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MICROBIALLY-MEDIATED REDUCTION AND OXIDATION OF URANIUM: IMPLICATIONS FOR THE BIOREMEDIATION OF U(VI)-CONTAMINATED AQUIFERS

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DEPARTMENT OF BOTANY AND MICROBIOLOGY

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Preface

Uranium contaminates sites where that element was mined, refined, and processed for the production of nuclear weapons and nuclear fuel. In humans, uranium may interfere with kidney and liver function and enriched uranium may cause cancer due to its radioactivity. Uranium predominantly exists at these sites as U(VI), which is quite soluble, is easily transported by groundwater, and therefore poses a threat to nearby drinking water sources and surface waters. An estimated 1.7 trillion gallons of contaminated groundwater exist at or near mining and processing sites, making uranium removal from groundwater by "pump-and-treat" an untenable option for restoration of these sites. Therefore, in situ reduction of soluble U(VI) to insoluble U(IV) has been proposed to limit the off-site migration of uranium with groundwater. Microorganisms are well known to catalyze this reaction via respiration of U(VI) or by catalyzing reactions that bring about geochemical conditions amenable to U(VI) reduction. Recent work has shown that stimulation of U(VI)-reducing activity by microorganisms native to U(VI)-contaminated aquifers may be a feasible strategy for uranium immobilization. Unfortunately, the stability of U(IV) after it has been immobilized is unclear. The work presented in this dissertation shows that U(IV) may be oxidized and remobilized by microbiological activity under nitrate-reducing conditions. The mechanisms of and microbiological and geochemical controls on this process (termed nitrate-dependent U(IV) oxidation) are also elucidated. The results presented here may be useful for predicting the stability of U(IV) after it has been reductively immobilized.

In Chapter 1, I demonstrate that microbiological activity can be exploited to reduce and immobilize uranium in situ. I also show that nitrate not only inhibits U(VI)

reduction, but that the addition of nitrate to U(IV)-containing sediments leads to the oxidation and remobilization of uranium. This process may be catalyzed by denitrification intermediates (especially nitrite and nitric oxide). Nitrate-dependent U(IV) oxidation could seriously threaten the stability of bioreduced U(IV) in bioremediated aquifers. This chapter was written with Dr. Jack Istok, Dr. Joseph Suflita, and Dr. Lee Krumholz in the style required by *Environmental Science and Technology*.

In Chapter 2, I examine the mechanism of nitrate-dependent U(IV) oxidation, and show that U(IV) is ultimately oxidized by Fe(III) produced during dissimilatory nitrate reduction. Fe(III) may be produced by the enzymatic activity of nitrate-dependent Fe(II)-oxidizing bacteria or by the oxidation of Fe(II) by nitrite. Crystalline Fe(III) minerals are formed by enzymatic nitrate-dependent Fe(II) oxidation, while amorphous Fe(III) is produced by the reaction of Fe(II) with nitrite. The amorphous Fe(III) is more reactive, and therefore a more effective oxidant of U(IV) than the crystalline biogenic Fe(III) minerals. This work has important implications for predicting the stability of U(IV) in bioremediated aquifers, since it may be possible to predict the mineralogy of biogenic Fe(III) minerals, and therefore predict the extent of nitrate-dependent U(IV) oxidation. This chapter was written with Dr. Yasser Mohammed, Dr. Thomas Dewers and Dr. Lee Krumholz in the style required by *Environmental Science and Technology*.

In Chapter 3, I examine controls on biogenic Fe(III) mineralogy, and show that Fe(III) produced by nitrate-dependent oxidation of FeS is amorphous, while nitrate-dependent oxidation of soluble Fe(II) produces goethite. The rate of nitrate-dependent Fe(II) oxidation controls biogenic Fe(III) mineralogy: high rates of nitrate-dependent Fe(II) oxidation give rise to amorphous Fe(III), while lower rates give rise to goethite.

This work has implications not only for the stability of U(IV) (where, for instance, high rates of Fe(II) oxidation would give rise to amorphous Fe(III) that will be a stronger U(IV) oxidant), but for the biogeochemical cycling of Fe, since crystalline Fe(III) minerals are poor electron acceptors for dissimilatory Fe(III)-reducing bacteria compared to amorphous Fe(III). This chapter was written with Dr. Thomas Dewers and Dr. Lee Krumholz as a short-form paper in the style required by *Applied and Environmental Microbiology*.

In Chapter 4, I determine what factors exert the strongest influence on nitrate-dependent U(IV) oxidation. I report the enrichment of microorganisms that can couple U(IV) oxidation to nitrate reduction for growth, but that these organisms appear to play a minor role in nitrate-dependent U(IV) oxidation compared to dissimilatory nitrate-reducing microorganisms that produce Fe(III), which is ultimately the oxidant of U(IV). I show that nitrate concentration controls the extent of U(IV) oxidation, but has a relatively minor impact on the rate of nitrate-dependent U(IV) oxidation. The addition of reductants (acetate, soluble sulfide, soluble Fe(II) and FeS) inhibited nitrate-dependent U(IV) oxidation. Soluble Fe(II) was the most effective inhibitor of nitrate-dependent U(IV) oxidation, possibly due to the production of crystalline Fe(III) with soluble Fe(II) addition as opposed to amorphous Fe(III) that may be produced in the presence of sulfide. This chapter was written with Dr. Joseph Suflita and Dr. Lee Krumholz in the style required by *Geomicrobiology Journal*.

Abstract

We assessed in situ microbial U(VI) reduction. U(VI) immobilization was observed in situ and confirmed as reduction in laboratory sediment incubations. Sulfate neither enhanced nor inhibited U(VI) reduction, but nitrate inhibited U(VI) reduction. U(VI) reduction occurred only after complete consumption of nitrate. The addition of nitrate to U(IV)-containing sediments lead to the oxidation and remobilization of uranium. The addition of intermediates of dissimilatory nitrate reduction (nitrite and nitrous oxide) to heat-inactivated U(IV)-containing sediment slurries lead to the oxidation of U(IV), suggesting that the accumulation of these reactive compounds during dissimilatory nitrate reduction is responsible for nitrate-dependent U(IV) oxidation. Nitrate-dependent U(IV) oxidation may also be catalyzed by Fe(III) that is produced by nitrate-dependent U(IV)-oxidizing bacteria, or by the direct, enzymatic, nitrate-dependent oxidation of U(IV).

Mechanisms of nitrate-dependent U(IV) were assessed by incubating biogenic U(IV) with nitrite or Fe(III). Fe(III) rapidly but incompletely oxidized U(IV) since Fe(II) accumulated during the reaction and inhibited any further U(IV) oxidation. Nitrite alone was a poor oxidant of U(IV), but the addition of a small amount of Fe(II) to nitrite- and U(IV)-containing incubations lead to complete U(IV) oxidation, suggesting that Fe shuttles electrons from U(IV) to nitrite. A nitrate-dependent acetate- and Fe(II)-oxidizing bacterium was isolated from a nitrate- and U(VI)-contaminated aquifer. U(IV) was oxidized by this organism in site groundwater at a greater rate and to a greater extent in the presence of acetate and Fe(II) (where nitrite accumulated to high levels) than under Fe(II)-oxidizing conditions. This activity was due to mineralogical difference in Fe(III) produced under the two conditions. Goethite was produced by enzymatic, nitrate-

dependent Fe(II) oxidation and amorphous Fe(III) was produced by the reaction of Fe(II) with nitrite. The latter Fe(III) mineral was a more effective oxidant of U(IV).

The effect of Fe(II) form and Fe(II) oxidation rate on Fe(III) mineralogy were determined. Fe(III) from FeS oxidation was amorphous, while Fe(III) from soluble Fe(II) was identified as predominantly goethite. High initial rates of Fe(II) oxidation lead to amorphous Fe(III), and with decreasing rates of Fe(II) oxidation, more crystalline goethite was formed.

Geochemical controls on nitrate-dependent U(IV) oxidation were determined in landfill leachate-impacted sediments. Organotrophic (glucose-oxidizing) and Fe(II)-oxidizing nitrate-reducing bacteria were more abundant in these sediments than organisms that coupled U(IV) oxidation to nitrate reduction for growth, suggesting that U(IV) is ultimately oxidized by Fe(III). In sediment incubations, nitrate concentration exerted a strong influence on the extent, but not the rate of nitrate-dependent U(IV) oxidation. The addition of reductants (acetate, sulfide, soluble Fe(II), and FeS) to sediment incubations lead to lower extents and rates of nitrate-dependent U(IV) oxidation. Fe(II) was the strongest inhibitor of nitrate-dependent U(IV) oxidation.

Chapter 1

In-situ evidence for uranium immobilization and remobilization

Abstract

The in-situ microbial reduction and immobilization of uranium was assessed as a means of preventing the migration of this element in the terrestrial subsurface. Uranium immobilization (putatively identified as reduction) and microbial respiratory activities were evaluated in the presence of exogenous electron donors and acceptors with field push-pull tests using wells installed in an anoxic aquifer contaminated with landfill leachate. Uranium(VI) amended at 1.5 μM was reduced to less than 1 nM in groundwater in less than 8 d during all field experiments. Amendments of 0.5 mM sulfate or 5 mM nitrate slowed U(VI) immobilization and allowed for the recovery of 10% and 54% of the injected element, respectively compared to 4% in the unamended treatment. Laboratory incubations confirmed the field tests and showed that the majority of the U(VI) immobilized was due to microbial reduction. In these tests, nitrate treatment (7.5 mM) inhibited U(VI) reduction, and nitrite was transiently produced. Further push-pull tests were performed in which either 1 or 5 mM nitrate was added with 1.0 μM U(VI) to sediments that already contained immobilized uranium. After an initial loss of the amendments, the concentration of soluble U(VI) increased and eventually exceeded the injected concentration, indicating that previously immobilized uranium was remobilized as nitrate was reduced. Laboratory experiments using heat-inactivated sediment slurries suggested that the intermediates of dissimilatory nitrate reduction (denitrification or dissimilatory nitrate reduction to ammonia), nitrite, nitrous oxide, and nitric oxide, were

all capable of oxidizing and mobilizing U(IV). These findings indicate that in-situ subsurface U(VI) immobilization can be expected to take place under anaerobic conditions, but the permanence of the approach can be impaired by dissimilatory nitrate reduction intermediates that can mobilize previously reduced uranium.

Introduction

When uranium is recovered from ore, H_2SO_4 and/or HNO_3 are frequently used as extractants (*1*). The waste from this process often contains high levels of soluble oxidized uranium (U(VI)) along with sulfate and/or nitrate, which are mobile in the environment and can contaminate groundwater. A proposed means of preventing the migration of U(VI) is the in-situ immobilization of soluble U(VI) (as UO_2^{2+}, usually complexed with carbonate in natural systems), via microbial reduction to insoluble U(IV) (as UO_2) (*2,3*). Several microorganisms are known to mediate this process, including the Fe(III)-reducing *Geobacter* sp. and *Shewanella* sp. (*2*), the Fe(III)- and sulfate-reducing *Desulfotomaculum* sp. (*4*), the sulfate-reducing *Desulfovibrio* sp. (*5,6*), and the fermentative anaerobic *Clostridium* sp. (*7*). Work to assess the influence of alternate terminal electron acceptors on U(VI) reduction has focused mostly on Fe(III) and sulfate, since the organisms that reduce these substances are expected to be primarily responsible for U(VI) reduction in contaminated aquifers. Spear et al. (*8*) reported that U(VI) reduction rates were greater in the presence of sulfate than without in both a pure culture of *Desulfovibrio desulfuricans* and a *D. vulgaris-Clostridium* sp. coculture. Ferrihydrite, but not goethite, was reported to inhibit U(VI) reduction by the Fe(III)-reducing *Shewanella alga* (BrY) (*9*). Several organisms are capable nitrate and U(VI) reduction,

and experiments performed by Ganesh et al. (*10*) suggested that U(VI) reduction by *D. desulfuricans* is slightly inhibited by the presence of 190 mM nitrate, but not by nitrate concentrations lower than 95 mM.

On the basis of potential for H_2 oxidation coupled to alternate terminal electron acceptors under standard conditions, the sequence of processes to be expected is denitrification (ΔE_o'= -1160 mV), uranium reduction (ΔE_o'= -820 mV), and sulfate reduction (ΔE_o'= -190 mV) (from ref *11*). This sequence of reductive processes was demonstrated in electron donor-amended groundwater and in a sediment core column from a U(VI) contaminated site in Tuba City, AZ (*12, 13*). Sediment slurry incubations from a uranium mill tailings disposal site in Shiprock, NM, revealed that uranium reduction will not commence until nitrate is completely depleted (*14*). Subsequently, U(VI), Fe(III), and sulfate reduction occur, with both Fe(III) and U(VI) reduction stimulated by glucose or acetate addition (*14*).

We determined the impact of electron donor and terminal electron acceptor amendments on the fate of U(VI) in anaerobic sediments from a landfill leachate impacted aquifer in Norman, OK. This aquifer had not been previously contaminated with U(VI). Field experiments and laboratory incubations reveal the potential for in-situ U(VI) immobilization via concerted microbiological and geochemical processes. We also present evidence that nitrate-reducing activity leads to the mobilization of previously immobilized uranium and may be due to oxidation of U(IV) by dissimilatory nitrate reduction intermediates.

Materials and Methods

Single well push-pull tests Single well push-pull tests were performed as described by Istok et al. (*15*). Each test consists of three phases: extraction of groundwater and amendment with test chemicals, injection of amended groundwater, and in-situ incubation with periodic extraction. Drive point wells were installed in an aquifer impacted by leachate from the Norman Municipal Landfill to a screen depth of 3.5 m. Sediments and groundwater from this site have been previously characterized microbiologically and geochemically (*16*). Groundwater (50 l) was pumped from the wells with a peristaltic pump into carboys and amended with electron donor or electron acceptor (below) while being constantly sparged with 80:20 N_2:CO_2. Norman landfill leachate has a pH of 6.9-7.2 when sparged with 80:20 N_2:CO_2. The groundwater was then injected back into the aquifer. Samples were taken during injection after every 10 l (of the 50 l total). All analytes were quantified as described below to see if any U(VI), sulfate, or nitrate loss occurred during injection. No transformation of amendments occurred during the injection phase of the tests (data not shown).

During the incubation phase, groundwater (2 l) was periodically removed from the wells and filtered (0.2 μm) into sterile tubes, stored at 4° C, and subsequently analyzed for U(VI), bromide, nitrate, sulfate and electron donor concentrations. These concentrations were normalized to account for dilution by the surrounding groundwater. This was accomplished by first calculating the ratio of the bromide concentration at each time point to the injected bromide concentration. This value was used to normalize the measured amendment concentration.

Table 1. Summary of amendments to groundwater for push-pull tests. All tests were amended with U(VI) and bromide as described in the Materials and Methods section.

Test	$NaNO_3$	Na_2SO_4	$NaCH_3COO$	$NaCH_3CHOHCOO$	NaCOO
Round 1					
1	-	-	-	-	-
2	-	-	11 mM	-	-
3	-	-	-	8 mM	-
4	-	-	-	-	17 mM
5	-	0.5 mM	-	-	-
6	5 mM	0.5 mM	-	-	-
Round 2					
7	-	-	-	-	-
8	5 mM	-	-	-	-
9	1 mM	-	-	-	-

Two rounds of tests were performed. Six different manipulations were performed in the first round where all wells were amended with 1 μM $UO_2(NO_3)_2$, 1.5 mM KBr and different amendments of electron donor or acceptor as summarized in Table 1. Approximately 2.5 months after completion of the first round of tests, a second round of tests were performed in wells that contained immobilized uranium from the previous round of tests. These tests were performed nearly identically to the previous round, with 1.0 μM U(VI), 1.5 mM bromide, and nitrate as summarized in Table 1. Approximately 1 month after the conclusion of the second round of tests, a core was collected approximately 0.5 m from each well. At the screened depth interval, 20 cm of core was divided into approximately 3 cm segments, and triplicate 1 g subsamples were taken from each segment and sequentially extracted as described below to determine soluble U(VI), solids-associated U(VI), and U(VI).

Laboratory incubations Sediments were collected from a shallow, alluvial, landfill leachate impacted-aquifer in Norman, OK using a hand auger. The depth from which the sediments were collected was slightly below the water table (approximately 2 m) in the anoxic zone, as indicated by blackening of the sediments. The sediments were transferred to sterile jars and transported back to the laboratory where they were flushed with N_2 and refrigerated prior to use. Sediments (5 g; 25% water) were placed in 25 ml serum bottles and stoppered while inside an anoxic glovebox. The bottles were then removed from the glovebox and flushed with 80:20 N_2:CO_2. Autoclaved (20 min) slurries served as negative controls. Amendments (0.1 ml) were made from sterile stock solutions to reach the following concentrations; uranyl acetate (2.5 μM), Na_2SO_4 (1 mM), or $NaNO_3$ (0.6 or 7.5 mM). NaCl was added to bottles, if needed, to achieve a final Na^+

concentration of 7.5 mM. After addition of groundwater (1.05 ml) and amendments (0.2 ml total), the final liquid volume of the incubations was 2.5 ml. All sediment incubations were performed in duplicate; incubated at room temperature in the dark; and periodically sacrificed for analysis of nitrate, nitrite, and sulfate as well as soluble U(VI), solids-associated U(VI), and U(IV) as described below.

U(IV) oxidation For U(IV) oxidation experiments, autoclaved sediment slurries were prepared as described above and amended with uranyl acetate (U(VI)) or uraninite (UO_2) (U(IV)) (Alfa Products, Danvers, MA) to an initial concentration of 75 nmoles/g sediment slurry. Fine-grained, clayey aquifer material was used, as opposed to the sandier sediments used for the sediment slurry incubations. Samples were allowed to equilibrate for 1 week, and representative bottles were sacrificed and uranium was sequentially extracted as described below to determine any changes in the uranium pool (i.e. reduction or adsorption) over the week-long

equilibration period. After equilibration, $NaNO_3$ (5 μmoles/g sediment), $NaNO_2$ (5 μmoles/ g sediment), NO (0.4 μmoles/g of sediment), N_2O (5 μmoles/g of sediment), or N_2 (5 ml to the 20 ml 80:20 N_2:CO_2 headspace) were added to the sediment slurries. For the incubations in which gas was added, NaCl (5 μmoles/g sediment) was added to produce an ionic strength equivalent to the nitrate and nitrite amended bottles. Subsamples were removed periodically from the soluble fraction and analyzed for U(VI) as described below. At the conclusion of the experiments, any remaining insoluble uranium was characterized by the sequential extraction procedure described below.

Analytical techniques Uranium speciation was determined by sequential extraction of soluble U(VI), solids-associated U(VI), and U(IV) in samples from non-sterile and heat

inactivated laboratory sediment incubations and cores collected at the conclusion of the push-pull tests. This was accomplished using a variation of methods described by Phillips et al. (*17*) and Fredrickson et al. (*18*) which has been shown to effectively remove solids-associated U(VI) from neutral pH sediments with a bicarbonate solution. Sediments were separated from porewater by centrifugation (6000*g* for 10 min), and the supernatant, containing the soluble U(VI) fraction was removed. To solubilize any solids-associated U(VI), a volume of anoxic sodium bicarbonate (100 mM; pH 8.3) equivalent to the supernatant removed (0.5 ml) was added to the remaining pellet (1 g sediment) under anoxic conditions. After incubation overnight in the sodium bicarbonate solution, samples were centrifuged again, the supernatant removed, and nitric acid was added under aerobic conditions in a volume equivalent to that of supernatant removed to oxidize and solubilize remaining uranium. Phillips et al. (*17*) reported that bicarbonate extracted 20% to 94% of the uranium extracted by nitric acid. The mean extraction efficiency for the first two extractions (soluble and bicarbonate extraction) was at least 67% for heat-inactivated incubations, assuming no reduction took place. Therefore, the maximum U(VI) potentially measured as U(IV) was 33% of the total uranium quantitated. Furthermore, when commercial UO_2 was added to sterile incubations (U(IV) oxidation experiments described above), the vast majority (approximately 96%) of uranium could only be recovered in the nitric acid extractable fraction, and 62-100% of the UO_2 added to the bottles was recovered. On the basis of these results, we were thus able to conclude that the nitric acid-extractable fraction was mainly U(IV). This extraction method allowed us to quantify the uranium in three pools: the soluble fraction, the solids-associated fraction (i.e. adsorbed), and the reduced fraction. While

spectroscopic evidence for U(VI) reduction was not obtained in these experiments, uranium that could only be recovered in the nitric acid-extractable fraction was putatively identified as reduced, and is referred to as reduced in the Results and Discussion section. U(VI) concentration in all samples was determined by kinetic phosphorescence analysis (KPA) (KPA-11; ChemChek Instruments, Richland, WA) (*19*). Bromide, sulfate, and nitrate were quantified by ion chromatography (Dionex DX 500 fitted with an AS-4A column and conductivity detector; Dionex Corporation, Sunnyvale, CA). Electron donors (formate, acetate, and lactate) were quantified by ion chromatography using the same system fitted with an AS-11 column.

