Bioreduction of Uranium in a Contaminated Soil Column

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The bioreduction of soluble uranium [U(VI)] to sparingly soluble U(IV) species is an attractive remedial technology for contaminated soil and groundwater due to the potential for immobilizing uranium and impeding its migration in subsurface environments. This manuscript describes a column study designed to simulate a three-step strategy proposed for the remediation of a heavily contaminated site at the U.S. Department of Energy's NABIR Field Research Center in Oak Ridge, TN. The soil is contaminated with high concentrations of uranium, aluminum, and nitrate and has a low, highly buffered pH (∼3.5). Steps proposed for remediation are (i) flushing to remove nitrate and aluminum, (ii) neutralization to establish pH conditions favorable for biostimulation, and (iii) biostimulation for U(VI) reduction. We simulated this sequence using a packed soil column containing undisturbed aggregates of U(VI)-contaminated saprolite that was flushed with an acidified salt solution (pH 4.0), neutralized with bicarbonate (60 mM), and then biostimulated by adding ethanol. The column was operated anaerobically in a closed-loop recirculation setup. However, during the initial month of biostimulation, ethanol was not utilized, and U(VI) was not reduced. A bacterial culture enriched from the site groundwater was subsequently added, and the consumption of ethanol coupled with sulfate reduction immediately ensued. The aqueous concentration of U(VI) initially increased, evidently because of the biological production of carbonate, a ligand known to solubilize uranyl. After ∼50 days, aqueous U(VI) concentrations rapidly decreased from ∼17 to <1 mg/L. At the conclusion of the experiment, the presence of reduced solid phase U(IV) was confirmed using X-ray absorption near edge structure spectroscopy. The results indicate that bioreduction to immobilize uranium is potentially feasible at this site; however, the stability of the reduced U(IV) and its potential reoxidation will require further investigation, as do the effects of groundwater chemistry and competitive microbial processes, such as methanogenesis.

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Introduction

One legacy of the Cold War is uranium contamination of soil and groundwater at U.S. Department of Energy (DOE) waste disposal areas and other sites (*1*−*4*). Uranium is of particular concern because of its carcinogenicity, long half-life (∼10^9 years), and potential mobility in the environment. Methods are needed to impede its movement into water bodies that provide ecological and human services.

Uranium commonly exists as either U(VI) or U(IV) in the environment, and its fate and transport is governed by the oxidation states. In near-surface and oxic groundwater, it is generally present as hexavalent U(VI) in the form of uranyl (UO_2^{2+}), which is particularly soluble and mobile at a relatively low pH (<6). At intermediate pH (near 6), it forms hydroxide precipitates or surface complexes with soil minerals (assuming that complexing ions such as Ca^{2+} and CO_3^{2-} are not present in the system) and thus becomes relatively immobile. At higher pH values, the solubility of U(VI) increases by several orders of magnitude due to complexation with carbonate in groundwater, and it again becomes highly mobile (*5*, *6*). By contrast, reduced U(IV) species are only sparingly soluble and thus immobile due to precipitation from solution or retention by soils and sediments (*7*−*9*). Under anaerobic conditions, a diverse set of microorganisms reduce U(VI) species to highly insoluble uraninite UO_2 (*9*−*15*). These observations have prompted the investigation of biological reduction as a means of immobilizing uranium in the environment, thereby limiting its migration (*8*−*18*).

We evaluated the feasibility of this approach using a column packed with U(VI)-contaminated soil obtained from the S-3 waste disposal area at the U.S. DOE BWXT Y-12 site in Oak Ridge, TN. The soil and groundwater at the site are highly contaminated with uranium and other pollutants discharged into the S-3 ponds (*19*). Near the source (∼20 m), contaminated groundwater has a low, highly buffered pH (3.5) and a high concentration of U(VI) (up to ∼60 mg/L), nitrate (as high as 40 000 mg/L), sulfate (1000 mg/L), and aluminum (500 mg/L). Soil within this zone has high levels of precipitated or sorbed U(VI) (up to 800 mg/kg), calcium, aluminum, and phosphate. Although sorption or precipitation of U(VI) does provide a natural mechanism of U(VI) attenuation at the site, groundwater U(VI) levels remain unacceptably high, underscoring the need for a more effective means of immobilization. To date, studies of the bioreduction of U(VI) have mostly been performed in homogeneous solutions. These studies demonstrate that many microorganisms have the ability to rapidly reduce U(VI) to U(IV) (*9*−*11*, *13*, *14*, *16*). However, relatively few studies have examined the bioreduction of U(VI) in heterogeneous soil under flow-through conditions, particularly in such a highly contaminated soil (*20*). The present study was therefore aimed at evaluating the bioreduction of U(VI) in soil columns as part of a larger field-scale investigation.

