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In Situ Bioreduction of Technetium and Uranium in a Nitrate-Contaminated Aquifer

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The potential to stimulate an indigenous microbial community to reduce a mixture of U(VI) and Tc(VII) in the presence of high (120 mM) initial NO_3^- co-contamination was evaluated in a shallow unconfined aquifer using a series of single-well, push−pull tests. In the absence of added electron donor, NO_3^-, Tc(VII), and U(VI) reduction was not detectable. However, in the presence of added ethanol, glucose, or acetate to serve as electron donor, rapid NO_3^- utilization was observed. The accumulation of NO_2^-, the absence of detectable NH_4^+ accumulation, and the production of N_2O during in situ acetylene-block experiments suggest that NO_3^- was being consumed via denitrification. Tc(VII) reduction occurred concurrently with NO_3^- reduction, but U(VI) reduction was not observed until two or more donor additions resulted in iron-reducing conditions, as detected by the production of Fe(II). Reoxidation/remobilization of U(IV) was also observed in tests conducted with high (∼120 mM) but not low (∼1 mM) initial NO_3^- concentrations and not during acetylene-block experiments conducted with high initial NO_3^-. These results suggest that NO_3^--dependent microbial U(IV) oxidation may inhibit or reverse U(VI) reduction and decrease the stability of U(IV) in this environment. Changes in viable biomass, community composition, metabolic status, and respiratory state of organisms harvested from down-well microbial samplers deployed during these tests were consistent with the conclusions that electron donor additions resulted in microbial growth, the creation of anaerobic conditions, and an increase in activity of metal-reducing organisms (e.g., *Geobacter*). The results demonstrate that it is possible to stimulate the simultaneous bioreduction of U(VI) and Tc(VII) mixtures commonly found with NO_3^- co-contamination at radioactive waste sites.

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Introduction

Contamination of the subsurface by U and ^{99}Tc (Tc) is common (*1*), but the mobility of these radionuclides in groundwater largely depends on site-specific biogeochemical conditions. In oxidizing environments, U occurs as U(VI), which forms highly soluble and mobile complexes with carbonate at pH >5 (*2*) and Tc occurs as Tc(VII) in the form of the highly soluble and mobile pertechnetate anion (TcO_4^-) (*3*, *4*). In reducing environments, U and Tc occur as U(IV) and Tc(IV and V), respectively, which have much lower solubility and mobility than their oxidized forms (*2*, *4*). For this reason, *bio-immobilization*, the addition of nutrients to the subsurface to stimulate indigenous microorganisms to reduce U(VI) and Tc(VII) and thereby produce and precipitate U(IV) and Tc(IV and V) solid phases, has been proposed as a strategy for reducing U and Tc concentrations in groundwater (*3*, *4*).

U(VI) and Tc(VII) can act as respiratory electron acceptors for certain bacteria (*7*−*9*), and direct enzymatic reduction of U(VI) and Tc(VII) has been reported for pure cultures of Fe(III)- and sulfate-reducing reducing microorganisms (*4*, *7*−*12*). U(VI) and Tc(VII) may also be indirectly reduced by microbiologically generated Fe(II) (*9*) or sulfide (*14*), although the rate of these reactions may be slow compared to microbially catalyzed reduction (*7*). For this reason, the addition of nutrients to the subsurface to stimulate U(VI) and Tc(VII) reduction and immobilization by indigenous microorganisms has been proposed as a strategy for reducing U and Tc concentrations in groundwater (*5*). However, most information on the microbial reduction of U(VI) and Tc(VII) has been obtained under laboratory conditions, which may not be representative of in situ conditions (*15*, *16*).

Although organisms with the capability to reduce U(VI) and Tc(VII) are apparently widespread in the subsurface (*15*), their activity in heavily contaminated environments is likely often limited by the numbers of microorganisms, availability of a suitable electron donor, high concentrations of competing electron acceptors, low pH, the presence of toxic metals, and other site-specific factors (*4*, *7*−*12*). Many sites are aerobic, and the addition of an organic electron donor is necessary to create the anaerobic and reducing conditions required for the growth of metal-reducing microorganisms. However, this approach is complicated at many sites by the presence of high concentrations (>100 mM) of NO_3^- derived from the use of nitric acid or ammonium (subsequently oxidized to NO_3^-) (*17*) in ore processing and isotope separation processes (*1*). Nitrate serves as a competing and more energetically favorable electron acceptor, which must be removed to initiate or maintain U(VI) and Fe(III) reducing conditions (*16*).

Nitrate is also an effective oxidant of U(IV). Recent work has shown that addition of NO_3^- to U(IV)-containing sediments leads to the oxidation and remobilization of U(IV) (*13*, *17*). This is believed to occur in the subsurface via one or more of several possible mechanisms that follow: U(IV) is abiotically oxidized by NO_2^- or NO, which accumulate during denitrification (*13*); U(IV) is oxidized by Fe(III) minerals generated by nitrate-dependent Fe(II) oxidation (*19*, *20*) or by the nitrate-dependent, enzymatic oxidation of U(IV) (*18*). Previous research has shown that U(VI) reduction is inhibited in the presence of NO_3^- and that the addition of NO_3^- or denitrification intermediates (e.g., NO_2^-, N_2O) to previously bioreduced U(IV) will result in the production of U(VI) (*13*, *17*). It is also possible that lithotrophic NO_3^- reducing bacteria could be responsible for observed U(IV)

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oxidation following NO_3^- addition (*18*) or that U(IV) could be oxidized by Fe(III) minerals generated by nitrate-dependent Fe(II) oxidation (*18, 20*). Although it is possible that similar processes could result in the reoxidation and remobilization of bioreduced Tc(IV and V), the effect of NO_3^- on Tc(VII) reduction or Tc(IV and V) oxidation has not been studied. As U(VI) and Tc(VII) occur often as co-contaminants, we might also predict that each might have some effect on the reduction of the other. However, there have not been any previously documented examples of the simultaneous bioreduction of U(VI) and Tc(VII).