Results and Discussion

Push-pull tests: effect of electron donors and acceptors on U(VI) reduction. Single well push-pull tests were used assess the impact of electron donors and alternate terminal electron acceptors on U(VI) reduction. Acetate, lactate and formate were all degraded relative to bromide over approximately a 2-week period during these tests (data not shown). However, there was no detectable influence of electron donors on U(VI) immobilization. Complete U(VI) immobilization in the absence of exogenous electron donor or acceptor occurred within 10 d (Figure 1), and a substantial amount (≥ 44%) of immobilization occurred within a few hours of injection, due either to adsorption of U(VI) on sediments or to a rapid rate of reduction. When sulfate was injected as an alternate terminal electron acceptor with U(VI), a slight inhibition of the rate of U(VI) immobilization was evident (Figure 1A). In the presence of nitrate and sulfate, more extensive inhibition of U(VI) reduction was observed especially as nitrate reduction

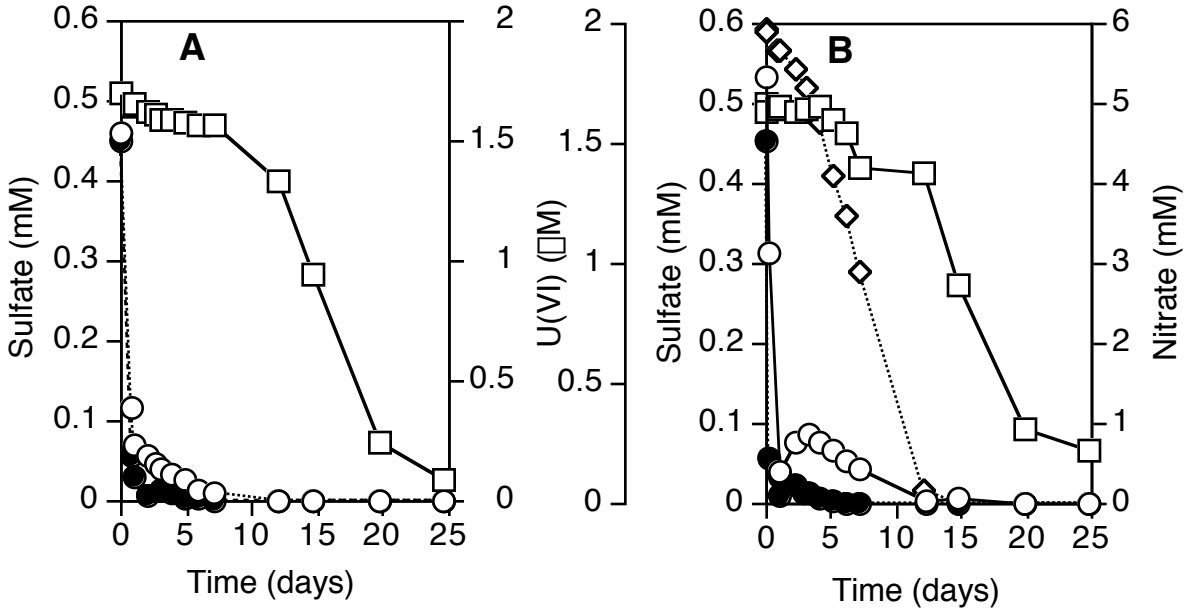

Figure 1. U(VI) immobilization in the presence of (A) 0.5 mM sulfate or (B) 5 mM nitrate and 0.5 mM sulfate, as assessed in-situ by push-pull tests. In both graphs, (●) represents U(VI) concentration in the terminal electron acceptor-unamended well, (○) U(VI) concentration in the amended wells, and (□) represents sulfate. Nitrate concentration is represented by (◇). All concentrations were adjusted for dilution by dividing measured concentrations by the C/C_o of bromide as described in the Materials and Methods.

proceeded (Figure 1B). Though the leachate-impacted sediments in this aquifer are generally considered to represent a sulfate-reducing, or methanogenic environment (*16, 20*), the presence of rapid nitrate reducing activity was far from surprising. Many anaerobic environments exhibit extensive nitrate reduction even though they do not receive regular inputs of nitrate (*21-23*), and several sulfate-reducing bacteria are capable of dissimilatory nitrate reduction to ammonia (*24*). Immobilization of U(VI) was not complete until nitrate was removed (~15 d). As is typical of anaerobic environments (*25-29*), significant sulfate reduction was not evident until the nitrate was depleted to low levels (Figure 1 B). The percent recovery of U(VI) was determined by dividing the amount removed from the well by the mass injected in 50 l of groundwater. This value was then normalized by the corresponding percent recovery of bromide to account for dilution of U(VI) by the surrounding groundwater. While the percent recovery of U(VI) in the sulfate amended well was only slightly higher than that of the untreated well, nearly half of the injected U(VI) was recovered from the nitrate-amended well (Table 2).

Laboratory incubations Laboratory U(VI) immobilization profiles in sediment incubations were similar to the in-situ tests. Uranium was reduced in the sediments, with near complete immobilization of uranium observed after 13 d (Fig. 2A & B). Upon addition of U(VI) to the sediment slurries, a near immediate (approx. 1 hour) immobilization of U(VI) was observed in both live incubations and in heat-inactivated incubations. As solids associated U(VI) increased at the start of the incubations, the initial decrease in soluble U(VI) is partly due to sorption. This was also evident initially in the heat-inactivated sediments, possibly as a result of surface-catalyzed U-reduction by Fe(II) or on iron-sulfides. The sum of all uranium pools has been plotted in Figure 2 to

Table 2. Percent recoveries for wells treated with U(VI) and nitrate or sulfate. All percentages are adjusted by dividing by the percent recovery of bromide to account for dilution as described in the Results and Discussion.

Treatment	% recovery U(VI)/ % recovery Br^-
U(VI) only	4%
U(VI) and 0.5 mM sulfate	10%
U(VI), 0.5 mM sulfate, and 5 mM nitrate	54%
U(VI) and 0.5 mM nitrate*	40%
U(VI) and 5 mM nitrate**	80%

*well had been treated with 1.5 μM U(VI) and 17 mM sodium formate in the previous round of tests

**well had been treated with 1.5 μM U(VI), 0.5 mM sodium sulfate, and 5 mM sodium sulfate in the previous round of tests

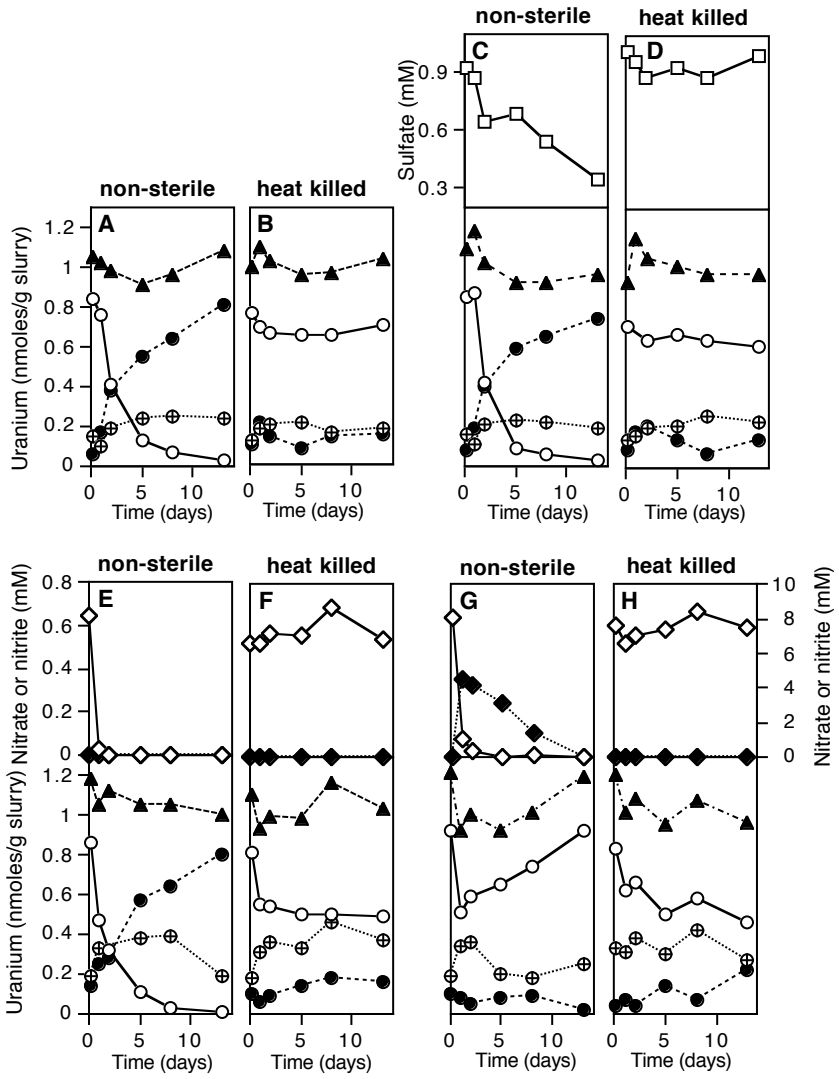

Figure 2. U(VI) immobilization in non-sterile and heat killed sediment incubations with (A) U(VI) and no alternate terminal electron acceptor and (B) heat-killed control; (C) U(VI) with 1 mM sulfate and (D) heat killed control; (E) U(VI) with 0.6 mM nitrate and (F) heat-killed control; (G) U(VI) with 7.5 mM nitrate and (H) heat-killed control. Bottom panels represent uranium species found in sequential extractions: (○) soluble U(VI), (⊕) solids associated U(VI), (●) U(IV), and (▲)total U. Top panels represent alternate terminal electron acceptors; either (□) sulfate, (▯) nitrate, or (◆) nitrite. All uranium concentrations are expressed as nmoles/g of sediment slurry. Each gram of sediment slurry contained 0.6 g dry sediment and 0.4 ml of water.

demonstrate that no pools of uranium are unaccounted for. Abiotic immobilization of uranium (via adsorption or reduction) has been well documented, occurring on iron sulfides and iron oxyhydroxides by both adsorption and reduction (*12, 30-36*), and by adsorption onto clay minerals (*37, 38*). However, biological activity was required for complete immobilization of U(VI) in our experiments. It has also been suggested that Fe(II) produced by iron-reducing bacteria can reduce U(VI) and other radionuclides on Fe(III) minerals (*17, 39*). Whether the biological reduction we observed in non-sterile incubation is a result of direct enzymatic reduction or is from the evolution of reducing bacterial endproducts is unknown. Finneran et al. (*14*) have suggested that Fe(II) and sulfide do not significantly contribute to U(VI) reduction in Shiprock NM sediments. Furthermore, both sulfate and microbially reducible Fe(III) were not abundant (100 □M and 200 nmoles/g sediment, respectively) in the sediment samples used for these experiments, while Fe(II) levels were high in both sterile and live sediments and minimal abiotic reduction occurred in the heat killed incubations. Uranium and sulfate reduction occurred concomitantly in sediment incubations, but sulfate appeared to neither stimulate nor inhibit U(VI) reduction relative to the unamended incubations (Figure 2 A-D). This suggests that U(VI) reduction did not occur as a result of sulfide generated by sulfate reduction as the addition of sulfate would likely have stimulated U(VI) reduction. As noted above, slight "inhibition" of U(VI) immobilization by sulfate was observed in the push-pull tests, but this was not observed in the laboratory incubations. Based on the sediment incubations, it appears that the apparent "inhibition" of U(VI) reduction by sulfate that occurred in the push-pull tests may be due to slightly different sorbtive characteristics of the sediments surrounding the push-pull test wells.

When 0.6 mM nitrate was added to sediment slurries, no inhibition of U(VI) reduction occurred (Figure 2 E & F). Nitrate was completely reduced within 2 d and no accumulation of nitrite was evident. In this case, nitrate was present in relatively low concentration, reduced quite rapidly, and had no detectable effect on U(VI) reduction. However, in sediment incubations with U(VI) and 7.5 mM nitrate, an initial immobilization of U(VI) was observed, but remobilization occurred as nitrate was reduced and nitrite, and likely other denitrification intermediates accumulated (Figure 2 G & H).

Abdoulas et al. (*40*) performed column experiments suggesting terminal electron acceptors were consumed in sequence. Nitrate reduction was followed by U(VI) reduction, and finally sulfate reduction. Complete reduction of nitrate occurred in an acetate and trimetaphosphate amended column after 7.5 d, with the transient accumulation of nitrite. U(VI) loss began after nitrate had been consumed and while nitrite reduction occurred. There was no evidence for the remobilization of U(VI) in these experiments. While our experiments also show that U(VI) reduction will not commence until nitrate is consumed, we present both field and laboratory evidence (below) that remobilization/oxidation of U(IV) occurs only after nitrate reduction has commenced.

Oxidation and remobilization of uranium under nitrate-reducing conditions. The inhibition of U(VI) reduction by nitrate in sediment incubations and the first round of push-pull tests could be interpreted simply as a result of nitrate controlling the redox potential, rather than remobilization of previously reduced uranium. An increase in the nitrous oxide to N_2 ratio can result from the addition of nitrate to environmental samples

(*41-43*), indicating that denitrification is occurring along with the transient accumulation of denitrification intermediates. To test the hypothesis that U(IV) was oxidized and remobilized by dissimilatory nitrate reduction intermediates, a second set of push-pull tests were performed in the wells from the previous experiments in which uranium was coinjected with two different nitrate concentrations in separate wells (1 mM and 5 mM). The sediments surrounding these wells already contained reduced uranium based both on our calculated recoveries and the apparent reduction observed in the laboratory experiments. A control well was tested with U(VI) only, and exhibited a similar pattern of immobilization to the first round of tests (Figure 3 A & B). However, when 1 mM or 5 mM nitrate accompanied U(VI), initial immobilization occurred over the first 1 to 2 d. Remobilization subsequently occurred during the period of active nitrate reduction. In the well with 5 mM nitrate, the dilution-adjusted U(VI) actually reached a higher concentration than that of the injected U(VI) (Figure 3B); percent recoveries of U(VI) in both wells were quite high (Table 2).

After completion of push-pull tests, 0.5 m cores were collected from two wells, and uranium was sequentially extracted. The majority of the U(VI) extracted from the cores was reduced (Table 3), suggesting that the immobilization observed in the push-pull tests was a result of reduction and precipitation. The cores were collected from the well used to conduct tests 1 and then 7 (see Table 1) and the well used to conduct tests 4 and then 9 (see Table 1). Interestingly, less total uranium was present in the core collected adjacent to a well which received an input of nitrate (during test 9). This would be expected, as this well had been treated with nitrate, and the resulting remobilized uranium would either be extracted or transported away from the well vicinity by regional

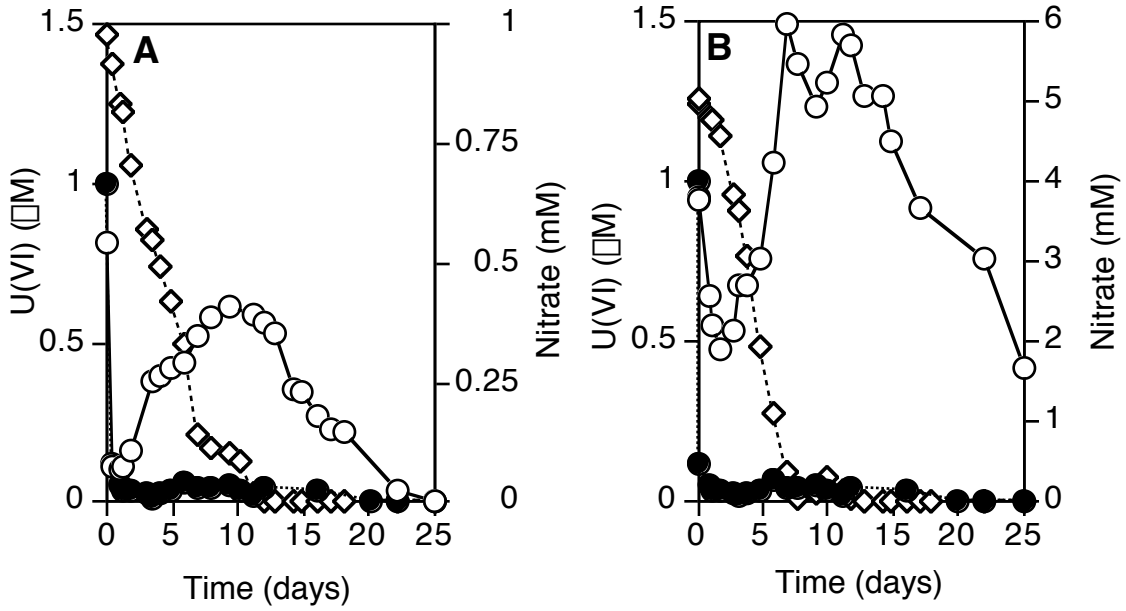

Figure 3. U(VI) immobilization, and remobilization as (A) 1 mM and (B) 5 mM nitrate is reduced. Nitrate concentration is represented by (□), U(VI) concentration in nitrate amended wells is represented by (○), and U(VI) concentration in the unamended well is represented by (●). All concentrations were adjusted for dilution by dividing measured concentrations by the C/C_o of bromide as described in the Materials and Methods.

Table 3. Sequential extraction of oxidized and reduced uranium species at conclusion of push-pull tests

	Well A (μmoles U/ kg sediment)*	Well D (μmoles U/ kg of sediment)**
U(IV) (nitric acid extractable fraction)	784	460
Solids Associated U(VI) (bicarbonate extractable fraction)	61	36
Soluble U(VI) (water extractable fraction)	48	32

*well had been treated with 1.5 μM U(VI) for two rounds of tests.

**well had been previously treated with 1.5 μM U(VI) and 17 mM formate.

groundwater flow, as opposed to the unamended well where U(VI) was immobilized in close proximity to the well. This observation, coupled with the slight lag in uranium remobilization until nitrate reduction had commenced, suggested that highly reactive nitrate reduction intermediates might be oxidizing previously reduced uranium.

To test the hypothesis that U(IV) was oxidized by intermediates as nitrate reduction proceeded, U(VI) and U(IV) were added independently to autoclaved sediments. The sediments used in these tests contained higher amounts of clay and fine-grained material to achieve more U(VI) adsorption and allow us to differentiate between remobilization via U(IV) oxidation or via liberation from solid phases to which U(VI) adsorbed. Within 30 min, 51% of the U(VI) had adsorbed to these sediments (as opposed to 17% in sandy sediments used for the non-sterile incubations to assess biological activity). After a 7 d equilibration period, 68% of the U(VI) was reduced, and 24% was solids-associated. More abiotic U(VI) reduction was observed in the heat-killed sediments in these experiments than the previous set which were used to assess biological reduction. This was likely a result of different mineralogical characteristics in the sediments, and may be attributed to a combination of the abiotic pathways of U(VI) reduction noted above. Sediments surrounding the wells have been shown to contain high levels of iron sulfides (*20*), which could be involved in U(VI) reduction. Marsh et al. (*44*) also observed abiotic Cr(VI) reduction in dark clayey sediments from the same aquifer, but abiotic Cr(VI) reduction did not occur in light colored, sandy sediments. When dissimilatory nitrate reduction intermediates (NO_2^-, N_2O, NO) were injected into these bottles, rapid U(VI) oxidation/remobilization occurred, providing evidence that the remobilization observed in laboratory experiments and push-pull tests was due to

oxidation of U(IV) and reduced adsorbent minerals (i.e. iron-sulfides) by denitrification intermediates (Figure 4). Nitrite and nitric oxide lead to the most extensive U(IV) oxidation/remobilization. When nitrite and nitric oxide were added to bottles initially treated with U(VI), all of the U(IV) present was oxidized, with any U(VI) not in solution being in the solids associated fraction (20% solids associated, 80% soluble). In incubations initially treated with U(IV), approximately 18% of the U(IV) was oxidized by nitrite and nitric oxide. Preliminary light-microscopic examinations of biogenic UO_2 and commercial UO_2 (Alfa Products, Danvers, MA) revealed that commercial UO_2 is considerably larger (approx. 5-7 μm diameter) than that recovered from resting cell incubations (less than 1 μm in diameter) of *D. desulfuricans*, performed as described by Lovley and Phillips (*6*). Based on particle diameter, it is reasonable to predict that biogenic UO_2 would present a greater surface area than commercial UO_2, and might be more easily oxidized.