Materials and Methods

Soil Column Preparation. A contaminated soil core sample (FWB 104-00-38) was collected from a depth of 11.6−13.1 m from the DOE NABIR field research center (FRC) site in Oak Ridge, TN. The groundwater at this depth is strongly acidic (pH ∼3.5) as a result of nitric acid leached from the S-3 ponds. The soils are derived from weathered, interbedded shale and

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limestone. The solid phase is dominated by clay and silt sized particles with illite being the primary phyllosilicate. Fe(III) and Al(III) (hydr)oxides are abundant (>3%), typically present as coatings on the clay minerals. The soil was initially characterized for its carbonate-extractable U(VI) (by 0.1 M Na_2CO_3 in 72 h) because carbonate is known to be effective in extracting U(VI) from contaminated soils (*21*, *22*). The contents of extracted U, NO_3^-, SO_4^{2-}, and PO_4^{3-} in soil were about 450, 780, 900, and 940 mg/kg, respectively.

The moist soil (containing ~12% water) was gently crushed into small aggregates, which were then carefully packed into a glass column (25 × 150 mm) fitted with two end plugs and polypropylene meshes. The amount of soil added to the column was 89.3 g on a dry weight basis, and the porosity of the packed soil was approximately 45%. The column was purged with CO_2 for 1 h before equilibration with the initial influent solution to minimize entrapped air bubbles during wet-up.

All solutions were stored in Tedlar bags and purged with a CO_2/N_2 (20:80% vol/vol) gas mixture prior to injection. The upward flow velocities through the column were maintained at 0.2 mL/min (~1.34 m/day) for all influent solutions using an ISCO high-precision pump. The column effluent was collected periodically using a fraction collector to monitor pH and the concentrations of NO_3^-, SO_4^{2-}, Cl^-, Br^-, U(VI), and other metal ions. The column was first saturated with a solution of 10 mM KCl and 10 mM NaCl and adjusted to pH 4 with 0.1 M HCl. To determine the hydrodynamic flow characteristics of the column, 0.5 mM KBr was added to the acidic salt solution after column saturation and was subsequently fed through the column for 3 days. The equilibration and tracer studies resulted in the flushing of indigenous pore water metals and anions including U, Al, Mg, Ca, and NO_3^-. The soil was then conditioned for ~20 h by flushing with a solution consisting of 30 mM $NaHCO_3$, 30 mM $KHCO_3$, and 5 mM Na_2SO_4 at pH 7.0 (adjusted by CO_2). On the basis of the concentration of U(VI) in the column effluent, less than 3% of the solid-phase U(VI) was removed during the preceding steps.

Soil Column Operation for U(VI) Reduction. After flushing and conditioning, the soil column was operated anaerobically with a closed-loop continuous recirculation of effluent back through the column. All flow-paths were made of nonmetallic PEEK, stainless steel, or glass to keep the system anaerobic. A 150-mL serum bottle with a butyl rubber stopper was used as the reservoir for recirculation of the column leachate. The reservoir was filled with 80 mL of the bicarbonate−nutrient solution consisting of $NaHCO_3$ (30 mM), $KHCO_3$ (30 mM), trimetaphosphate (3 mg/L), and NH_4Cl (10 mg as N/L) with a nitrogen headspace. Ethanol was added to the reservoir to an initial concentration of 3 mM, and the system recirculation was then initiated (day 1) at a constant flow rate of 0.2 mL/min. Samples (2 mL) were withdrawn from the reservoir using a sterilized syringe weekly and replaced by the same volume of oxygen-free bicarbonate solution. Neither ethanol consumption nor U(VI) bioreduction were observed in the soil column between days 1 and 30.

The column was subsequently inoculated with 2 mL of a denitrifying culture (approximately 5.5 mg dry weight as biomass) via the three-way inlet valve. The culture was from an anaerobic, denitrifying fluidized bed reactor (FBR) inoculated with a denitrifying bacterial enrichment from a monitoring well at the same FRC site. Recent studies have shown that the FBR biomass was capable of rapidly reducing both sulfate and U(VI) and contained diverse microbial populations (*15*). The column was operated continuously after inoculation, and the effluent was sampled weekly. To maintain a constant volume in the reservoir, 2 mL of bicarbonate solution was used to replace the sample volume after each sampling event. Ethanol was supplemented once a week after day 64. In this case, the sample volume was replaced by adding 0.2−0.4 mL of 1 M ethanol and 1.6−1.8 mL of bicarbonate solution.

A subsample (0.1 mL) was immediately transferred to a 10% phosphoric acid solution and analyzed for U(VI) concentration. Another subsample (0.5 mL) was acidified with 1 M HCl (0.05 mL) and sealed in a glass vial for the analyses of ethanol and acetate. The sample pH was measured, and major anion concentrations were monitored. Selected headspace samples were also analyzed for methane production when a positive pressure was noted in the reservoir.