Effective implementation of bio-immobilization requires that methods be developed to monitor and quantify the effect of nutrient additions on the activity of U(VI)- and Tc(VII)-reducing microbial communities under actual field conditions. In situ testing is clearly preferred because of the well-known difficulties of obtaining representative subsurface samples and preserving them in the laboratory under environmentally relevant conditions (*21, 22*). In this study we investigated the in situ bioreduction of U(VI) and Tc(VII) in the presence of NO_3^- co-contamination in a shallow unconfined aquifer at a U.S. Department of Energy (DOE) legacy waste site. The geochemistry of groundwater at this site is complex and heterogeneous with high and variable concentrations of NO_3^- (up to 170 mM), U(VI) (up to 20 μM), and Tc(VII) (up to 30000 pM). The aquifer is aerobic (0.1−0.25 mM O_2), and microbial activity is believed to be electron donor limited. Single-well, push−pull tests (*13, 23*) and down-well microbial samplers (*24*) were used to monitor the response of the indigenous microbial community to electron donor additions. In situ rates of donor utilization and NO_3^-, U(VI), and Tc(VII) reduction were measured as well as viable biomass, community composition, metabolic status, and respiratory state of the in situ community. Through a series of injections of ethanol, acetate, or glucose we were able to stimulate the growth and activity of indigenous denitrifying and metal-reducing organisms and thus demonstrate that bio-immobilization is a viable remediation strategy at a site contaminated with mixtures of U(VI) and Tc(VII). To our knowledge, this is the first field study of the mechanisms and kinetics of U(VI) and Tc(VII) reduction in a NO_3^--contaminated aquifer.

Materials and Methods

Field Site. The field site was the U.S. Department of Energy's Natural and Accelerated Bioremediation Research Program's Field Research Center, which is located on the western edge of the Y-12 plant at the Oak Ridge Reservation in Oak Ridge, TN. At this site, subsurface contamination resulted from liquid waste discharges to former disposal ponds. While in operation, the ponds received acidic (pH <2) liquid wastes containing nitric acid and high concentrations of U, Tc, and other dissolved metals. Waste disposal ended in 1983, and the ponds were capped in 1988. Field tests were conducted ~50 m down-gradient of the former ponds in monitoring wells installed in the shallow unconfined aquifer formed in the unconsolidated silty-clayey saprolite overlying the Nolichucky shale (*25, 26*). Wells were installed by direct-push methods to a depth of ~7 m and were constructed of 3 cm PVC with a 1.5 m screened interval at the bottom of the well. Pretest well sampling indicated spatially variable groundwater geochemistry (Table 1). The groundwater pH ranged from 3.3 to 6.8 with U(VI), NO_3^-, and Tc(VII) at concentrations up to 5.8 μM, 168 mM, and 18000 pM, respectively; SO_4^{2-} concentrations were <1 mM. Prior to testing, groundwater was aerobic (0.1−0.25 mM dissolved O_2) and NO_2^- and Fe(II) were not detectable. These geochemical data suggest that microbial activity in the aquifer is electron donor limited. The combination of low pH and high and variable concentrations of NO_3^-, U(VI), and Tc(VII) in an aerobic subsurface environment is representative of many legacy waste sites in the DOE complex including uranium mill tailing reclamation areas, ore processing, and isotope separation facilities (*1*).

TABLE 1. Pretest Groundwater Concentrations in Wells Used in Field Tests

well	pH	NO_3^- (mM)	SO_4^{2-} (mM)	U (μM)	Tc (pM)
FW21	3.3	142	0.4	5.8	18000
GW835	6.4	1	0.8	4.9	410
FW19	6.6	8	0.6	0.7	2300
FW27	5.4	168	0.0	0.1	15000
FW30	3.5	145	0.0	4.2	13000
FW31	5.7	63	0.1	0.0	1200
FW32	5.2	23	0.0	0.0	940
FW33	5.9	14	0.7	0.3	1300
FW34	6.8	1	0.8	0.5	39

Push−Pull Tests. Preliminary microcosm experiments (data not shown) using sediments and groundwater from portions of the site with pH >5 indicated that the addition of various electron donors (acetate, ethanol, and glucose) could stimulate microbial activity. For this study, a series of single-well, push−pull tests were conducted in six wells with moderate pretest pH (5.2−6.8) and various pretest concentrations of NO_3^-, U(VI), and Tc(VII) (Table 1) to measure the effect of electron donor additions on rates of donor utilization and on rates of NO_3^-, U(VI), and Tc(VII) reduction. The test sequence is summarized in Table 2; 5 tests were conducted without added electron donor in wells FW19, FW27, and FW31, and 35 tests were conducted with added glucose, ethanol, or acetate in wells FW19, FW32, FW33, and FW34. Injected test solutions were prepared from groundwater obtained from well FW21, well GW835, or distilled water, depending on the purpose of the test (Table 2). In wells that received electron donor, two tests, ~2 months apart, were initially conducted in each well using test solutions prepared from FW21 groundwater with added tracer, bicarbonate, and electron donor as described below (e.g., tests 14 and 22 in well FW34, Table 2). Then three additional tests, ~1 month apart, were conducted in each well using test solutions prepared from distilled water as described below (e.g., tests 28, 32, and 36 in well FW34) to increase microbial biomass and activity and stimulate metal-reducing conditions in the subsurface. After biostimulation, two additional tests were conducted using test solutions prepared from FW21 groundwater with (tests 39−42) and without (tests 44 and 46) added electron donor. Tests 47−50 were conducted as described below using test solutions prepared from GW835 groundwater as described below, which had lower initial NO_3^- and Tc(VII) concentrations (Table 1). Finally, tests 56 and 57 were conducted in wells FW34 and FW33 using test solutions prepared from FW21 groundwater as described below.