The Gibbs free energies were calculated for some reactions in which U(IV) is oxidized with the reduction of inorganic nitrogen compounds involved in denitrification. These calculations reveal that U(IV) oxidation by nitrate and dinitrogen are less favorable than U(IV) oxidation by nitrite, nitric oxide, and nitrous oxide (Table 4). The presence of sulfides in the test sediments may also have accentuated the effects of nitric oxide and nitrous oxide. Nitrous oxide and nitric oxide reductases are inhibited by sulfide (*45*) and the respective intermediates have been shown to accumulate in transition zones from nitrate reduction to sulfate reduction in coastal marine sediments (*46*).

Our results suggest that U(VI) can be reduced through the concerted action of microbiological and geochemical processes. However, dissimilatory nitrate reduction

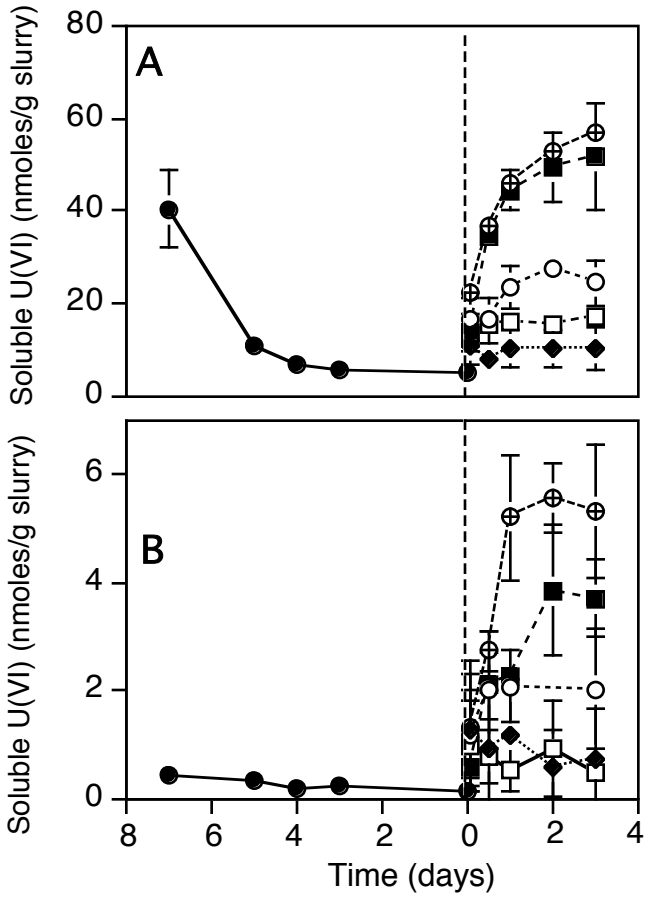

Figure 4. Oxidation and remobilization of uranium in the presence of denitrification intermediates. U(VI) was added to heat killed sediments (A) and allowed to equilibrate for 7 d (●), during which time, 68% of the U(VI) was reduced, and 24% adsorbed to sediments. (B) U(IV) was added in parallel sediment incubations. The dashed line represents the point at which intermediates were added, and soluble U(VI) was monitored in the presence of (□) nitrate, (■) nitrite, (⊕) nitric oxide, (○) nitrous oxide, and (◆) nitrogen gas. The dashed line at 0 d represents the point at which nitrogen oxides were added. Error bars represent standard deviation of duplicate incubations.

Table 4. Gibbs free energies of reactions coupling the oxidation of amorphous uraninite (UO_2(am)) to the reduction of various compounds involved in denitrification. Values calculated from Dean (10).

Reaction	$\Delta G^{o'}_R$ (kJ/mole)
$UO_2 + 0.33\ N_2 + 2.67\ H^+ \rightarrow UO_2^{2+} + 0.33\ NH_4^+$	+80
$UO_2 + N_2O + 2\ H^+ \rightarrow UO_2^{2+} + N_2 + H_2O$	-235
$UO_2 + 2NO + 2H^+ \rightarrow UO_2^{2+} + N_2O + H_2O$	-200
$UO_2 + NO + 2H^+ \rightarrow UO_2^{2+} + 0.5\ N_2 + H_2O$	-218
$UO_2 + 0.4\ NO + 2.2\ H^+ \rightarrow UO_2^{2+} + 0.4\ NH_4^+ + 0.4\ H_2O$	-47
$UO_2 + 0.33\ NO_2^- + 2.67\ H^+ \rightarrow UO_2^{2+} + 0.33\ NH_4^+ + 0.67\ H_2O$	-159
$UO_2 + 0.67\ NO_2^- + 2.67\ H^+ \rightarrow UO_2^{2+} + 0.33\ N_2 + 1.33\ H_2O$	-40
$UO_2 + NO_3^- + 2H^+ \rightarrow UO_2^{2+} + NO_2^- + H_2O$	-57
$UO_2 + 0.25\ NO_3^- + 2.5\ H^+ \rightarrow UO_2^{2+} + 0.25\ NH_4^+ + 0.75\ H_2O$	-44
$UO_2 + 0.4\ NO_3^- + 2.4\ H^+ \rightarrow UO_2^{2+} + 0.2\ N_2 + 1.2\ H_2O$	-118

could lead to conditions in which U(IV) is oxidized. We do not know if the oxidation observed in the non-sterile incubations and push-pull tests is due exclusively to abiotic oxidation by denitrification intermediates or if there is a role for lithotrophic sulfide, Fe(II), or U(IV) oxidation coupled to nitrate reduction. The simplest explanation for our observations is that uranium remobilization during nitrate reduction is an abiotic process, occurring as dissimilatory nitrate reduction intermediates accumulate. Fe(II) is oxidized by nitrite and nitric oxide in the same manner (*47*). If Fe(II) is oxidized by denitrification intermediates, it is possible that the Fe(III) produced may oxidize U(IV) (*48*). Several denitrifying organisms have been shown to couple Fe(II) oxidation to nitrate reduction either lithotrophically or mixotrophically at neutral pH (*49-52*). Interestingly, the organisms isolated by Straub et al. (*49,52*) were able to oxidize insoluble Fe(II)-phosphates and –carbonates, suggesting insoluble reduced metal minerals like UO_2 can act as electron donors for lithotrophic organisms. An instance of lithotrophic U(IV) oxidation by *Thiobacillus ferrooxidans* under aerobic and acidic conditions has been reported (*53*). Therefore, lithotrophic U(IV) oxidation coupled to nitrate reduction is conceivable, but not documented under neutral conditions.

This work provides an explanation for persistent U(VI) solubility in contaminated sites in which nitrate is present. Evolution of the dissimilatory nitrate reduction intermediates apparently creates a highly oxidizing environment, leading to the oxidation of U(IV), reversing the reducing conditions required for uranium immobilization. If electron donor is injected into a U(VI) impacted aquifer as part of a remediation strategy, nitrogen could be cycled in the sediments, maintaining conditions favorable for U(IV)

oxidation. Therefore, it will be necessary to stimulate the removal of nitrogen from systems via denitrification before the immobilization of U(VI) can commence.

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Chapter 2

A Role for Fe(III) Minerals in Nitrate-Dependent Microbial U(IV) Oxidation.

Abstract

Microbiological reduction of soluble U(VI) to insoluble U(IV) is a means of preventing the migration of that element in groundwater, but the presence of nitrate in U(IV)-containing sediments leads to the U(IV) oxidation and remobilizaton. Nitrite or Fe(III)-oxyhydroxides may oxidize U(IV) under nitrate-reducing conditions and we determined the rate and extent of U(IV) oxidation by these compounds. Fe(III) oxidized U(IV) at a greater rate than nitrite (130 and 10 □M U(IV)/day, respectively). In aquifer sediments, Fe(III) may be produced during microbial nitrate-reduction by oxidation of Fe(II) by nitrite or by enzymatic Fe(II) oxidation coupled to nitrate reduction. To determine which of these mechanisms was dominant, we isolated a nitrate-dependent acetate- and Fe(II)-oxidizing bacterium from a U(VI)- and nitrate-contaminated aquifer. This organism oxidized U(IV) at a greater rate and to a greater extent under acetate-oxidizing (where nitrite accumulated to 50 mM) than under Fe(II)-oxidizing conditions. We show that the observed differences in rate and extent of U(IV) oxidation are due to mineralogical differences between Fe(III) produced by reaction of Fe(II) with nitrite (amorphous) and Fe(III) produced enzymatically (goethite or lepidocrocite). Our results suggest the mineralogy and surface area of Fe(III) minerals produced under nitrate-reducing conditions affects the rate and extent of U(IV) oxidation. These results may be useful for predicting the stability of U(IV) in aquifers.

Introduction

The reduction of soluble U(VI) to insoluble U(IV) has been proposed as a strategy for preventing the migration of that toxic element with groundwater (*1, 2*). The strategy entails the injection of physiological electron donors to stimulate U(VI) reduction by microbial communities native to contaminated aquifers (*3, 4*). U(VI) reduction should occur under Fe(III) and/or sulfate reducing conditions (*4-6*). Nitrate (a frequent co-contaminant with U(VI)), if present, must be reduced before U(VI), Fe(III), and sulfate reduction will commence (*4,6-8*). Recent laboratory and field-scale studies have shown the potential of microbial communities at contaminated sites to reduce nitrate and U(VI) if exogenous electron donor is supplied (*3, 4*).

While nitrate reduction must be complete before U(VI) will be reduced, the addition of nitrate to U(IV)-containing sediments leads to oxidation and subsequent remobilization of U (*4, 7, 8*). Therefore, nitrate inputs to aquifers where uranium has been reductively immobilized could seriously threaten U(IV) stability. Fe(III) oxidizes U(IV) under environmentally relevant conditions (*8-12*), and may play a role in U(IV) oxidation under nitrate-reducing conditions. Finneran et al. (*8*) hypothesized that Fe(III) produced by nitrate-dependent Fe(II)-oxidizing (lithotrophic) bacteria oxidizes U(IV). Alternatively, intermediates in dissimilatory nitrate reduction are quite reactive and have been shown to abiotically oxidize U(IV) in sediments (*7*). Under this latter scenario, as organotrophic, dissimilatory nitrate reduction proceeds, these intermediates (especially nitrite) transiently accumulate (*7, 13-15*) and react with U(IV), oxidizing it to U(VI). It is unclear which of these is the predominant mechanism of U(IV) oxidation. Finneran et al.

(*8*) suggested that U(IV) oxidation by nitrite in heat-killed sediment incubations proceeds slowly compared to U(IV) oxidation that occurred in non-sterile sediment incubations, but in studies by Senko et al., (*7*), nitrite (5 μmole/g sediment) completely oxidized U(IV) (75 nmole/g sediment) in heat-killed sediments within 3 days.

To establish the predominant mechanism of U(IV) oxidation under nitrate-reducing conditions, we examined rates and extents of "abiotic" U(IV) oxidation by nitrite and Fe(III), and show here that nitrate-dependent U(IV) oxidation is likely to proceed via abiogenic or biogenic Fe(III). We propose a new mechanism of U(IV) oxidation, in which U(IV) is oxidized by Fe(III) produced by nitrite oxidation of Fe(II). This mechanism of U(IV) oxidation appears to be more effective than U(IV) oxidation mediated by biogenic Fe(III) due to differences in mineralogy and surface area between the Fe(III) minerals formed by the two processes.

Materials and Methods

Abiotic Incubations. All incubations to assess abiotic U(IV) oxidation were performed in anoxic bicarbonate buffer (3.5 g/l, pH 6.8) under a headspace of 80% N_2 and 20% CO_2 contained in serum bottles that were sealed with thick rubber stoppers (*16*). Where appropriate, biogenic U(IV) (*17*), $NaNO_2$, $FeCl_2$, or Fe(III) minerals (described below) were added from sterile or pasteurized concentrated stock solutions.

Bacterial Culturing Techniques. *Desulfovibrio vulgaris* Hildenborough was grown on lactate-sulfate medium described by Rapp and Wall (*18*). An autotrophic, anaerobic, nitrate-dependent, FeS-oxidizing enrichment culture was obtained from a landfill leachate-impacted aquifer (Senko et al., in preparation) and maintained in a bicarbonate-

buffered (3.5 g/l, pH 6.8) liquid medium containing 0.8 g/l NaCl, 1 g/l NH_4Cl, 0.1 g/l KCl, 0.03 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7H_2O$, and 0.04 g/l $CaCl_2.2H_2O$, vitamins, and trace metals (*18*). The enrichment culture was maintained in serum tubes or bottles containing a headspace of 80% N_2 and 20% CO_2 and sealed with thick rubber stoppers (*16*). FeS (5 mM) was the sole electron donor in this medium and $NaNO_3$ (10 mM) was the sole electron acceptor.

An acetate- and Fe(II)-oxidizing pure culture was isolated from nitrate- and U(IV)-contaminated groundwater at the U.S. Department of Energy's Field Research Center (FRC) in Oak Ridge, TN. During tests to stimulate microbiological nitrate- and U(VI)-reducing bacterial activity, successive electron donor additions were made to the aquifer (*4*), and extensive biomass was observed in water extracted from injection wells. Biomass was recovered from a well (FW33) that received glucose stimulation. Water samples were collected in sterile centrifuge tubes, refrigerated, and shipped to the laboratory where they were serially diluted and spread onto solid medium. Organisms were isolated on the minimal medium described above with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (50 mM, pH 7) instead of bicarbonate under a N_2 headspace and agar (20 g/l) as a solidifying agent (*19*). Acetate (30 mM) was the sole electron donor and nitrate (20 mM) was sole electron acceptor. Plates were prepared under oxic conditions before transfer to an anoxic glovebag (Coy Laboratory Products, Grass Lake, MI) where they equilibrated overnight under anoxic conditions. Colonies exhibiting distinct morphologies were picked and transferred to anoxic HEPES-buffered acetate-nitrate liquid medium (as above but with no agar) in serum tubes with a N_2 headspace (*16*). One isolate (designated FW33AN) was chosen for further study due to

its robust nitrate-reducing activity in FRC groundwater and its ability to mediate nitrate-dependent Fe(II) oxidation. The purity of the isolate was assessed microscopically.

Biologically-Mediated Nitrate-Dependent U(IV) Oxidation. The pH of FRC groundwater (from well FWO21, starting pH 3.7, approx. 130 mM nitrate) was adjusted to approximately 7.0 with sodium bicarbonate (approx. 10 g/l) as described by Istok et al. (*4*). Neutralization resulted in the formation of extensive precipitates that were removed by filtration. Oxygen was removed from neutralized FRC groundwater by boiling under a stream of 80:20 N_2:CO_2. Groundwater (50 ml) was transferred to 120 ml serum bottles with a headspace of 80% N_2 and 20% CO_2, and bottles were sealed with rubber stoppers (*16*). Acetate (95 mM CH_3COONa), Fe(II) (0.4 mM $FeCl_2$), and U(IV) (0.4 mM biogenic U(IV)) were added by syringe from sterile stocks. Strain FW33AN was grown to mid-log phase in HEPES-buffered acetate-nitrate medium described above, harvested by centrifugation, washed three times with anoxic bicarbonate buffer (3.5 g/l, pH 6.8), and resuspended in bicarbonate buffer before addition to groundwater to a final concentration of 0.1 mg protein/ml. During groundwater incubations, samples were periodically removed by needle and syringe and analyzed as described below.

Fe(III) Mineral Preparation. Poorly crystalline Fe(III) oxyhydroxide and magnetite were prepared as described by Lovley and Phillips (*21*) and Schwertmann and Cornell (*22*), respectively. The precipitates were washed three times to remove ions, bubbled with N_2 to remove O_2, and pasteurized. Lepidocrocite and goethite were purchased from Alfa Aesar (Alfa Aesar, Ward Hill, MA) and stock suspensions were prepared similarly. Fe(III) from oxidation of Fe(II) by nitrite was prepared by reacting 5 mM $FeCl_2$ with 5 mM $NaNO_2$ in bicarbonate buffer. Biogenic Fe(III) (later identified as goethite) was

prepared by incubating strain FW33AN (0.1 mg protein/ml) with 5 mM $FeCl_2$ and 5 mM $NaNO_3$ in bicarbonate buffer. Another biogenic Fe(III) mineral (later identified as lepidocrocite) was obtained from the nitrate-dependent, FeS-oxidizing culture (described above). All anaerobically prepared Fe(III) minerals were harvested by centrifugation under anoxic conditions, washed three times and resuspended in water. This final suspension served as a stock suspension that was added to U(IV)-containing bicarbonate-buffered incubations.

Sampling and Analytical Techniques. Samples were periodically removed from incubations by syringe in an anoxic glovebag. Solids were removed by centrifugation and U(VI), Fe(II), nitrate, and nitrite were quantified in the soluble fraction. Fe(II) was preserved in 0.5 M HCl. Solid phase U and Fe species were extracted from pellets and quantified as described below. Solids-associated U(VI) and U(IV) were extracted with a bicarbonate solution and nitric acid, respectively, as described by Elias et al. (*17*). Solid-phase Fe(II) was extracted with 0.5 M HCl (*21*)

Nitrate and nitrite were quantified by ion chromatography with conductivity detection (Dionex DX 500 fitted with an AS-4A column; Dionex Corporation, Sunnyvale, CA) (*7*). U(VI) was quantified by kinetic phosphorescence analysis (KPA) on a KPA-11 (ChemChek Instruments, Richland, WA) (*23*). Fe(II) was quantified by ferrozine assay (*21*). Due to the poor extractability of crystalline Fe(III) minerals (*21*), Fe(III) minerals from stock suspensions were dried in an anoxic glovebag and Fe(III) concentration was determined gravimetrically. Protein was quantified using the bicinchoninic assay (Pierce Biotechnology, Inc., Rockford, IL). X-ray diffraction of

powdered mineral samples was determined on an automated Rigaku diffractometer (Rigaku/MSC, The Woodlands, TX).

Results and Discussion

Abiotic oxidation of U(IV) under anoxic conditions. We compared rates of U(IV) oxidation by chemically prepared poorly crystalline Fe(III) oxyhydroxide (*20*) (Equation 1) and nitrite (Equation 2).

