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Pilot-Scale in Situ Bioremediation of Uranium in a Highly Contaminated Aquifer. 2. Reduction of U(VI) and Geochemical Control of U(VI) Bioavailability

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In situ microbial reduction of soluble U(VI) to sparingly soluble U(IV) was evaluated at the site of the former S-3 Ponds in Area 3 of the U.S. Department of Energy Natural and Accelerated Bioremediation Research Field Research Center, Oak Ridge, TN. After establishing conditions favorable for bioremediation (Wu, et al. *Environ. Sci. Technol.* **2006**, *40,* 3988−3995), intermittent additions of ethanol were initiated within the conditioned inner loop of a nested well recirculation system. These additions initially stimulated denitrification of matrix-entrapped nitrate, but after 2 months, aqueous U levels fell from 5 to ∼1 μM and sulfate reduction ensued. Continued additions sustained U(VI) reduction over 13 months. X-ray near-edge absorption spectroscopy (XANES) confirmed U(VI) reduction to U(IV) within the inner loop wells, with up to 51%, 35%, and 28% solid-phase U(IV) in sediment samples from the injection well, a monitoring well, and the extraction well, respectively. Microbial analyses confirmed the presence of denitrifying, sulfate-reducing, and iron-reducing bacteria in groundwater and sediments. System pH was generally maintained at less than 6.2 with low bicarbonate level (0.75−1.5 mM) and residual sulfate to suppress methanogenesis and minimize uranium mobilization. The bioavailability of sorbed U(VI) was manipulated by addition of low-level carbonate (<5 mM) followed by ethanol (1−1.5 mM). Addition of low levels of carbonate increased the concentration of aqueous U, indicating an increased rate of U desorption due to formation of uranyl carbonate complexes. Upon ethanol addition, aqueous U(VI) levels fell, indicating that the rate of microbial reduction exceeded the rate of desorption. Sulfate levels simultaneously decreased, with a corresponding increase in sulfide. When ethanol addition ended but carbonate addition continued, soluble U levels increased, indicating faster desorption than reduction. When bicarbonate addition stopped, aqueous U levels decreased, indicating adsorption to sediments. Changes in the sequence of carbonate and ethanol addition confirmed that carbonate-controlled desorption increased bioavailability of U(VI) for reduction.

Introduction

Uranium is a major groundwater contaminant at U.S. Department of Energy (DOE) sites. To investigate strategies to minimize uranium migration in the subsurface, we constructed a test facility in Area 3 of the Field Research Center (FRC) of the DOE Natural and Accelerated Bioremediation Research (NABIR) program. Area 3 has high levels of uranium in the groundwater (∼50 mg/L) and aquifer solids (up to 800 mg kg^{-1}) (*1*).

Microbial reduction of U(VI) to sparingly soluble and immobile U(IV) is one promising strategy for control of uranium migration (*2*−*6*, *46*). Under anaerobic conditions, many microorganisms mediate this transformation (*7*, *8*), including Fe(III)-reducing bacteria (FeRB), such as *Shewanella* spp. and *Geobacter* spp. (*9*−*11*); sulfate-reducing bacteria (SRB), such as *Desulfovibrio* spp. (*12*−*17*) and *Desulfosporosinus* spp. (*18*, *19*); a butyrate-utilizing *Desulfotomaculum* spp. (*20*); *Clostridium* spp. (*21*); *Salmonella* (*22*); *Cellulomonas* (*23*); and denitrifying *Acidovorax* spp. (*24*). However, maintenance of a stable U(VI)-reducing microbial community in a complex anaerobic ecosystem is not straightforward. Subsurface heterogeneity affects electron donor delivery, geochemical processes control partitioning of U(VI), and ecological interactions between microbial populations affect process control. Some ecological interactions are of particular concern. Microbial populations that reduce U(VI) may compete with populations that do not. Methanogens may compete with U(VI)-reducing SRB and FeRB, for instance. This is significant given that methane production has correlated with remobilization of U(VI) (*25*, *26*). Similarly, U(VI)-reducing SRB, such as *Desulfovibrio* spp. (which degrades ethanol and lactate), may compete for sulfate with SRB that do not reduce U(VI), such as the acetate-utilizing *Desulfobacter* spp. and *Desulfotomaculum acetoxidans* and the propionate-utilizing *Desulfobulbus propionicus* (*13*). The nature of the electron donor (such as ethanol or lactate rather than acetate) may influence the selection of SRB type (*13*). For instance, *Geobacter* spp. may compete for acetate with SRBs that do not reduce U(VI). Solid-associated U(VI) resisted reduction when acetate was provided (*27*). Addition of acetate in a field experiment at Rifle, CO initially stimulated *Geobacter*

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spp., and soluble uranium concentrations decreased, but acetate-utilizing SRB subsequently became prevalent, and dissolved U(VI) levels rebounded (*28*). In addition, bioreduced U(IV) can be reoxidized to U(VI) by oxygen, especially in the presence of a high level of bicarbonate (1 M) (*29*). Oxidation of bioreduced U(IV) in a column test using FRC Area 2 sediments occurred together with high methanogenic activity even though typical U(VI)-reducing bacteria (*Geobacter* spp.) were still present (*26*). A thermodynamic analysis (*26*) suggested that high levels of bicarbonate (15 mM) and Ca^{2+} (1 mM) can create conditions suitable for oxidation of U(IV) by residual Fe(III). Development of technologies for bioremediation of uranium thus calls for an integrated understanding of site hydrogeology, geochemistry, and factors influencing microbial ecology (competition, selection, and prevention of reoxidation of bioreduced U(IV)).

Prior to the field test, batch laboratory experiments were used to select the electron donor and to characterize U(VI) sorption/desorption properties of the soil. Ethanol supported faster U(VI) reduction than acetate or lactate (Supporting Information, Figure S1). The results were consistent with those of others who have reported that ethanol supports growth of U(VI)-reducing *Desulfovibrio* spp. (*30*), *Geobacter* spp. (*31*), and *Shewanella* spp. (*32*). Ethanol was therefore selected as the electron donor for field experiments. Sorption and desorption studies using FRC soils revealed that U(VI) adsorption was highly pH dependent (Supporting Information, Figure S2). Peak sorption occurred at ~pH 6.0, consistent with prior studies with uncontaminated FRC soils (*33*). These results indicated that small pH changes near the U(VI) sorption edge, close to pH 6, cause large changes in the concentration of dissolved U(VI).