Test solutions prepared from FW21 groundwater were amended with ~80−130 mM sodium bicarbonate and mixed with compressed 80% N_2/20% CO_2 gas. These latter additions, which served to increase the test solution pH to approximately match the pH of groundwater in each test well (Table 1), also ensured that all of the U(VI) was in the form of soluble uranyl carbonate complexes (*2*). These forms of U(VI) are those most commonly encountered in wastewaters at DOE sites (*27*). Because the uranyl carbonate [$UO_2(CO_3)_2^{2-}$] complex is highly soluble and is less likely to sorb to aquifer sediments than the uncomplexed form (*10*), dilution adjustments can be performed using measured concentrations of a co-injected Br^- tracer as described below (*28*). pH adjustment resulted in the formation of ~2 g/L precipitate, consisting primarily of calcium, aluminum, and manganese hydroxides (*27*), which settled to the bottom of the drum. The supernatant, containing ~120 mM NO_3^-, ~6 μM U(VI), and ~20000 pM Tc(VII), was transferred to a second drum and amended with

TABLE 2. Effect of Electron Donor Additions on Rates of Donor Utilization, Rates of NO_3^-, Tc(VII), and U(VI) Reduction, and Maximum NO_2^- Concentration

well	test	source water for test solution	treatment	donor rate (mM/h)	NO_3^- rate (mM/h)	Tc(VII) rate (pM/h)	U(VI) rate (μM/h)	max NO_2^- (mM)
FW019	13	FW21	no donor		0	0	0	0
FW031	18, 24	FW21	no donor		0	0	0	0
FW027	20, 26	FW21	no donor		0	0	0	0
FW019	15	FW21	+ acetate	0.14	0.12	0	0	75
	21	FW21	+ acetate	0.40	0.36	107	0	8
	27, 31, 35	$CaCl_2$ solution	+ acetate	donor addition only—no rate data available				
	39	FW21	+ acetate	24	0.51	9	0	10.6
	43	FW21	+ acetate	0.8	0.69	39	0	10
	47	GW835	+ acetate		>0.01		0.021	0.08
FW034	14	FW21	+ ethanol	0.04	0.28	32	0	130
	22	FW21	+ ethanol	0.25	0.4	150	0.003	12
	28, 32, 36	$CaCl_2$ solution	+ ethanol	donor addition only—no rate data available				
	40	FW21	+ ethanol	7.6	3.1	189	0.008	6
	44	FW21	no donor		0.2	91	0	3
	48	GW835	+ ethanol	0.16	0.011	4.8	0.024	0.04
	56	FW21	+ ethanol + acetylene	0.82	1.5	104	0.002	2.5
FW033	17	FW21	+ glucose	0.08	0.76	10		61
	23	FW21	+ glucose	0.16	0.59	37		31
	29, 34, 38	$CaCl_2$ solution	+ glucose	donor addition only—no rate data available				
	41	FW21	+ glucose	0.94	3.2	134	0.019	23
	45	FW21	+ glucose	4.3	0.04	45	0.025	18
	49	GW835	+ glucose	0.1	0.012		0.041	0.06
	57	FW21	+ glucose + acetylene	1.4	1.1	25	0	7.1
FW032	19	FW21	+ glucose	0.02	0	0	0	1
	25	FW21	+ glucose	0.11	0.44	143	0.001	9
	30, 33, 37	$CaCl_2$ solution	+ glucose	donor addition only—no rate data available				
	42	FW21	+ glucose	4.1	0.51	460	0.015	3
	46	FW21	no donor		0.12	2	0	7
	50	GW835	+ glucose	0.31	>0.05		0.034	0.09

~1.3 mM Br^- tracer and ~20−200 mM (depending on the test) acetate, ethanol, or glucose (Table 2).

Test solutions prepared from distilled water were amended with 15 mM $CaCl_2$ (to increase ionic strength and avoid dispersing sediment clay minerals) and ~400 mM glucose, ethanol, or acetate. Test solutions prepared from GW835 groundwater were amended with 10 mM sodium bicarbonate and ~1.3 mM Br^- tracer and contained ~1 mM NO_3^-, 5 μM U(VI), and 410 pM Tc(VII). Test solutions for tests 56 and 57 were prepared as described for other tests using FW21 groundwater except that, prior to injection, the test solution was mixed with equal parts compressed acetylene and 80% N_2/20% CO_2 gas to achieve a dissolved acetylene concentration of ~180 mM.

Approximately 200 L of test solution was injected into each well for each test using a siphon over a period of 0.5−2 days. Five samples of the test solution were collected during injection. Following injection, groundwater samples were periodically collected from the same well for up to 1000 h. Samples were filtered (0.2 μm) in the field and stored at 4 °C until analyzed as follows.

