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

WEI-MIN WU[1], JACK CARLEY[2], JIAN LUO[1], MATTHEW A. GINDER-VOGEL[3], ERICK CARDENAS[4], MARY BETH LEIGH[4], CHIACHI HWANG[5], SHELLY D. KELLY[6], CHUANMIN RUAN[2], LIYOU WU[7], JOY VAN NOSTRAND[7], TERRY GENTRY [2], KENNETH LOWE[2], TONIA MEHLHORN[2], SUE CARROLL[2], WENSUI LUO[2], MATTHEW W. FIELDS[5], BAOHUA GU[2], DAVID WATSON[2], KENNETH M. KEMNER[6], TERENCE MARSH[4], JAMES TIEDJE[4], JIZHONG ZHOU[7], SCOTT FENDORF[3], PETER K. KITANIDIS[1], PHILIP M. JARDINE[2], AND CRAIG S. CRIDDLE[1*]

[1] Department of Civil and Environmental Engineering , Stanford University, Stanford, CA 94305.

[2] Environmental Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831.

[3] Department of Geological and Environmental Sciences, Stanford University, Stanford, CA 94305.

[4] Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1325.

[5] Department of Microbiology, Miami University, Oxford, OH 45056.

[6] Biosciences Division, Argonne National Laboratory, Argonne, IL, 60439.

[7] Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019

[*] Corresponding author. Email: criddle@stanford.edu; Phone:650-723-9032; Fax: 650-725-3164

ABSTRACT. Over a two-year period, in-situ bioreduction of U(VI) decreased the levels of dissolved uranium in groundwater to submicromolar levels and enabled immobilization of uranium as U(IV) at a site located at U.S. Department of Energy (DOE) Environmental Remediation Sciences Program (ERSP) Field Research Center (FRC) at Oak Ridge, TN. The groundwater at this site was contaminated with U(VI) up to 135 µM. Bioreduction was achieved by stimulating growth of denitrifying, Fe(III)-

reducing, and sulfate-reducing bacteria through weekly additions of ethanol for two-day periods. Following sulfite additions to remove dissolved oxygen, aqueous U(VI) concentrations at the monitoring wells fell below the US EPA maximum contaminant limit (MCL) for drinking water (< 30 μg L^{-1} or 0.126 μM). The low concentrations were stable under anaerobic conditions, even in the absence of added ethanol. However, when sulfite additions stopped, and 4.0-5.5 mg L^{-1} dissolved oxygen (DO) was allowed into the injection well over a 60-day period, spatially variable changes in aqueous U(VI) occurred, 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 a MLS well located further away. Resumption of ethanol addition after DO exposure restored reduction of Fe(III), sulfate, and U(VI) within 36 hours at all MLS wells. After two years of ethanol addition, X-ray absorption near-edge structure spectroscopy (XANES) analyses indicated that reduced U(IV) made up 60-80 % of the total uranium in sediment samples. U concentrations in MLS were reduced at below 0.1 μM at the end of the project (1260 days). 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 predominant Fe(III)-reducing species were *Geothrix* spp.

KEYWORDS Bioremediation, groundwater, uranium, reduction and oxidation, biostimulation, sulfate reduction, Fe(III) reduction.

BRIEFS Two-year intermittent addition of ethanol stimulated microbial reduction of aqueous U(VI) to levels below 30 μg L^{-1}, with 60-80% of the total uranium present in the sediment as U(IV). Bioreduced U(IV) was stable under anaerobic conditions but re-oxidized following exposure to dissolved oxygen.

INTRODUCTION

Bioreduction of multivalent metals can convert dissolved, oxidized forms of multivalent heavy metals and radionuclides, such as U(VI) to reduced forms that readily precipitate from solution (*1*). U(VI) reduction/immobilization has been evaluated in batch serum bottles (3-*5*), microcosms (6), sediment columns (7, 8), and field studies (*2,3,9-11*). The process is mediated by iron(III)-reducing bacteria (FeRB), sulfate-reducing bacteria (SRB), and a diverse range of other bacteria (*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 no fixed target levels, concentrations below the US EPA maximum contaminant level (MCL) for drinking water of 0.126 μM (30 μg L^{-1}) (*12*) would be desirable. Pure culture kinetic studies raise concerns about the feasibility of achieving such low concentrations. Researchers reported rapid reduction at high U concentrations (500 to 1200 μM) but the half saturation

coefficients ranged from 130 to 880 μM for SRB and FeRB (*13-16*). These high values imply first order kinetics and slow rates at concentrations near the EPA MCL and a relatively high threshold U(VI) concentration for bioreduction. But biology alone does not control the aqueous concentrations. Physical processes, such as desorption/diffusion limitations and sorption, abiotic reduction also affect the aquerous concentrations (*1*). Sulfide, a reductant generated by sulfate respiration, can reduce U(VI) to U(IV) (*17*), as can microbially generated green rust (*18*).

