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In Situ Bioreduction of Uranium (VI) to Submicromolar Levels and Reoxidation by Dissolved Oxygen

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Groundwater within Area 3 of the U.S. Department of Energy (DOE) Environmental Remediation Sciences Program (ERSP) Field Research Center at Oak Ridge, TN (ORFRC) contains up to 135 μM uranium as U(VI). Through a series of experiments at a pilot scale test facility, we explored the lower limits of groundwater U(VI) that can be achieved by in-situ biostimulation and the effects of dissolved oxygen on immobilized uranium. Weekly 2 day additions of ethanol over a 2-year period stimulated growth of denitrifying, Fe(III)-reducing, and sulfate-reducing bacteria, and immobilization of uranium as U(IV), with dissolved uranium concentrations decreasing to low levels. Following sulfite addition to remove dissolved oxygen, aqueous U(VI) concentrations fell below the U.S. Environmental Protection Agengy maximum contaminant limit (MCL) for drinking water ($<30\ \mu g\ L^{-1}$ or 0.126 μM). Under anaerobic conditions, these low concentrations were stable, even in the absence of added ethanol. However, when sulfite additions stopped, and dissolved oxygen (4.0−5.5 mg L^{-1}) entered the injection well, spatially variable changes in aqueous U(VI) occurred over a 60 day period, with concentrations increasing rapidly from <0.13 to 2.0 μM at a multilevel sampling (MLS) well located close to the injection well, but changing little at an MLS well located further away. Resumption of ethanol addition restored reduction of Fe(III), sulfate, and U(VI) within 36 h. After 2 years of ethanol addition, X-ray absorption near-edge structure spectroscopy (XANES) analyses indicated that U(IV) comprised 60−80% of the total uranium in sediment samples. At the completion of the project (day 1260), U concentrations in MLS wells were less than 0.1 μM. The microbial community at MLS wells with low U(VI) contained bacteria that are known to reduce uranium, including *Desulfovibrio* spp. and *Geobacter* spp., in both sediment and groundwater. The dominant Fe(III)-reducing species were *Geothrix* spp.

Introduction

Reduction of multivalent metals can convert dissolved, oxidized forms of multivalent heavy metals and radionuclides, such as U(VI), to reduced forms of low solubility that precipitate from solution (*1*). U(VI) reduction/immobilization has been investigated in batch serum bottles (*3-5*), microcosms (*6*), sediment columns (*7*, *8*), and field studies (*2*, *3*, *9*−*11*). The process is largely mediated by iron(III)-reducing bacteria (FeRB), sulfate-reducing bacteria (SRB), and a few other microorganisms (*1*). A concern is whether low levels of aqueous phase U can be achieved and maintained under field conditions. While the U.S. Department of Energy has not established targets for U concentrations in water, target concentrations below the U.S. Environmental Protection Agency (USEPA) maximum contaminant level (MCL) for drinking water of 0.126 μM (30 $\mu g\ L^{-1}$) (*12*) would be desirable. Pure culture kinetic studies raise doubts about the feasibility of achieving such low concentrations. While researchers have reported rapid reduction at high U concentrations (500−1200 μM), the reported half saturation coefficients range from 130 to 880 μM for SRB and FeRB (*13*−*16*). These values imply first-order kinetics, and slow rates at concentrations near the EPA MCL, and they suggest high U(VI) threshold concentrations for microbial transformation of U(VI). But biology alone does not control aqueous U concentrations. Diffusive mass transfer coupled to sorption and desorption limit aqueous concentrations, and abiotic reductions are also important (*1*). Sulfide, a reductant generated by sulfate respiration, can reduce U(VI) to U(IV) (*17*), as can microbially generated green rust (*18*).

The long-term stability of bioreduced and immobilized uranium is an additional issue. Suzuki et al. (*19*) reported that *Desulfosporosinus* spp. reduced U(VI) to nanometer-size uraninite (UO_2) particles. They expressed concern that these particles could be mobile in porous sediments and susceptible to oxidation. Oxygen oxidizes U(IV) and does so rapidly in the presence of high levels of bicarbonate (1 M) (*20*). Nitrate also promotes oxidation of bioreduced U(IV) to U(VI) (*4*). The intermediates of dissimilatory nitrate-reduction−nitrite, nitrous oxide, and nitric-oxide−oxidize U(IV) and can mobilize U(VI) (*9*). These reports justify efforts to remove oxidants, and particularly nitrate because it can potentially be present at high concentrations (*10*). Even Fe-(III) species can oxidize U(IV). Aqueous U(VI) concentrations

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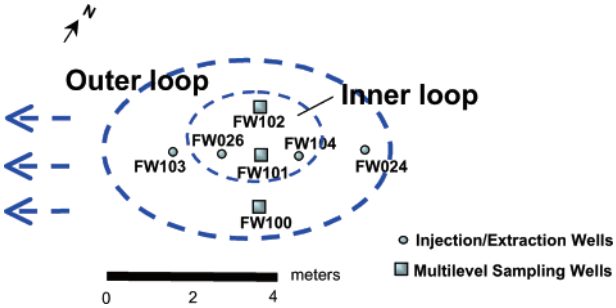