Bench experiments also previewed field operations. Initial studies focused on nitrate removal. Denitrifying biomass from a pilot-scale fluidized bed reactor (FBR) removed nitrate efficiently with slow reduction of U(VI) (*34*). Biomass from this reactor was a source of organisms for start-up of a full-scale FBR at the field site. The full-scale reactor removed bulk nitrate from water extracted from the treatment zone, and treated effluent from this reactor was used to flush the subsurface (*1*, *35*). To approximate expected field conditions in preliminary laboratory studies, simulated FBR effluent was added to batch microcosms containing sediment that had been washed to remove aluminum, calcium, and bulk nitrate; neutralized; amended with ethanol; and incubated. Reduction of U(VI) occurred concurrently with sulfate reduction in most microcosms, but in 2 of 17 microcosms, sulfate and electron donors were depleted, and U(VI) levels rebounded after an initial decrease (*24*). The results suggested the importance of adequate sulfate and electron donor. Biostimulation operations were also previewed by recirculating a buffered nutrient solution (60 mM bicarbonate, pH 7.0) through a packed column containing sediment from Area 3 and intermittently adding ethanol to the recirculating fluid. Ethanol additions stimulated reduction and immobilization of uranium with aqueous levels decreasing to less than 1.3 μM, but after 200 days of operation, methane appeared and U rebounded to 3.4 μM (*25*). With the use of push−pull tests, another research team investigated immobilization of U(VI) at FRC site Areas 1 and 2. Acetate, ethanol, or glucose was injected along with bromide, a conservative tracer. After a period of incubation, water was extracted, and acetate, ethanol, or glucose ratios relative to bromide were computed. Evidence for removal of nitrate and aqueous U (VI) was obtained, but the complex sorptive interactions of U and the absence of solid-phase data prevented unambiguous determination of U(VI) reduction to U(IV) as a removal mechanism (*5*, *36*).

In this study, we explored the feasibility of in situ bioreduction and immobilization of U(VI) and some of the mechanisms involved. Findings from our preliminary studies and from other researchers informed our choice of baseline operating conditions. An important decision was the operational pH. In general, we operated at a pH of 6.0−6.2—a range that is optimal for sorption but inhibitory to methanogenesis. We also operated at low bicarbonate levels (1.0 to a maximum of 5.0 mM) to minimize desorption. In this article, we provide evidence that this strategy combined with intermittent ethanol addition (1−1.5 mM) stimulated microbial reduction of U(VI) to U(IV) within a hydraulically well-controlled and chemically conditioned treatment zone.

Materials and Methods

Chemicals. Chemicals used in laboratory studies were analytical grade. Chemicals used for fieldwork were industrial grade. Solutions of K_2CO_3 (50%, w/w) and HCl (20%, w/w) were obtained from The Dycho Company (Niota, TN). Industrial-grade ethanol (containing 88.12% ethanol, 4.65% methanol, and 7.23% water) was obtained from Aaper Alcohol and Chemical Co., Shelbyville, KY.

System Design. Figure 1 of the companion article (*1*) illustrates the overall scheme for the in situ well system and its connection to the above-ground treatment system. The above-ground system removed aluminum and calcium by precipitation and nitrate by denitrification (*1*). The below-ground system consisted of a nested recirculation system with a protective outer loop surrounding an inner loop where electron donor was added to stimulate growth of U(VI)-reducing bacteria. Groundwater flow modeling was used to estimate required flow rates (*37*). Components of the below-ground system included an outer loop injection well (FW024), an inner loop injection well (FW104), an inner loop extraction well (FW026), an outer loop extraction well (FW103), and three multilevel sampling (MLS) wells (FW100, FW101, and FW102). The recirculation wells were of diameter 10.16 cm and depth 14.3 m, with 1.9 m screened intervals. Each MLS well contained seven separate sampling tubes (diameter = 1.9 cm) at different depths below ground surface (bgs). MLS wells FW101-2 (sampling at 13.7 m bgs), FW101-3 (12.2 m bgs), FW102-2 (13.7 m bgs), and FW102-3 (12.2 m bgs) were selected for routine monitoring, based on a tracer study (*1*). The flow rate of the inner loop recirculation between FW026 and FW104 was 0.45 L min^{-1}. The outer loop enabled hydraulic control over the treatment zone and prevented invasion of untreated ambient groundwater. The flow rate of the outer loop recirculation from well FW103 to FW024 was 0.45 L min^{-1}. To further minimize entry of ambient groundwater, additional clean water (0.9 L min^{-1}) was injected into FW024 (*1*). The injected water was thus a mixture of treated groundwater (extracted at well FW105 at a rate of 0.25−0.4 L min^{-1}) and Y-12 Plant tap water (pH 8.0, containing 2.82−3.38 mM Cl^-, 0.04−0.048 mM NO_3^-, 0.24−0.26 mM SO_4^{2-}, 0.68−0.75 mM Ca^{2+}, and <0.007 mM aluminum). This water was adjusted to pH ~6 and injected at FW024. Solutions of ethanol and/or K_2CO_3 were periodically added using metering pumps (series MP, Pulsafeeder Inc., Punta Gorda, FL). Extraction of groundwater was accomplished using bladder pumps (QED Environmental Systems, Inc., Ann Arbor, MI).

System Layout and Operational Phases. Field studies began August 24, 2003 (day 1) with start-up of the FBR. From days 9 through 137, the treatment zone was conditioned to create an environment favorable for microbial activity. A detailed description of this period is provided in the companion article (*1*). Start-up of the FBR is also described elsewhere (*35*). The effect of these operations was creation of a hydraulically and chemically distinct region containing lower aqueous phase concentrations of calcium (<1 mM), aluminum (<0.03 mM), nitrate (<1 mM), and pH at ~5−6. Increasing pH to this range increased sorption of U compared

to that of the initial pH of ~3.4. As a result, the concentration of U in the groundwater decreased from ~300 to ~5 μM, but high levels remained in the soil. Ethanol addition began on day 138 (January 7, 2004) and ended on day 535 (February 7, 2005). During this period, water from the outer loop extraction well was reintroduced directly into the outer loop injection well along with additional makeup water, prepared as described above. To minimize accumulation of N_2 within the inner loop, a vacuum stripper removed dissolved N_2 from water recirculated within the inner loop. Degassed water was stored in a tank with He headspace, then reinjected at inner loop injection well FW104. On day 268, nitrate levels in inner loop extraction well FW026 were deemed sufficiently low (<1 mM) to justify removal of the vacuum stripper, and it was taken off line. From day 138 through day 535, ethanol was injected 56 times into FW104. An ethanol stock solution (6.9 g COD L^{-1}) was injected via a metering pump to achieve the desired target concentration (1.0−1.5 mM). No other nutrients were added. To minimize clogging, ethanol injections were intermittent, with a frequency of approximately once per week. From days 137 to 268, injection of ethanol was maintained for 3−7 days, and the average rate of ethanol injection was 163 g as COD per week. The purpose of these prolonged ethanol injections was to expand the biologically active zone. From days 268 to 535, the duration of ethanol addition was decreased to 1−3 days, and the average rate of ethanol injection was 58 g as COD/week. During this period, bioavailability experiments were performed in which a solution of K_2CO_3 (375 mM) was periodically introduced into the inner loop to increase pH and enhance the bioavailability of U(VI). Depending on the experiment, carbonate and ethanol additions were either stopped simultaneously or carbonate addition was allowed to continue for one additional day.