The stability of bioreduced and immobilized uranium is a concern. Suzuki et al. (*19*) reported that *Desulfosporosinus* spp. reduced U(VI) to form nanometer-size uraninite (UO_2) particles. They were concerned that these particles could be mobile in porous sediments and susceptible to oxidation. Nitrate promotes oxidation of bioreduced U(IV) to U(VI) in sediments (*4*). The incompletely reduced intermediates of dissimilatory nitrate reduction - nitrite, nitrous oxide and nitric oxide – can oxidize U(IV) and remobilize U(VI) (*9*). These reports justify efforts to remove of high levels of nitrate (*10*), and O_2 removal may also be necessary. Oxygen oxidizes U(IV), and the reaction is rapid in the presence of high levels of bicarbonate (1M) (*20*). Even Fe(III) species can oxidize U(IV) when conditions are appropriate. A rebound of U(VI) was observed under a lactate-limited sulfate-reducing conditions inoculated with *Desulfovibrio desulfuricans* G20 in the presence of Fe(III)(hydr)oxides (*21*). Oxidation of bioreduced U(IV) and elevated methanogenesis occurred in a column study using FRC Area 2 sediments, even though known U(VI)-reducing bacteria - *Geobacteraceae* - were present (*8*,*22*). The hypothesized oxidant was residual Fe(III). A thermodynamic analysis (*8*) established that a high level of bicarbonate (15 mM) and Ca^{2+} (1 mM) adversely affect the stability of bioreduced U(IV) by alterring solution thermodynamics to favor U(IV) oxidation by Fe(III) solids. Thermodynamic analyses predicted and laboratory experiments confirmed that oxidation of biogenic UO_2 by Fe(III)(hydr)oxides was favorable under certain conditions (*23*).

Previously, a test facility for bioremediation strategies was constructed in Area 3 of the DOE ERSP FRC site, located adjacent to the former S-3 Ponds and containing high levels of uranium 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 has 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) that can be achieved 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 US EPA MCL (<0.126 μM) can be achieved *in situ*, that the 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 uranium.

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, the system consisted of an outer recirculation loop (from FW024 to FW103) protecting a nested inner recirculation loop (from FW026 to FW104) from penetration by highly contaminated groundwater from the source zone. Ethanol additions stimulated reduction of U(VI) to U(IV) in the inner loop. 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) were used for routine monitoring because these depth levels had the highest groundwater flowrates, and highest U in the water and on the solid phase (*10,24*). The recirculation flow rate in both the inner and outer loops 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 (*10,24*). The additional water was Y-12 Plant tap water (pH 8.0 with 2.82 to 3.38 mM chloride; 0.04 to 0.048 mM nitrate; 0.24 to 0.26 mM sulfate; 0.68 to 0.75 mM Ca, < 0.007 mM Al). Prior to injection at FW024, the pH of the blended water was adjusted to 5.4 to 5.6 with HCl (20%, w/w) in a storage tank. The DO in the clean water vaeried from 9 mg L^{-1} in summer and 12 mg L^{-1} in winter. After day 638, Na_2SO_3 (approximately 0.9 mM) was added to the storage tank, The added sulfite removed oxygen by the reaction $2SO_3^{2-} + O_2 \rightarrow 2\ SO_4^{2-}$, decreasing DO to near zero, but did not reduce U(VI). This water was injected into the outer loop, extracted at the inner loop extraction well, and recirculated into the inner loop. When sulfite was not added, DO-containing water entered the outer loop, then the inner loop via the same route.

Ethanol and its collective metabolites were monitored as Chemical Oxygen Demand (COD), where 8 g of oxygen demand contains one mole of available electrons. Ethanol, prepared as a 9.8 g COD L^{-1} stock solution, was normally injected at FW104 over a 48-hour period each week, resulting 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. Akalinity at the MLS wells ranged from 0.8 to 4 mM as HCO_3^-, depending on K_2CO_3 additions.

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

extraction well FW026, 50% of the captured flow came from the inner injection well FW104, 44% from the outer injection well FW024, and 6% from regional flow.