Analytical Methods. Br^-, NO_3^-, SO_4^{2-}, NO_2^-, and acetate were measured by ion chromatography (Dionex, model DX-120). U(VI) was measured by a kinetic phosphorescence analyzer (Chemcheck, KPA-11) (*29*), Tc(VII) by liquid scintillation analysis (Packard, Tricarb 2900) (*30*), pH by glass electrode (Accumet, model 25), ethanol by gas chromatography (Hewlett-Packard, model 5880) with flame ionization detection, glucose by the phenol−sulfuric acid method (*31*), and Fe(II) by the ferrozine assay (*32*). For tests 56 and 57, additional groundwater samples were collected in 37 mL crimped-top serum bottles containing no headspace. A N_2O-free bubble of N_2 was added to each bottle, gases were allowed to equilibrate, and the gas headspace was analyzed for N_2O by gas chromatography with electron capture detection (Varian, model 4000) (*33*). Rates of donor utilization and of NO_3^-, U(VI), and Tc(VII) reduction were computed by least-squares fitting of dilution-adjusted concentrations versus time. Dilution-adjusted concentrations for donor, NO_3^-, Tc(VII), and U(VI) were computed as the measured concentration of those solutes divided by the relative concentration (C/C_0) of the Br^- tracer (*28*), where C is the Br^- concentration in a sample and C_0 is the average Br^- concentration in the injected test solution. Dilution-adjusted concentrations were similarly calculated for Fe(II), NO_2^-, and N_2O formed in situ. Rates could not be computed for tests in which a $CaCl_2$ solution was injected because the Br^- tracer was not added in these tests, thus making it impossible to distinguish microbial utilization from dilution in those tests.

Microbial Samplers. The effects of electron donor additions on microbial biomass, community composition, metabolic status, and respiratory state were independently monitored by deploying solid phase microbial samplers in each well prior to push−pull testing. As described in ref *24*, the samplers remained in the well throughout the field testing and were subsequently retrieved for analysis. Microbial samplers were constructed from 1.25 cm o.d. Teflon PFA (perfluoroalkoxy) tubing loaded with Bio-Sep biocarrier beads (*34*, *35*) and deployed as described by Peacock et al. (*24*). Once recovered, the solid phase samplers were frozen at −20 °C until analyzed. Phospholipid fatty acid (PLFA) analysis was performed on thawed samples using previously reported precautions (*36*). The beads were extracted with the single-phase chloroform−methanol buffer system of Bligh and Dyer (*37*), as modified (*38*). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (*39*). The polar lipids were transesterified to the fatty acid methyl esters (FAMEs) by a mild

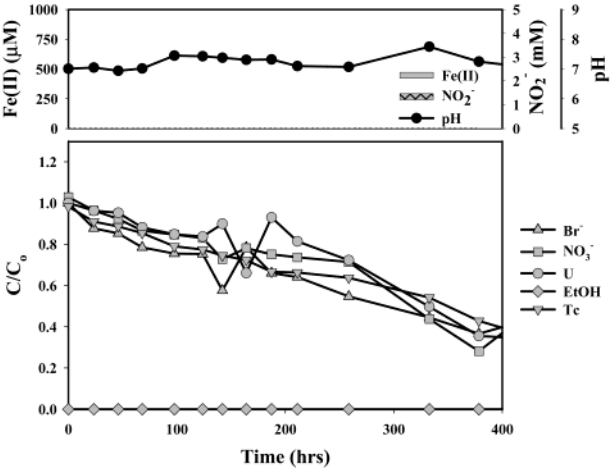

FIGURE 1. Concentration history for test 13 in well FW19 showing simple dilution of injected test solution components and no NO_2^- or Fe(II) production in the absence of added electron donor.

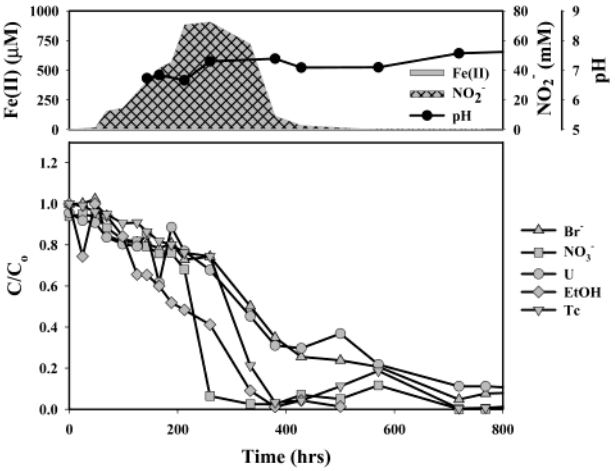

FIGURE 2. Concentration history for test 14 in well FW34 showing ethanol utilization, NO_3^- and Tc(VII) reduction, and NO_2^- and Fe(II) production following first ethanol injection.

alkaline methanolysis (*39*) with the Mayberry and Lane (*40*) method to protect cyclopropane PLFA and release plasmalogen ethers as dimethylacetals (DMAs). The FAMEs were analyzed by gas chromatography/mass spectroscopy as described (*24*). The neutral lipid fraction of the Bligh and Dyer (*37*) extract after fractionation on silicic acid columns was examined for respiratory ubiquinone and menaquinone isoprenologues by high-performance liquid chromatography/atmospheric pressure photoionization tandem mass spectrometry (HPLC/APPI/MS/MS) (*41*, *42*).

Results and Discussion

Control (No Added Donor) Tests. Microbial activity was not detected in control tests conducted without added electron donor. For example, in test 13 (conducted in well FW19, Figure 1), relative concentrations (C/C_0, where C is the measured concentration in a sample and C_0 is the average concentration of the same component in the injected test solution) of injected NO_3^-, U(VI), and Tc(VII) decreased similarly to injected Br^-. This indicates that changes in NO_3^- and radionuclide concentrations were largely due to dilution of the test solution as it gradually drifted away from the injection well and that intrinsic rates of NO_3^-, U(VI), and Tc(VII) reduction (i.e., supported by endogenous electron donors) are not detectable and are likely very small. The results also confirm that, under the conditions of these tests (with added bicarbonate), sorption of injected NO_3^-, U(VI), and Tc(VII) to aquifer sediments at this site is negligible. This was confirmed in laboratory batch experiments conducted with sediment collected during well construction (data not shown). Dilution-adjusted concentrations for NO_3^-, Tc(VII), and U(VI) showed no statistically significant decrease with time (data not shown). Similar results were obtained for five tests conducted without added donor in three separate wells at the site (Table 2).