Well Cleaning and Sediment Sampling. To clean clogged well screens, a PVC surge block (10 cm × 15 cm) was attached to a threaded rod and inserted into the fouled well. The block was then lifted up and down 4−6 times in a rapid plunging motion, with a 2 m stroke. This plunging motion detached biofilm on the well screens and drew sediment from the soil matrix surrounding the well screens into the well. Both the sheared biomass and the sediment settled to the bottom of the well where it was available to be pumped out for disposal or collected for analysis in anaerobic serum bottles. The same procedure was used to collect sediment samples from all wells for microbial analysis and uranium analysis. This operation interrupted recirculation for 3−4 h. A small PVC surge block (1.9 cm × 5 cm) was used to retrieve sediment samples from the MLS wells.

Analytical Methods. Chemical oxygen demand (COD) was used as an overall indicator to monitor the consumption of electron donors (ethanol, its metabolite acetate, and others). COD, sulfide, and turbidity were determined using a Hach DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Anions (including NO_3^-, Br^-, Cl^-, SO_4^{2-}, and PO_4^{3-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale, CA), and cations (Al, Ca, Fe, Mn, Mg, U, K, etc.) were determined using an inductively coupled plasma mass spectrometer (ICPMS) (Perkin-Elmer ELAN 6100) as described elsewhere (*39*). Ethanol, acetate, and methane were analyzed with an HP5890 or HP6890 gas chromatograph equipped with an FID detector, as described previously (*40*). Methanol was at low concentrations (<0.1 mM) and so was not routinely monitored.

Groundwater samples for uranium analysis were centrifuged at 10 000 rpm for 5 min and then acidified with 16 N HNO_3 (0.03 mL in 1.2 mL of sample). U(VI) concentration was determined by kinetic phosphorescence analysis using a KPA-11 analyzer (ChemChek Instruments, Richland, WA) (*41*). Uranium content in solids was determined using nitric acid extraction (*38*). X-ray absorption near-edge structure spectroscopy (XANES) was used to determine the oxidation state of uranium. Wet sediment samples were mounted on a Teflon plate and sealed with Kapton polymide film in an anaerobic glovebox to prevent oxidation and then stored anaerobically until analysis. XANES data for samples obtained on days 258, 271, 333, and 409 were collected on beamline 13-BM-C (GSE-CARS) at the Advanced Photon Source (APS), while samples retrieved on day 535 were analyzed on beamline 11-2 at the Stanford Synchrotron Radiation Laboratory (SSRL). Energy selection at the APS was accomplished with a water-cooled Si(111) monochromator, while a liquid N_2 cooled Si(220) monochromator was used at SSRL. Higher order harmonics were rejected by detuning the monochromator 10% at the APS or through the use of a collimating mirror at SSRL. Fluorescence spectra were recorded by monitoring the U L_{III} with either a 13 (APS) or a 30 (SSRL) element Ge semiconductor detector. Incident and transmitted X-ray intensities were measured with in-line ionization chambers. The energy range studied was −200 to +500 eV about the L_{III}-edge of U (17.166 keV). All samples were internally referenced to a U(VI) nitrate standard placed between the second and third in-line ionization chambers. Spectra were collected at ambient temperature and pressure, with 2−4 individual spectra averaged for each sample. Spectra were analyzed using the SixPACK (*42*) interface to IFEFFIT (*43*). Fluorescence spectra were normalized, background subtracted, and the atomic adsorption was normalized to unity. The absorption edge was defined as the half-height position of the XANES spectrum after background subtraction and normalization and was referenced against the edge position of U(VI) nitrate. The relative amount of reduced uranium in sediment samples was determined by comparison of the half-height edge position of each sample to a standard curve (*44*) obtained from samples with varying known mole ratios of U(IV)/U(VI). The uncertainty of this fitting routine is ±5%.

Microbiological Analyses. Denitrifying bacteria, FeRB, and SRB were enumerated using the most probable number (MPN) technique with five tubes for each dilution. The protocol is described in the Supporting Information.

Results and Discussion

Overview of Biostimulation. Biostimulation proceeded through two phases: (1) a denitrification phase, with removal of residual matrix-associated nitrate, followed by (2) a sulfate and U(VI) reduction phase, in which aqueous U concentrations in the groundwater decreased from 5 to 1 μM. In the first phase (days 137−184), nitrate concentrations declined, but sulfate and U concentrations remained relatively unchanged. In the second phase (days 184−535), U concentrations decreased, and beginning on day 201, sulfate concentrations also decreased (Figure 1).

The pH of inner loop injection well FW104 depended upon the addition of K_2CO_3 and varied from 5.2 to 7.9 (Figure 1A). At the inner loop extraction well FW026, pH gradually increased from 5.2 to ~6.3 from day 137 to day 230. Thereafter, the pH of the extraction well was maintained at 5.8−6.2, a range that is below the optimum range of 6.5−7.6 for methanogenesis (*45*). Avoiding methanogenesis was deemed desirable because methanogens compete with U(VI)-reducing SRB and FeRB, possibly preventing stable reduction of U(VI) (*25*); reoxidation of U(IV) seemed to correlate with the onset of methanogenesis (*26*), and poorly soluble methane gas could contribute to aquifer plugging. In the absence of added carbonate, bicarbonate levels ranged from 0.75 to 1.5 mM. When carbonate was added at FW104, bicarbonate levels were maintained at less than 5.0 mM. Maintenance of generally low carbonate levels ensured higher U(VI) adsorption to sediments. Low carbonate levels also prevented