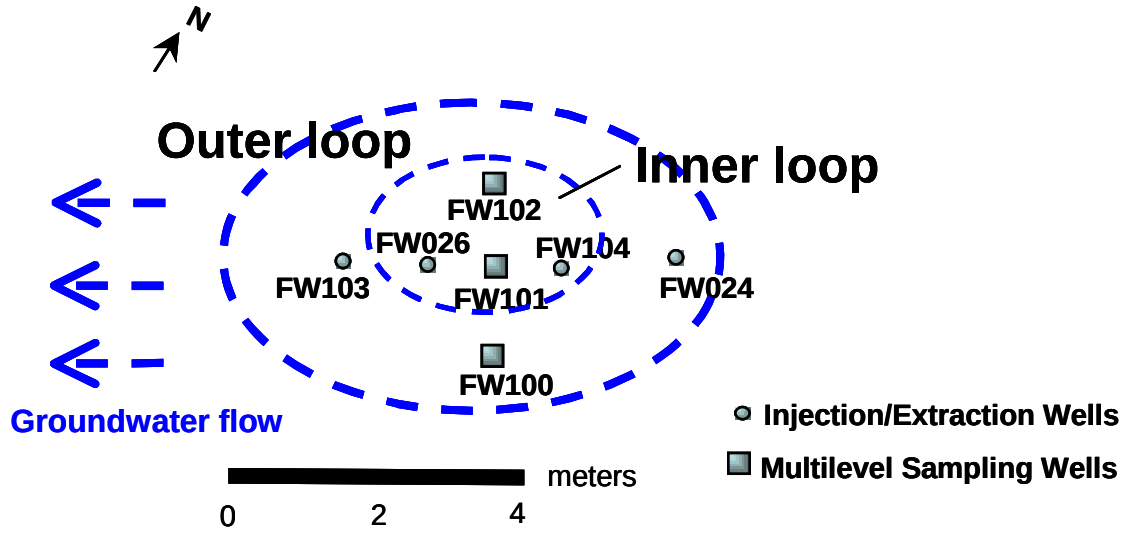

FIGURE 1. Pilot-scale bioremediation well system.

Chemicals and analytical methods. Previous publications (*10,11*) give detailed information on the source and quality of chemicals used at the field site; 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; use of a kinetic phosphorescence KPA-11 analyzer for U analysis (Chemchek Instruments, Richland, WA); and groundwater and sediment sample collection. The oxidation state of U in sediments was determined by XANES (see 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 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. In previous work (days 137 to day 532), ethanol addition stimulated bioreduction of uranium. Aqueous U concentrations at FW104 and FW026 decreased from 5 µM to

around 1 µM while at MLS wells decreased to 0.2-0.5 µM (*11*). In the present study, four tests were performed: (1) U(VI) reduction without DO control (days 533 to 637); (2) U(VI) reduction with DO control (days 638 to 688); (3) a test of immobilized U stability with no added ethanol and no added DO (days 713 to 754); and (4) a test of stability with added DO but no added ethanol (day 806 to 884). DO removal and weekly two-day ethanol additions were then resumed and continued by day 1266 to test stabilities of the reduced site (data not presented in this paper). The pH in the inner loop injection well FW104 increased from 5.8-6.0 to 6.6-7.0 during the injections and remained at 5.9 to 6.4 in the MLS wells with biocarbonate. Without injection of biocarbonate, the pH was maintained at 5.7-6.1. The temperature of the subsurface ranged from 12 ^{o}C in the winter to 21^{o}C in the summer. From day 137 to day 1266, ethanol was injected for 140 times with a total amount of 8.0 kg.