Analytical Methods. An inductively coupled plasma atomic emission−mass spectrometer (ICP−MS) (Thermo Jarrell Ash PolyScan Iris Spectrometer) was used for the analysis of total uranium and metal concentrations in the initial acidic and bicarbonate flushing effluent samples. However, during the biostimulation experiments, U(VI) concentration was determined by the steady-state phosphorescence technique, which is specific for the detection of hexavalent U(VI) since tetravalent U(IV) does not phosphoresce (*18*). The method involves the addition of an aliquot of sample into deoxygenated phosphoric acid (10%) in a quartz cell. The measured phosphorescence intensity is directly proportional to the concentration of U(VI) in solution. All measurements were performed with a Fluorolog-3 fluorescence spectrometer equipped with both excitation and emission monochromators (Johin-Yvon-SPEX instruments, New Jersey). Emission spectra were collected from 482 to 555 nm with an excitation wavelength of 280 nm. The peak intensity (515.4 nm) was used to calculate the solution U(VI) concentration.

Anions (including NO_3^-, 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) (*7*). Ethanol, acetate, and methane were analyzed with a HP6890 gas chromatograph equipped with a FID detector as described elsewhere (*23*).

To validate that the bioreduction of U(VI) was occurring on the soil, X-ray absorption near edge structure (XANES) spectroscopy was used to determine the oxidation state of uranium after completion of the experiment. The column was sacrificed, and soil samples were removed from the bottom, middle, and top sections of the column. Samples were dried in an anaerobic glovebox, mounted on a Teflon plate, and sealed with a Kapton polymide film to prevent oxidation while minimizing X-ray absorption. XANES spectra were collected on beamline 13-BM-C (GSE-CARS) at the Advanced Photon Source (APS). The APS ring was operated at 7 GeV with a current of 100 mA, and energy selection was accomplished with a water-cooled Si(111) monochromator. Higher-order harmonics were eliminated by detuning the monochromator ~40%. Fluorescence spectra were recorded by monitoring the U $L_{III}\alpha$ fluorescence with a 16-element Ge semiconductor detector. Incident and transmitted intensities were measured with in-line ionization chambers. The energy range studied was −200 to +500 eV about the $L_{III}\alpha$-edge of U (17.166 keV). Between two and four individual spectra were collected and averaged for each sample. The spectra were then analyzed using IFEFFIT and WinXAS software (*24*, *25*). Fluorescence spectra were normalized and background subtracted, and the atomic absorption was normalized to unity. First derivative XANES spectra were smoothed with 17.6% Savitsky−Golay smoothing. The relative amount of reduced U(IV) in each sample was determined by fitting a series of Gaussian functions to the smoothed derivative spectra using PeakFit v4 (AISN Software Inc). The ratio of the amplitudes of the Gaussian functions centered at the U(IV) and U(VI) first derivative inflection points (17.173 and 17.176

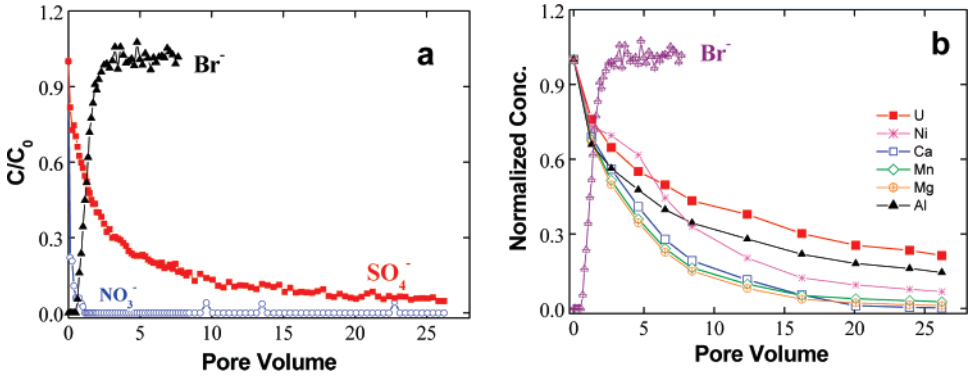

FIGURE 1. Elution profiles of major anions, uranium, and metal ions from a contaminated soil column (2.5 × 15 cm) by a mixture of 10 mM KCl and 10 mM NaCl at pH 4. KBr (0.5 mM) was added as a tracer. C/C_0 is the normalized concentration either to the initial influent concentration (for Br^-) or to the initial effluent concentration for other anions and metals, which were not present in the influent solution.

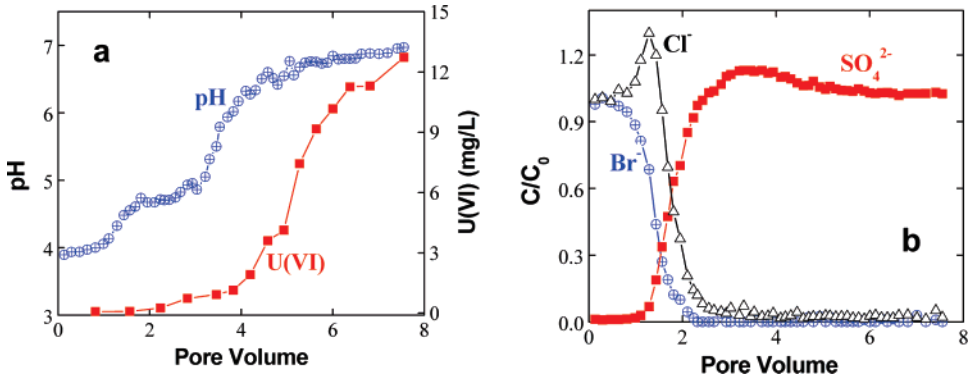

FIGURE 2. pH and concentration profiles of U(VI), Br^- tracer, Cl^-, and SO_4^{2-} during leaching of column with a mixture of 30 mM $NaHCO_3$, 30 mM $KHCO_3$, and 5 mM Na_2SO_4 at pH ~7. C/C_0 is the normalized concentration either to the initial influent concentration (for SO_4^{2-}) or to the initial effluent concentration for Br^- and Cl^-, which were not present in the influent solution.