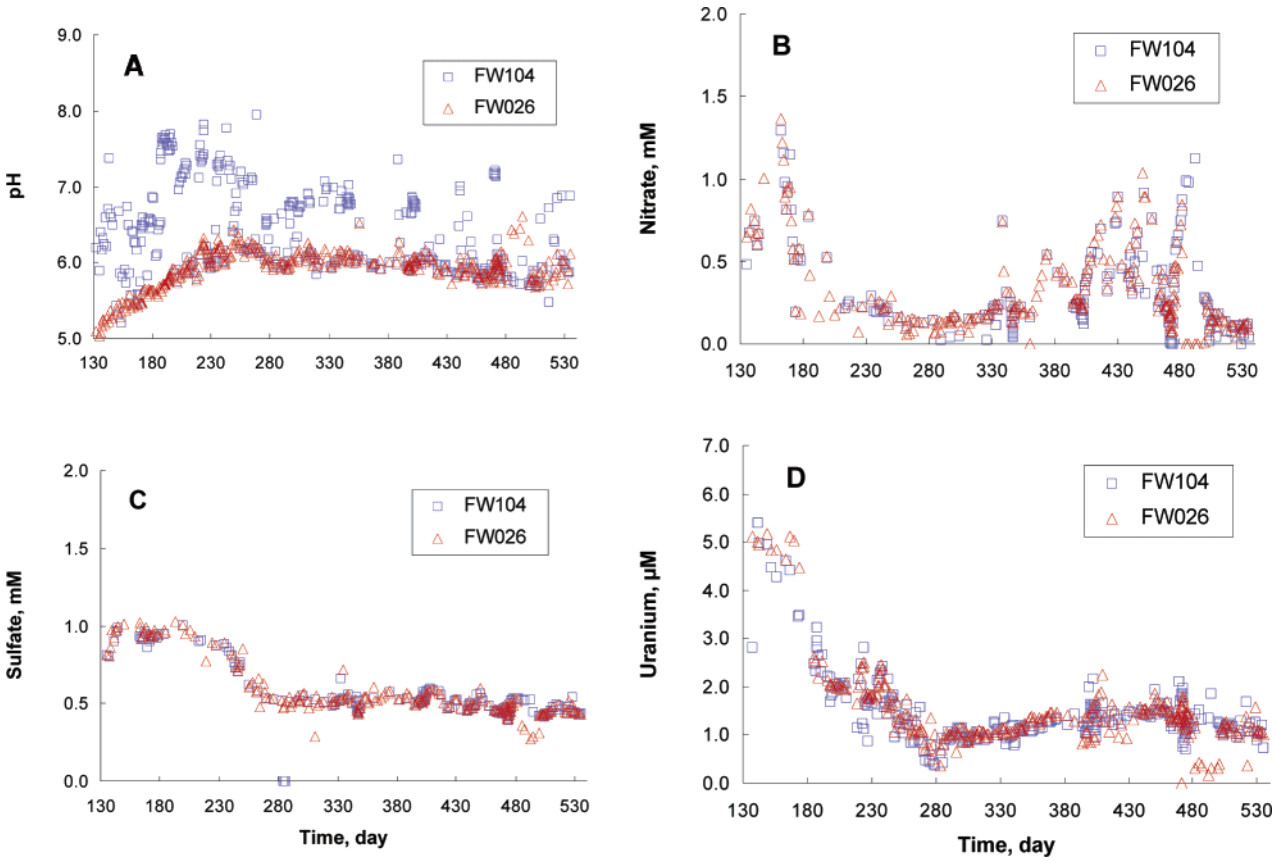

FIGURE 1. Geochemical changes in the inner loop injection well FW104 and extraction well FW026 during in situ biostimulation: (A) pH, (B) nitrate, (C) sulfate, (D) uranium.

conditions favorable for reoxidation of bioreduced U(IV), as noted by Wan et al. (*26*). When ethanol injections began, nitrate concentrations decreased from an initial range of 0.5−1.4 mM to <0.3 mM by day 185 and remained low (0.07−0.3 mM) through day 330 (Figure 1B), suggesting efficient denitrification. From days 330 to 500, nitrate concentrations occasionally increased from low levels of ~0.1 to 0.5 mM or higher, then returned to low levels within a period of 24−48 h, with no ethanol addition. These spikes were attributed to groundwater recharge and influx of water from rain events and/or the release of nitrate trapped within the soil matrix. Afterward, nitrate eventually dropped to 0.1 mM or less. Sulfate concentrations remained unchanged for 2 months after the initiation of ethanol injection (Figure 1C), but beginning on day 201, they began to decrease, indicating development of an SRB population. Sulfate concentrations continued to decrease with time, eventually leveling off around day 265 at around 0.5 mM. The incoming sulfate likely came from the aquifer matrix of the outer loop. Aqueous U concentrations in FW026 (Figure 1D) did not change appreciably prior to day 177, likely because high nitrate levels prevented net U(VI) reduction, but dropped from 5 to <1 μM by day 268. During this period, the average ethanol injection rate was 163 g COD/week. Sequential reduction of nitrate, sulfate, and U(VI) is consistent with results from sediment microcosms (*24*, *25*, *47*). From days 268 to 480, U concentrations in FW026 and FW104 increased slightly then stabilized at 1.4−1.5 μM for several months, possibly due to the lower ethanol injection rate during that period (58 g COD/week) and continuous release of U(VI) from the sediment matrix near FW026. U concentrations dropped below 1 μM by day 535. Studies performed after day 535 to assess the limits of U(VI) reduction and stability of reduced U(IV) are the subject of future reports (in preparation).

Well clogging occurred in both the injection and extraction wells of the inner loop but mainly on the screen of the inner loop injection well FW104. Two types of clogging agents were identified. During the early period of biostimulation (days 69−184), clogging was caused by deposition of a small amount of aluminum hydroxide precipitate (*1*). Thereafter, clogging was due to formation of microbial biofilms. On day 212, a pink-colored biofilm appeared in the tubing of the recirculation line between FW026 and FW104. A submerged camera confirmed that biofilm was present on the injection well screen on day 422. Cleaning of the well with a surge block every ~1.5 months restored hydraulic conductivity and generated a small volume of wastewater (20−30 L). Figure S3 in the Supporting Information illustrates changes in water level in the inner loop wells for days 140−480: an increase in the water level in the injection well and a drop in the water level within extraction well FW026 indicated clogging. After cleanup, water levels returned to baseline levels. As expected, the inner loop injection well FW104 clogged more frequently than extraction well FW026. Clogging was not observed in the outer loop injection well FW024 or extraction well FW103, although seasonal water level fluctuations were observed (Figure S3B).