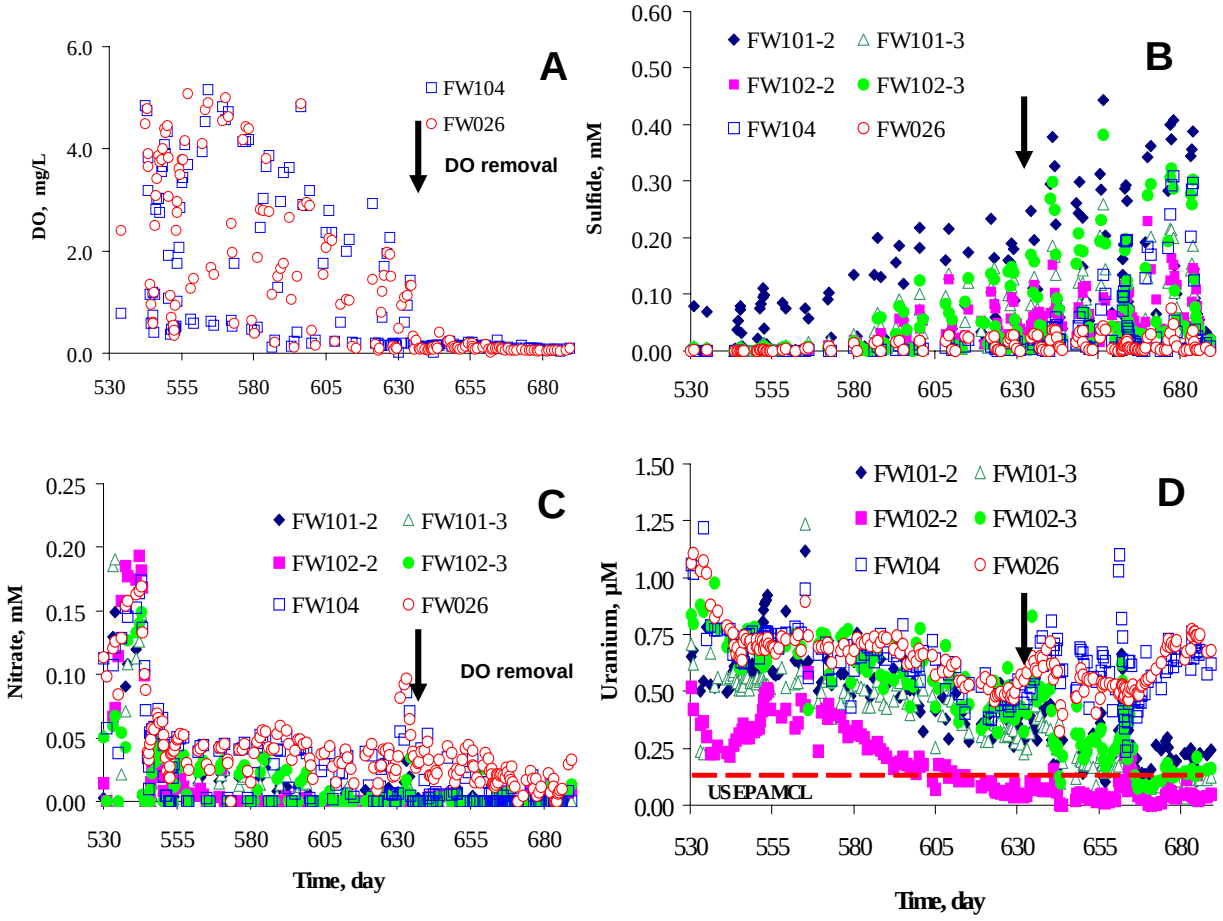

FIGURE 2. Geochemical changes during biostimulation in groundwater from the inner loop recirculation and MLS wells before and after DO control on day 637. A. DO concentrations in the inner loop extraction (FW026) and injection wells (FW104). DO

concentrations were maintained below 0.15 mg L^{-1} in FW104 after day 637. B. Sulfide. C. Nitrate. D. Uranium.

U(VI) Reduction to below the US EPA MCL (<0.126 µM). FIGURE 2 summarizes results from initial two tests (day 530 to day 688). By day 637, DO concentrations in FW104 were around 0.5 to 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 continuously increased at the MLS wells (FIGURE 2B). After day 637, DO was removed by addition of Na_2SO_3 to water that entered the outer loop. DO concentrations decreased to less than 0.15 mg L^{-1} (FIGURE 2A). In the absence of DO, 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 even prior to DO control due to weekly ethanol injections (FIGURE 2D). Removal of DO inputs from the outer loop coincided with further declines in U(VI) concentrations in the groundwater and likely enabled U(VI) reduction to concentrations at or below the US 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 were maintained thereafter (Table 1). In FW101-2 and FW102-3, U concentrations fell below the EPA MCL during ethanol injection but rebounded slightly in its absence. Low U concentrations were maintained for days to months in wells FW101-3 and FW102-2 but were variable in FW101-2 and FW102-3, likely because these wells were most closely connected to the injection well where U(VI) from the outer loop was continuously injected (FIGURE 2D).