FIGURE 1. Pilot-scale bioremediation well system.

rebounded after depletion of electron donor (lactate) in cultures of lactate-fed sulfate-reducing *Desulfovibrio desulfuricans* G20 that also contained Fe(III)(hydr)oxides (*21*). In a bench-scale column study using sediment from ORFRC Area 2, oxidation of bioreduced U(IV) and methanogenesis occurred despite the fact that known U(VI)-reducing bacteria (*Geobacteraceae*) were present (*8*, *22*). The hypothesized oxidant was Fe(III). A thermodynamic analysis (*8*) established that a high level of bicarbonate (15 mM) and Ca^{2+} (1 mM) changed solution thermodynamics to favor U(IV) oxidation by Fe(III) solids. Thermodynamic analyses predicted that Fe(III)(hydr)oxides oxidize biogenic UO_2 under certain geochemical conditions, and laboratory experiments confirmed those predictions (*23*).

Previously, we constructed a test facility in Area 3 of the DOE ERSP Oak Ridge FRC, a site located adjacent to the former S-3 Ponds and containing high levels of U on the sediments (up to 800 mg kg^{-1}) and in groundwater (as high as 250 μM). Testing began on August 24, 2003 (day 1) (*10*, *11*) and continued to the present. Reduction of U(VI) was stimulated by weekly 2 day injections of ethanol. X-ray absorption near-edge structure spectroscopy (XANES) analysis of the sediment confirmed partial reduction of U(VI) to U(IV) (*11*). In this report, we focus on the lower limits of U(VI) concentration achievable through in situ bioreduction and on the stability of U(IV) in the presence and absence of dissolved oxygen. The results demonstrate that aqueous U concentrations below the USEPA MCL (<0.126 μM) can be achieved in situ, that bioreduced/immobilized uranium is stable under anaerobic conditions, and that infiltration of DO into the reduced area promotes spatially variable oxidation of U(IV) and mobilization of U(VI).

Materials and Methods

Field Subsurface System. The overall scheme for the in situ well system was similar to that reported previously (*10*, *24*) with some modifications (Figure 1). Briefly, it consisted of an outer recirculation loop in which water continuously recirculated between wells FW024 and FW103 protecting a nested inner recirculation loop, in which water continuously recirculated between wells FW026 and FW104, from the highly contaminated groundwater exiting the adjacent source zone. The recirculation flow rate in the inner and outer loops was 0.45 L min^{-1}. Water injected into the outer loop was blended 50:50 with Y-12 Plant tap water (pH 8.0 with 2.82−3.38 mM chloride; 0.04−0.048 mM nitrate; 0.24−0.26 mM sulfate; 0.68−0.75 mM Ca, < 0.007 mM Al) in a storage tank. This water was then injected into the outer loop at 0.9 L min^{-1}. The pH of the blended water was adjusted to 5.4−5.6 with HCl prior to injection (*10*, *24*). The DO of the blended water ranged from 9 mg L^{-1} in the summer to 12 mg L^{-1} in the winter. After day 638, Na_2SO_3 (approximately 0.9 mM) was added to the storage tank. The added sulfite removed oxygen ($2SO_3^{2-} + O_2 \rightarrow 2\,SO_4^{2-}$), decreasing DO to near zero, but did not reduce U(VI). During periods of sulfite addition, some of the DO-free water injected into the outer loop was extracted by the inner loop extraction well then pumped into the inner loop by the inner lump injection well. During periods when sulfite was not added, DO-containing water entered the inner loop via the same route.

Within the inner loop, ethanol additions were used to stimulate reduction of U(VI) to U(IV). Multilevel sampling (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) located between the injection and extraction wells enabled routine monitoring of chemical additions and U levels in solution. The monitored MLS wells were those located at the level that had the highest ambient groundwater flowrates and the highest concentrations of U in the water and on the solid phase (*10*, *24*).

Ethanol and its collective metabolites were monitored as chemical oxygen demand (COD), where 8 g of COD is equivalent to one mole of reducing equivalents. Ethanol, prepared as a 9.8 g COD L^{-1} stock solution, was normally injected at FW104 over a 48 hour period each week. This resulted in a COD of 120−150 mg L^{-1} at FW104. A solution of K_2CO_3 (375 mM) was also injected to manipulate pH and carbonate concentrations. Depending on the levels of carbonate added, alkalinity at the MLS wells ranged from 0.8 to 4 mM as HCO_3.

Two bromide tracer studies were performed. The first (days 801−803) investigated the extent of hydraulic communication between the inner loop injection well and the MLS wells. The second (days 869−873) characterized breakthrough of dissolved oxygen as oxygenated water passed from the outer loop into and through the inner loop. The results confirmed connectivity of the MLS wells to the inner loop injection well (*25*). FW101-2 responded rapidly, with arrival of bromide tracer within 2.8 h of the initiation of injection and >95% recovery of injected bromide. The results also indicated that after 2 years of biostimulation, pathways for transport of fluids through the subsurface remained open, despite changes in hydrogeology and sediment structure (*25*). However, the flow captured at the extraction wells was of variable origin. At the inner loop extraction well, 50% of the captured flow came from the inner injection well, 44% came from the outer injection well, and 6% came from the regional flow.