Effect of Electron Donor Addition on NO_3^-, Tc, and U Reduction. In the first test with added donor in each well (tests 14, 15, 17, and 19, Table 2) utilization of injected electron donor was observed in all tests, NO_3^- reduction to NO_2^- was observed in three of four tests, and Tc(VII) reduction was observed in two tests (Table 2). For example, in test 14 (conducted in well FW34, Figure 2) injected ethanol was rapidly utilized and relative concentrations of ethanol, NO_3^-, and Tc(VII) decreased more rapidly than the Br^- tracer (Figure 2). Dilution-adjusted concentrations decreased linearly with time (Figure 3) and fitted rates for test 14 were 0.04 mM/h for ethanol utilization, 0.28 mM/h for NO_3^- reduction, and 32 pM/h for Tc(VII) reduction (Table 2). In situ production of NO_2^- was also observed (Figure 2), indicating that added ethanol stimulated NO_3^--reducing activity. No NH_4^+ production was detected in any test (data not shown). Fe(II) production was not observed (Figure 2), and dilution-adjusted SO_4^{2-} concentrations remained constant (data not shown). U(VI) reduction was also not observed (Figures 2 and 3).

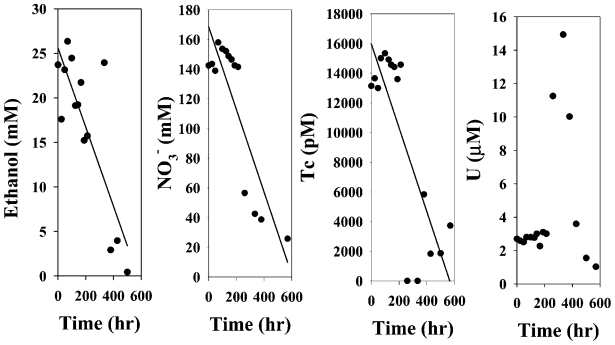

FIGURE 3. Dilution-adjusted concentrations used to compute rates of ethanol utilization, NO_3^- reduction, and Tc(VII) reduction.

The first series of tests demonstrated that although donor additions could stimulate microbial activity, the results were variable and initial donor additions were not successful in stimulating Fe(III)- and U(VI)-reducing activity. We hypothesized that subsequent donor additions would increase the population size of the metal-reducing microorganisms and consequently create conditions favorable for simultaneous Fe(III), U(VI), and Tc(VII) reduction in all wells. A second series of tests was conducted (tests 21−23 and 25) under identical conditions; rates of donor utilization and NO_3^- and Tc(VII) reduction increased in all wells, but U(VI) reduction was observed in only two tests (Table 2). In well FW34, for example, the rate of ethanol utilization increased from 0.04 to 0.25 mM/h, the rate of NO_3^- reduction increased from 0.28 to 0.4 mM/h, the rate of Tc(VII) reduction increased from 32 to 150 pM/h, and the initial rate of U(VI) reduction increased from 0 to 0.003 μM/h (Table 2).

Three additional electron donor additions were performed in each well to further increase microbial activity (e.g., tests 28, 32, and 36 in well FW34). Because these donor additions were performed using test solutions prepared from distilled water (see Materials and Methods) and did not contain a tracer, it was not possible to monitor microbial activity during these tests. However, rates of microbial activity increased substantially in subsequent tests performed with test solutions prepared from FW21 groundwater in these wells. For

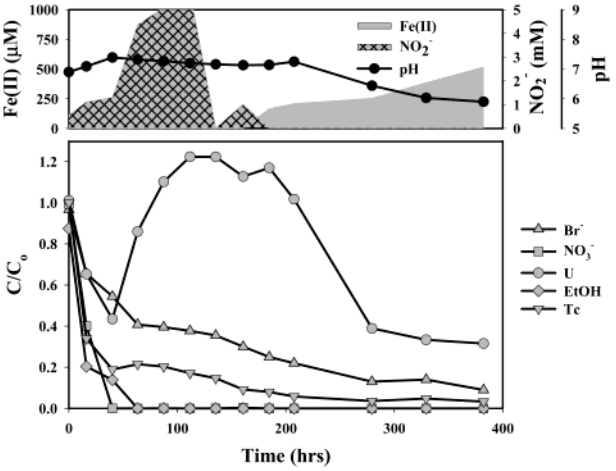

FIGURE 4. Concentration history for test 40 in well FW34 showing ethanol utilization, NO_3^- and Tc(VII) reduction, initial U(VI) reduction followed by U(IV) reoxidation, and NO_2^- and Fe(II) production following seventh ethanol injection.

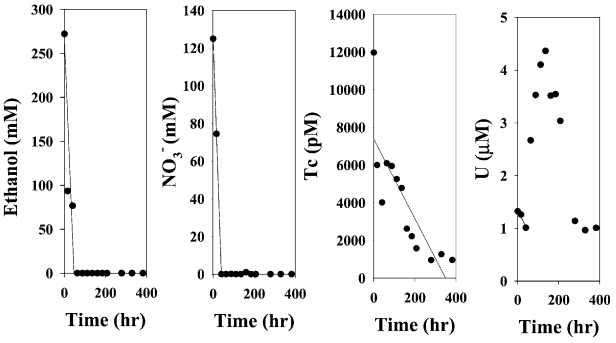

FIGURE 5. Dilution-adjusted concentrations for test 40 in FW34 showing regressions used to estimate rates of ethanol utilization and NO_3^-, Tc (VII), and U(VI) reduction. U(VI) reduction rate was estimated from data for the first 50 h of the test only.