keV, respectively) was related to U(IV)/(VI) proportions using five standards having U(VI) percentages ranging from 10 to 90%. The uncertainty of the fitting routine is ±10%.

Results and Discussion

Leaching of Metal Ions and Anions during Soil Conditioning. The soil column was initially leached with an acidic NaCl/KCl solution that was spiked with a Br^- tracer to quantify the hydrodynamic flow conditions of the column and to remove metal ions, NO_3^- and SO_4^{2-}. The Br^- breakthrough curve was fairly symmetric exhibiting only slight tailing near equilibrium (at $C/C_0 = \sim 1$) (Figure 1). The location of $C/C_0 = 0.5$ occurred at ~1.28 pore volumes (PVs) rather than 1 PV, indicating the possibility of sorptive retardation and/or slight physical nonequilibrium processes, the latter of which is the more likely explanation because (i) the influent solution contained nearly 2 orders of magnitude higher concentrations of Cl^- than Br^- and (ii) the soil column was packed with moist soil aggregates. This conclusion was also supported by previous studies that these soils in an undisturbed state can exhibit physical nonequilibrium due to a time-dependent diffusion process into matrix aggregates (*26*, *27*). The soil was then treated with bicarbonate to raise the pH to a value of ~6.5. This sequence was similar to the proposed field operations because direct neutralization of the soil without the prior acidic flush would have resulted in precipitation of large quantities of aluminum hydroxide and calcium or magnesium carbonates, clogging flow paths within the field soil and the column. The initial acidic flush also removed the bulk of the nitrate, an oxidant and inhibitor of U(VI) bioreduction (*8*, *28*).

During the initial phase of soil conditioning, the desorption and leaching of SO_4^{2-} was significantly retarded, relative to NO_3^- and the breakthrough of the Br^- tracer (Figure 2a). More than 99% of nitrate was removed in <2 PVs. However, significant amounts of SO_4^{2-} remained in the soil even after it was washed with ~27 PVs of the acidic NaCl/KCl solution. This observation may be partially attributed to a slow desorption of sorbed SO_4^{2-} because the soil contained significant iron- and aluminum-oxyhydroxide coatings that possess positively charged surface sites below pH 8. More importantly, perhaps, it may have originated in part from the dissolution of sulfate-bearing minerals such as basaluminite [$Al_4(OH)_{10}SO_{4,am}$], as noted next. Desorption and leaching of metals (such as Ca, Al, Mg, Mn, and Ni) were also significantly retarded (Figure 2b), as expected based on their known reactive behavior with soil minerals. Additionally, the leaching of Ca, Mg, and Mn followed a pattern similar to that of SO_4^{2-}, suggesting that these ions are likely associated or coprecipitated with sulfate and Al-oxyhydroxides, such as basaluminite. Basaluminite is a metastable solid phase, but its precipitation is kinetically favored under the site-specific conditions at the FRC where the groundwater has very high Al and SO_4^{2-} concentrations (*19*, *29*).

During the acidic flush, the effluent concentration of U(VI) decreased slowly from ~13 to ~1.7 mg/L, but the total amount of U(VI) leached constituted <3% of the total carbonate-extractable U(VI) in the soil. The rate of U(VI) desorption was the slowest among the metal ions (Figure 1b). These observations may be explained partly by a slow dissolution of solid-phase U(VI) species such as uranyl-phosphate minerals, the dominant solid-phase U(VI) species in this soil (*30*). In particular, the U(VI) concentration could have been controlled by the slow dissolution of calcium−uranyl−phosphate or autunite [$Ca(UO_2)_2(PO_4)_2 \cdot 10H_2O$] since the soil contained relatively high concentrations of phosphate and calcium. Autunite precipitates from natural groundwater and is generally insoluble at about neutral pH conditions (in the