Chemicals and Analytical Methods. Previous publications (*10*, *11*) give detailed information on the methods used to measure COD, sulfide, anions (including NO_3^-, Br^-, Cl^-, SO_4^{2-}, and PO_4^{3-}), cations (Al, Ca, Fe, Mn, Mg, U, K, etc.), methane, ethanol, and acetate; the kinetic phosphorescence KPA-11 U analysis method (Chemchek Instruments, Richland, WA); the source and quality of chemicals; and methods of groundwater and sediment sample collection. The oxidation state of U in sediments was determined by XANES (see the Supporting Information). Fe(II) was measured colorimetrically using a HACH DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). DO was measured directly using a HACH Q10 DO meter.

Bacterial Community Analysis. The composition and structure of the bacterial communities were characterized by constructing clonal libraries of small-subunit (SSU) rRNA gene sequences, analyzing DNA samples by functional gene microarrays (FGA), and enumerating cells by most probable number (MPN) analyses (see the Supporting Information). Groundwater (2 L) was collected and filtered through a 0.2 μm filter to obtain biomass for DNA extraction.

Results and Discussion

Summary of Field Tests. Two operational phases preceded the experiments described in this paper. In the first phase (days 1−136), a region of the subsurface was prepared for biostimulation by flushing at low pH to remove bulk nitrate and aluminum. Subsequent flushing with added base

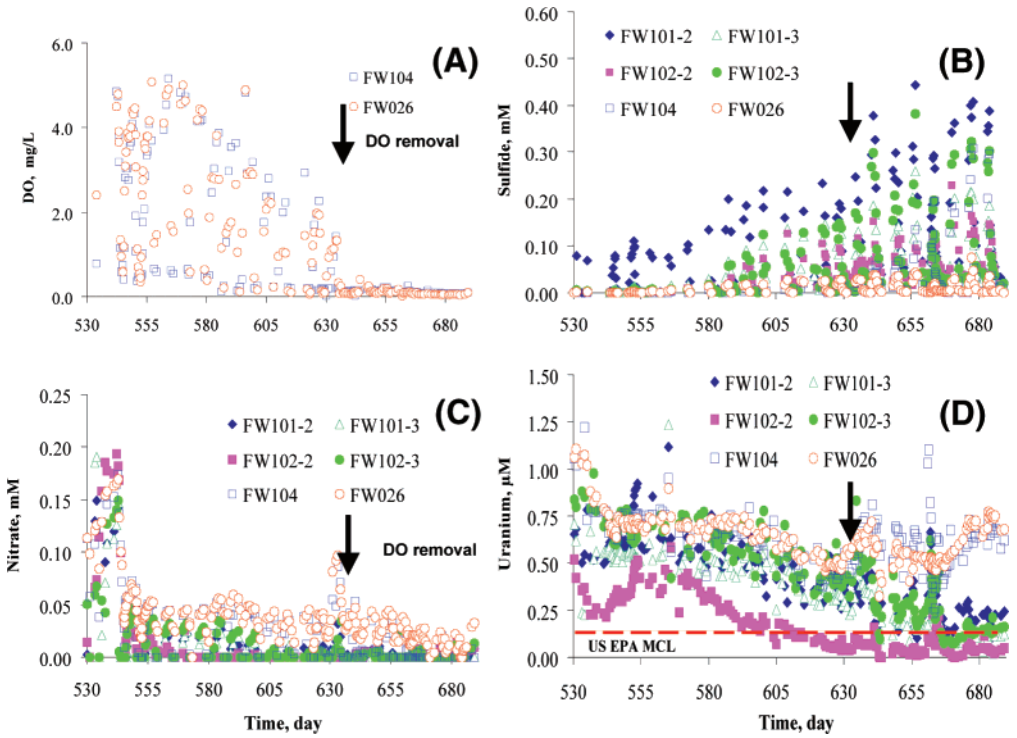

FIGURE 2. Geochemical changes during biostimulation in groundwater from the inner loop recirculation and MLS wells before and after DO control on day 637. (A) DO concentrations in the inner loop extraction (FW026) and injection wells (FW104). DO concentrations were maintained below 0.15 mg L^{-1} in FW104 after day 637. (B) Sulfide. (C) Nitrate. (D) Uranium.

increased groundwater pH in the inner recirculation loop from 3.4 to 5.7−6.1, which is a pH range favorable for microbial activity and for U(VI) sorption. As a result of these operations, aqueous U(VI) concentrations fell from 168 to 5 μM at the inner loop injection and extraction wells. In the second phase, weekly carbonate additions increased pH to 6.6−7.0, which is a range that enhanced desorption and bioavailability of U(VI). Weekly ethanol additions thereafter stimulated bioreduction of U(VI) to U(IV), with aqueous phase U concentrations falling from 5 to 1 μM at the injection and extraction wells and from 0.2 to 0.5 μM at the MLS wells (*11*).