Equation 1: $2\ Fe_5HO_8 \bullet 4H_2O + 5UO_2 + 30\ H^+ \rightarrow 10\ Fe^{2+} + 5\ UO_2^{2+} + 24\ H_2O$

Equation 2: $2\ NO_2^- + 2\ UO_2 + 6H^+ \rightarrow N_2O + 2UO_2^{2+} + 3H_2O$

Nitrous oxide has not been shown experimentally to be the product of U(IV) oxidation by nitrite (Equation 2), but it has been proposed as the most likely product of nitrite reduction by Fe(II) (*24-28*). In our experiments, U(IV) was rapidly but incompletely oxidized by poorly crystalline Fe(III) oxyhydroxide (130 □M U(IV) oxidized/day), while U(IV) oxidation by nitrite proceeded slowly (10 □M U(IV) oxidized/day) (Figure 1 A). Magnetite did not oxidize U(IV) (not shown). We hypothesized that incomplete oxidation of U(IV) by Fe(III) oxyhydroxide occurred as a result of Fe(II) accumulation during reduction of Fe(III) by U(IV) and subsequent passivation of the reaction between Fe(III) and U(IV). Similar surface passivation has been reported for microbiological Fe(III) reduction, where adsorption/precipitation of Fe(II) on Fe(III) mineral surfaces inhibits microbiological reduction of Fe(III) (*29-33*). Fe(II) that was evolved from the reduction of Fe(III) by U(IV) could have actually reduced the U(VI) that was produced by the reaction (*34, 35*). Bacterial reduction of Fe(III) also results in partially reduced Fe minerals like magnetite and green rust that will not oxidize U(IV) or which could actually

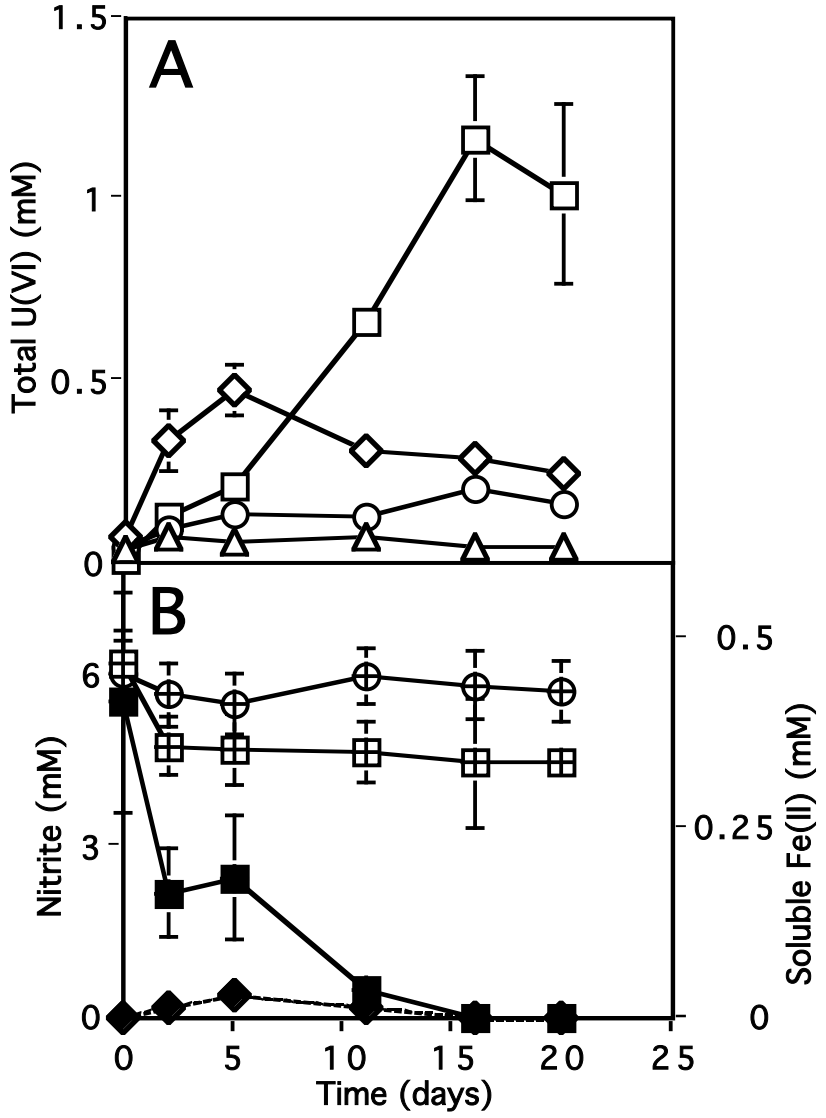

Figure 1. Anaerobic U(IV) oxidation in the presence of no oxidant (triangles), 6 mM nitrite (circles), 5 mM poorly crystalline Fe(III) oxyhydroxide (diamonds), or 6 mM nitrite and 0.4 mM Fe(II) (squares). Total U(VI) concentrations are shown in panel A and soluble Fe(II) and nitrite are shown in panel B. Open shapes represent total U(VI) concentrations, crossed shapes represent nitrite concentrations, and closed shapes represent soluble Fe(II) concentrations.

reduce U(VI) (this study, *36*). While we could detect the release of Fe(II) into solution as Fe(III) oxidized U(IV) (Figure 1 B), an electron equivalent amount of Fe(II) was not released into solution during Fe(III) reduction by U(IV), suggesting that Fe(II) was adsorbed to the Fe(III) surfaces or was incorporated into Fe(II)/Fe(III) minerals.

We then determined whether accumulation of Fe(II) inhibited U(IV) oxidation, we incubated Fe(III) oxyhydroxide with various ratios of Fe(II) to Fe(III). Less U(IV) oxidation occurred in the presence of progressively higher ratios of Fe(II)/Fe(III) (Figure 2). U(IV) was oxidized by 5 mM and 3 mM Fe(III) at similar rates and to similar extents (not shown), indicating that Fe(III) concentration did not limit U(IV) oxidation in these incubations. The addition of nitrite to incubations rapidly oxidized Fe(II) (Figure 2 B), and alleviated the inhibition of U(IV) oxidation by Fe(II) (Figure 2 A).

While nitrite alone was a poor oxidant of U(IV), the addition of 400 μM Fe(II) to U(IV)- and nitrite-containing incubations stimulated complete U(IV) oxidation (Figure 1 A). Fe(II) was oxidized by nitrite in these incubations (Figure 1 B), suggesting that Fe was acting as a catalyst for U(IV) oxidation by nitrite, where nitrite oxidized Fe(II) to Fe(III) which in turn oxidized U(IV) (Equations 3 and 1).

Equation 3: $10\ NO_2^- + 20\ Fe^{2+} + 33\ H_2O \rightarrow 5\ N_2O + 4\ Fe_5HO_8 \bullet 4H_2O + 30\ H^+$

Fe(II) reduces nitrite to ammonium or nitrous oxide (*24-28, 37*), and we hypothesized that Fe acts as a "electron shuttle" from U(IV) to nitrite. If this hypothesis is correct, levels of U(IV) oxidation by nitrite should be independent of Fe(II) concentration, if Fe(II) is present at adequate concentration to react at the maximum rate and provided nitrite is present at levels sufficient to oxidize all Fe(II) and U(IV). U(VI) oxidation by nitrite was stimulated at all Fe(II) concentrations tested (Figure 3), suggesting that nitrite

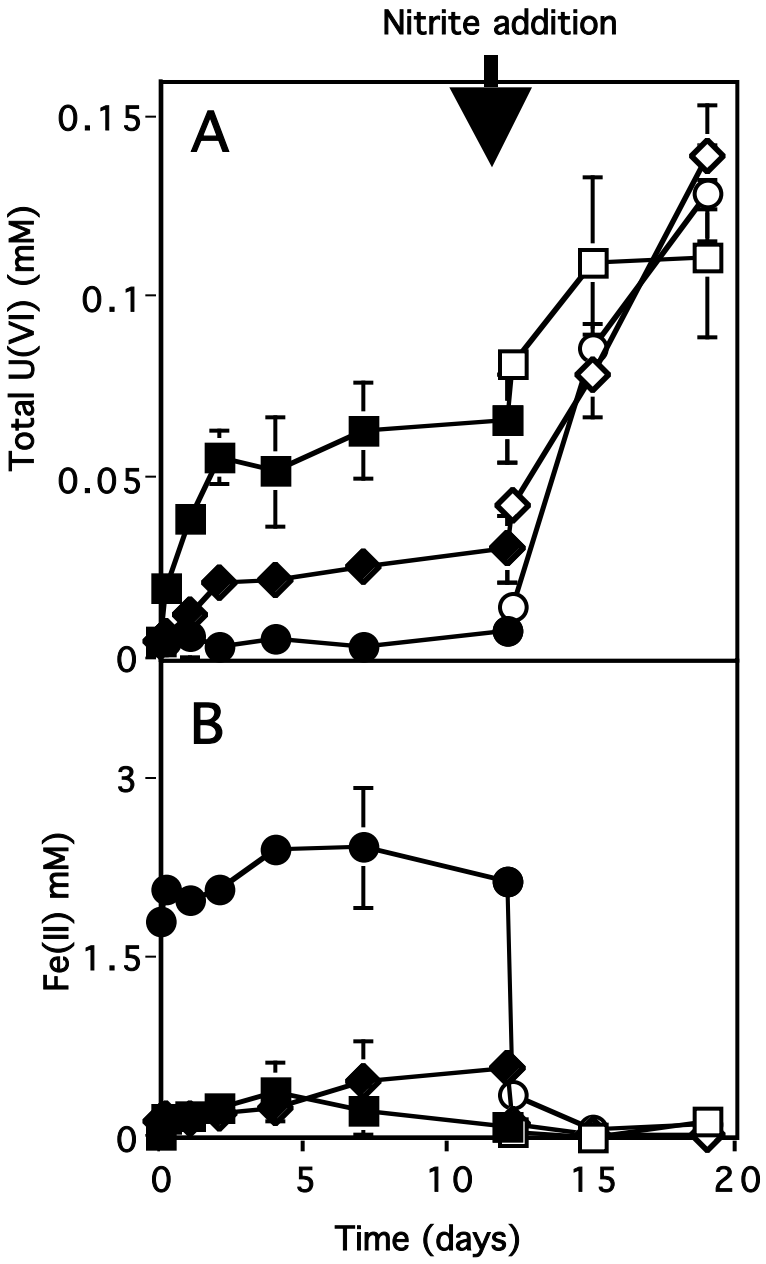

Figure 2. Anaerobic U(IV) oxidation by Fe(III) at Fe(II)/Fe(III) concentrations of 0 mM/5 mM (squares), 0.2 mM/4.8 mM (diamonds), and 2 mM/3 mM (circles). Total U(VI) concentrations are shown in panel A and total Fe(II) concentrations are shown in panel B. Open shapes represent total U(VI) or Fe(II) concentrations after nitrite addition.

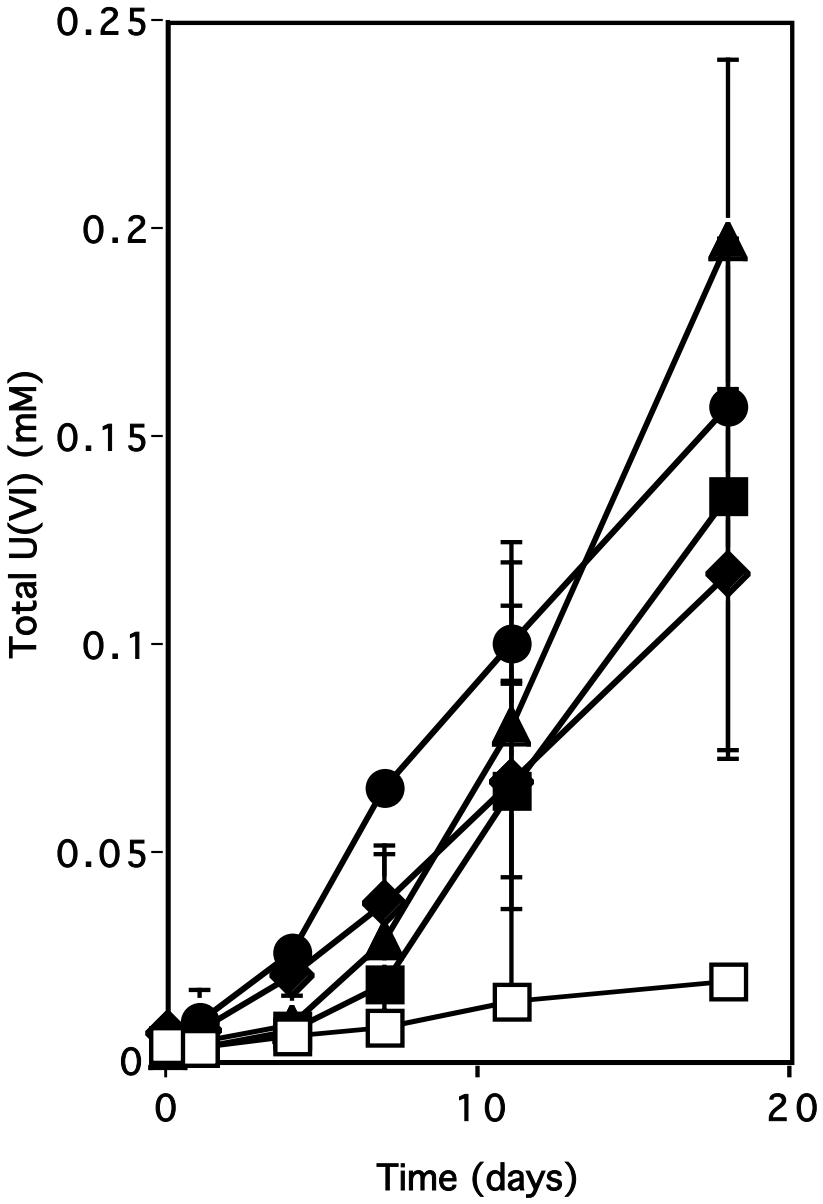

Figure 3. Anaerobic U(IV) oxidation by nitrite in the presence of 0 mM (□), 0.01 mM (◆), 0.1 mM (●), 1 mM (▲), and 10 mM (■) Fe(II).

oxidized Fe(II) to Fe(III), which in turn oxidized U(IV). This regenerated Fe(II) that was again oxidized by nitrite. Fe(II) in excess of nitrite (100 mM) inhibited U(IV) oxidation (not shown). We use the term "electron shuttling" because Fe-mediated U(IV) oxidation by nitrite appears to proceed in a manner similar to the shuttling of electrons by humic material from iron-reducing bacteria to Fe(III) minerals (*12, 38*). Like this latter process, the extent of U(IV) oxidized by nitrite is largely independent of the amount of electron shuttle (Fe) present. Nitrite oxidizes Fe(II) to Fe(III) (Equation 3) which in turn oxidizes U(IV) (Equation 1). The Fe(II) produced by this reaction can then be oxidized again by remaining nitrite. Work by Weber et al. (*39*) showed that nitrite is a poor oxidant of Fe(II), but experiments conducted by these workers used nitrite concentrations at molar ratios of 1:4 nitrite:Fe(II). In all of our experiments and most likely in aquifers undergoing bioremediation (*4*), nitrite:Fe(II) ratios were not lower than 1:1, which almost certainly enhanced the reaction rate.

Evaluation of mechanisms of nitrate dependent U(IV) oxidation by a pure culture isolated from the Oak Ridge National Laboratory Field Research Center (FRC). While it was clear from the preceding chemical experiments that U(IV) was ultimately oxidized by Fe(III), it was not clear whether biogenic Fe(III) and abiogenic Fe(III) (i.e. produced by oxidation of Fe(II) by nitrite) were equally effective oxidants of U(IV). To establish this, we isolated an acetate- and Fe(II)-oxidizing, nitrate-reducing bacterium from FRC groundwater. We could then compare rates and extents of U(IV) oxidation under organotrophic (where Fe(II) is oxidized by nitrite produced during acetate oxidation) and lithotrophic (where Fe(II) is oxidized by microbial respiration) conditions.

During experiments to stimulate in-situ nitrate and U(VI) reduction (*4*), water was extracted from an injection well (FW33) in a nitrate and U(VI)-contaminated aquifer at the Oak Ridge Field Research Center (FRC). Water produced from this well contained extensive microbial growth as visually determined by turbidity. This water was serially diluted and colonies were isolated on plates under anoxic conditions with acetate as the carbon source and sole electron and nitrate as the sole terminal electron acceptor. Due to its robust nitrate-reducing activity in neutralized FRC groundwater, one isolate (termed FW33AN) was chosen for further study. Strain FW33AN was able to couple the oxidation of Fe(II) to the reduction of nitrate (Figure 4), a mode of metabolism that is widespread among diverse dissimilatory nitrate-reducing bacteria (*8, 40-49*). Molecular microbial community analyses of FRC sediment and groundwater revealed the presence of microorganisms whose relatives have been shown to be capable of dissimilatory nitrate reduction and nitrate-dependent Fe(II) oxidation (*50-53*).

We compared U(IV) oxidation by this organism under acetate-oxidizing (organotrophic) and Fe(II)-oxidizing (lithotrophic) conditions. Strain FW33AN was incubated in neutralized FRC groundwater (which contains approx. 130 mM nitrate) with U(IV) and amended with 0.4 mM Fe(II) or 0.4 mM Fe(II) and 95 mM acetate. We hypothesized that if U(IV) oxidation was catalyzed by Fe(III) produced by lithotrophic activity, the addition of Fe(II) alone would stimulate U(IV) oxidation. Alternatively, if U(IV) was oxidized predominantly by the shuttling of electrons from U(IV) to biologically produced nitrite through Fe, we would expect to see U(IV) oxidation only in the presence of both Fe(II) and acetate.

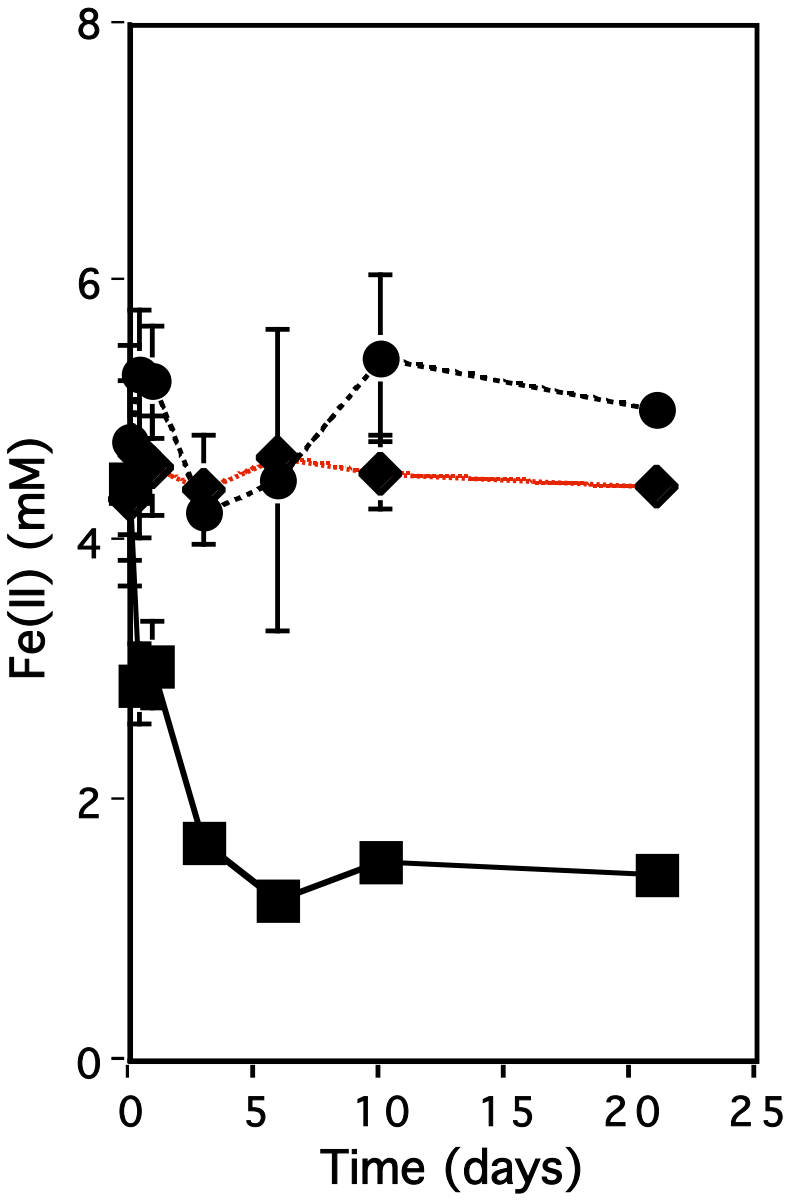

Figure 4. Nitrate-dependent Fe(II) oxidation by strain FW33AN. (■) cells + nitrate; (◆) no nitrate; no cells (●).

Resting cells of FW33AN rapidly and near-completely oxidized U(IV) in neutralized FRC groundwater with acetate and Fe(II) amendment (Figure 5 A; Figure 6 A). U(IV) oxidation occurred concurrently with nitrate reduction (Figure 5 D), nitrite accumulation (Figure 5 C) and rapid Fe(II) oxidation (Figure 5 B; Figure 6 B). Nitrite transiently accumulated to 50 mM, a concentration consistent with in-situ tests at the FRC where nitrite accumulated up to 130 mM (*4*). Fe(II) was also rapidly oxidized in acetate-free incubations (Figure 5 B; Figure 6 B). In these incubations, U(IV) was oxidized to a greater extent than in acetate- and Fe(II)-unamended controls, but at a lower rate and to a lesser extent than in acetate- and Fe(II)-amended incubations (Figure 5 A; Figure 6 A). Due to the low levels of electron donor relative to available nitrate in acetate-free incubations, nitrate reduction could not be assessed (Figure 5 D). Nitrite did not accumulate in acetate-free incubations (Figure 5 C), suggesting that Fe(II) oxidation in acetate-free incubations was due to the direct, enzymatic oxidation of Fe(II) coupled to reduction of nitrate to N_2 or NH_4^+ by strain FW33AN.

These results are consistent with in-situ results where organic electron donor was required for nitrate-dependent U(IV) oxidation; no U(IV) oxidation occurred when nitrate-containing, electron donor-free groundwater was injected (*4*). While U(IV) was clearly oxidized to a greater extent under organotrophic conditions than lithotrophic conditions, it was unclear why this was occurring, since Fe(III) was generated in both cases. We hypothesized that differences in Fe(III) mineralogy between "abiotically" formed and biologically formed Fe(III) could explain this observation.