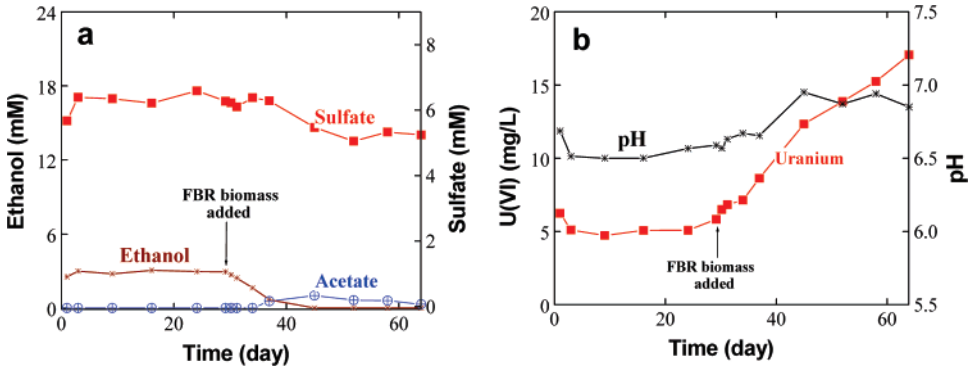

FIGURE 3. Changes in (a) the concentrations of ethanol, acetate, and SO_4^{2-} and (b) the U(VI) concentration and solution pH during the first 2 months of the biostimulation experiment. Inoculation of column with FBR biomass was performed on day 30.

absence of carbonates). In a study of uranium phases in contaminated Fernald soil in Ohio, Buck et al. (*2*) also reported that the $Ca(UO_2)_2(PO_4)_2{\cdot}xH_2O$ mineral was among the most abundant uranium phase identified using energy-dispersive X-ray spectroscopy and electron diffraction techniques.

During the bicarbonate flush, the effluent pH increased gradually, and an inflection occurred between pH ~4.5 and 5 due to speciation changes of surface bound Al or Al−oxyhydroxides in the soil, which buffered the system (Figure 2a). As the pH increased, desorption of U(VI) increased, with a rapid increase in effluent U(VI) when the pH increased above 6. Metal ions other than U(VI) decreased to low or nondetect levels at pH >6 (data not shown), but U(VI) concentrations continued to increase until the flow was stopped. A likely explanation is carbonate-mediated dissolution of calcium−uranyl−phosphate minerals at higher pH conditions (pH >6) (*19*) and the formation of soluble uranyl carbonate species such as $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$. The overall dissolution reaction can be written as

$$Ca(UO_2)_2(PO_4)_{2(s)} + CO_3^- \rightarrow UO_2(CO_3)_3^{4-} + Ca_3(PO_4)_{2(s)}$$

In fact, carbonate and bicarbonate are commonly used for the complexation and extraction of U(VI) in soil, and the efficiency of extraction increases with increasing concentration of carbonate (*8*, *19*, *31*).

The elution behavior of Br^-, Cl^-, and SO_4^{2-} was intriguing because a snow-plow effect was observed for Cl^- and SO_4^{2-} (Figure 2b). After flushing with approximately one pore volume of bicarbonate solution (supplemented with 5 mM SO_4^{2-}), the effluent Cl^- concentration increased greatly (C/C_0 ~1.3), suggesting a significant desorption of sorbed Cl^- from soil solids. This phenomenon is attributed to the competitive sorption of SO_4^{2-} on variable, positively charged soil minerals at pH below ~5 and thus the displacement of previously sorbed Cl^- during the initial bicarbonate wash. Such a snow-plow effect has been described numerically by Barry and co-workers (*32*). Variable charge sites in this soil are likely from Fe−oxides and precipitated Al−oxyhydroxides. Indeed, the breakthrough of SO_4^{2-} was significantly retarded, with a complete breakthrough after ~2 PVs (Figure 2b). However, the effluent SO_4^{2-} concentration continued to increase and reached a maximum at ~3 PVs (C/C_0 ~1.2, where C_0 in this case is the final effluent concentration). This elevated SO_4^{2-} concentration (C/C_0 >1) can be attributed to the increase in soil pH (>6 at PV = 3) during the bicarbonate wash. This caused a decrease in surface positive charge (or fewer sorption sites) on Al− and/or Fe−oxyhydroxides and thus increased the desorption of SO_4^{2-}.

Biostimulation and U(VI) Reduction. During bicarbonate conditioning of the soil, the column effluent pH increased from ~4 to 6.7 (Figure 2a). The column was then maintained in a closed-recirculation state with an in-line reservoir where 3 mM ethanol was periodically added. Ethanol was selected as the electron donor because it supports the growth of sulfate-reducing bacteria (SRB) such as *Desulfovibrio* (*23*) and iron-reducing bacteria (FeRB) such as *Geobacter* spp. (*9*, *33*). Both SRB and FeRB are known to be effective in the reduction of U(VI) to U(IV) (*10*, *11*, *33*). Microbial enrichments with FRC soil in close proximity to the field site demonstrated the presence of both iron- and sulfate-reducing microorganisms. In addition, serial dilutions of contaminated soil (13−15 m) indicated that iron-reducers were present at 50−100 cells mL^{-1} and that sulfate reducers were present at a level of 250−1000 cells mL^{-1}. These results indicated that FeRB and SRB were present in the soil but at low levels. During the first month of column operation, the pH of the recirculated fluid slowly decreased from ~6.7 and stabilized at 6.6 for more than 20 days (Figure 3a). This initial decrease in pH was likely caused by the residual acidity from soil aggregates. Throughout this biostimulation period, there was no evidence of ethanol consumption or reduction of U(VI) or SO_4^{2-}. This suggested that the initial populations of ethanol-degrading microorganisms and microorganisms capable of uranium reduction were either removed or killed during the column construction and flushing or that the organisms did not respond to stimulation under the tested conditions. High toxic metal concentrations and low pH in the soil may have severely limited the viable populations initially present. These observations suggested that inoculation of the soil was required. This step also simulated field operations in which the treated effluent from a denitrifying fluidized bed reactor was to be recirculated through the subsurface.