The present report describes how DO controls affect aqueous U(VI) concentration and stability of immobilized U(IV). More specifically, these experiments evaluated (1) U(VI) reduction without DO control (days 530−637); (2) U(VI) reduction with DO control (days 638−688); (3) the stability of immobilized U in the absence of ethanol and DO (days 713−754); and (4) the stability of immobilized U in the absence of ethanol but in the presence of DO (days 806−884). To further assess the stability of immobilized U, DO removal and weekly ethanol additions continued until day 1266. Over the entire time period of biostimulation (days 137−1266), a total of 8 kg of ethanol was added in 140 separate biostimulation events. Subsurface temperatures ranged from 12 °C in the winter to 21 °C in the summer.

U(VI) Reduction to Below the USEPA MCL (<0.126 μM). Figure 2 summarizes results from the initial two tests (days 530−688). By day 637, DO concentrations in FW104 were around 0.5−1.0 mg L^{-1} during ethanol injection and increased to 3−5 mg L^{-1} in its absence (Figure 2A). DO in the MLS wells was low (<0.2 mg L^{-1}) or absent (data not shown). When ethanol was injected, sulfide concentrations increased continuously at the MLS wells (Figure 2B). After day 637, the addition of Na_2SO_3 removed DO from water injected into the outer loop. DO concentrations fell to less than 0.15 mg L^{-1} (Figure 2A). Sulfide concentrations increased rapidly at FW104, indicating enhanced SRB activity. Nitrate diffused from the sediment matrix (*26*) but decreased from 0.2 mM to 0.05 mM after day 540 (Figure 2C). Uranium concentrations varied at FW104, and decreased continuously at the MLS wells due to weekly injections of ethanol, even prior to the implementation of DO control measures (Figure 2D). Removal of DO from the outer loop coincided with further declines in aqueous U(VI) concentrations and likely enabled U(VI) reduction to concentrations at or below the U.S. EPA MCL. The concentration of U in FW102-2 fell to the EPA MCL of 0.126 μM by day 615, and to that same level in FW101-3 by day 640. Low concentrations persisted thereafter (Table 1). In FW101-2 and FW102-3, U concentrations fell below the EPA MCL during ethanol injection but rebounded slightly when ethanol injection stopped. Low U concentrations persisted for days to months in wells FW101-3 and FW102-2 but varied in FW101-2 and FW102-3, likely because these wells were most closely connected to the injection well where U(VI) drawn from the outer loop was continuously injected (Figure 2D).

Stability of Uranium without Added Ethanol. Ethanol was injected into the inner loop from days 710 to 713. By day 713, aqueous U concentrations were below the EPA MCL at all MLS wells (Figure 3 A). From days 713 to 754, no ethanol was injected. Aqueous U continuously entered the inner loop through FW104 at concentrations of 0.5−0.7 μM. There is evidence of a sink for this added U(VI) in the zone between the injection well and the MLS wells. U concentration at the MLS wells slowly increased, but never to the levels observed in the inner loop injection (FW104) and extraction wells (FW026) (Figure 3A).

Unlike uranium, sulfate concentrations at the MLS wells did increase to levels found in the injection well (Figure 3B). Sulfide concentrations increased during ethanol injection, then decreased, but remained at significant levels throughout the test period (Figure 3C). The values observed (within the 0.01 mM range)) are indicative of an active sulfate-reducing conditions. Total soluble Fe (Figure 3D) was used as an indicator of Fe(II) concentrations. Soluble Fe concentrations initially fell, perhaps due to FeS formation. Concentrations

TABLE 1. Uranium in Groundwater and Sediments from the Inner Loop Injection and MLS and XANES Analyses of U(IV) Content[a]

well	day pulled	pH	aqueous U (μM)	U in sediments (g/kg solids)	days storage at 4° C	% U(IV) XANES
FW104	258[b]	6.15	1.20	2.60	>4 weeks	36
	409[b]	5.98	1.25	2.79	>4 weeks	42
	535	5.88	0.73	4.32	45	43
	774	5.82	0.51	10.3	45	61
	898	5.7	0.50	4.64	47	61
	935	5.8	0.52	ns		ns
FW101-2	535	6.35	0.54	0.91	30	35
	774	6.08	0.12	1.25	45	51
	935	6.09	0.21	0.89	9	74
FW101−3	535	5.83	0.23	1.02	9	9
	774	6.04	0.11	1.83	45	53
	935	6.19	0.12	1.37	9	67
FW102−2	774	6.25	0.05	0.52	45	30
	935	6.28	0.12	0.86	9	78
FW102−3	774	5.84	0.06	0.86	45	17
	935	5.78	0.42	1.32	9	82

[a] Analytical errors of XANES for U(IV) is about ± 10%. U(VI) reduction in sediment samples continued in serum bottles stored in a refrigerator and the U(IV) content increased significantly during 1 year storage (*34*). Thus, the measured U(IV) content of stored sediment samples may be greater than the values obtained when the samples were first removed from the subsurface. ns, not sampled. [b] See ref *11*.