Confirmation of Subsurface Reduction of U(VI) to U(IV). Well sediment samples were not available for analysis until the onset of fouling, but core samples obtained during drilling of the inner loop injection well (FW104) were analyzed. U concentrations ranged from 30 to 500 mg U kg^{-1} soil (*25*, *38*). The concentrations of U in surged sediment recovered from the inner loop wells during biostimulation are summarized in Table 1. From day 247 to 535, the U content of sediments from extraction well FW026 ranged from 1000 to 1400 mg U kg^{-1} soil and U content of sediments from injection well FW104 ranged from 2600 to 4300 mg U kg^{-1} soil, indicating

TABLE 1. XANES Analyses of Sediment Samples Taken from Inner Loop Injection Well FW104, MSL FW101-2, and Extraction Well FW026 Show Partially U(VI) Reduction to U(IV)

day	well	uranium (g/kg solids)	% U(IV)
258	FW104	2.60	36
271	FW104	1.03	44
333	FW104	NP[a]	49
409	FW026	1.29	0
409	FW104	2.79	42
535	FW026	1.14	28
535	FW101-2	0.91	35
535	FW104	4.32	51

[a] NP: analysis was not performed.

reduction throughout the zone of biostimulation, with accumulation near the injection well. The increase in solid-phase U within the inner loop was likely due to continuous recirculation of groundwater containing U(VI) followed by reduction to U(IV) and immobilization. This scenario would account for the observed accumulation of U near the injection well, but U reduction was also observed meters away from the injection well. Successive cleaning operations generated smaller quantities of sediment, and it is likely that repeated surging removed sediment from different locations along the well screen. On day 168, FW104 sediments were brown; by day 258, however, they were dark green, indicative of reduced conditions or formation of green rustlike precipitate. On day 535, FW026 sediment appeared slightly green. A similar color change was observed in columns studies using core sample from FW104 (*25*). XANES was used to determine the uranium oxidation state in sediments generated by surge block operations. Reduced uranium was not detected in all wells prior to biostimulation. Partial reduction of U(VI) to U(IV) occurred after biostimulation. As shown in Table 1, U(IV) was first found in sediment recovered from the inner loop injection well FW104 on day 258. XANES analysis revealed that the 39% of the U was present as U(IV). On days 271, 333, and 409 in FW104, the solid-phase uranium was 54%, 51%, and 53% U(IV), respectively. U(IV) was not detected in extraction well FW026 sediment on day 409, but was detected on day 535 (Table 1), indicating progressive expansion of the reduction zone. On day 535, the fraction of U(IV) in FW104, FW101-2, and FW026 was 51%, 35%, and 28%, respectively, decreasing with distance away from the injection well (Figure 2). XANES analyses of sediment samples provides definitive proof of reduction of U(VI) to U(IV) as a mechanism of U(VI) removal.

The structure of U(IV) generated at the site remains uncertain. Suzuki et al. described nanometer-sized U(IV) precipitated on SRB cells and expressed concern that these particles could be transported as colloids (*18*). For groundwater samples taken during U(VI) reduction, there was no change ($<\pm 5\%$) in U concentrations before and after microfiltration (0.3 μm) or centrifugation. This suggests that few such particles were produced under field conditions.

In Situ Denitrification (Days 137−184). During this period, intermittent ethanol additions stimulated denitrification. K_2CO_3 was added to increase pH to a range better suited for microbial growth and, later, to increase the dissolved concentration of U(VI). Figure 3 illustrates a 22-day time series (days 162−184) that is representative of this period. Addition of ethanol increased the COD in injection well FW104, then at the MLS wells FW101-2 and FW102-3 (Figure 3A). The COD in water removed from extraction well FW026 was low, indicating that the added ethanol was consumed in transit. Ethanol consumption was accompanied by nitrate removal. While ethanol was added, nitrate

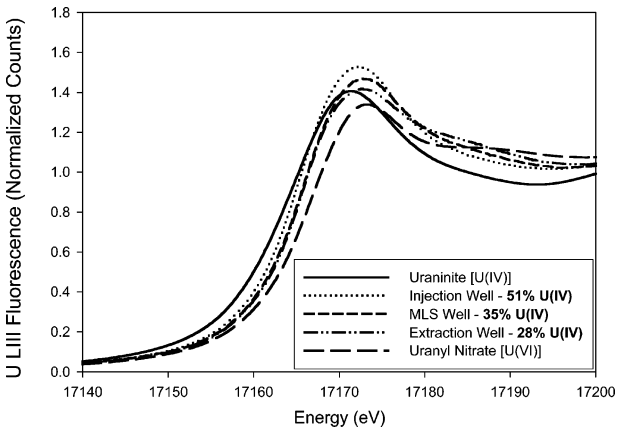

FIGURE 2. XANES analyses confirmed the presence of partially reduced uranium (IV) in sediment samples obtained on day 535 using the surge block technique at the injection well FW104 (51% U(IV)), monitoring well FW101-2 (35% U(IV)), and extraction well FW026 (28% U(IV)).

concentrations at the MLS wells rapidly decreased to 0.1 mM or less. When ethanol injection stopped, nitrate concentrations rebounded, likely due to diffusive release of nitrate from the sediment matrix. With successive ethanol additions, the rebounds became less pronounced. In the extraction well FW026, for example, the peak rebound concentration gradually decreased (Figure 3B). No nitrite was detected. Another FRC research team detected nitrite when they injected acetate, ethanol, or glucose into a region containing high nitrate without prior adjustment of subsurface pH (*5*).

During denitrification, the pH at MLS wells FW101-2 and FW102-3 mirrored changes in pH at the injection well FW104 (Figure 3A). These changes can be attributed to the addition of K_2CO_3 plus alkalinity that is generated by denitrification (Figure 3C). Sulfate concentrations in the MLS wells and at extraction well FW026 remained stable (Figure 3D), and there was no sulfide odor. U(VI) removal was not observed during this period. The solubility of nitrogen gas is 0.675 mmol L^{-1} water at 1.0 atm and 20 °C. On the basis of Henry's law and the stoichiometry of denitrification (0.5 mol N_2 per mol NO_3 consumed, ignoring biomass synthesis), nitrogen gas could be expected to accumulate when nitrate concentration exceeds 1.4 mM, if no provisions are made for removal of N_2. Nitrate concentrations in the MLS and extraction wells normally were less than 1.0 mM after the clean water flush, but there were occasional spikes to 2−3 mM, as discussed earlier. To remove dissolved N_2 and other volatile substances, vacuum stripper operation continued until day 268.

