Among the summed features, features 1 (i15:0 and a15:0) and 3 (i17:1ω7 and 10Me16:0) showed high levels of incorporation, features 2 (i16:0, 16:1ω9, 16:1ω7c, 16:1ω7t, 16:1ω5, and 16:0) and 5 (18:1ω9, 18:1ω7c, 18:1ω7t, 18:1ω5, 18:0, and br19:0a) showed moderate incorporation, and feature 4 (a17:0, cy17:0, and 17:0) showed little or no incorporation. Combined, fatty acids 14:0, i15:0, a15:0, i17:1ω7, 10Me16:0, and i17:0 defined the strains of bacteria that showed the highest levels of ^{13}C incorporation.

Some of the moderately ^{13}C-enriched PLFAs (16:1ω7c, 16:0, and 18:1ω7c) are abundant in DIRB such as *Geobacter* and *Shewanella* (32, 33); however, none of the most ^{13}C-enriched PLFAs are dominant fatty acids in these organisms. Although no single described species of SRB contains all of the most ^{13}C-enriched PLFAs as their major fatty acids, each of these is a major fatty acid for at least one described species within the SRB genus *Desulfovibrio* (see Table S1 in the supplemental material), and a mixed community containing several *Desulfovibrio* species would show the observed pattern. Several other SRB, including *Desulfoarculus*, *Desulfotomaculum*, *Thermodesulfovibrio*, *Desulfoluna*, *Desulfobulbus*, *Desulfobacter*, *Desulfomicrobium*, and *Desulfobacula*, also contain one or more of the highly labeled fatty acids. Thus, a mixed community containing several members of these genera, with or without *Desulfovibrio* species, would show the observed PLFA pattern. The moderate incorporation of ^{13}C-labeled substrates into summed features 2 and 5 is consistent with the active community being composed of SRB, as many members of the aforementioned genera bear one or more of the fatty acids (par-

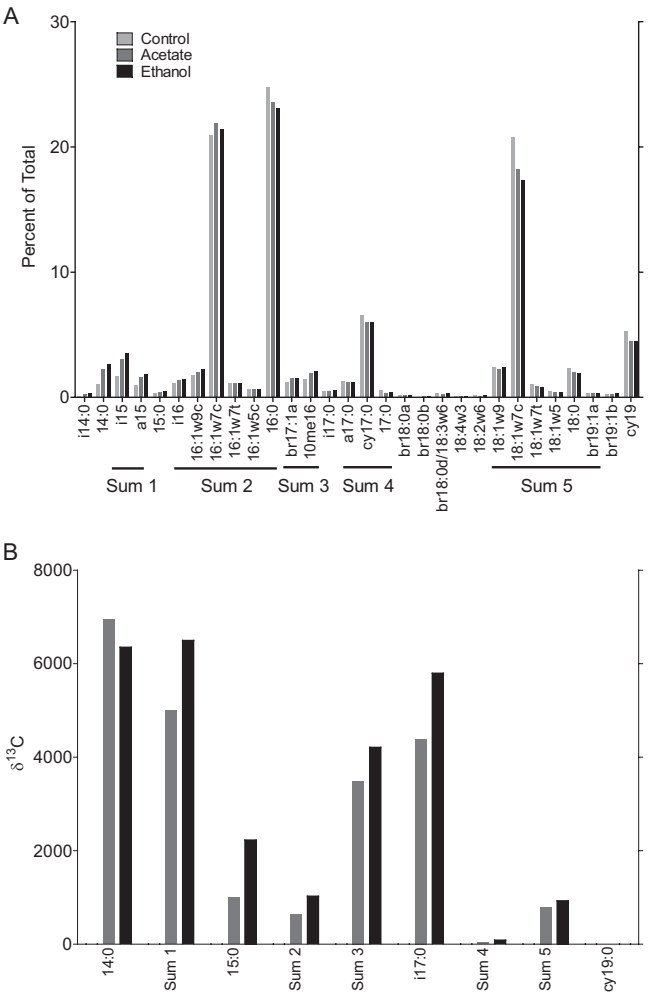

FIG 3 (A) Relative abundances determined by GC-FID. (B) $\delta^{13}C$ values for PLFAs determined by GC-IRMS. All data are from the ^{13}C-labeled ethanol- and ^{13}C-labeled acetate-amended slurries from the second phase of the experiment. All $\delta^{13}C$ values for the control (unamended) slurries were between 0 and −21‰ and are not shown in panel B. Individual components of the summed PLFA ^{13}C peaks, as inferred from parallel PLFA mass quantifications (see the text), are indicated in panel A.

ticularly 16:1ω7 and 18:1ω7) contained within summed features 2 and 5. Fatty acids a17:0, cy17:0, 17:0, and cy19:0 represented dormant genera. These fatty acids are typically not associated with SRB, except for cy17:0, which is found in members of the genera *Desulfobacter* and *Desulfobacterium*.

High copy numbers of the *dsrA* gene, together with the incorporation of ^{13}C-labeled substrates at high levels into fatty acids representative of a mixed SRB community, suggest that SRB capable of ethanol and acetate metabolism proliferated during the second phase of the experiment. These results in turn are consistent with the idea that SRB were involved in U(VI) reduction under sulfate-reducing conditions. A recent study suggested that U(VI) reduction in acetate-stimulated sulfate-reducing sediments may be been linked to residual DIRB activity (34). However, no measurable Fe(III) reduction took place in the sulfate-reducing slurries, which argues against DIRB-linked U(VI) reduction. Of the genera potentially represented by fatty acids containing high levels of ^{13}C incorporation, only members of the genera *Desulfovibrio*, *Desulfotomaculum*, and *Desulfomicrobium* and the species *Desulfarculus baarsii* are known to reduce U(VI) (2). Of these, *Desulfovibrio* spp. are well known for their ability to reduce U(VI) when electron donors such as ethanol and H_2 are supplied (2). The proliferation of these organisms therefore provides a simple explanation for U(VI) reduction in the ethanol-amended slurries consistent with the inferred involvement of *Desulfovibrio* in U(VI) reduction during *in situ* ethanol stimulation (10, 14, 19, 20).

To date, no acetate-oxidizing SRB are definitively known to reduce U(VI). *D. baarsii* can oxidize acetate (35) and reduce U(VI) (21); however, utilization of acetate as an electron donor for U(VI) reduction has not been tested in this organism. *Desulfotomaculum reducens* is known to reduce U(VI) but appears unable to utilize acetate (36), while the closely related *Desulfotomaculum acetoxidans* oxidizes acetate (37) but cannot reduce U(VI) (21). Of the other highly labeled genera identified in this study, *Desulfobacula* and *Desulfobacter* can utilize acetate as an electron donor (38, 39), but there is no evidence that members of these genera can reduce U(VI). Thus, the identity of organisms responsible for U(VI) reduction in the acetate-stimulated sulfate-reducing slurries remains unclear. Nevertheless, the qPCR, ^{13}C-enriched PLFA, and geochemical results together strongly suggest that a community composed primarily of SRB was responsible for U(VI) reduction in both the ethanol- and acetate-amended sediments. To our knowledge, this is the first report to directly implicate acetate-oxidizing SRB in U(VI) reduction. Subsequent long-term flow-through reactor experiments have confirmed the capacity for both ethanol- and acetate-stimulated Area 2 sediments to carry out sustained U(VI) reduction under sulfate-reducing conditions (40).

ACKNOWLEDGMENT

This research was supported by grant DE-SC0001180 from the Subsurface Biogeochemistry Research Program, Office of Biological and Environmental Research, U.S. Department of Energy.

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