Characterization of biogenic Fe(III) minerals and their reactivity toward U(IV). During the above experiments and others, we have noted that different methods of

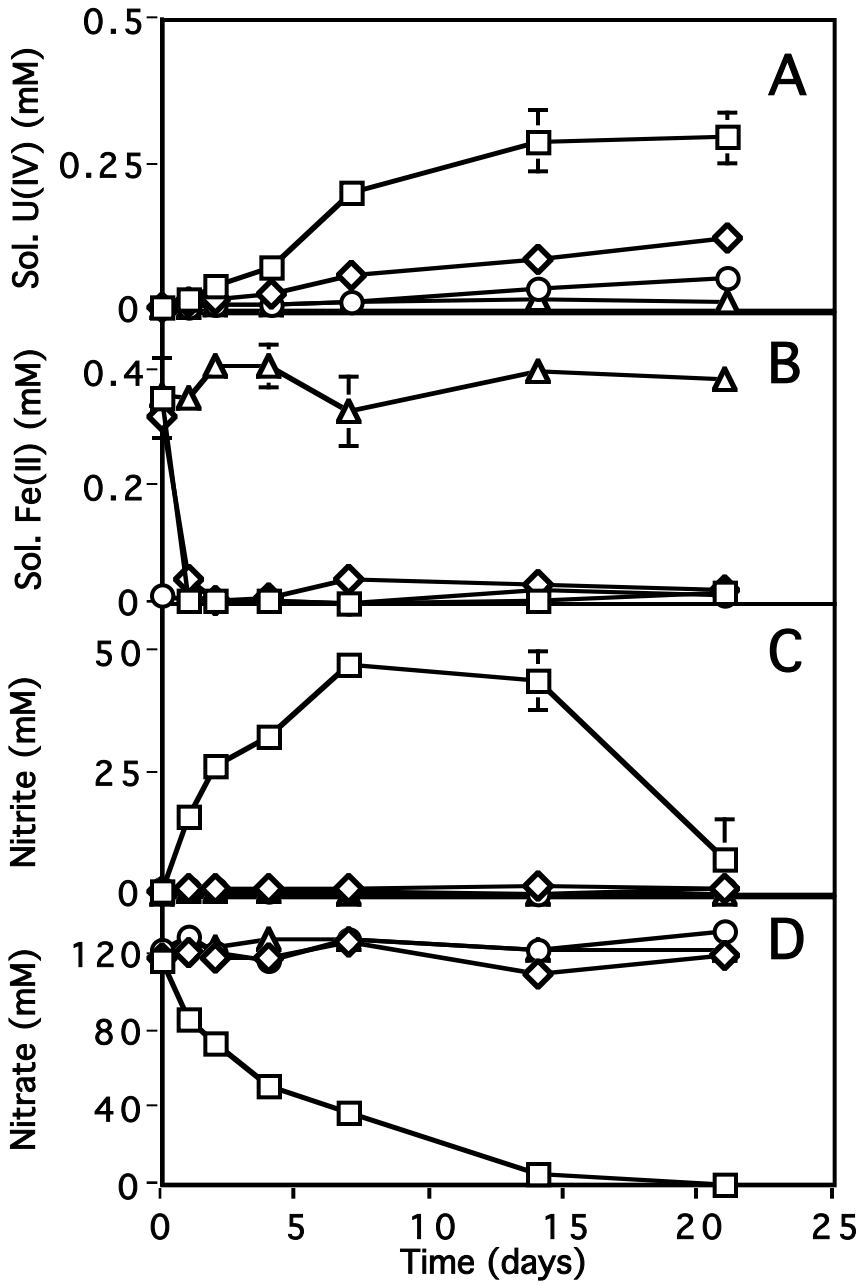

Figure 5. U(IV) oxidation in FRC groundwater by strain FW33AN with (○) no amendment , (□) acetate- and Fe(II)-amendment, (▯) Fe(II)-amendment, and (△) in uninoculated groundwater. Soluble U(VI) concentrations are shown in panel A, soluble Fe(II) in panel B, nitrite in panel C, and nitrate in panel D.

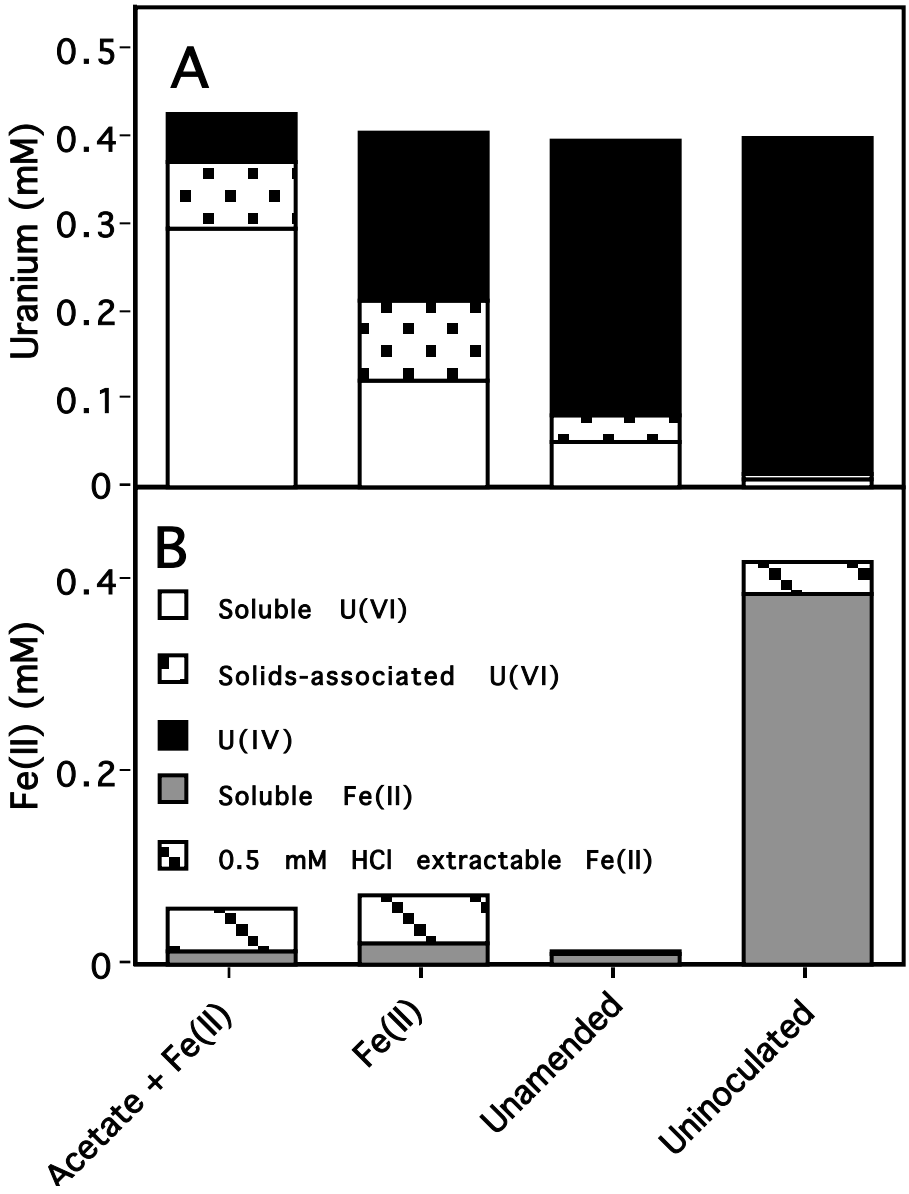

Figure 6. Extraction of solid-phase U (A) and Fe(II) (B) species in incubations of strain FW33AN in FRC groundwater at t = 21 days.

biological and abiotic Fe(III) production yielded Fe(III) minerals of different shades of red/orange/brown. Fe(III) mineralogy is known to strongly influence reactivity and susceptibility of Fe(III) to both biological and chemical reduction (*31, 32, 54-61*), which might explain the differences in U(IV) oxidation in the preceding experiments. To test the hypothesis that Fe(III) mineralogy was responsible for the observed differences in U(IV) oxidation rates we produced Fe(III) minerals by 1) hydrolysis of $FeCl_3$ under oxic conditions (the poorly crystalline Fe(III) oxyhydroxide used for previous experiments) (*21*), 2) oxidation of Fe(II) by nitrite under anoxic conditions, 3) nitrate-dependent oxidation of soluble Fe(II) by resting cells of strain FW33AN, and 4) nitrate-dependent oxidation of FeS by an enrichment culture obtained from a landfill leachate impacted aquifer (Senko et al., in preparation). Fe(III) produced by hydrolysis of $FeCl_3$ was identified as poorly crystalline goethite (Figure 7 C), Fe(III) produced by oxidation of Fe(II) by nitrite was amorphous hydrous ferric oxide (HFO) (Figure 7 D), Fe(III) produced by nitrate-dependent Fe(II) oxidation by strain FW33AN was goethite (Figure 7 B), and Fe(III) produced by nitrate-dependent oxidation of FeS by an enrichment culture was lepidocrocite (Figure 7 A). The surface area of these minerals were determined and are shown in Table 1. These minerals were harvested, washed, and incubated with U(IV). HFO produced by reaction of Fe(II) with nitrite and poorly crystalline goethite were the most effective oxidants of U(IV), while the more crystalline biogenic lepidocrocite and goethite were the least effective oxidants of U(IV) (Figure 8). The extent of U(IV) oxidation by Fe(III) minerals corresponded with the predicted effects of the redox potential of the individual minerals and their surface area (Figure 7 and Table 1). These results provide an explanation for why U(IV) was oxidized to a greater extent

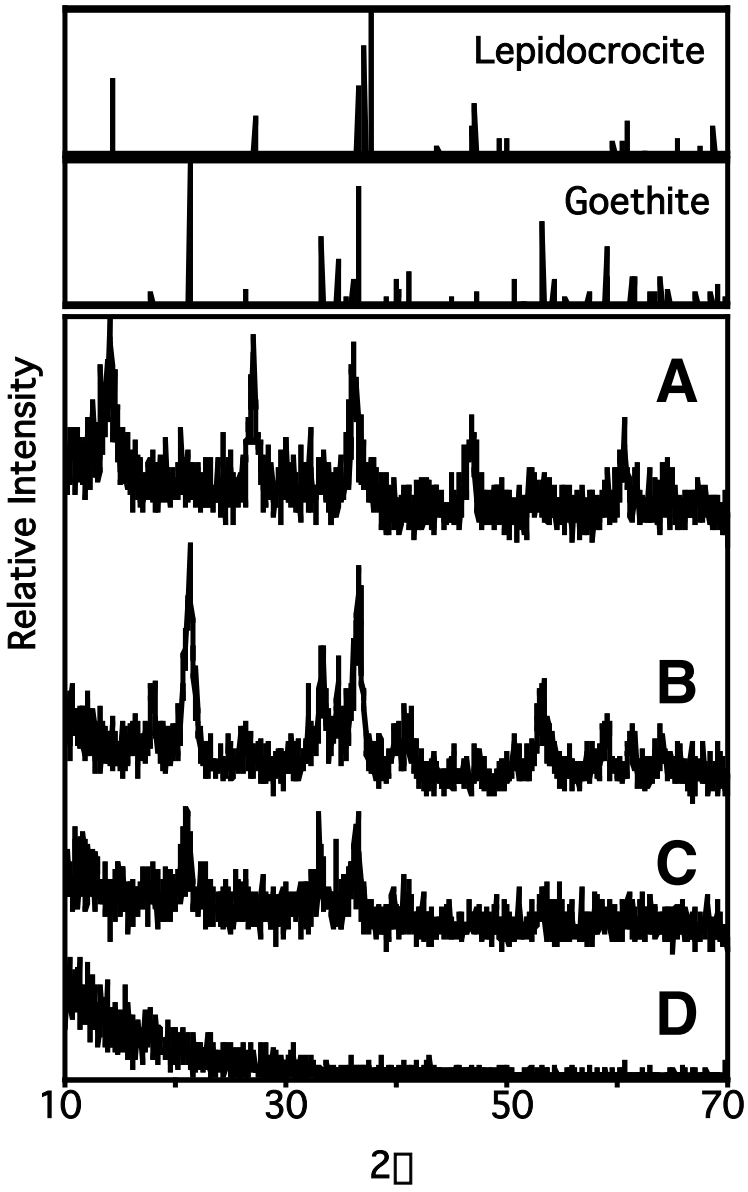

Figure 7. X-ray diffraction spectra for Fe(III) minerals: Fe(III) produced by nitrate-dependent FeS-oxidizing enrichment culture (A), Fe(III) produced by nitrate dependent Fe(II) oxidation by strain FW33AN (B), Fe(III) prepared by hydrolysis of $FeCl_3$ (*21*) (C), Fe(III) produced by oxidation of Fe(II) by nitrite (D). Reference plots for goethite and lepidocrocite are from JADE 3.1.

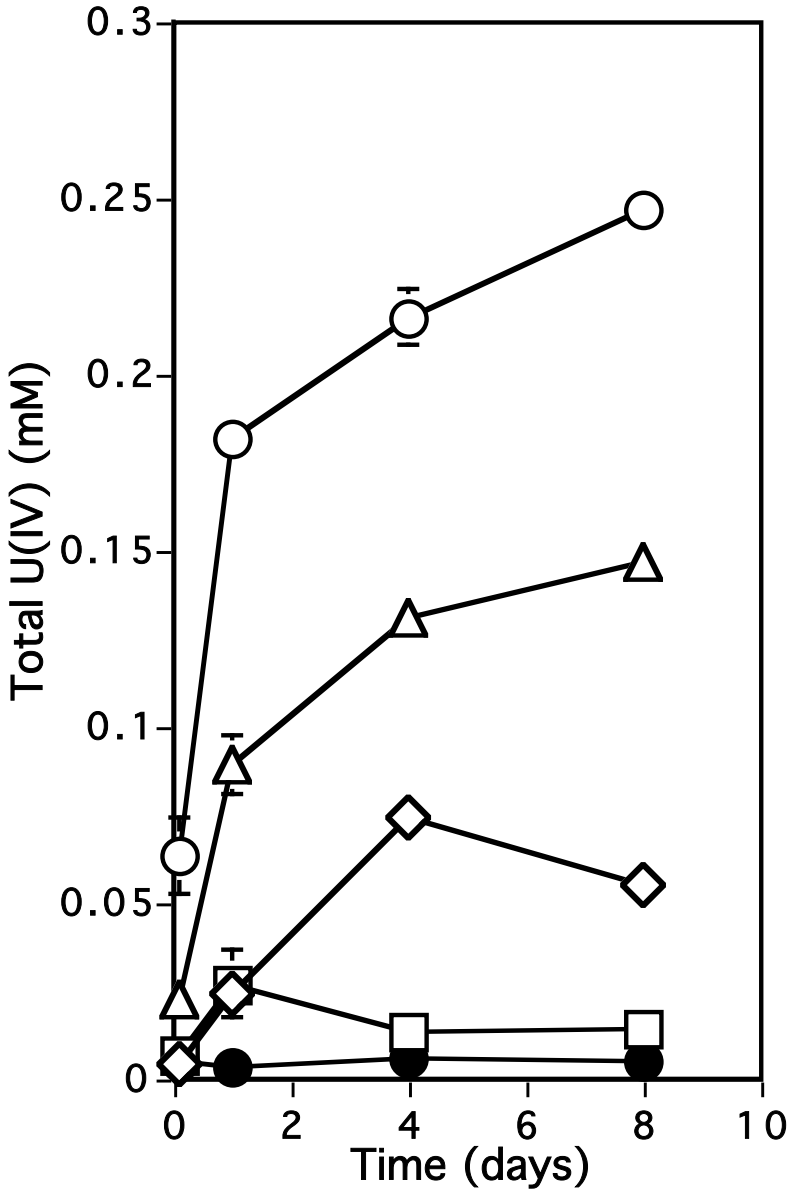

Figure 8. Anaerobic U(IV) oxidation with no oxidant (●), with anoxically prepared hydrous ferric oxide (○), poorly crystalline goethite (△), biogenic lepidocrocite (▯), and biogenic goethite (□).

Table 1. Characteristics of Fe(III) minerals used in U(IV)-oxidizing incubations.

Method of Fe(III) mineral preparation	**XRD identity**	**E_H^f (V)[a]**	**Surface area (m^2/g)**
Hydrolysis of $FeCl_3$ solution (20)	Poorly crystalline goethite[b]	+0.34	87.9
Fe(II) oxidation by nitrite	Hydrous ferric oxide (HFO)	+0.34	113.8
Nitrate-dependent Fe(II) oxidation by strain FW33AN	Goethite	-0.14	26.1
Nitrate-dependent FeS oxidation by enrichment culture	Lepidocrocite	-0.04	109.3

[a]From Roden (*31*).

[b]This mineral was identified as poorly crystalline goethite based on XRD, but the method of synthesis should yield HFO (*21, 31*), therefore we use the E_H^f value for HFO.

under organotrophic conditions than under lithotrophic conditions. Fe(III) was generated under both conditions, but due to its amorphous nature and higher surface area, the Fe(III) produced by abiotic Fe(II) oxidation by nitrite (HFO) was more reactive than biogenic Fe(III) (goethite). These results are consistent with the observation by Roden (*31*) that the susceptibility of Fe(III) minerals to chemical reduction by ascorbate is dependent on surface area and the redox potential as determined by mineralogy. Commercially available lepidocrocite was a more effective U(IV) oxidant than commercially available goethite (not shown).

The biogenic production of Fe(III) minerals is well known. Goethite (*58, 62-64*), lepidocrocite (*64*), green rust (*45*), akaganeite (*65*), ferrihydrite (*47, 58, 62, 63*), schwertmannite (*62*) and magnetite (*45*) have been found to be produced by the enzymatic activity of aerobic and/or anaerobic Fe(II) oxidizing bacteria. It is not clear if biogenic Fe(III) mineralogy is specific to the organism producing the Fe(III) mineral. While we observed the formation of goethite by one bacterial culture and lepidocrocite by another, it is important to note that biogenic goethite was formed from soluble Fe(II) by resting cells while biogenic lepidocrocite was formed from FeS in growth medium. Therefore, whether biogenic Fe(III) mineralogy is bacterial species-dependent is difficult to assess. It is likely that biogenic Fe(III) mineralogy is dependent on oxidation rate, prevailing geophysical and geochemical conditions, Fe(II) substrate, and characteristics of bacterial cell surfaces (*22, 62, 66-76*). In similar studies with *Dechlorosoma suillum* biogenic magnetite and green rust were produced during growth on Fe(II) with nitrate as the terminal electron acceptor, but resting cells of this organism produced ferrihydrite under nitrate-reducing conditions (*45, 47*). In this case, we predict based on

thermodynamic considerations and experimental results that ferrihydrite could effectively oxidize U(IV), while the Fe(II)/Fe(III) minerals green rust and magnetite would not (*31*, *36*, this work). Further work to elucidate controls on microbiological Fe(II)-oxidizing activity and biogenic Fe(III) mineralogy may allow us to better predict the stability of U(IV) in aquifers in which uranium has been reductively immobilized.

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Chapter 3.

The effect of the form of Fe(II) and oxidation rate on microbial nitrate-dependent Fe(III) mineralogy

Abstract

We tested the effect of Fe(II) chemical form and nitrate-dependent oxidation rate on biogenic Fe(III) mineralogy. Nitrate-dependent oxidation of FeS yielded amorphous Fe(III), while soluble Fe(II) oxidation yielded goethite. More crystalline goethite minerals were produced at slower rates of Fe(II) oxidation.

Body

Microbiological nitrate-dependent Fe(II) oxidation to Fe(III) is widespread and may play an important role in Fe cycling in oxygen-limited microbial ecosystems (2, 12, 13, 17, 22, 26, 27, 30-33, 35). The mineralogy of Fe(III) strongly influences its susceptibility to microbiological and chemical reduction as well as sorbtive characteristics (10, 11, 16, 19-21, 25, 36). Fe(III) mineralogy can therefore have a profound impact on organic and inorganic contaminant migration as well as biological Fe(III) respiration. Different biogenic Fe(III) minerals (including goethite, lepidocrocite, magnetite, green rust, and poorly crystalline Fe(III) oxyhydroxide) are formed by anaerobic Fe(II) oxidation under similar physical and chemical conditions (6, 15, 17, 33, Senko et al., submitted for publication). The reason for these mineralogical differences is unclear, but the chemical form of Fe(II) and Fe(II) oxidation rate exert control on the mineralogy of abiotically

produced Fe(III) (9). We therefore assessed the effect of these two parameters on the mineralogy of biogenic Fe(III) produced by nitrate-dependent Fe(II) oxidation.