U(VI) Reduction and Bioavailability (Days 185−535). During this period, U (VI) and sulfate were reduced when ethanol was injected. To test the bioavailability of U(VI), more than 10 experiments were performed over a range of conditions. Due to seasonal ambient temperature variations, groundwater was heated and/or cooled as it recirculated through the aboveground system, affecting subsurface temperatures. Figure 4 illustrates a summer experiment from day 345 to day 349 (August 2004, groundwater temperature of 19−21 °C). Prior to day 345, the pH at injection well FW104 and MLS well FW101-2 was 6.1 and the U concentration was 1.2 μM. Following carbonate addition, the pH increased to 6.6, and by day 345, U concentrations had increased to 2 μM. This indicates that water passing from FW104 to FW101-2 came into contact with carbonate-extractable U(VI). In FW104, U concentrations remained stable at 1.2−1.3 μM (Figure 4, parts C and E). The likely explanation is that the regions surrounding FW104 had a higher fraction of reduced U that was not susceptible to solubilization by carbonate.

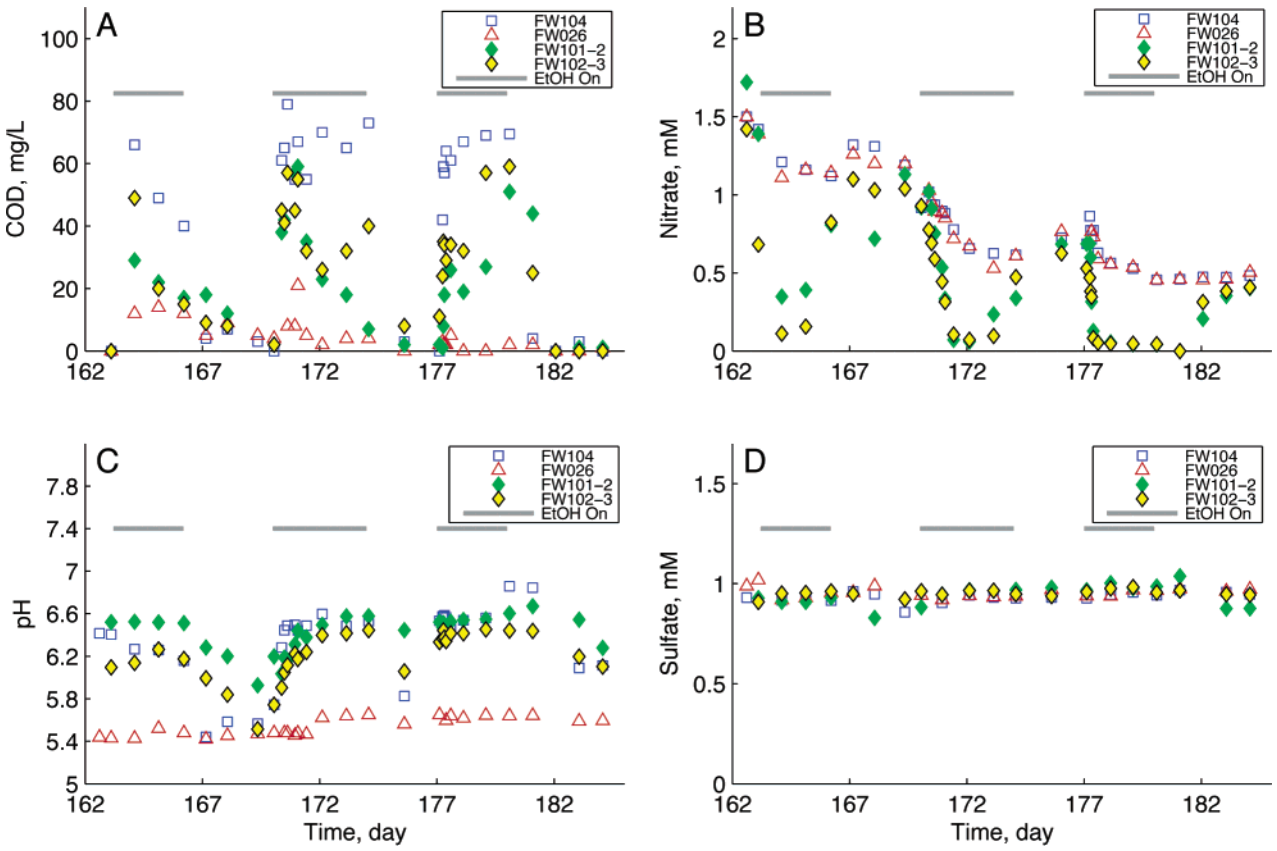

FIGURE 3. Time course of in situ denitrification for days 162−182 showing rapid nitrate removal but not sulfate removal when ethanol was injected: (A) COD, (B) nitrate, (C) pH, (D) sulfate.

When ethanol was added, COD levels quickly increased to 90 mg L^{-1} at FW101-2, but the increase at FW102-3 was delayed and less (Figure 4A). MLS well FW 101-2 also responded more strongly to a pH increase at the injection well (Figure 4C). Tracer studies indicated good communication between FW101-2 and FW102-3 and injection well FW104 before this experiment (Figure 3 of the companion article, *1*) and after this experiment (Figure S4, in the Supporting Information). Thus, low COD levels at FW102-3 were likely due to nearly complete consumption of ethanol from the injection well to FW102-3. At MLS well FW 101-2 nitrate was rapidly removed, disappearing within just 4 h of injection, but at FW102-3, nitrate removal was delayed and less extensive (Figure 4B). Sulfate and U(VI) removal were extensive at FW101-2 but also limited and delayed at FW102-3. Thus, the quantity of ethanol transported along flow paths from FW104 to FW101-2 was sufficient to support denitrification, sulfate reduction, and U(VI) reduction, but ethanol delivery via the flow path from FW104 to FW 102-3 was only sufficient to support denitrification and limited sulfate reduction (Figure 4D). There was a distinctive pattern of ethanol consumption and U(VI) removal. When ethanol was added, the concentration of U at FW101-2 decreased from 1.85 to 0.74 μM (Figure 4E), a 60% decrease. Sulfate concentrations likewise decreased, and sulfide accumulated (Figure 4, parts D and F). After ethanol injection stopped on day 347, carbonate injection continued until day 349. During that period, the U concentration increased from 0.74 to 1.70 μM. No dissolved oxygen was detected during this period, and sulfide was present in the groundwater. This is evidence that the increase in aqueous U occurred because the rate of desorption of U(VI) exceeded the rate of reduction and not because of reoxidation by DO. On day 349, carbonate injection also stopped, and the pH in FW101-2 decreased to 6.3 after 2 days. Rates of sorption to the solid phase increased, and aqueous U levels decreased to 0.73 μM. These patterns were highly reproducible.