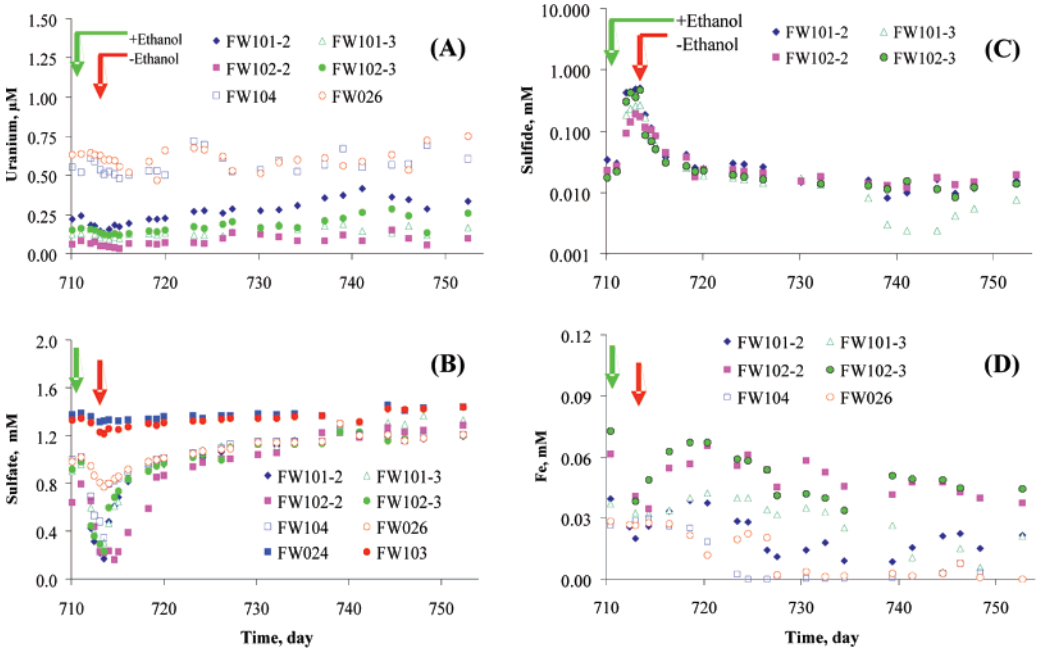

FIGURE 3. Changes in groundwater quality in the absence of ethanol addition. (A) Uranium. (B) Sulfate. (C) Sulfide. (D) Dissolved Fe.

then increased until day 718, suggesting Fe(III) reduction and accumulation of Fe(II). A gradual decrease in Fe (II) thereafter may reflect decreasing rates of reduction of Fe-(III). DO at FW104 was <0.2 mg L^{-1} d, so DO likely had little or no effect on soluble Fe.

Active SRB were present and viable after 41 days of starvation. When ethanol was injected into the inner loop injection wells on day 754, sulfide concentrations increased at all MLS wells within 6 h. After 12 h, sulfide levels increased from 0.014 to 0.27 mM at FW101-2, and from 0.014 to 0.29 mM at FW102-3. U reduction also continued: after two weeks of weekly 2 day ethanol additions, U concentrations fell below the EPA MCL at all four MLS wells (data not shown).

Impact of DO. Prior to introduction of oxygen, the site was first reduced by ethanol injection into the inner loop from days 801 to 803. No ethanol was added from days 804 to 866. From day 811 until day 884, DO (9−11 mg L^{-1}) entered the outer loop (Figure 4A). DO concentrations increased at the inner loop injection well to 1.7 mg L^{-1} by day 815 and to 3.0 mg L^{-1} by day 817 (Figure 4A). By day 866, DO in the inner loop extraction and injection wells had increased to 5.2 mg L^{-1}, about half the concentrations of the outer loop injection well. On day 823, measured DO concentrations in MLS wells FW101-2, 101-3, 102-2, and 102-3 were less than 0.6, 0.6, 0.22, and 0.25 mg L^{-1}, respectively, and, by day 866, levels were less than 2.0, 0.8, 0.3, and 0.33 mg L^{-1} respectively. The actual DO concentrations were likely less than these values given that DO measurement entailed slow pumping of groundwater through an aboveground glass vial containing a DO probe, where some oxygen likely diffused through the sample tubing. Nevertheless, DO differences between the water injected and the water from the MLS wells indicated continuous consumption of DO as it passed through the reduced zone between the injection and MLS wells.