Accordingly, on day 30, the soil column was inoculated with a low level of biomass suspension (~5.5 mg) obtained from a pilot-scale denitrifying FBR that would later serve as an innoculum for the field study (*15*). Immediately after inoculation, ethanol degradation commenced with complete consumption by day 45 (Figure 3a). During the same period, up to 1 mM acetate accumulated in the pore water, and the SO_4^{2-} concentration simultaneously decreased from 6.1 to 5.4 mM. The consumption of ethanol associated with acetate accumulation together with sulfate removal is typical of sulfate reduction by ethanol-degrading SRB (e.g., by *Desulfovibrio* spp.). The reaction may be written as (*23*, *34*)

$$2CH_3CH_2OH + SO_4^{2-} = 2CH_3COO^- + 2H_2O + H_2S \quad (1)$$

SRB were present in the FBR biomass used as an inoculum, as confirmed by inhibition of sulfate reduction in the presence of molybdate, a specific SRB inhibitor, and by an increase in the level of the dissimilatory sulfite reductase gene (*dsr*A) in the biomass (with ~80% nucleotide identity to *dsr*A of *Desulfovibrio vulgaris*) (*15*).

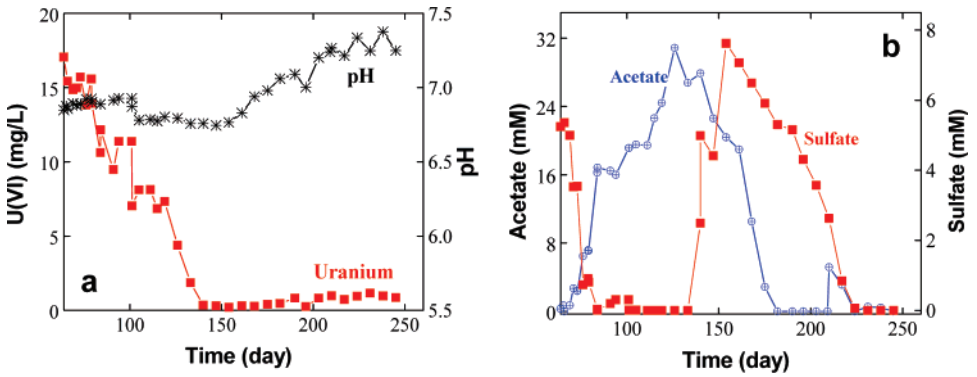

FIGURE 4. Changes in (a) the concentration of U(VI) and solution pH and (b) sulfate and acetate concentrations during biostimulation with ethanol.

From days 30−45, pore water SO_4^{2-} concentrations decreased by 0.77 mM with a concomitant increase in pore water pH. During the consumption of 0.77 mM sulfate, reaction 1 predicts the consumption of ~1.5 mM ethanol and production of ~1.5 mM acetate. In addition, if reaction 1 alone controlled the solution pH, the expected pH would have decreased (based on the equilibrium expressions, mole balances on acetate and bisulfide, and a charge balance). But, the actual ethanol consumption was 3 mM, only 1 mM acetate accumulated, and the pore water pH increased from 6.6 to 6.9. These observations indicate that some acetate was consumed in a reaction that generates alkalinity. One possibility is the coupled consumption of acetate and sulfate by reaction 2 as described by Wu et al. (*23*) and Hansen (*34*)

$$CH_3COO^- + SO_4^{2-} = 2HCO_3^- + HS^- \quad (2)$$

If reaction 2 by itself controlled solution pH, the expected pH would be 9.5, but the soil buffering capacity could explain a lesser increase. A second likely acetate sink and alkalinity source is iron respiration as described by reaction 3

$$CH_3COO^- + 8Fe(OH)_3 = 8Fe^{2+} + 13OH^- + 2CO_3^{2-} + 7H_2O \quad (3)$$

Reaction 3 generates large amounts of alkalinity and also tends to increase the system pH. Although $Fe(OH)_3$ is shown as the assumed electron acceptor in reaction 3, a similar stoichiometry could be obtained for other Fe(III) mineral phases. Fe(III) −oxides are present in the FRC soil, and iron-reducing bacteria such as *Geobacter* spp. and others consume acetate (*33*). *Geobacter* spp. has been recently detected in the field samples relevant to this study, and it has also found in a different area at the FRC (*35*). Moreover, the inoculum used for the soil column came from a FBR that contained FeRB (isolated initially from the same groundwater used to create the enrichment culture seed for the FBR). Sequence analysis of SSU rRNA genes from the FBR biomass indicated the presence of a small number of G + C bacteria capable of iron reduction (*15*). Additional evidence of FeRB activity was the presence of Fe(II) in the effluent and changes in soil color over time. Initially, the soil color was tan to brown as a result of Fe(III)−oxide coatings. After the onset of ethanol degradation, the soil became gray, suggesting the loss of Fe(III)−oxide coatings on the soil, and at longer times the soil became dark green or black. These latter changes are consistent with the formation of ferrous minerals such as ferrous sulfide precipitates and green rusts.