Repeated experiments with variations in the sequence and duration of carbonate and ethanol addition revealed that dissolved U(VI) levels were set by the relative rates of desorption and reduction. Carbonate concentrations, and therefore pH and inorganic carbon, controlled the rate of desorption, while ethanol concentration controlled the rate of reduction. When these rates balanced, there was no net increase or decrease in the concentration of dissolved U(VI). Addition of carbonate but not ethanol led to increases in U(VI) concentration because desorption rates exceeded the rates of sorption and reduction. When ethanol was then added, the reduction rate exceeded the rate of desorption, and dissolved U(VI) concentrations fell. When ethanol injection stopped but carbonate levels remained stable, the desorption rate exceeded the rate of reduction, and dissolved U(VI) concentrations again increased. Sulfate reduction accompanied U(VI) reduction. Microbial conversion of 1 mol of sulfate generates approximately 1 mol of sulfide, but the measured molar decline in sulfate was greater than the molar increase in sulfide. This is likely due to the formation of sulfide precipitates.

This pattern of desorption and reduction was repeated 50 times, with some variations in the on/off sequence for base and ethanol additions, such as ethanol and carbonate on/off at the same time, but with similar outcomes. A sequence for winter operations is provided in the Supporting Information. Removal of nitrate, sulfate, and U(VI) was observed during winter operation at a groundwater temperature of 12−14 °C. During ethanol injection, U concentrations fell by >50% in FW101-2, though at a slightly slower rate than during the summer (Figure S5 in the Supporting Information).

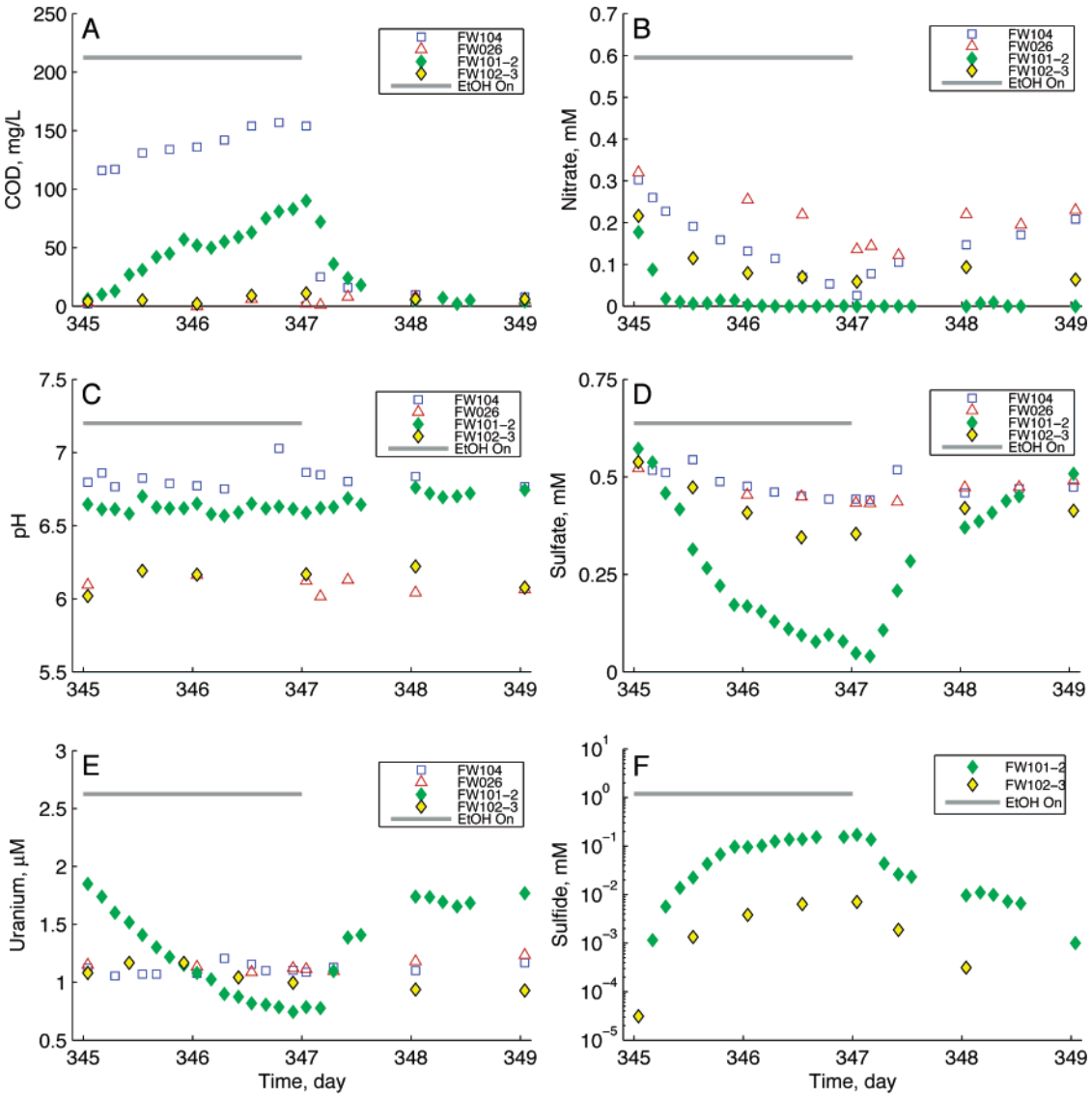

FIGURE 4. Changes in groundwater chemistry within the inner loop recirculation and MLS wells when carbonate and ethanol were added during the period of U(VI) and sulfate reduction in the summer; groundwater temperature = 18−20 °C (days 345−349). Carbonate was added just prior to and throughout the experiment time period shown. The period of ethanol addition is indicated by the horizontal bars. (A) COD, (B) nitrate, (C) pH, (D) sulfate, (E) uranium, (F) sulfide.

The results of this work differ from those reported for a recent field study at Rifle, CO, where acetate was added to stimulate U(VI) reduction (*28*). At the Rifle site, a rebound in aqueous U(VI) concentrations occurred when acetate-utilizing SRB became prevalent. At our site, SRB activity correlated with U(VI) reduction. This may reflect a difference in selection pressures imposed by the electron donors used.