Stability of Uranium without Ethanol Injection. Ethanol was injected into the inner loop from day 710 to day 713. By day 713, aqueous U concentrations had fallen below the EPA MCL at all MLS wells (FIGURE 3 A). From day 713 to day 754, no ethanol was injected. Aqueous U continuously entered the inner loop through FW104 at concentrations of 0.5 to 0.7 µM. The concentration of U at MLS wells slowly increased, but never to the levels of the inner injection (FW104) and extraction wells (FW026) (FIGURE 3A), indicating a sink for U(VI) in the zone between the injection well and the MLS wells.

Sulfate concentrations in FW104 and FW026 increased then stabilized (FIGURE 3B). Unlike uranium, sulfate at the MLS wells increased to the level of FW104 and FW026 (FIGURE 3B). Sulfide concentrations in MLS increased to peak concentrations of about 0.5 mM during ethanol injection, then decreased, but remained at significant levels (0.01 mM) throughout the test period (FIGURE 3C),

indicating persistence of anaerobic conditions. During this period, total soluble Fe (FIGURE 3D) was used as an indicator of Fe(II) concentrations. Soluble Fe concentrations initially fell during addition of ethanol, perhaps due to FeS formation. Concentrations then increased until day 718, suggesting Fe(III) reduction and accumulation of Fe(II). The gradual decrease thereafter may reflect decreasing rates of Fe(III) reduction. DO at FW104 was < 0.2 mg L^{-1}d, so DO had little or no effect on soluble Fe.

Active SRB were thus 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 hours. After 12 hours, sulfide levels in FW101-2 had increased from 0.014 to 0.27 mM, and levels in FW102-3 increased from 0.014 to 0.29 mM. U reduction also continued: after two weeks of weekly 2-day ethanol additions, U concentrations dropped below the EPA MCL in all four MLS wells (data not shown).

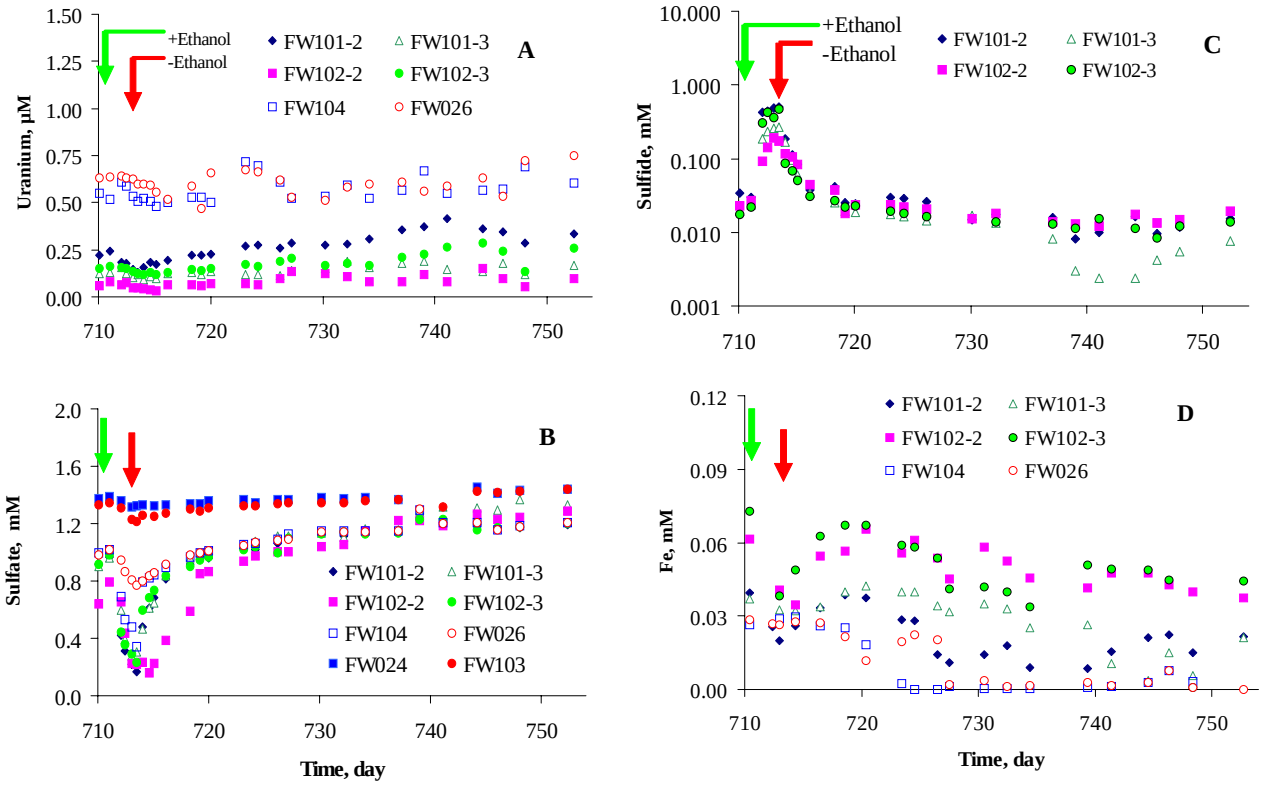

FIGURE 3. Changes in groundwater quality in the absence of ethanol addition. A. Uranium. B. Sulfate. C. Sulfide. D. Dissolved Fe.