The effect of Fe(II) form on biogenic Fe(III) mineralogy. A nitrate-dependent Fe(II)-oxidizing bacterium (designated strain FW33AN) was isolated from a nitrate- and U(VI)-contaminated aquifer (Senko et al., submitted for publication). FW33AN was grown anaerobically (under N_2) on a mineral medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (50 mM, pH 7) as the buffer, acetate (30 mM CH_3COONa) as the electron donor, and nitrate (20 mM $NaNO_3$) as the sole terminal electron acceptor (Senko et al., submitted for publication). Cells were harvested by centrifugation, washed three times with bicarbonate buffer (3.5 g $NaHCO_3$/l, pH 6.8) equilibrated under 80:20 N_2:CO_2, and resuspended in the same buffer. Washed cells (0.1 mg protein/ml) were incubated in anoxic bicarbonate buffer containing 4 mM $NaNO_3$ and either soluble Fe(II) (3.5 mM $FeCl_2$) or FeS (2.5 mM FeS) under a headspace of 80:20 N_2:CO_2. All steps were carried out in an anoxic glovebag (Coy Laboratory Products, Grass Lake, MI). Geochemical modeling using PHREEQC (23) revealed that Fe(II) in $FeCl_2$-amended incubations was predominantly present as $FeHCO_3^+$ (51%) and Fe^{2+} (45%), with $FeCO_3$ (siderite) representing a minor fraction of the total Fe(II) (3%). Samples were periodically removed and acidified with anoxic 0.5 M HCl to liberate insoluble Fe(II) (18). Fe(II) was quantified by the ferrozine assay (18). Despite its relative insolubility, FeS was oxidized at a rate comparable to soluble Fe(II) (Fig. 1 A). At the conclusion of the incubations (t = 14 d), acid volatile sulfide and total reduced inorganic sulfur (TRIS) were extracted (34) and quantified by methylene blue assay (7).

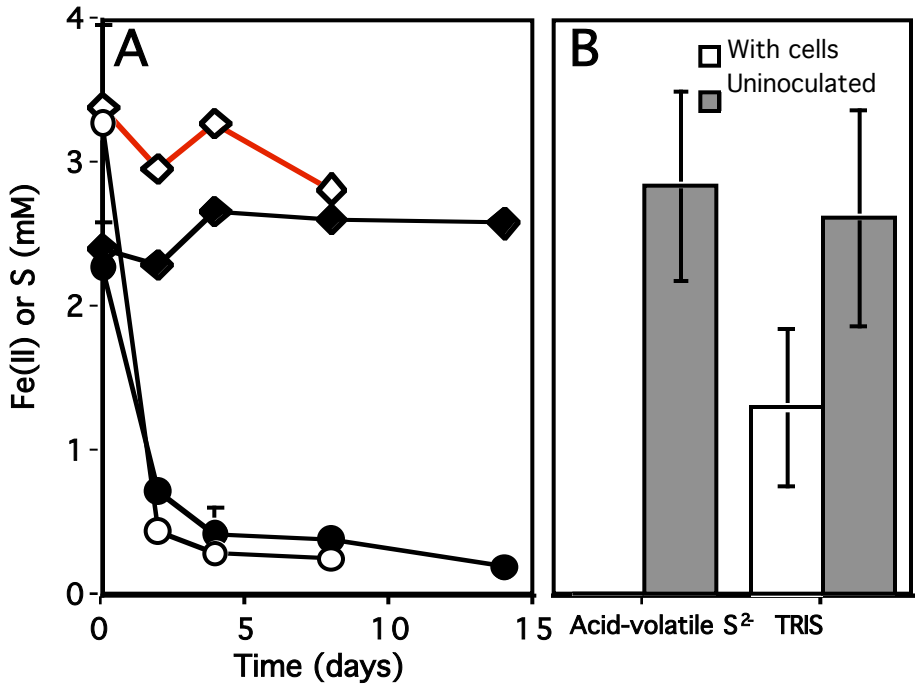

FIG. 1. Fe(II) oxidation by strain FW33AN with soluble Fe(II) (○) or FeS (●) under nitrate-reducing conditions (A). Fe(II) concentrations in uninoculated incubations are represented by ◇ and ◆ for soluble Fe(II) and FeS, respectively. Acid volatile sulfide and total reduced inorganic sulfur (TRIS) were extracted from FeS oxidizing incubations at t = 14 days and quantified (B).

Sulfide was likely oxidized by Fe(III) to elemental sulfur, polysulfide or polythionate by Fe(III) (27) (Fig. 1 B). No thiosulfate or sulfate were detected in the incubations.

Solids were removed at the conclusion of incubations, dried on glass slides overnight in an anoxic glovebag and X-ray diffraction (XRD) of powdered minerals was carried out on a Rigaku automated diffractometer (Rigaku/MSC, The Woodlands, TX). Incubations with soluble Fe(II) yielded goethite (Fig. 2, spectrum D), while incubations with FeS (identified as poorly crystalline mackinawite (Fig. 2, spectrum A and B)) yielded amorphous Fe(III) (Fig. 2, spectrum C). The proportion of crystalline product was determined by extraction with HCl (for Fe(II)) (18), hydroxylamine-HCl (for amorphous Fe(III)) (18), and ammonium citrate in room light (for total Fe(III)) (24, 29). These extractions revealed that all Fe(III) produced by bacterial oxidation of FeS was amorphous, while 44% of the Fe(III) produced by oxidation of soluble Fe(II) was not extractable with hydroxylamine-HCl, indicating that a large fraction was crystalline (Table 1) (18).

These results suggest that different forms of Fe(II) substrate give rise to different Fe(III) minerals upon nitrate-dependent Fe(II) oxidation. Similarly, Kappler and Newman (15) observed amorphous Fe(III) from anaerobic FeS oxidation by an anoxygenic, Fe(II)-oxidizing phototrophic bacterium, but goethite and lepidocrocite from oxidation of soluble Fe(II) by the same organism. In those experiments, goethite and lepidocrocite were formed after 12 days of maturation, but no such mineral transformation was observed in Fe(III) produced by FeS oxidation over a similar time period. The lack of crystallinity in FeS-oxidizing incubations may be the result of a low level of soluble Fe(II) in solution. Soluble Fe(II) stimulates the transition of poorly

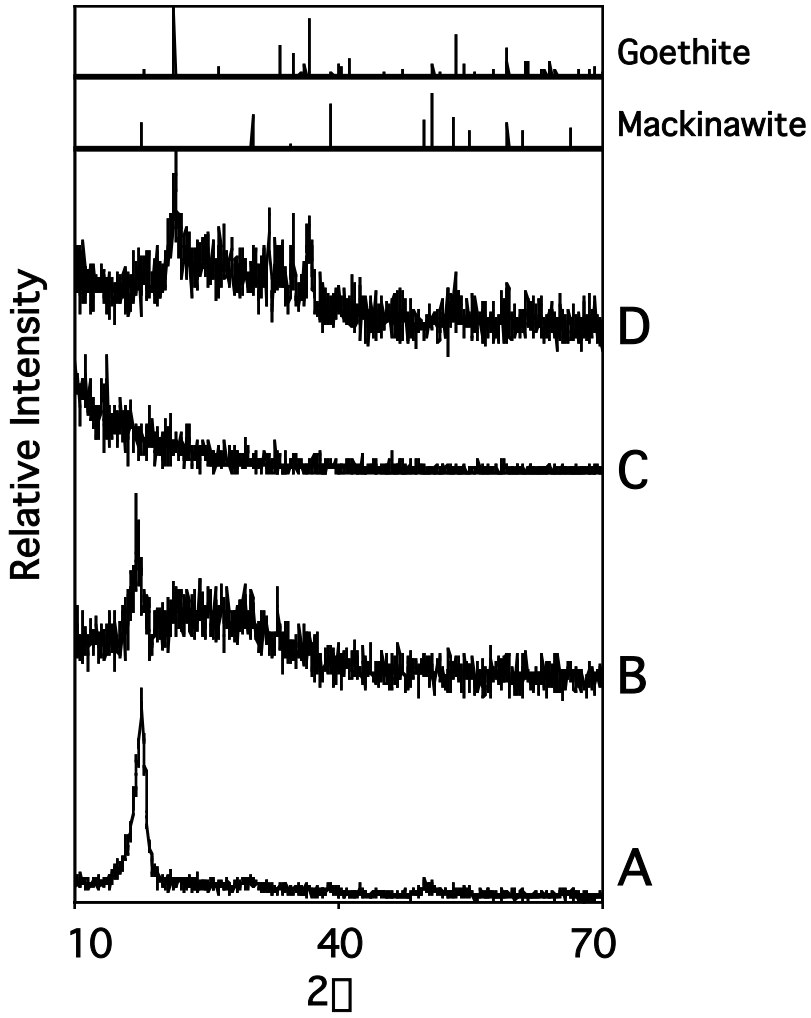

FIG. 2. X-ray diffraction spectra for Fe minerals in FeS-oxidizing incubations at t = 0 days (A), t = 8 days (B), t = 14 days, and in soluble Fe(II)-oxidizing incubations at t = 8 days (D). Reference plots for goethite and mackinawite are from JADE 3.1 (Materials Data Inc., Livermore, CA).

Table 1. Composition of Fe(III) minerals formed by nitrate dependent oxidation of soluble Fe(II) or FeS.

Treatment (Fe(II) form or Fe(II) oxidation rate)	**Fe(II) (%)[a]**	**Crystalline Fe(III) (%)[b]**
Soluble Fe(II)	4.1 ± 0.5	44.1 ± 5.8
FeS	3.9 ± 0.5	1.6 ± 7.7
0.12 mmole Fe(II)/day	4.7 ± 2.1	63.2 ± 13.3
0.24 mmole Fe(II)/day	4.3 ± 0.9	45.7 ± 8.6
0.42 mmole Fe(II)/day	3.5 ± 0.5	1.0 ± 5.0

[a]Fe(II) = [HCl-extractable fraction]

[b]Crystalline Fe(III) = [ammonium citrate-extractable fraction] – [Hydroxylamine-HCl-extractable fraction]

crystalline Fe(III) to more crystalline products (8, 9), and the lack of it in the FeS-oxidizing incubations may have limited goethite formation.

The effect of Fe(II) oxidation rate on biogenic Fe(III) mineralogy. Resting cells of strain FW33AN were prepared by washing as described above and added to soluble Fe(II)- and nitrate-containing bicarbonate-buffered 20 ml reaction solutions at protein concentrations of 0, 0.8, 2, and 4 mg protein/ml. Fe(II) was oxidized at initial rates of 0, 0.12, 0.24, and 0.42 mmole/day, respectively (Fig. 3). Specific initial Fe(II) oxidation rates were 0.12 ± 0.03 mmole/mg protein/day. After 7 days of incubation, solids were removed, dried, and characterized by XRD. Fe(II) oxidation at the most rapid initial rate yielded amorphous Fe(III) (Fig. 4 A), but with progressively lower rates of Fe(II) oxidation, a stronger goethite signal was observed (Fig. 4 B and C) and a progressively larger proportion of Fe(III) was crystalline (Table 1). Similarly, the crystallinity of Fe(III) oxyhydroxides has been shown in abiotic experiments to increase with decreasing rate of formation (9).

Residual Fe(II) present in slow-oxidizing incubations may have enhanced goethite crystal formation (8, 9). In this scenario, biogenic Fe(III) in all incubations may have initially been amorphous, but with the slower rate of Fe(II) consumption in slow-oxidizing incubations, this Fe(II) was available to catalyze goethite formation (8, 9). It is unlikely that increased cell material altered Fe(III) dissolution/reprecipitation (15), since the Fe(III) was removed for XRD analysis soon after Fe(II) oxidation ceased (t=7 days), and we would expect that if cell material played a role in Fe(III) dissolution/precipitation, higher levels of cells would yield more crystalline Fe(III) products, since cell material

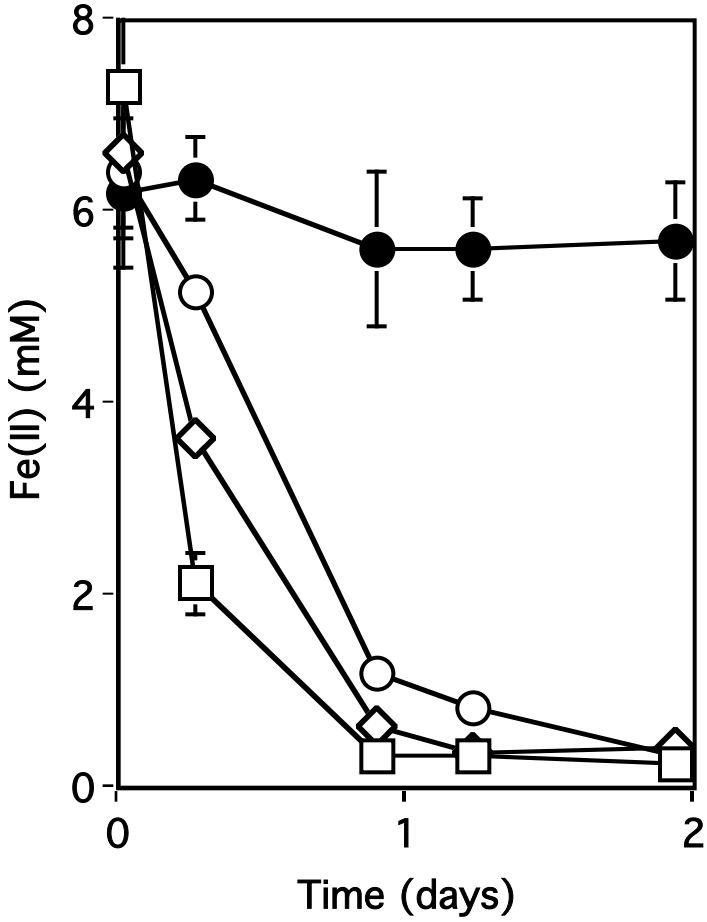

FIG. 3. Nitrate-dependent Fe(II) oxidation by strain FW33AN at protein concentrations of 0 mg protein/ml (●), 0.04 mg protein/ml (○), 0.1 mg protein/ml (◇), and 0.2 mg protein/ml (□).

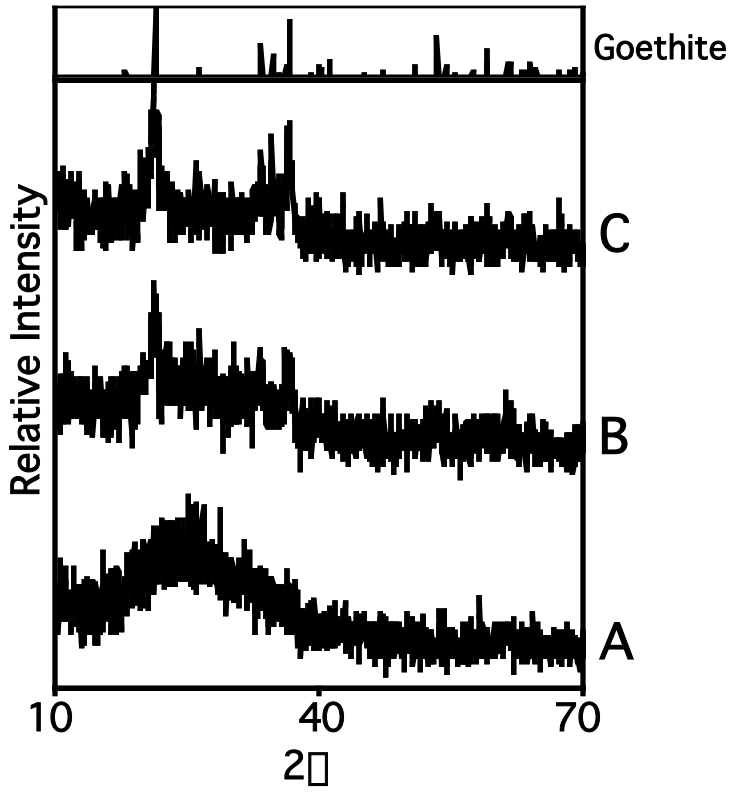

FIG. 4. X-ray diffraction spectra for biogenic Fe(III) minerals produced by strain FW33AN at Fe(II) oxidation rates of 0.42 mmole/day (A), 0.24 mmole/day (B), and 0.12 mmole/day (C). Reference plot for goethite is from JADE 3.1 (Materials Data Inc., Livermore, CA).

might enhance Fe(III) dissolution and reprecipitation. The rapidity of goethite formation (within 1 week) by strain FW33AN is striking. In experiments by Kappler and Newman (15), minerals collected 5 days after complete Fe(II) oxidation were amorphous. Goethite and lepidocrocite formation only appeared after extended incubation (≥12 days) in the growth medium. We conclude that as with abiotic Fe(III) mineral formation (9), the rate of biological nitrate-dependent Fe(II) oxidation exerts a strong influence on biogenic Fe(III) mineralogy. Indeed, ferrihydrite was formed by *Dechlorosoma suillum* at an initial nitrate-dependent Fe(II) oxidation rate of approximately 72 mM/day (17), while magnetite was formed when this organism oxidized Fe(II) at a rate of approximately 0.576 mM/day (6).

Our results suggest that Fe(II) form and Fe(II) oxidation rate exert a strong influence on biogenic Fe(III) mineralogy. However, Fe(II) form and oxidation rate are by no means the exclusive determinants of biogenic Fe(III) mineralogy. For instance, phosphate may be an especially important factor in controlling Fe(III) mineralogy and crystallinity (5, 14, Roden, personal communication). Geophysical and geochemical conditions (e.g. temperature or pH) and the presence of cellular material will certainly affect biogenic Fe(III) mineralogy (1, 3-5, 8, 9, 28). Further work is necessary to determine the relative contributions of these variables to Fe(III) mineralogy in-situ.

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Chapter 4

Geochemical Controls on Microbial Nitrate-Dependent U(IV) Oxidation

Abstract

After reductive immobilization of uranium, the element may be oxidized and remobilized in the presence of nitrate by the activity of dissimilatory nitrate-reducing bacteria. We examined controls on microbially mediated nitrate-dependent U(IV) oxidation in landfill leachate-impacted sediments. Nitrate-dependent U(IV)-oxidizing bacteria were at least two orders of magnitude less numerous in these sediments than organotrophic or Fe(II)-oxidizing nitrate-reducing bacteria and grew more slowly than the latter organisms, suggesting that U(IV) is ultimately oxidized by Fe(III) produced by dissimilatory nitrate-reducing activity. We examined the effect of nitrate and reductant concentration on nitrate-dependent U(IV) oxidation in sediment incubations and used the initial reductive capacity (RDC = [reducing equivalents] – [oxidizing equivalents]) of the incubations as a unified measurement of the nitrate or reductant concentration. When we lowered the RDC with progressively higher nitrate concentrations, we observed a corresponding increase in the extent of U(IV) oxidation, but did not observe this relationship between RDC and U(IV) oxidation rate, especially when RDC > 0, suggesting that nitrate concentration strongly controls the extent, but not the rate of nitrate-dependent U(IV) oxidation. On the other hand, when we raised the RDC in sediment incubations with progressively higher reductant (acetate, sulfide, soluble Fe(II), or FeS) concentrations, we observed progressively lower extents and rates of nitrate-dependent U(IV) oxidation. Acetate was a relatively poor inhibitor of nitrate-dependent U(IV) oxidation, while Fe(II)

was the most effective inhibitor. Based on these results, we propose that it may be possible to predict the stability of U(IV) in a bioremediated aquifer based on the geochemical characteristics of that aquifer.

Introduction

Uranium contaminates aquifers near sites where that element was mined and processed in the production of nuclear weapons and fuel (McCullough et al., 2003). These aquifers are generally oxidized, so uranium predominantly exists as U(VI) (UO_2^{2+}, usually complexed with carbonate (Grenthe, 1992)), which is quite soluble and easily migrates with groundwater. Reduced uranium (U(IV), usually as uraninite) is relatively insoluble, and stimulation of bacteria capable of reducing U(VI) has been proposed as a means of preventing the off-site migration of uranium with groundwater (Lovley et al., 1991; McCullough et al., 2003). U(VI) reduction occurs anaerobically and concurrently with Fe(III) and/or sulfate reduction (Finneran et al., 2002a; Senko et al., 2002; Elias et al., 2003a; Istok et al., 2004). Therefore, reductive uranium immobilization will generally require the addition of organic electron donors (e.g. acetate or ethanol) to produce the anoxic conditions necessary for U(VI) reduction (Anderson et al., 2004; Istok et al., 2004). This will leave the bioremediated aquifer in a more reduced state than it was before the initiation of biostimulation.