example, in test 40 in well FW34, injected ethanol, NO_3^-, and Tc(VII) were rapidly utilized concomitantly with the production of NO_2^- and Fe(II) (Figure 4). Relative concentrations of U(VI) initially decreased from 1 to 0.25 within the first 50 h following injection, then increased to a maximum value of $C/C_0 = 1.22$ at 140 h, and then decreased to $C/C_0 = 0.17$ by the end of the test (390 h) (Figure 4), a pattern that was consistently observed in all tests conducted with test solutions prepared from FW21 groundwater with high initial NO_3^- concentration (~120 mM). Computed rates from this test increased compared to tests conducted prior to the three donor additions (i.e., test 22) from 0.25 to 7.6 mM/h (ethanol utilization), from 0.4 to 3.1 mM/h (NO_3^- reduction), from 150 to 189 pM/h [Tc(VII) reduction], and from 0.003 to 0.008 μM/h [U(VI) reduction]. The rates of U(VI) reduction reported for this test and all other tests were based on the initial reduction in dilution-adjusted U(VI) concentrations that occurred during the first 50 h after injection (Figure 5).

It seems likely that the increase in U(VI) concentration that occurred after the initial decrease is due to the reoxidation and resolubilization of U(IV). Senko et al. (*13*) reported that the addition of NO_3^- resulted in the remobilization of U(IV) in field tests and that the addition of NO_3^-, NO_2^-, NO, and N_2O led to the abiotic oxidation of U(IV) in laboratory sediment incubations. It is also possible that chemolithotrophic bacteria directly oxidized U(IV) using NO_3^- or NO_2^- as the electron acceptor or oxidized Fe(II) to Fe(III), which may then have chemically oxidized U(IV) (*18*). To address the hypothesis that microbially mediated U(IV) oxidation was responsible for the effect of electron donor

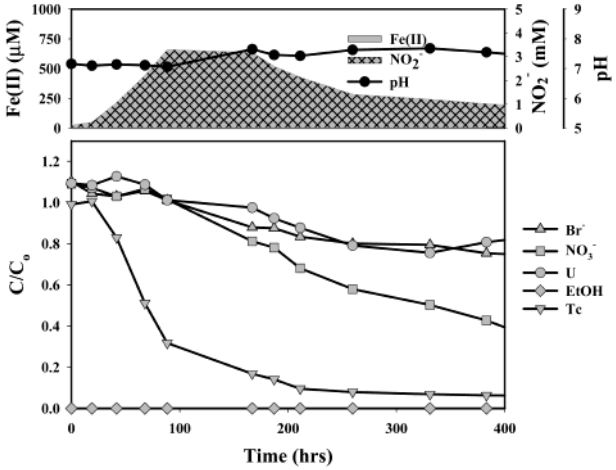

FIGURE 6. Concentration history for test 44 in well FW34 showing reduced rate of NO_3^- and Tc(VII) reduction, the absence of U(VI) reduction, and Fe(II) production after biostimulation but in the absence of added ethanol.

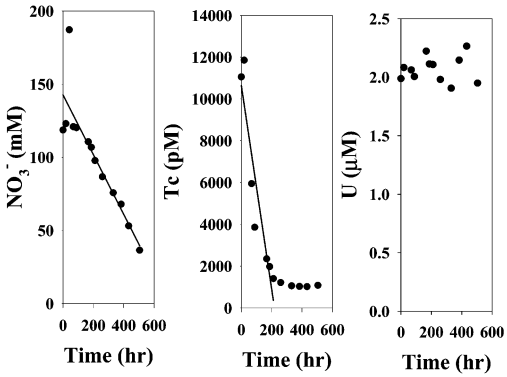

FIGURE 7. Dilution-adjusted concentrations for test 44 in FW34 showing regressions used to estimate rates of NO_3^- and Tc(VII) reduction.

and NO_3^- concentration on the observed remobilization of U(VI), additional tests were conducted by injecting test solutions consisting of (a) high NO_3^- (~120 mM) FW21 groundwater without added electron donor, (b) low NO_3^- (1 mM) GW835 groundwater with added electron donor, and (c) high NO_3^- FW21 groundwater with added electron donor and dissolved acetylene.

After microbial activity had been stimulated by seven previous donor additions, tests 44 and 46 were conducted using test solutions prepared from FW21 groundwater but without added ethanol. In both cases, NO_3^- and Tc(VII) reduction occurred and NO_2^- was produced, but U(VI) reduction followed by U(IV) reoxidation/remobilization and Fe(II) production was not observed (Figures 6 and 7). The computed rate of NO_3^- reduction decreased from 3.1 to 0.2 mM/h and the rate of Tc(VII) reduction decreased from 189 to 91 pM/h compared to the previous test (test 40) conducted with added ethanol (Table 2). Similar behavior and rates were observed for tests 42 and 46 (Table 2). These results suggested to us that, in this system, active donor oxidation is needed to maintain the highest rates of NO_3^- and Tc(VII) reduction and to initiate Fe(III)-reducing conditions suitable for detectable U(VI) reduction and that the U(IV) reoxidation/remobilization observed in previous tests with higher initial NO_3^- concentrations was a biological process somehow linked to donor oxidation. Moreover, the reduced rate of Tc(VII) reduction in the absence of added donor suggests that previously observed Tc(VII) reduction is likely primarily a biological process and not due exclusively to abiotic reactions with Fe(II) bound to mineral surfaces.