Before the introduction of DO, U concentrations were near or below the EPA MCL (Figure 4B). When DO entered the inner loop (~day 816), U concentrations increased first at FW101-2 and FW102-3 and then at FW101-3 (Figure 4B). On day 816, U concentrations were 0.46 μM levels at FW101-2 and FW102-3 but increased to 0.86 μM on day 817, while the

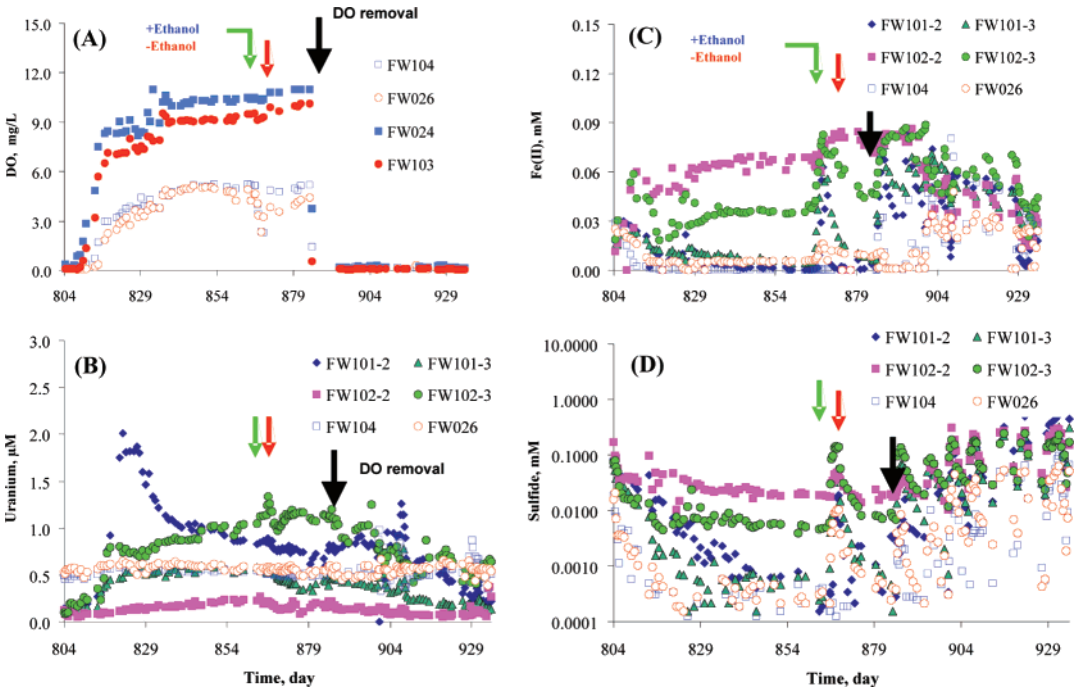

FIGURE 4. Impact of DO on stability of the bio-reduced subsurface within the inner loop (days 811−884). The changes of concentrations in groundwater: (A) DO of outer loop and inner loop wells. (B) Uranium. C. Fe(II). (D) Sulfide.

concentration injected at FW104 remained at (~0.5 μM). The increase in U concentration was thus due to mobilization of solid-associated uranium and not a change in the input U concentration. A strong response occurred at FW101-2, where aqueous U concentrations increased continuously, peaking at 1.87 μM on day 826. Levels decreased gradually thereafter, but remained higher than concentrations at the injection well FW104. The large response at FW101-2 is consistent with tracer study results indicating that this well was hydraulically well connected to the injection well (*25*). Aqueous U concentrations in FW101-3, increased to the same level as FW104 and remained essentially unchanged thereafter. In FW102-3, aqueous U concentrations increased rapidly to 0.8 μM but slowly thererafter. In 102-2, U levels remained low. A likely explanation is the presence of a more extensive reduced zone near that well.

When DO entered the outer loop, Fe(II) concentrations in the outer loop fell to below the detection limit (<0.002 mM). After DO entered the inner loop on day 811, Fe(II) concentrations fell to below the detection limit at the inner loop injection well (Figure 4C). But water from the inner loop extraction well continued to have detectable but low levels of Fe(II) (0.005−0.006 mM) throughout the period of DO addition. Fe(II) levels at MLS well FW101-2 were somewhat lower (0.003 mM or less), while Fe(II) levels at MLS wells FW102-3 and FW102-2 remained at relatively high levels (0.03 mM). Sulfide concentrations were also sensitive to DO (Figure 4D). Sulfide in FW104 and FW026 dropped to below 0.001 mM or near the detection limit (<0.0002 mM). At the MLS wells, sulfide decreased but remained above the detection limit.

On day 866, the recovery of Fe(III)-, sulfate-, and U(VI)-reduction was evaluated by a brief (2 day) period of ethanol addition to the inner loop. DO concentrations fell when ethanol was added, and DO levels increased when ethanol addition stopped (Figure 4A). Fe(III) and sulfate reduction were also stimulated: Fe(II) concentration inceased within 12−24 h at the MLS wells; this was followed by an increase in sulfide concentration (Figure 4C and D). From days 866 to 868, the concentration of U at the MLS wells increased slightly likely due to release of U(VI) sorbed to Fe(III) oxides.

On day 884, DO was again removed, and seven more ethanol additions were performed. Levels of U gradually decreased at the MLS wells, returning to levels near or below the EPA MCL (0.126 μM) by day 935 (Table 1). With continued ethanol injections, aqueous U concentrations fell at the four MLS wells, and levels below the EPA MCL were again achieved. Over the five week period from days 1237 to 1273, the average U concentrations was 0.24 ± 0.03μM in FW104, 0.30 ± 0.02 μM in FW026, 0.079 ± 0.026 μM in FW101-2, 0.054 ± 0.024 μM in FW101-3, 0.052 ± 0.015 μM in FW102-2, and 0.072 ± 0.023 μM in FW102-3, respectively.