Accumulation of acetate in the pore water indicated that reactions 2 and 3 were initially slower than reaction 1. This was likely because of the small numbers and slow growth of acetate-utilizing SRB and FeRB initially present. During the initial period of biostimulation, the concentration of U(VI) in the pore water increased from 5 to ~12.3 mg/L, peaking on day 64 at ~17 mg/L (Figure 3b). These results appear to contradict the notion that increased microbial activity would decrease aqueous U(VI) concentrations; however, during the same period, the pH of the pore water increased from 6.5 to 6.9 (Figure 3b). These observations suggest that, during this time period, the rate of U(VI) desorption or dissolution exceeded the rate of microbial reduction. U(VI)−pH adsorption envelopes for these soils indicate that U(VI) sorption is strongly pH and bicarbonate concentration dependent (*36*). As noted earlier, carbonate and bicarbonate solutions are commonly used to extract U(VI) from contaminated soils (*22*). If microbial reduction of U(VI) was slower than the desorption or dissolution rates, the aqueous U(VI) concentration should increase as observed. With sufficiently high microbial activity, the rate of reduction would balance the rate of uranium solubilization, and the U(VI) concentration in solution would be expected to plateau. With continued electron donor addition and an increase in microbial biomass, the reduction rate would be expected to exceed the desorption/dissolution rate, and soluble U(VI) should decrease. Accordingly, additional ethanol was provided after day 64. The ethanol was consumed within 1 week; thus, a weekly feeding frequency was adopted thereafter. A concentration of 3 mM ethanol was provided each week for 3 weeks followed by a reduction in concentration to 1.5 mM ethanol for the duration of the study.

On day 85, the aqueous U(VI) concentration dropped from ~17 to 12.2 mg/L after the introduction of ethanol. The pore water U(VI) concentration decreased to 0.3 mg/L by day 140 (Figure 4a), and the pH remained steady at 6.8 until day 160. With continued ethanol additions, sulfate concentrations continuously decreased reaching nondetect levels by day 85 (Figure 4b). As the sulfate concentration decreased, acetate accumulated, reaching concentrations as high as 31 mM. To stimulate acetate-degrading SRB and minimize acetate accumulation, sulfate was added to the soil column 3 times (on days 133, 140, and 147). By day 153, the sulfate concentration reached ~7.5 mM. Pore water acetate concentrations fell rapidly with concomitant reduction of sulfate. By day 182, pore water acetate concentrations were negligible, and by day 223, no sulfate remained in solution. During this period, the pore water U(VI) concentration remained relatively low (~0.3 mg/L), although it increased to ~0.8 mg/L as the system pH increased from 6.8 to ~7.4 by day 245. The lack of significant U(VI) in solution as the pH increased is an indirect indication that solid phase U(VI) had been reduced to sparingly soluble U(IV). This observation was later confirmed with XANES analysis.

On day 216, gas pressure within the headspace of the reservoir increased significantly, and methane (~5%) was detected. By day 245, the reservoir headspace contained

~50% methane, indicating significant methanogenesis. Methanogens are strict anaerobes and prefer a pH between 6.5 and 7.8. The growth of methanogens is relatively slow, and acetate-utilizing methanogens would be out-competed by SRB in the presence of sulfate. Therefore, methane production was detected after ~200 days when the sulfate levels declined. Although their impact on the rate of U(VI) reduction is not known, H_2-utilizing methanogens can indirectly compete for available electron donors via syntrophic ethanol degradation (*23*, *34*). When methanogenesis begins, SRB could function as acetogens (reaction 4). The reaction byproduct, acetate, could then be utilized by methanogens (reaction 5), resulting in the formation of methane (1.5 mol per mol of ethanol consumed) and a net decrease in pH due to carbon dioxide formation.

$$2CH_3CH_2OH = CH_3COOH + 2CH_4 \quad (4)$$

$$CH_3COOH + H_2O = CO_2 + CH_4 \quad (5)$$

The formation of methane could have several adverse effects on U(VI) reduction: (i) methanogens are not known to reduce U(VI); however, they compete for the electron donor with organisms that do reduce U(VI); and (ii) methane has a low solubility (1.37 mM at 20 °C); therefore, a gas phase can develop within the porous media—in this case, the packed column. This scenario could cause a decrease in hydraulic conductivity, clog the soil media, or shield mineral surfaces, thereby reducing U(VI) bioavailability. The slightly increased concentration of U(VI) in the pore water after day 216 (Figure 4a) may have been due to such interactions. These observations suggest that, although it might prove technically challenging, the control of methanogenesis would likely be beneficial to the bioreduction of U(VI) and its subsequent immobilization in soil. One possible solution might be to maintain constant, low levels of a terminal electron acceptor (e.g., SO_4^{2-}).