Impact of DO. The site was first reduced by injecting ethanol into the inner loop from days 801 to 803. No ethanol was added from day 804 to day 866. DO (9-11 mg L^{-1}) was introduced into the outer loop from day 811 through day 884. (FIGURE 4A). DO at the inner loop injection well FW104 increased 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 FW104 and FW026 was 5.2 mg L^{-1}, which was approximately 50% of DO in FW024. Because the diameter of the MLS wells was too small for insertion of a DO probe, DO was measured by slowly pumping groundwater through an aboveground glass vial containing a DO probe. On day 823, measured DO levels were less than 0.6, 0.6, 0.22, and 0.25 mg L^{-1} in FW101-2, 101-3, 102-2 and 102-3, respectively, and on day 866, they were less than 2.0, 0.8, 0.3 and 0.33 mg L^{-1} respectively. Given that some oxygen likely diffused through the tubing used for sampling, the actual DO was probably less than these values. 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 monitoring wells.

Prior to 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). The levels at FW101-2 and FW102-3 (0.76 µM) exceeded the values from the inner loop injection well FW104 (~0.57 µM) on day 817, indicating that these increases were due to mobilization of solid-associated uranium and not to 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 those in FW104. These observations are consistent with tracer results indicating that FW101-2 was hydraulically well connected to the injection well (*25*). Aqueous U concentrations in FW 101-3, increased to the same level as FW104 and remained essentially unchanged thereafter. In FW102-3, aqueous U concentrations initially increased rapidly to 0.8 µM and then slowly increased at approximately 0.01 µM d^{-1}. In 102-2, U levels remained low. A possible explanation is a more extensive reduced zone near well 102-2.

At FW104, Fe(II) decreased to zero when DO entered on day 811 (FIGURE 4C), but groundwater at FW026 contained low levels of Fe(II) (0.005-0.006 mM) throughout the test. The Fe(II) at FW101-2 was even lower (0.003 mM or less). Fe(II) in both FW102-3 and FW102-2 remained at a relatively high levels (0.03 mM). Fe(II) concentrations in the outer loop were zero or below the detection limit (<0.002 mM). Sulfide concentrations were sensitive to DO (FIGURE 4D). Sulfide in FW104 and FW026 dropped to below 0.001 mM or near the detection limit (< 0.0002 mM). In MLS wells, sulfide decreased but remained above the detection limit.

241 On day 866, ethanol was injected into the inner loop for two days to test survival of FeRB and
242 SRB and recovery of reduction activity. DO concentrations in FW104 and FW026 decreased but
243 rebounded when ethanol injection stopped (FIGURE 4A). FeRB and SRB were stimulated: at the MLS
244 wells, Fe(II) concentration inceased within 12-24 hours, and this was followed by an increase in sulfide
245 concentrations (FIGURE 4C and 4D). From day 866 to 868, initially, U concentrations at the MLS
246 wells increased slightly - likely due to the release of U(VI) sorbed to Fe(III) oxides. After DO was
247 removed on day 884, ethanol injection was performed for 7 times and U concentrations gradually
248 returned to near or below the EPA MCL in three MLS wells by day 935 (Table 1). U in FW102-3
249 remained relatively high (0.4μM) for 10 months due to changes in groundwater flow pattern *(25)* with
250 invasion of low pH water from local source. Based on flow rates, and DO concentrations, from day 811
251 to 884, total DO injected to FW024 was approx. 1560 g and about 240 g was estimated to enter to the
252 extraction well FW026. From day 866 to 934, ethanol was injected at a total amount of 1360 g COD to
253 innerloop in order to restore the low U concentrations. This suggests that almost the same amount of
254 electron equivalent as oxygen injected to the system is needed to restore orginal treatment conditions.