The biogeochemical processes likely to occur after the reductive immobilization of uranium has been achieved (and electron donor addition has ceased) are poorly understood, so the stability of U(IV) in a bioremediated aquifer is difficult to predict. U(IV) may be oxidized abiotically by oxygen (Abdelouas et al., 1999; Duff et al., 1999),

and recent work has shown that the addition of nitrate (a common co-contaminant with uranium) to U(IV)-containing sediments leads to the oxidation and remobilization of U(IV) (Senko et al., 2002; Finneran et al., 2002b; Istok et al., 2004). Nitrate-dependent U(IV) oxidation is believed to proceed via oxidation of U(IV) by Fe(III) (Nevin and Lovley, 2000; Finneran et al., 2002b; Senko et al., submitted for publication). Fe(III) may be produced under dissimilatory nitrate-reducing conditions by nitrate-dependent Fe(II)-oxidizing microorganisms or by the reaction of nitrite (that accumulates during dissimilatory nitrate reduction) with Fe(II) (Finneran et al., 2002b; Senko et al., 2002; Senko et al., submitted for publication). Another possible mechanism of nitrate-dependent U(IV) oxidation is the direct nitrate-dependent oxidation of U(IV) by microorganisms (DiSpirito and Touvinen, 1982; Finneran et al., 2002b), but this mechanism likely plays a relatively minor role in U(IV) oxidation compared to U(IV) oxidation by Fe(III) (Finneran et al., 2002b; Senko et al., submitted for publication). Regardless of the mechanism of nitrate-dependent U(IV) oxidation, influxes of nitrate may reverse the reducing conditions brought about by electron donor addition.

Bioremediated aquifers are unlikely to be impervious to influxes of nitrate. Nitrate sources may include adjacent nitrate-contaminated regions of the aquifer, nitrate-containing municipal and agricultural runoff, or the seasonal turnover of organic matter (Burden, 1982; Portnoy et al., 1998, Nowicki et al., 1999; Smith et al., 2002; Istok et al., 2004; Walvoord et al., 2003). Nitrate levels entering an aquifer may vary over a wide range of concentrations, depending on the nitrate source. Nitrate concentration may control the activity of dissimilatory nitrate-reducing bacteria (DNRB) which may in turn control the extent and rate of U(IV) oxidation. To what extent nitrate concentration

controls nitrate-dependent U(IV) oxidation is unclear, so it is not known what levels of nitrate can be considered benign and what levels can be considered a serious threat to the stability of U(IV).

Since U(VI) reduction occurs concurrently with Fe(III) and sulfate reduction, bioremediated aquifers should contain abundant Fe(II) and/or sulfide. Remnant exogenous electron donor or cell biomass represent other electron donors contributing to the net reducing conditions of a bioremediated aquifer. Whether organic or inorganic, reductants could abiotically or biologically reduce U(IV) as its oxidized (Wersin et al., 1994; Liger et al., 1999; Fredrickson et al., 2000) or act as competitive electron donors for available nitrate (Marchand and Silverstein, 2002; Marchand and Silverstein, 2003), thus influencing the stability of U(IV). Indeed, work by Abdelouas et al. (1999) has illustrated that the presence of FeS and biomass that result from biostimulation in sediments inhibits U(IV) oxidation by oxygen, and therefore "protects" U(IV) from reoxidation. It is unclear what reductants might be the most effective inhibitors of U(IV) oxidation.

We hypothesize that the principal factor controlling the rate and extent of nitrate-dependent U(IV) oxidation is the net reducing/oxidizing capacity of the bioremediated aquifer, which will be controlled by nitrate and reductant concentration. In this study we determined the presence and abundance of DNRB that could catalyze nitrate-dependent U(IV) oxidation in sediments where this process was shown to occur (Senko et al., 2002), and determined the effect of nitrate and reductant concentration on the rate and extent of nitrate-dependent U(IV) oxidation. We show that nitrate concentration strongly controls the extent of U(IV) oxidation, but has a relatively minor effect on the rate of nitrate-

dependent U(IV) oxidation. Reductants (acetate, FeS, and soluble Fe(II) or sulfide) exert strong control on both the extent and rate of U(IV) oxidation, with soluble Fe(II) being the most effective inhibitor of nitrate-dependent U(IV) oxidation.

Materials and Methods

Sediment and groundwater collection. Sediments from a shallow, alluvial, landfill leachate-impacted aquifer in Norman, OK were collected as previously described (Senko et al., 2002). Groundwater was collected from previously installed wells at a depth of 3.5 m, using a peristaltic pump and transferred to sterile bottles with no headspace. Sediment and groundwater were transported to the laboratory and stored at 4°C before further processing (≤ 2 weeks). Fe(II) and total reduced inorganic sulfur (TRIS) were extracted from sediment and quantified as described below. Fe(II) and TRIS concentrations in sediments were 4.0 and 8.3 μmole/g sediment, respectively. Dissolved Fe(II) in groundwater was 100 μM. Dissolved organic carbon (DOC) from this region of the leachate plume has been previously reported to be approximately 8 mM (Cozzarelli et al., 2000).

Microbial culture medium, enrichments, and enumerations. Microbial enrichments and enumerations were carried out using 5 ml of a bicarbonate-buffered (3.5 g/l) mineral medium containing 0.8 g/l NaCl, 1 g/l NH_4Cl, 0.1 g/l KCl, 0.03 g/l, $MgSO_4.7H_2O$, and 0.04 g/l $CaCl_2.2H_2O$, vitamins, and trace metals (Tanner, 1997). Phosphate (KH_2PO_4) was provided at a lower concentration (30 μM) than suggested by Tanner (1997) to minimize precipitation of U(VI) as uranyl phosphate. Nitrate (3 mM $NaNO_3$) was provided as a terminal electron acceptor and where appropriate, yeast extract (0.5 g/l)

was provided. The medium was boiled under a stream of 80% N_2 and 20% CO_2 to remove oxygen and dispensed into serum tubes under a headspace of 80% N_2 and 20% CO_2 (Balch et al., 1979). Glucose (2 mM), FeS (1.5 mM), biogenic U(IV) (2 mM), or $FeCl_2$ (4 mM) were provided as electron donors from sterile, anoxic, concentrated stock solutions or suspensions.

Microbial enumerations in sediments were conducted using a three-tube most probable number (MPN) assay (Colwell, 1979) using the media described above, with uninoculated media serving as negative controls. FeS-oxidizing assays were scored based on color change of the black FeS precipitate to an orange Fe(III) precipitate, and Fe(II) oxidizing assays based on production of an orange-brown precipitate. U(IV)-oxidizing assays were scored based on clearing of the black precipitate accompanied by a color change in the medium from clear to pale yellow. Glucose-oxidizing assays were scored by the appearance of turbidity. Growth and activity in all enrichments and MPN series were confirmed by monitoring the loss of nitrate and an increase in protein concentration. Protein concentration was not monitored in FeS-oxidizing incubations because sulfide interfered with the assay. Biogenic U(IV) was produced by incubating resting cells of *Desulfovibrio vulgaris* Hildenborough with 4 mM uranyl acetate and 4 mM sodium lactate in a bicarbonate-buffered (3.5 g/l, pH 6.8) solution, and recovered as previously described (Elias et al., 2003b).

Sediment incubations. To assess the effect of nitrate concentration on the rate and extent of nitrate-dependent U(IV) oxidation, 25 g of sediment were placed in 120 ml serum bottles in an anoxic glovebag (Coy Laboratory Products, Inc., Grass Lake, MI). Sterile anoxic groundwater (50 ml) was added to the sediments and bottles were sealed

with thick rubber serum stoppers. These were subsequently removed from the glovebag and flushed with an oxygen-free mixture of 80% N_2 and 20% CO_2. Final pH of the sediment slurries was 6.8-7.1. Autoclaved sediment slurries served as negative controls. Nitrate ($NaNO_3$) was provided from a sterile, anoxic, concentrated stock solution to final concentrations of 0.1, 1, 4, or 40 mM. Biogenic U(IV) was added to a final concentration of 150 μM. Samples were periodically removed by syringe in an anoxic glovebag and nitrate, nitrite, sulfate, soluble U(VI), and Fe(II) were quantified as described below.

For experiments to assess the effects of different reductants on U(IV) oxidation, sediments were washed three times with anoxic bicarbonate (3.5 g/l, pH 6.8) solution and prepared as described above. Bicarbonate buffer solution was used in lieu of groundwater, which allowed us to manipulate the organic electron donor concentration. Nitrate ($NaNO_3$) was added to a final concentration of 4.5 mM, and biogenic U(IV) was added to a final concentration of 230 μM. Soluble Fe(II) ($FeCl_2$), soluble sulfide (Na_2S), acetate (CH_3COONa), and FeS were added to incubations from concentrated, sterile, anoxic stock solutions or suspensions. Samples were periodically removed and nitrate, nitrite, sulfate, soluble U(VI), and Fe(II) were quantified as described below.

Analytical methods. Solid phase Fe(II) was extracted from sediments with 0.5 M HCl, and quantified spectrophotometrically using the ferrozine assay (Lovley and Phillips, 1987). Total reduced inorganic sulfur (TRIS) was extracted from sediment as described by Ulrich et al. (1997) and quantified spectrophotometrically by methylene blue assay (Cline et al., 1969).

Samples from microbial cultures and sediment incubations (0.2-0.5 ml) were removed from serum bottles or tubes by syringe and insoluble material was removed by

centrifugation. Nitrate, nitrite, sulfate, soluble Fe(II), glucose, and U(VI) in the supernatant were quantified. Samples for Fe(II) analysis were preserved in 0.5 M HCl and Fe(II) was quantified by the ferrozine assay (Lovley and Phillips, 1987). Nitrate, nitrite, and sulfate concentrations were determined by ion chromatography with conductivity detection (Dionex DX 500 fitted with an AS-4A column and conductivity detector; Dionex Corporation, Sunnyvale, CA). Soluble U(VI) was quantified by Kinetic Phosphorescence Analysis (KPA) on a KPA-11 (ChemChek Instruments, Richland, WA) (Brina and Miller, 1992). Glucose was quantified by the phenol-sulfuric acid method (Daniels et al., 1994). Protein was quantified using the bicinchoninic assay (Pierce Biotechnology, Inc., Rockford, IL).

Calculations. Reductive capacity (RDC) is an indicator of the total concentration of species that may donate electrons to a given oxidant, in this case, nitrate (Scott and Morgan, 1990). The initial RDC of sediment incubations was calculated as described by Scott and Morgan (1990) using the following equation:

[reducing electron equivalents at t = 0] – [oxidizing equivalents at t = 0] (1)

and reported as milliequivalents (meq). Therefore, net reducing (relative to nitrate) sediment incubations contain reductants in excess of nitrate and have a positive RDC, while net oxidizing incubations contain nitrate in excess of available reductants and have a negative RDC. Nitrate was considered to be the sole oxidant in the incubations. Denitrification was assumed with a molar equivalent of 5 electrons involved in nitrate reduction to N_2. Reducing equivalents were considered Fe(II), sulfide, U(IV), DOC and acetate. Sulfide oxidation to sulfate was assumed, which involves an 8 electron transfer. DOC was assumed to be CH_2O, which can be oxidized to CO_2 with a 4 electron transfer.

The percent U(IV) oxidized was calculated by the following equation:

$$\% \text{ U(IV) oxidized} = [U(VI)_{max}]/[U(IV)_{initial}] \times 100 \quad (2)$$

The maximum soluble U(VI) concentration ($U(IV)_{max}$) was used for these calculations since U(VI) reduction was observed in some incubations after nitrate had been completely consumed.

Pseudo-first-order rate coefficients (k) were determined for nitrate-dependent U(IV) oxidation by linear least squares regression fitting of ln[(U(IV)] versus time using the following equation:

$$\ln[U(IV)_{initial} - U(VI)_t] = -kt + \ln[U(IV)_{initial} - U(VI)_{initial}] \quad (3)$$

Since U(VI) reduction was observed in some incubations after nitrate was completely consumed, any U(VI) concentrations after U(VI) reduction began were not used for calculation of k. Standard errors for pseudo-first-order rate coefficients were calculated as described by Rosenkrantz (1997).

Results and Discussion

Enumeration of dissimilatory nitrate-reducing bacteria. Nitrate-dependent U(IV) oxidation may be catalyzed by Fe(III) produced under nitrate-reducing conditions or by the direct, enzymatic oxidation of U(IV) by DNRB (Finneran et al., 2002b; Senko et al., submitted for publication). Nitrate-dependent U(IV) oxidation via Fe(III) requires the activity of organotrophic dissimilatory nitrate-reducing bacteria and/or nitrate-dependent Fe(II)-oxidizing bacteria, both of which have been shown to be active in the sediments used for these studies (Caldwell et al., 1999; Marsh et al., 2000; Senko et al., 2002). Organisms capable of coupling enzymatic U(IV) oxidation to nitrate reduction for growth

have not been reported in this aquifer or any others. To gain insight into the potential contributions of organotrophic dissimilatory nitrate-reducing bacteria, nitrate-dependent Fe(II)-oxidizing bacteria, and nitrate-dependent U(IV)-oxidizing bacteria to nitrate-dependent U(IV) oxidation, we compared the abundance of these organisms in the sediments by most probable number assay.

Nitrate-dependent FeS- and U(IV)-oxidizing microorganisms could only be cultured under autotrophic conditions, while soluble Fe(II)-oxidizing bacteria could only be recovered from the sediments with yeast extract in the medium, suggesting that these organisms are heterotrophic, like most characterized nitrate-dependent Fe(II)-oxidizing bacteria (Straub et al., 1996; Straub and Buchholz-Cleven, 1998; Straub et al., 2001; Lack et al., 2002: Shelobolina et al., 2003). Organotrophic (glucose-oxidizing), FeS-oxidizing, and soluble Fe(II)-oxidizing nitrate-reducing bacteria were most abundant in the sediments used for these experiments, while low numbers of U(IV)-oxidizing nitrate-reducing bacteria were present (Table 1). The enrichments obtained from the MPN assays were maintained through at least 10 transfers. After the third transfer from the initial enrichment, we assessed the time each culture required to completely oxidize its respective electron donor. Glucose oxidation was determined by glucose loss, Fe(II) oxidation was determined by Fe(II) loss, FeS oxidation was determined by sulfate production, and U(IV) oxidation was determined by U(VI) production. The U(IV)-oxidizing enrichment completely oxidized its electron donor more slowly than the other groups of organisms cultured (Table 1). The relatively high numbers and fast growth of organotrophic and Fe(II)-oxidizing dissimilatory nitrate-reducing bacteria, and the relatively poor free energy yield from nitrate-dependent U(IV) oxidation (Table 1)

Table 1. Reactions, enumeration and growth of dissimilatory nitrate reducing bacteria that could catalyze nitrate-dependent U(IV) oxidation.

Reaction catalyzed by enrichment	Most Probable Number (cells/g sediment)	Time to completely oxidize substrate[b]	ΔG^{o}_{R}' (kJ/mol)[a]
$0.2\ C_6H_{12}O_6 + NO_3^- \rightarrow 1.25\ HCO_3^- + 0.5\ N_2 + 0.5\ H_2O + 0.25\ H^+$	2.76×10^{7}	≈ 3 d	-568
$0.56\ FeS + 0.89\ H_2O + NO_3^- \rightarrow 0.56\ Fe(OH)_3 + 0.56\ SO_4^{2-} + 0.5\ N_2 + 0.11\ H^+$	2.05×10^{6}	9 d	-433
$5\ Fe^{2+} + NO_3^- + 12\ H_2O \rightarrow 5\ Fe(OH)_3 + 0.5\ N_2 + 9\ H^+$	4.62×10^{5}	9 d	-491
$2.5\ UO_2 + NO_3^- + 3H^+ \rightarrow 2.5\ UO_2^{2+} + 0.5\ N_2 + 3\ H_2O$	2.76×10^{3}	17 d	-401

[a]Determined by monitoring consumption of glucose, or Fe(II) or production of U(VI) or sulfate.

[b]Values calculated from Dean (1985).

suggest that U(IV) is ultimately oxidized by Fe(III) in these sediments. This Fe(III) may be produced by reaction of nitrite with Fe(II) or by microbiological oxidation of Fe(II). While nitrate-dependent growth with U(IV) as the sole electron donor has not previously been reported, the minor role for these organisms that we predict is consistent with previous results. U(IV) oxidation by Fe(III) may be quite rapid (130 μM U(IV)/day) (Senko et al., submitted for publication), and while *Geobacter metallireducens* was shown to enzymatically oxidize U(IV) with nitrate reduction, U(IV) was oxidized more rapidly when the organism oxidized Fe(II) to Fe(III) which in turn oxidized U(IV) (Finneran et al., 2002b).

The effect of nitrate concentration on the rate and extent of nitrate-dependent U(IV) oxidation. The amount of nitrate entering a bioremediated aquifer may control the activity of DNRB and consequently the rate and extent of nitrate-dependent U(IV) oxidation. Groundwater nitrate concentrations can range from undetectable - 6 mM in uncontaminated aquifers (Burden et al., 1982; Portnoy et al., 1998; Nowicki et al., 1999) to 0.1 – 1 mM in agricultural/municipal/residential runoff-contaminated aquifers (Burden et al., 1982; Portnoy et al., 1998; Nowicki et al., 1999; Smith et al., 2001) to 100 mM or more in radionuclide-contaminated aquifers and some desert subsoils (Elias et al., 2003a; Walvoord et al., 2003; Istok et al., 2004).

To determine how nitrate concentration influences U(IV) oxidation, we incubated sediments with biogenic U(IV) and 0.1, 1, 4, and 40 mM nitrate. Nitrate-dependent U(IV) oxidation proceeded as long as nitrate was available, and after complete reduction of nitrate, U(VI) was reduced (Figure 1). Nitrite accumulation was only observed in sediment incubations receiving 40 mM nitrate (Figure 1). Reductive capacity (RDC) was

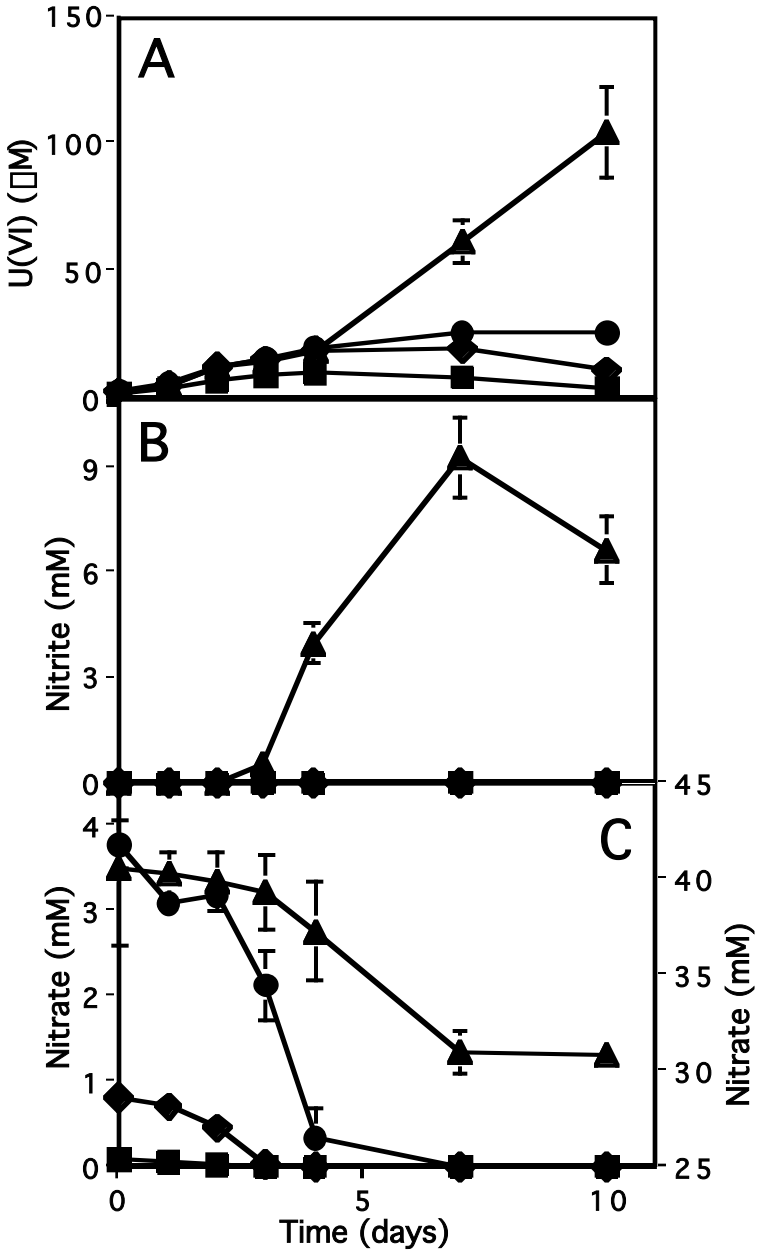

Figure 1. U(IV) oxidation (A), nitrite accumulation (B), and nitrate reduction (C) in sediment incubations amended with (■) 0.1 mM, (◆) 1 mM, (●) 4 mM, or (▲) 40 mM nitrate.

used as an indicator of the nitrate concentration relative to the concentration of reductants (Fe(II), sulfide, DOC, and U(IV)) present in the sediment incubations. We compared the extent of nitrate-dependent U(IV) oxidation with the RDC of the sediment incubations and observed a strong correlation between RDC and the extent of nitrate-dependent U(IV) oxidation (Figure 2 A), suggesting that the extent of nitrate-dependent U(IV) oxidation is dependent on the amount of nitrate entering an aquifer. While higher nitrate concentrations generally lead to an increase in the rate of nitrate reduction, this relationship was weak under net reducing conditions (RDC > 0) (Figure 2). Under net oxidizing conditions (RDC < 0; 40 mM nitrate amendment), U(IV) oxidation occurred in two phases: an initial phase before nitrite accumulation (Figure 1) where the k value was similar to those observed under reducing conditions (crossed circle, Figure 2 B), and a second phase that occurred as nitrite accumulated (Figure 1), where the k value was much higher (open circle, Figure 2 B). This suggests that nitrate-dependent U(IV) oxidation may initially occur via Fe(III) produced by enzymatic nitrate-dependent Fe(II) oxidation and later by Fe(II) oxidation by nitrite. These results suggest that if nitrate is present well in excess of available electron donor, nitrite will accumulate (Oh and Silverstein, 1999) and enhance U(IV) oxidation. It appears that nitrate concentration controls the rate of nitrate-dependent U(IV) oxidation only under net oxidizing conditions, where nitrite accumulates to high levels. Under conditions where nitrite does not accumulate, nitrate concentration exerts weak control on the rate of nitrate-dependent U(IV) oxidation.