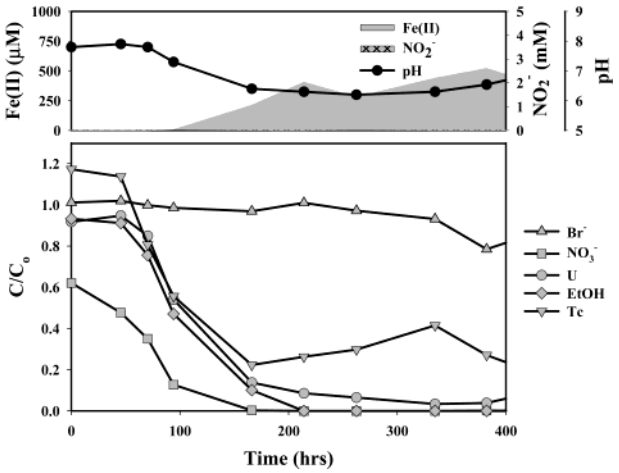

FIGURE 8. Concentration history for test 48 in well FW34 showing ethanol utilization, NO_3^-, Tc(VII), and U(VI) reduction, and Fe(II) production when test solution was prepared with an initial NO_3^- concentration of 1 mM compared to ~120 mM used in previous tests.

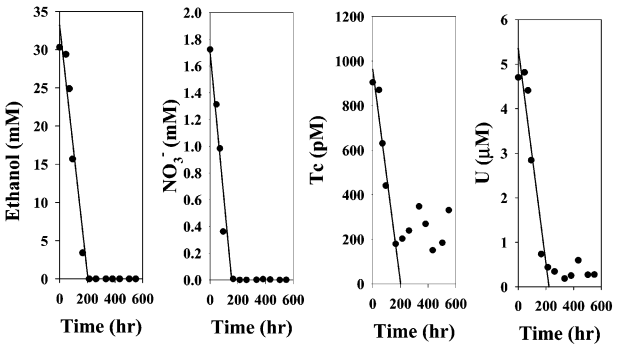

FIGURE 9. Dilution-adjusted concentrations for test 48 in FW34 showing regressions used to estimate rates of ethanol utilization and NO_3^-, Tc(VII), and U(VI) reduction.

Tests 47−50 were conducted using test solutions prepared from groundwater from well GW835, which had a much smaller initial NO_3^- concentration (~1 mM) (Table 1). During these tests, the small quantity of injected NO_3^- was rapidly reduced and NO_2^- was detectable in only a few samples at a maximum concentration of 0.07 mM (Table 2). Moreover, in contrast to tests conducted with much higher initial NO_3^- concentrations, relative concentrations of U(VI) decreased continuously during these tests. For example, in test 48 conducted in FW34, injected NO_3^- was completely removed within ~180 h following injection and only trace levels of NO_2^- were detected (Figure 8). Relative concentrations of U(VI) decreased continuously from $C/C_0 = 1$ to 0.03 by the end of the test (~400 h) with no apparent remobilization. Computed rates of donor utilization, NO_3^- reduction, and Tc(VII) reduction were all smaller than in previous tests conducted with added donor, but the rate of U(VI) reduction increased from 0.008 to 0.024 μM/h in test 48 compared to test 44 (Figure 9; Table 2). Similar results were observed for all tests in this series (Table 2), although it was not possible to compute a rate of Tc(VII) reduction for tests 47, 49, and 50 because Tc(VII) concentrations were reduced below detection limits in all samples from those tests.

Tests 56 and 57 were conducted to determine whether the loss of NO_3^- was due to denitrification or the reduction of NO_3^- to NH_4^+. We determined the effect of co-injected dissolved acetylene on the patterns and rates of reductive processes observed in previous tests. Acetylene is a known inhibitor of N_2O reductase (*43*), and the acetylene-block procedure is widely used to measure denitrification rates

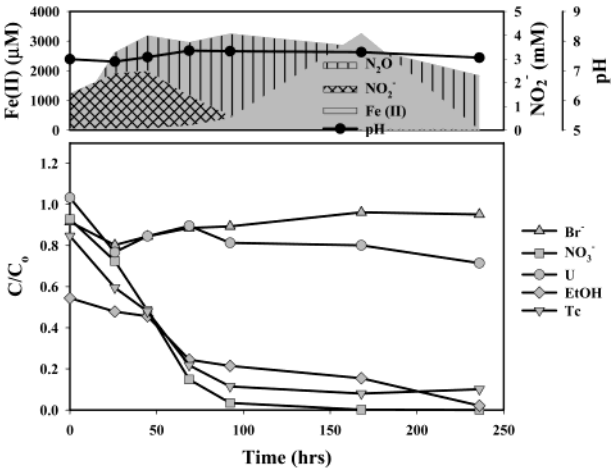

FIGURE 10. Concentration history for test 56 in well FW34 showing ethanol utilization, NO_3^- and Tc(VII) reduction, and Fe(II), N_2O, and NO_2^- production in the presence of dissolved acetylene gas.

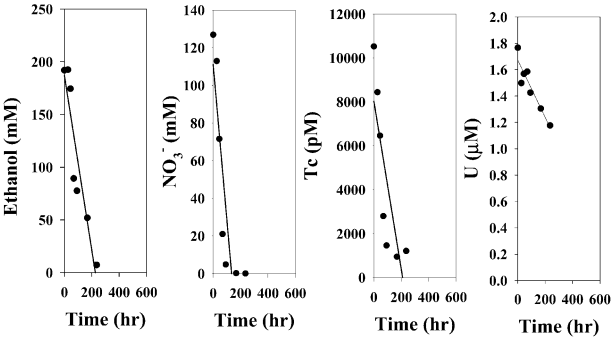

FIGURE 11. Dilution-adjusted concentrations for test 56 in FW34 showing regressions used to estimate rates of ethanol utilization and NO_3^-, Tc(VII), and U(VI) reduction.