Validation of U(VI) Reduction by XANES. XANES spectroscopic analysis confirmed the presence of reduced U(IV) species on the solid phase following dissection of the column upon completion of biostimulation (Figure 5). The soil column was disassembled and sectioned into three parts (bottom, middle, and top) inside an anaerobic chamber. Reduced U(IV) was present in all sections, particularly in the middle of the soil column, where approximately 47% of the total uranium was present as a U(IV) species (Figure 5). Lower percentages of U(IV) were found in the bottom and top sections (~10 and 20%, respectively). These results provided direct evidence that U(VI) in the contaminated soil is biologically reduced and immobilized.

The fact that residual U(VI) remained in the soil column after more than 8 months of biostimulation (even after a low effluent U(VI) concentration was detected at day 140) may be due to one or more of the following factors: (i) a large portion of U(VI) is sorbed inside soil aggregates and not bioavailable; (ii) methanogenesis decreased the rate of U(VI) bioreduction; (iii) methane gas production decreased the bioavailability of U(VI) at some locations within the column; or (iv) some U(IV) might have been reoxidized to U(VI). Some reoxidation is likely because the column sat static for approximately 1 month prior to destructive sampling and analysis by XANES. Although the entire system was kept in a closed loop system, oxygen might have slowly diffused through the Teflon end plugs at the bottom and top of the column. This might explain the lower percentages of U(IV) in the top and bottom sections of the column. Moreover, reoxidation of U(IV) species can be rapid in the presence of O_2. Previous studies have shown that reoxidation of U(IV) can occur within a few hours to days upon contact with air (*6*, *22*). In a recent study (*22*), we observed rapid reoxidation,

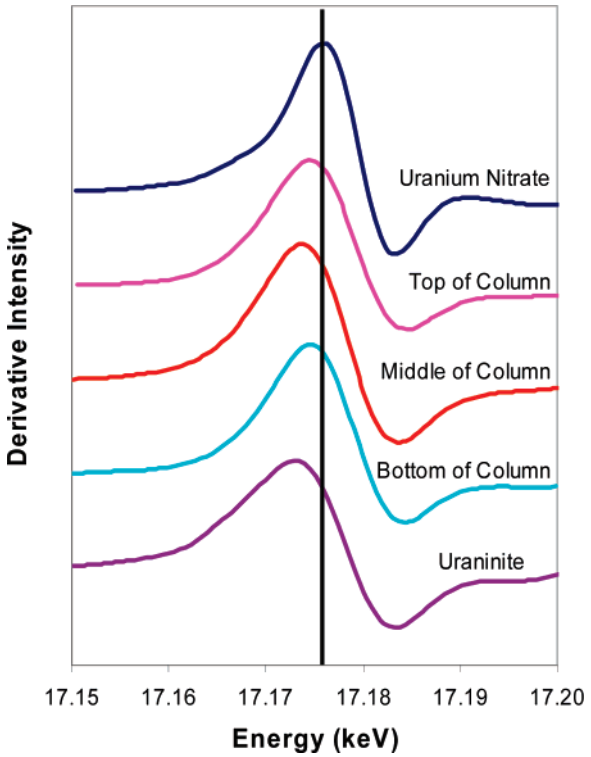

FIGURE 5. First derivative X-ray absorption near edge structure (XANES) spectra of uranium in soil samples taken from the top, middle, and bottom sections of the soil column, which had been subjected to biostimulation for approximately 9 months. Reference standard spectra of uraninite [U(IV)] and uranyl U(VI) nitrate were included for comparison.

with a half-life on the order of ~1 h when microbially reduced U(IV) precipitates were vigorously stirred in open air. Other oxidants such as Mn−oxides or nitrate and nitrite can inhibit microbial reduction of U(VI) or cause reoxidation of reduced U(IV), although we have no evidence that such oxidants played a role in this study (*8*, *37*).

This study demonstrates that oxidized forms of U(VI) (either sorbed or precipitated) in contaminated soil can be reduced to relatively insoluble U(IV) by the recirculation of groundwater amended electron donors when competent microbial populations are present and active. The results are consistent with previous findings that microbial reduction of U(VI) to U(IV) offers a potentially effective remediation strategy to immobilize uranium in soil and groundwater (*8*, *9*, *13*, *16*, *18*), and they support the proposed sequence of field operations planned for the NABIR FRC field site. During microbial reduction of U(VI), other reduced minerals, such as iron sulfides, are produced. These minerals help to maintain a low redox condition in soil and may serve as a reservoir of reducing power capable of scavenging oxygen and other oxidants that may enter the system (*38*−*40*). However, our results indicate that significant technical hurdles remain; future studies must address the stability of reduced U(VI), prevention of U(IV) reoxidation, and potential competitive processes for available electron donor(s).

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