255 As ethanol injection continued, U in the four MLS eventually dropped below EPA MCL all the
256 time. From day 1237 to 1266, the average U concentrations over four week period was 0.25 ± 0.03μM
257 in FW104 and were 0.10 ± 0.024, 0.033 ±0.0065, 0.058±0.014 and 0.078±0.023μM in FW101-2, 101-3,
258 102-2 and 102-3, repectively.

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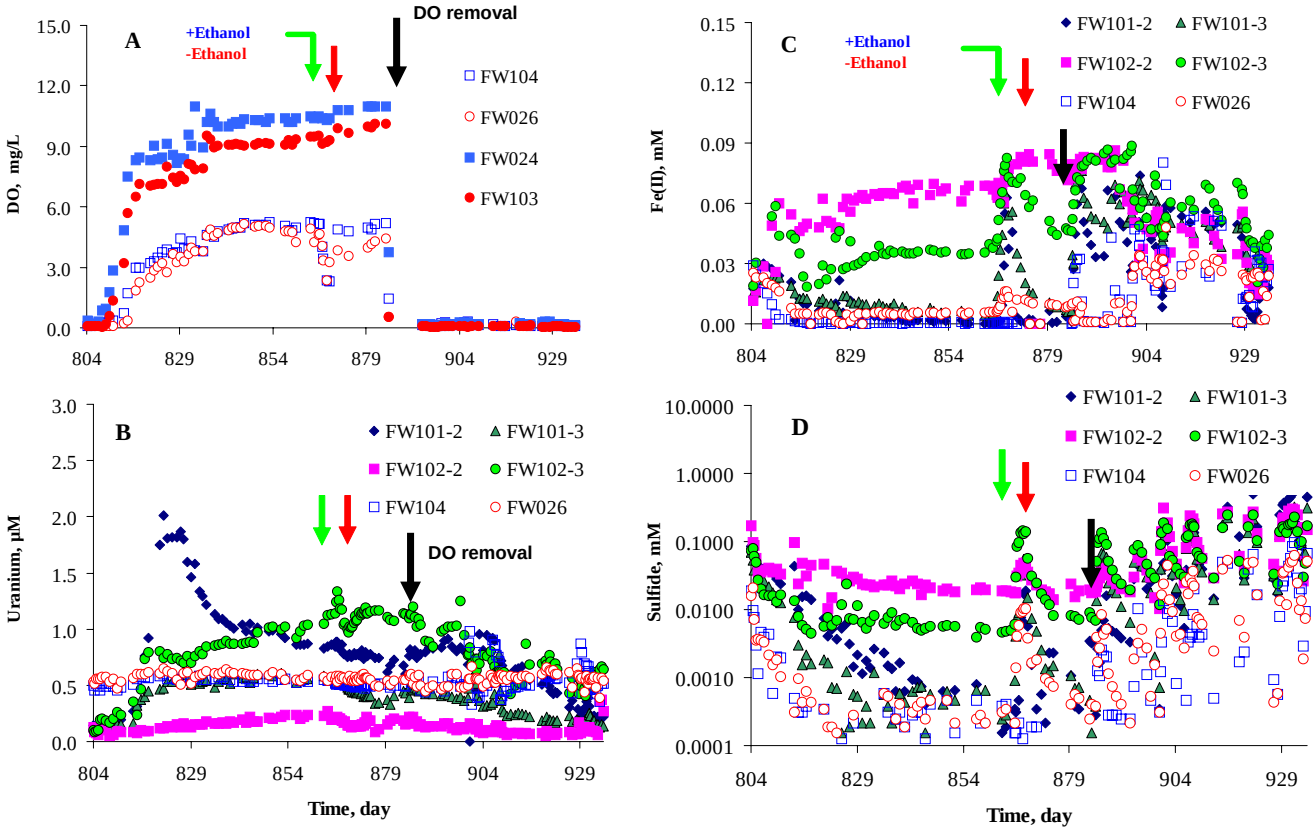

FIGURE 4. Impact of DO on stability of the bio-reduced subsurface within the inner loop (day 811-884). The changes of concentrations in groundwater: A. DO of outer loop and inner loop wells. B. Uranium. C. Fe(II). D. Sulfide.