From flow rates and the measured DO concentrations during the ethanol consumpotion period, about 1560 g of DO were injected at FW024, and about 240 g were withdrawn at extraction well FW026, a difference of 1320 g. This value was close to the amount of COD that ultimately had to be added (~1360 g) to restore aqueous U levels similar to those prior to oxygen addition.

Sediment Uranium Levels and XANES Results. Table 1 gives U concentrations in groundwater and sediment from the inner loop wells and percentages of total sediment U present as U(IV). The data suggest a spatially heterogeneous response to dissolved oxygen. Prior to the introduction of dissolved oxygen (day 811), aqueous U concentrations had fallen to low levels at the inner loop injection well FW104 and at MLS wells FW101-2 and FW101-3. After oxygen exposure (days 811−884), the measured U concentration in sediment from the injection well (4.64 g/kg on day 898) was less than the value measured prior to oxygen exposure (10.3 g/kg on day 774). The same pattern was true of MLS wells FW101-2 and FW101-3 (Table 1, days 935 and 774). This suggests a loss of immobilized U during the oxidation period. At MLS wells FW102-2 and 102-3, however, U concentrations on the sediment increased from days 774 to 935, suggesting sustained U immobilization and/or less loss of U during the oxidation period. This may be because wells further from the point of oxygen input are less affected by the introduction of DO: the FW101 MLS wells are closer to the injection well than the FW102 MLS wells (Figure 1).

XANES analysis of day 935 samples indicated that a significant fraction of U, up to 60−80% of total U, was present

TABLE 2. Predominant Bacterial Community Members in MLS Wells Where U Levels Decreased below the EPA MCL[a]

trophic group	genus	U(VI) reduction	relative abundance (% of total clones)	
			sediment	groundwater
SRB	*Desulfovibrio*	yes	4−15	13−28
FeRB	*Geobacter*	yes	1−11	2−7
FeRB	*Geothrix*	unknown	4−10	nd
FeRB/DNB	*Ferribacterium*	no report	6−38	nd
DNB	*Acidovorax*	yes	1−2	0 or <1
DNB	*Sphingomonas*	no report	0−2	nd
DNB/FeOB	*Thiobacillus*	no report	0−27	nd
others	*Duganella*	no report	2−11	nd
	Rhodanobacter		0−5	nd
	Actinobacterium		nd	3−8
	Phyllobacterium		nd	0−6
	Variovorax		nd	0−12

[a] SRB, sulfate reducing nacteria; FeRB, Fe (III) reducing bacteria; DNB, denitrifying bacteria; FeOB, Fe (II)-oxidizing bacteria. Sediment was Sampled on day 775. Groundwater was sampled on days 622 and 670. nd, not detected.

as U(IV) at the MLS wells. However, some U in the sediment was still present as U(VI) even when uranium in the aqueous phase was at low concentrations, as in FW101-3 and 102-2. These results suggest that complete reduction may not be necessary for adequate remediation of U-contaminated sediments.

Extent and Stability of U reduction/immobilization. After day 884, delivery of ethanol to the subsurface stimulated in situ bioreduction of aqueous phase U to levels below <0.05−0.1 μM at the MLS wells. Even lower levels—below the KPA method detection limit of 0.01 μM—were observed in batch tests using groundwater from FW101-2 and FW102-3 at pH 6.6 (data not shown). Separate microcosm tests using reduced sediments from FW104 and four MLS wells indicated that the aqueous U concentrations can be maintained below the U.S. EPA MCL at room temperature and at low temperature (4 °C) for more than 2 years without addition of electron donor at pH 6.6−6.8 (data not shown). These results establish that extremely low concentrations are achievable. The results also indicate that mobilization of nanometer-size UO_2 particles are not significant for this system (*19*). The low aqueous phase U concentrations in the field were much less than the previously reported half-saturation coefficients of 130−880 μM for U(VI) reduction by FeRB and SRB (*13*−*16*) and thus also much lower than the expected threshold values that would be expected for growth-associated reduction of U(VI).

To date most studies of U(VI) bioreduction have been performed at pH values >7. Values greater than 7 negatively affect U(VI) reduction by sulfide species (*17*). They are also less favorable for oxidation of U(IV) by Fe(III)(hydr)oxides (*8*, *23*). We found that reduced/immobilized uranium was stable under anaerobic, quiescent conditions at pH values near 6, even without added ethanol. There was also no evidence of abiotic reoxidation of U(IV) by solid Fe(III) (*23*) or bioreoxidation by SRB (*21*). Our results also differ from those reported for a sediment column experiment (*8*, *22*) where U(VI) levels rebounded even though electron donor (lactate) was available and FeRB (*Geothrix fermentans*) were present. But conditions in the column study (*8*, *22*) were significantly different from those of the present study in terms of pH (<6.8 vs 7.0 for the column study), bicarbonate (<5 vs 15 mM), electron donor (ethanol vs lactate), sulfate (present vs absent), and methanogenic activity (little or insignficant vs extremely high). These differing results suggest that more research is needed to resolve key biogeochemical factors, such as pH, carbonate, divalent cations, sulfide species, and methanogenesis.