Microbiology. Microbiological data support the conclusion that conditioning and ethanol addition resulted in effective biostimulation. As shown in Table 2, denitrifying bacteria, SRB, and FeRB were detected in groundwater samples from inner loop wells. SRB or FeRB were not detected at wells FW106 and FW112. Wells FW106 and FW112 are located within Area 3 but were not conditioned and did not receive ethanol injections. Consequently, geochemical conditions at these wells are similar to those at FW104 and FW026 prior to conditioning.

During the initial denitrification phase, the only organic compound detected was ethanol. With the onset of sulfate and uranium reduction, acetate was also detected in FW101-2 but not FW102-3 (Figure S6, Supporting Information). Its presence may be due to the activity of SRB, such as *Desulfovibrio* spp., that incompletely oxidize ethanol to acetate (*30*, *40*). The presence of both SRB and FeRB likely indicates a diversity of U(VI)-reducing populations. Because

TABLE 2. Most Probable Number (MPN) Estimates for Denitrifiers, SRB, and FeRB in Groundwater (Number of Cells/mL)

well	date[a]	denitrifiers	SRB	FeRB
FW026 (extraction well)	day 278	NP[b]	7.2×10^2	4.9×10^3
	day 354	3.4×10^5	1.3×10^5	2.4×10^3
	day 453	5.2×10^4	7.9×10^2	7.6×10^2
FW101-2 (MLS well)	day 278	NP[b]	6.2×10^3	7.4×10^4
	day 354	5.6×10^2	1.7×10^5	5.9×10^2
	day 453	9.2×10^6	2.0×10^4	3.4×10^3
FW102-3 (MLS well)	day 278	NP[b]	NP[b]	NP[b]
	day 354	9.6×10^4	3.1×10^5	6.1×10^2
	day 453	2.4×10^4	6.9×10^2	6.9×10^3
FW106 (control well)	day 278	3.3×10^0	0	0
FW112 (control well)	day 453	3.3×10^0	0	0

[a] Day 278 was May 27, 2004; day 354 was August 11, 2005; day 453 was November 11, 2005. [b] NP indicates that the test was not performed.

groundwater MPN data do not reflect biomass distribution, MPN analyses of sediment samples were performed near the inner loop injection well FW104 on day 453. Counts were as follows (cell/g dry weight): denitrifying bacteria, 9.2×10^9; SRB, 2.9×10^{10}; FeRB, 9.0×10^7. These data suggest that denitrifying bacteria and SRB were present at approximately the same level, ~100 times higher levels than the FeRB, but additional characterization is needed. Molecular studies of the dynamics of microbial communities are in progress. Recent DNA analyses of groundwater samples from the MLS wells revealed several types of organisms that are known to reduce U(VI), including denitrifying *Acidovorax* (*24*), *Desulfovibrio*-like, and *Geobacter*-like species. *Geobacter* spp. was previously detected in FRC Area 2 solids (*36*).

Implications and Further Studies. Several aspects of this work are of interest for field-scale remediation activities. Removal of potential clogging agents and inhibitors (nitrate, aluminum, and calcium in this case), control of pH with low level bicarbonate (<5 mM), and maintenance of a sulfate residual should have general value at other sites. Well-surging restored well performance and allowed routine collection and monitoring of contaminant levels in the sediment. Control over carbonate addition enabled manipulation of U(VI) bioavailability and stability of reduced U(IV). Operation at a pH level below the optimum for methanogens and maintenance of a sulfate residual appeared to suppress methanogenesis, favoring long-term maintenance of desirable SRB populations.

Challenges for future field research include the development of strategies to achieve even lower concentrations of dissolved uranium while also ensuring long-term resistance to uranium reoxidation and remobilization. While the U.S. Department of Energy has no fixed targets for uranium concentrations in groundwater, it would be desirable to achieve concentrations that are less than the EPA maximum contaminant level for drinking water of 0.126 μM (30 μg L^{-1}) (*49*). Rapid reduction of U(VI) occurs at high uranium concentrations (500−1200 μM) (*9*, *17*, *50*, *51*), but the reported half-saturation coefficients for U(VI) reduction range from 130 to 880 μM for SRB and FeRB (*50*−*54*). When dissolved uranium concentrations fall below these values, volumetric removal rates become proportional to U(VI) concentration and slower as concentration continues to fall, even in the absence of mass transfer limitations. But as this work demonstrates, the concentration of dissolved U(VI) is set by the relative rates of mass transfer and reduction, and both rates can be manipulated. Low dissolved U(VI) concentrations should be possible if adequate biomass levels are maintained at low mass transfer rates. While complete reduction of U(VI) to U(IV) may not be feasible due to complexities of geology and structure of the solids that form, a goal of immobilization and low levels of U in the aqueous phase may be achievable. Research is needed to understand the extent of solid-phase U(VI) reduction needed to ensure sufficiently low levels of U(VI) in the water. Studies of reoxidation are also needed. Reoxidation and remobilization of reduced U(IV) to U(VI) by oxygen was rapid in the presence of high concentrations of bicarbonate (1 M) (*29*). Dissolved oxygen concentrations in the background groundwater at FRC Area 3 are low or near zero. However, long-term stability of reduced U(IV) will require assessment of dissolved oxygen effects. Reoxidation of U(IV) was also observed under methanogenic conditions at high bicarbonate levels (15 mM) and a pH of 7.2 (*26*). This led Wan et al to propose that residual Fe(III) species can oxidize U(IV) when conditions are favorable for the formation of calcium uranyl carbonate species (*26*). U(VI) levels rebounded under lactate-limited sulfate-reducing conditions with *Desulfovibrio desulfuricans* G20 (*55*), suggesting that reduced U(IV) can be reoxidized by Fe(III)(hydr)oxides. Other oxidants of concern are nitrogen oxides (*48*). While further testing is needed to evaluate the magnitude of these effects, the strategy used in this work—elimination of bulk nitrate and operation at low calcium and carbonate levels—should decrease the potential for U(IV) reoxidation.

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Supporting Information Available

Methods and results for uranium adsorption and desorption experiments; methods and results for batch microcosm experiments used to select the electron donor; methods used for MPN analysis; tracer study with ethanol and bromide; methods and results for a typical bioavailability experiment performed in the winter months. This material is available free of charge via the Internet at http://pubs.acs.org.

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