Sediment Uranium Levels and XANES Results. TABLE 1 gives U concentrations in both groundwater and sediment from the inner loop wells and percentages of total U present as U(IV). Prior to oxidation test, U contents in injection well FW104 and MLS wells FW101-2 and FW101-3 increased significantly by day 774. This was likely due to bioreduction and immobilization. On day 774, U content in the sediment from FW104 were higher than the value on day 898, and U concentration in sediments from FW101-2 and FW101-3 were higher than values on day 935. This could be attributed to a loss of immobilized U during the reoxidation period (days 811-884), but the U content of sediments at FW102-2 and 102-3 increased from day 774 to 935. This suggests that more U was immobilized and/or that less was lost during the oxidation period. The distance between the FW102 wells and FW104 was longer than the distance between FW101 and FW104 (FIGURE 1). Thus, DO would be expected to have less impact near FW102. XANES analysis of day 935 samples indicated that a significant fraction of U, up to 60-80% of total U, was present as U(IV) at the MLS wells. However, a part of the sediment U was still present as U(VI) even when uranium in the aqueous phase was at very low concentrations such as in FW101-3 and 102-2. This suggests 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 in MLS. 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 indicated that the aqueous U concentrations can be maintained below US EPA MCL at room temperature and at low temperature (4 ^{o}C) for more than two years without addition of electron donor at pH 6.6-6.8 (data not shown). These results demonstrate that extremely low concentrations can be achieved *in situ*. The results also indicate that the mobilization of nanometer-size UO_2 particles in this system is not a significant factor as concerned (*19*). The low aqueous phase U concentrations in the field were much less than the half-saturation coefficients (Km) of 130-880 µM previously reported for U(VI) reduction by FeRB and SRB (*13-16*). If U(VI) reduction were a purely bioreduction there could be a threshold concentration, which could be much higher than US EPA MCL because of Km from 130-880 µM and impact the extent of U(VI) reduction. Most tests reported to date were carried out at pH>7.0. A pH>7.0 negatively affects U(VI) reduction by sulfide (*17*) but is less favorable for re-oxidation of U(IV) by Fe(III)(hydr)oxides (*8, 23*). We found that reduced/immobilized uranium was stable under anaerobic, quiescent conditions. It was also stable *in situ*, even without added ethanol. Neither clear evendience of abiotic re-oxidation of U(IV) by solid Fe(III) (*23*) nor bioreoxidation by SRB (*21*) was observed in our system. Our results also differ from those reported for a sediment column test (*8, 22*), where U(VI) rebounded, even though electron donor (lactate) was available and FeRB (*Geothrix fermentans*) were present. However, conditions in the column study (*8, 22*) were quite different from those evaluated in this fieldwork. Differences of this study vs the colum test (*8, 22*) include pH (<6.8 vs 7.0), bicarbonate (<5 mM vs 15 mM), electron donor (ethanol vs lactate), sulfate (present vs. absent), and methanogenic activity (little or insignficant vs extremely high). Our results suggest that more research is needed to understand the role of geochemical factors such as pH, carbonate, divalent cations and sulfide species.

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 (*38*).

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 entered the inner loop injection well prior to day 638 and during re-oxidation 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. At end of 62-day oxygen exposure period, 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 to 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 can benefit from development of additional methods for DO removal, improved methods of chemical delivery, techniques to limit or prevent infiltration of water containing DO, and creation of solid-phase forms of uranium that resist oxidation by selecting cost-effective approaches.

Acknowledgments

This work was funded by the U. S. DOE Environmental Remediation Sciences Program (ERSP), under grant DOEAC05-00OR22725. The authors thank Hui Yan for analytical help and Julie Stevens for her daily work in project administration. Michael Fienen, Jennifer Nyman and Margaret Gentile provided valuable input for the field tests and assistance with data organization. US DOE ERSP and DOE Office of Science, Office of Basic Energy Sciences support for XANES investigations.

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.

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469 TABLE 1. Uranium in groud water and sediments from the inner loop injection and MLS and XANES
470 analyses of U(IV) content.
471

Well	Day Pulled	pH	Aqueous U (µM)	U in sediments (g/kg solids)	Days storage at 4° C	% U(IV) XANES
FW104	258*	6.15	1.20	2.60	> 4 weeks	36
	409*	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

472
473 Note: Analytical errors of XANES for U(IV) is about ± 10%. U(VI) reduction in sediment samples
474 continued in serum bottles stored in a refrigerator and the U(IV) content increased significantly during
475 one year storage (*34*). Thus, the measured U(IV) content of stored sediment samples may be greater than
476 the values obtained when the samples were first removed from the subsurface. * See reference (*11*). Not
477 sampled: ns.

TABLE 2. Predominant bacterial community members in MLS wells where U levels decreased below the EPA MCL. SRB = sulfate reducing bacteria. 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.

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