Community Structure Analyses of the microbial communities in groundwater and sediment confirmed the presence of U-reducing microorganisms. Clone libraries were dominated by protobacteria in all wells, and γ- and δ-protobacteria were the most abundant. Table 2 summarizes results for an MLS well after U concentrations decreased to near or below the EPA MCL. Sequences for FeRB (*Ferribacterium* and *Geobacter*), SRB (*Desulfovibrio* spp.) and denitrifying bacteria (*Acidovorax, Ferribacterium*) were obtained. FeRB *Geobacter* spp. and SRB *Desulfovibrio* spp. reduce U(VI) (*1*). Previously, *Geobacter* spp. was detected in FRC Area 2 solids (*29*). Fe(II) oxidizing species (*Thiobacillus*) were also present. *Acidovorax*, a denitrifying microorganism that can reduce U(VI) (*6*), was detected in sediment. This organism was previously detected in FRC groundwater and in the denitrifying fluidized bed reactor used to remove bulk nitrate (*30*). *Geothrix* spp., a dominant FeRB (*31*), was detected in sediment from the MLS wells but not the groundwater. This organism grows attached rather than free-swimming. *Geothrix fermentans* was previously found in column experiments on sediment from FRC Area 2 (*22*). It is not yet known whether *Geothrix* can reduce U(VI) (personal communication with D.R. Lovley and J.D. Coates). SRB are likely involved in the degradation of ethanol, production and consumption of acetate, and digestion of biomass. Microbial community analyses based on SSU rRNA clonal libraries indicated an increase in *Desulfovibrio* during the period when DO was removed in wells FW104, FW101-2, and FW102-2 (data not shown). MPN enumeration indicated low levels of methanogens (10^2 cells g^{-1}) at FW104 but none in the MLS wells. FGA analyses indicated that dominant sulfate-reducing genes were *Desulfovibrio* spp. while the dominant cytochrome C genes were from *Desulfovibrio*, *Geobacter*, and *Mycobacterium*. Methanogenic genes were not detected (*35*).

DO Consumption and Persistence of the U(VI)-Reducing Microbial Community. During ethanol biostimulation and the period without ethanol addition, small amounts of DO (about 0.03 mg L^{-1}) entered the inner loop by way of the aboveground recirculation line. DO (up to 5 mg L^{-1}) also enterred the inner loop injection well prior to day 638 and during reoxidation tests. In all cases, DO was consumed. This was likely due to the oxygen-scavenging activities of reduced inorganic solids, such as FeS, and decaying biomass. After 62 days of oxygen exposure, renewed ethanol addition stimulated rapid increases in Fe(II) and sulfide. Thus, oxygen exposure did not prevent rapid restoration of FeRB and SRB activity. Although SRB are classified as strictly anaerobic, *Desulfovibrio desulfuricans*, *D. vugarius*, and *Desulfobacterium autotrophicum* are capable of oxygen-dependent growth at low oxygen levels (up to 0.9−9 μM or 0.028−0.28 mg L^{-1}) (*32*). *Geobacter* spp. can also take advantage of slightly oxic conditions. *G. sulfurreducens* can grow with oxygen when

it is present at a headspace concentration that is 10% or less (*33*). *Geobacter* spp. appear even more oxygen tolerant than *Desulfovibrio* spp. This may explain why *Geobacter*-related sequences were recovered more frequently than *Desulfovibrio*-related sequences. Yet even though *Geobacter*-related sequences were present, U(VI) levels increased when DO was present. After sulfite addition removed DO, *Desulfovibrio* populations recovered and became prevalent. It appears possible that oxygen consumption by SRB and FeRB could protect immobilized U(IV) from oxidation by low levels of DO.

Implications and Further Studies This is the first study to demonstrate that U levels below the EPA MCL can be achieved and maintained in situ. The immobilized uranium is stable under anaerobic, quiescent conditions, and U levels can continue to decline under these conditions. DO oxidizes U(IV) to mobile U(VI), but the response is spatially heterogeneous, likely because of variability in the lengths of flow paths and uneven distribution of reducing agents. Sulfite addition scavenged oxygen and prevented DO entry into the reduction zone. Remediation strategies for the long-term stewardship of U contaminated sites will benefit from the development of additional methods for DO removal, improved methods of chemical delivery, techniques to limit or prevent infiltration of water containing DO, and practical, and cost-effective strategies for the creation of solid-phase forms of uranium that resist oxidation.

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Supporting Information Available

Bacterial community analysis. FGA analysis. MPN method. Methods of XANES measurements and analysis performed at MR-CAT including figure showing data and models. This material is available free of charge via the Internet at http://pubs.acs.org.

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