(*44*). Acetylene is also a known inhibitor of the monooxygenase enzyme system, (*45*, *46*), which is widespread among aerobic subsurface microorganisms. Thus, the production of N_2O in tests conducted with added acetylene is diagnostic of in situ microbial denitrification. Moreover, added acetylene could potentially block microbial reoxidation of U(IV) by organisms expressing monooxygenase (if oxygen were present). The results from test 56 conducted in well FW34 showed rapid utilization of injected donor, production of NO_2^-, Fe(II), and N_2O, and simultaneous reduction of NO_3^- and Tc(VII) (Figures 10 and 11). However, computed rates of these processes were smaller than in previous tests conducted without acetylene (Table 2). From these results we conclude that denitrification is the principal process contributing to observed NO_3^- reduction. As the decrease in the rate of NO_3^- consumption is likely due to inhibition of the process by acetylene, our results also suggest that Tc(VII) reduction may be carried out by the same organisms. U(VI) concentrations also continuously decreased, similar to those of Br^- without the reoxidation/remobilization previously observed. The lack of U(VI) reduction is likely due to the continuous presence of oxidized NO_3^- reduction intermediates.

A detailed description of microbial sampler results is presented in ref *24* and is only briefly summarized here. Biomass measured as either PLFA or DMA was higher in microbial samplers deployed in the wells that received additions of electron donors compared to control wells, indicating that donor additions stimulated the growth of indigenous microorganisms. For example, viable biomass in well FW34, which received several additions of ethanol during the sampler deployment, was 526 pmol of PLFA/bead

TABLE 3. Effect of Electron Donor Additions on Biomass, Community Composition, Metabolic Status, and Respiratory State of Organisms Extracted from Microbial Samplers Deployed in Test Wells

	FW27 (control)	FW19 (+ acetate)	FW34 (+ ethanol)	FW33 (+ glucose[Na])	FW32 (+ glucose[Ab])
Viable Biomass					
biomass (pmol of PLFA/bead)	213	468	526	309	517
biomass (pmol of DMA/bead)	0.2	0.3	2.7	2.3	8.0
Community Composition					
branched-chain saturated	14	11	11	15	11
monounsaturated	61	65	63	59	62
Metabolic Status					
total cyclopropyl/monounsaturated	1.6	2.6	1.1	1.0	2.4
total trans/cis	0.2	0.2	0.1	0.2	0.2
Respiratory State					
UQ/MK[c]	6.3	11.0	5.3	2.1	9.7

[a] Neutral water/acidic sediments. [b] Acid water/acidic sediments. [c] UQ/MK aerobic versus mostly anaerobic respiration.

compared with 213 pmol/bead in control well FW27 (Table 3). The samplers contained a diverse community profile that consisted of all major PLFA structural groups; however, no apparent differences were detected between wells using PLFAs (Table 3). The dominant PLFA group was the monounsaturates, which accounted for 59−65% of the PLFA profiles in the beads regardless of donor type (Table 3). DGGE profiles of amplified 16S V3 rDNA fragments (data reported separately in ref *24*) indicated that detected sequences were related to genera capable of using NO_3^- as a terminal electron acceptor and metal reduction (*15*). Sequences affiliated with *Geobacter* [a known U(VI) reducer (*15*)] were detected only in the wells that received donor additions, confirming that donor additions stimulated the growth and activity of metal-reducing organisms. The lipid composition of microorganisms is a product of metabolic pathways and so reflects the phenotypical response of the microbe to the environment. Gram-negative bacteria make trans fatty acids to modify their cell membranes against environmental stress; as such the physiological status of Gram-negative communities can be assessed by ratios of specific PLFAs. The total trans to cis isomer ratio for 16:1ω7 and 18:1ω7 was essentially unchanged in all wells. The starvation/toxicity biomarker cyclopropyl/monounsaturated precursor ratio increased with glucose (acid condition) and acetate additions but remained at the unstimulated level with ethanol and glucose (neutral condition) additions (Table 3). The proportion of aerobically respiring (using dissolved O_2 or NO_3^- as electron acceptor) organisms as indicated by the ubiquinone/menaquinone ratio (UQ/MK) was increased relative to the control by acetate and glucose additions (acid condition) and depressed by the addition of ethanol and glucose (neutral condition) (Table 3).

Implications

A recent field study (*47*) described the addition of acetate to stimulate U(VI) reduction in an aerobic aquifer in Colorado. This study has presented some of the first in situ experiments to demonstrate that it is possible to stimulate microbially mediated uranium and technetium reduction under conditions representative of DOE legacy waste sites, especially the presence of high NO_3^- concentrations. Groundwater geochemistry, push−pull tests, and analyses of microbial samples indicate that at this site, microbial activity is electron donor limited and, in the absence of added donor, activities are likely dominated by aerobic respiration. The addition of acetate, glucose, or ethanol stimulated the growth and activity of indigenous microorganisms, resulting in the development of an anaerobic and reducing environment that favored reduction of NO_3^-, Fe(III), Tc(VII), and U(VI). U(VI) reduction was not observed in tests conducted with added acetylene or in tests conducted without active donor oxidation. These results suggest that NO_3^--dependent, microbially mediated U(IV) oxidation is an important process in modulating the stability of bioreduced U(VI) at this site and at similar sites where U(VI) and Tc(VII) contamination is accompanied with high concentrations of NO_3^- co-contamination. Interestingly, we observed the presence of *Dechlorosoma* species in the microbial samplers, which have been shown to anaerobically oxidize Fe(II) to Fe(III) (*45*). By using NO_3^- to oxidize Fe(II) to Fe(III) that could subsequently oxidize U(IV), the activity of these organisms could be responsible for the U(IV) oxidation observed. Microbial oxidation of reduced Tc was not observed.

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