The greater rate of U(IV) oxidation after nitrite accumulated to high levels is consistent with recent work suggesting that amorphous Fe(III) produced by the reaction

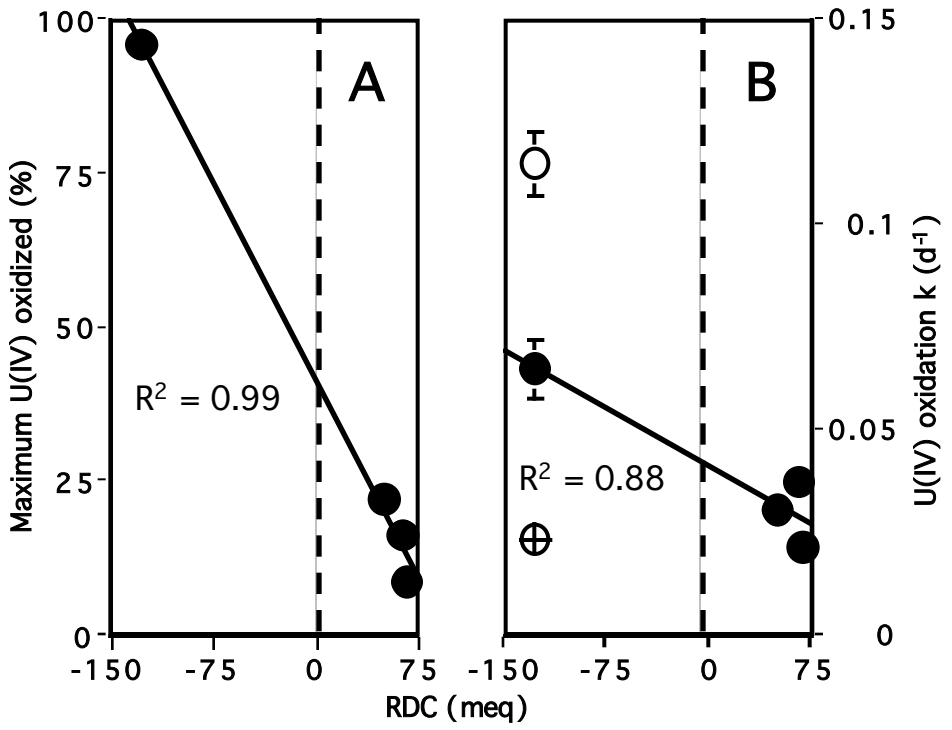

Figure 2. The relationship between RDC (manipulated by varying the nitrate concentration) in sediment incubations and the extent (A) and rate (B) of nitrate-dependent U(IV) oxidation. In panel B, closed circles represent pseudo-first-order rate coefficients (k) that were calculated as long as nitrate-dependent U(IV) oxidation continued. The crossed circle represents the k value for nitrate-dependent U(IV) oxidation in incubations receiving 40 mM nitrate before nitrite accumulation, and the open circle represents the k value for nitrate-dependent U(IV) oxidation after nitrite began to accumulate in the same incubations. The dashed line represents the point where RDC = 0. Points to the left of the line represent maximum U(IV) oxidized or U(IV) oxidation k values under net oxidizing conditions with respect to nitrate, while points to the right of the line represent values under net reducing conditions with respect to nitrate.

between nitrite and Fe(II) is more reactive with U(IV) than more crystalline Fe(III) minerals that are produced by the enzymatic, nitrate-dependent Fe(II) oxidation (Senko et al., submitted for publication). Our results suggest that while nitrate concentration relative to available reductant may be a good predictor of the extent of nitrate-dependent U(IV) oxidation, rates of nitrate-dependent U(IV) oxidation are largely independent of nitrate concentration, especially under net reducing conditions.

The effect of reductants/electron donors on U(IV) oxidation. We tested the effect of organic and inorganic reductants on nitrate-dependent U(IV) oxidation by incubating sediments with biogenic U(IV), nitrate, and various concentrations of reductants. Sediments were washed and bicarbonate buffer was used in lieu of site groundwater so that we could examine lithotrophic activity without interference by DOC in site groundwater that could support organotrophic nitrate-reducing activity. Inorganic reductants (soluble sulfide, soluble Fe(II), and FeS) were added at concentrations of 0, 0.1, 1, and 10 mM, and acetate was added at concentrations of 0, 0.3, 3, and 30 mM. These reductant amendments were in addition to insoluble Fe(II) and sulfide already present in the sediments that were not removed by washing. In all cases, quantitatively greater reductant additions yielded greater inhibition of nitrate-dependent U(IV) oxidation (Figure 3).

We plotted the RDC versus the extent and rate (k) of U(IV) oxidation as described above and fitted logarithmic trend lines to the data (Figure 3). Using these trend lines, the more negative the slope of the line, the more effectively (on an electron equivalent basis) the reductant inhibited nitrate-dependent U(IV) oxidation. For instance, soluble

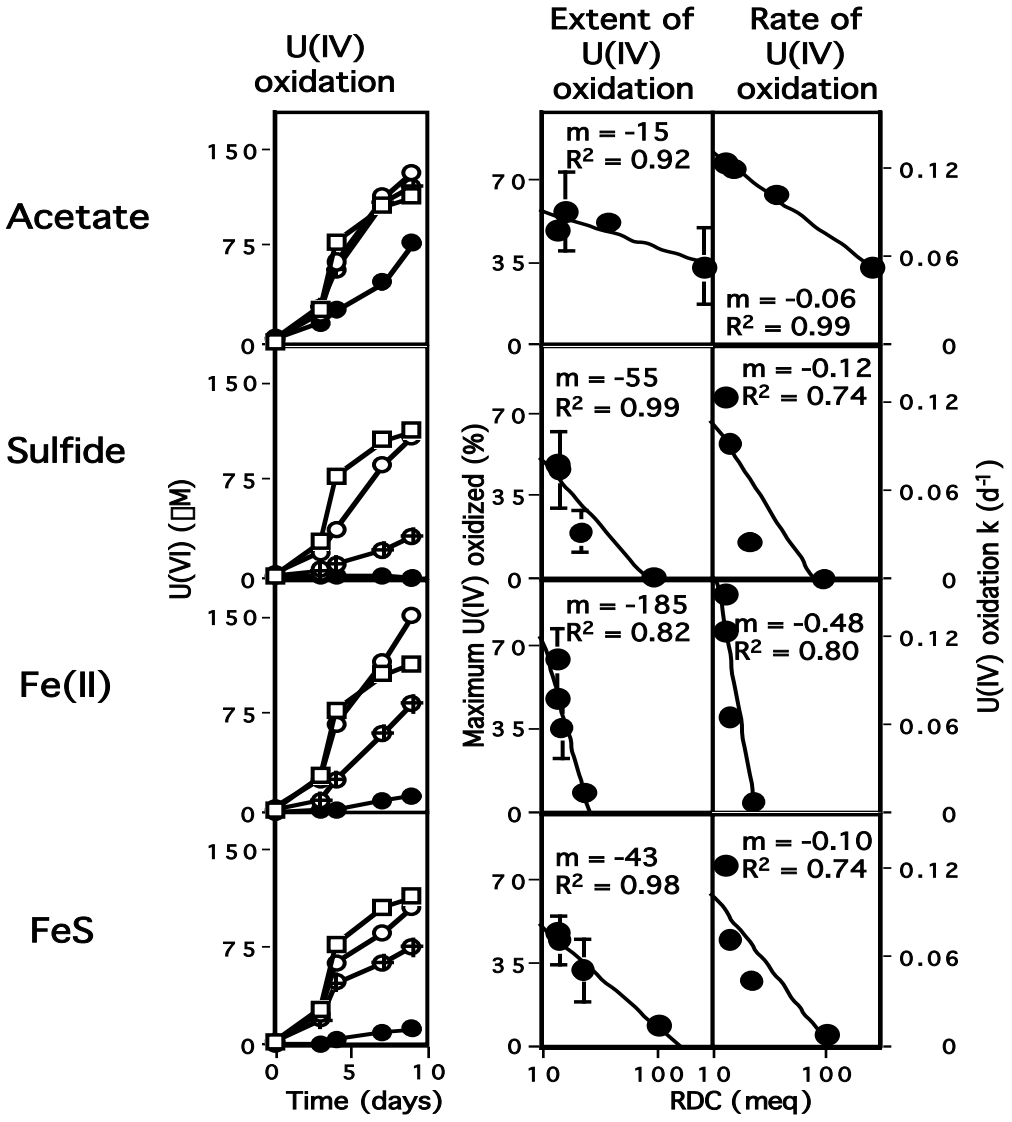

Figure 3. Nitrate-dependent U(IV) oxidation with the addition of (●) 30 mM acetate or 10 mM sulfide, soluble Fe(II), or FeS, (⊕) 3 mM acetate or 1 mM sulfide, soluble Fe(II), or FeS, (○) 3 mM acetate or 1 mM sulfide, soluble Fe(II), or FeS, or (□) with no reductant addition, and the relationship between RDC (manipulated by varying the reductant concentration) in sediment incubations and the extent and rate of nitrate-dependent U(IV) oxidation. Logarithmic regression trend lines are shown with the slope (m) of the lines.

Fe(II) was the most effective inhibitor of nitrate-dependent U(IV) oxidation, while acetate was the least effective inhibitor of nitrate-dependent U(IV) oxidation (Figure 3). Inhibition of nitrate-dependent U(IV) oxidation by acetate was probably due to organotrophic nitrate-reducing bacteria outcompeting Fe(II)-oxidizing nitrate-reducing bacteria for available nitrate (Marchand and Silverstein, 2002; Marchand and Silverstein 2003), inhibiting Fe(III) formation, and, consequently, inhibiting nitrate-dependent U(IV) oxidation.

Inhibition of nitrate-dependent U(IV) oxidation by inorganic reductants was probably due to decreased reactivity of biogenic Fe(III) caused by the reductants. While sulfide alone is an inefficient reductant of U(VI) in bicarbonate buffer (Finneran et al., 2002a), sulfide and FeS may have reduced Fe(III) as it was formed by nitrate-dependent Fe(II) oxidation (Nevin and Lovley, 2000; Schippers and Jørgensen, 2002) and inhibited oxidation of U(IV) by Fe(III). Free sulfide addition may also have been toxic to microorganisms, as the addition of 10 mM sulfide partially inhibited nitrate reduction (not shown).

Soluble Fe(II) was a more effective inhibitor of nitrate-dependent U(IV) oxidation than soluble sulfide or FeS. Inhibition of nitrate-dependent U(IV) oxidation by soluble Fe(II) addition may have been due to coating of Fe(III) minerals by Fe(II) (Royer et al., 2004), or the formation of less reactive Fe(III) minerals (Chaudhuri et al., 2001, Senko et al., submitted for publication), which are ineffective oxidants of U(IV) (O'Loughlin et al., 2003; Senko et al., submitted for publication). The reason that soluble Fe(II) was a more effective inhibitor of nitrate-dependent U(IV) oxidation could be that different Fe(III) mineral phases were formed during nitrate-dependent Fe(II) oxidation in the

presence of Fe(II) compared to those formed in the presence of soluble sulfide or FeS. Soluble Fe(II) enhances the formation of crystalline Fe(III) minerals from ferrihydrite or hydrous ferric oxide (Cornell et al., 1989; Cornell and Schwertmann, 2003). Anaerobic oxidation of Fe(II) sulfides leads to amorphous or poorly crystalline Fe(III) minerals compared to the anaerobic oxidation of soluble Fe(II) (Kappler and Newman, 2004). We hypothesize that more crystalline Fe(III) products were formed in the presence of soluble Fe(II), while amorphous Fe(III) products were formed in the presence of sulfide. Since crystalline Fe(III) minerals are less effective oxidants of U(IV) than amorphous Fe(III) minerals (Senko et al., submitted for publication), this would lead to lower extents and rates of nitrate-dependent U(IV) oxidation in the presence of soluble Fe(II).

The results we present here provide further evidence that nitrate-dependent U(IV) oxidation occurs through a reaction with Fe(III) that is produced during dissimilatory nitrate reduction. While nitrate concentration exerts a strong influence on the extent of nitrate-dependent U(IV) oxidation, it exerts a weak influence on the rate of this process, especially under reducing conditions. Both the rate and extent of nitrate-dependent U(IV) oxidation are strongly controlled by the concentration of inorganic and organic reductants, and Fe(II) is the most effective inhibitor of nitrate-dependent U(IV) oxidation. After uranium has been reductively immobilized in a contaminated aquifer, we propose that a simple geochemical characterization of a bioremediated site, including the reducing capacity of groundwater and sediment could prove to be a useful predictor of the stability of U(IV).

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Appendix 1

Barite deposition resulting from phototrophic sulfide-oxidizing bacterial activity

Abstract

Barite ($BaSO_4$) deposits generally arise from mixing of soluble barium-containing fluids with sulfate-rich fluids. While the role of biological processes in modulating barium solubility has been shown, no studies have shown that the biological oxidation of sulfide to sulfate leads to barite deposition. Here we present an example of microbially mediated barite deposition in a continental setting. A spring in the Anadarko Basin of southwestern Oklahoma produces water containing abundant barium and sulfide. As emergent water travels down a stream to a nearby creek, sulfate concentration increases from 0.06 mM to 2.2 mM while Ba^{2+} concentration drops from 0.4 mM to less than 7 □M. Stable isotope analysis, microbial activity studies, and in-situ experiments provide evidence that as sulfide-rich water flows down the stream, anaerobic, anoxygenic, phototrophic bacteria play a dominant role in oxidizing sulfide to sulfate. Sulfate then precipitates with Ba^{2+} producing barite as travertine, cements, crusts, and accumulations on microbial mats. Our studies suggest that phototrophic sulfide oxidation and concomitant sulfur cycling could prove to be important processes regulating the cycling of barium in continental sulfur-containing systems.

Introduction

Authigenic sea floor barite precipitation and diagenetic barite formation in the subsurface have been extensively studied due to their potential insight into oceanic paleoproductivity and economic importance, respectively. Barite deposits in these two environments have been well characterized, but there are few reports regarding the occurrence of recent surficial, continental barite deposits.

The role of biological activity in aquatic barite deposition is well known. Barite accumulation on the floor of large water bodies has been proposed as an indicator of productivity by planktonic organisms (Finlay et al., 1983, Stroobants et al., 1991, Cattaldo et al., 1998, Schenau et al., 2001). Planktonic microorganisms may actively or passively accumulate barium (as Ba^{2+} or barite) (Gooday and Nott, 1982, Finlay et al., 1983, Wilcock et al., 1989, Bertram and Cowen, 1997, Cattaldo et al., 1998). As planktonic organisms accumulate barium and eventually die, they settle to bottom sediments. As the organisms decompose, barite saturated microenvironments develop and barite then precipitates (Varvanas, 1987, Stroobants et al., 1991, Stamatakis and Hein, 1993, Cattaldo et al., 1998; Naehr et al., 2000).

Another somewhat different seafloor system has been described in which massive barite deposits occur (up to 10 m in depth) (Greinert et al., 2002). In this system, referred to as a "Giant Cold Seep", sulfide and barium laden seep water mixes with oxygen and sulfate-rich sea water, resulting in barite precipitation (Cecile et al., 1983, Greinert et al., 2002). Both a decrease in sulfide concentration as seep water reaches surface sediments,

and the presence of chemoautotrophic tube worms, suggests that sulfide may be acting as a substrate for chemolithotrophic bacterial formation of sulfate (Greinert et al., 2002).

Barite deposits are also found in terrestrial subsurface environments. Two general mechanisms have been proposed for their formation. In the first case, barium and sulfide laden brines mix with oxidizing meteoric water, resulting in abiotic oxidation of sulfide to sulfate which results in diagenetic barite formation (Plummer, 1971). Alternatively, barium laden brines may mix with sulfate-containing meteoric water, also resulting in barite formation (Kaiser, 1987, Williams-Jones et al., 1992). Although microbial processes have not been previously shown to be directly involved in sulfide oxidation and resultant barite formation in subsurface systems, stable isotope data has shown that sulfate or thiosulfate reducing bacteria may be involved in formation of sulfide and sulfite, respectively (Kaiser, 1987, Spirakis, 1991). These sulfur compounds can then be oxidized to form sulfate and subsequently barite.

These studies on both aquatic sediments and terrestrial subsurface sediments suggest that biological activity plays an important role in modulating the solubility of barium. It is also clear that the redox cycling of sulfur (via biological or abiotic mechanisms) strongly influences barium solubility. Microorganisms play an important role in the sulfur cycle via the reduction of sulfate and elemental sulfur to sulfide (Pfennig and Widdel, 1982, Trüper, 1984), the disproportionation of elemental sulfur and thiosulfate to sulfate and sulfide (Bak and Pfennig, 1987, Bak and Cypionka, 1987, Jørgensen, 1990), and the oxidation of sulfide to elemental sulfur or sulfate, and further oxidation of sulfur to sulfate (Brune, 1995, Van Gemerden, 1983, Friedrich, 1998, Trüper, 1984). The role of sulfate reducing bacteria in barium cycling is already well

established: Barite-SO_4^{2-} can be reduced to sulfide leading to the release of soluble Ba^{2+} (Bolze et al., 1974, Torres et al., 1996; Phillips et al., 2001; Karnachuk et al., 2002). Therefore, it stands to reason that sulfide-oxidizing bacteria could also play an important role in barite formation. However, to our knowledge, no previous reports of the role of sulfur or sulfide oxidizing bacteria in this process exist. Here we report barite formation in a sulfide and sulfur-rich artesian spring. The oxidation of sulfide to sulfate (which precipitates with barium) in this spring is apparently catalyzed by the activity of anaerobic, anoxygenic, phototrophic bacteria.

Materials and Methods

Sampling and analytical methods. For analysis of major ions (except sulfide) in spring water, samples were collected by syringe, filter-sterilized (0.22 μm filtered) and stored on ice before analysis. Nitrate, sulfate, and thiosulfate concentrations were determined using a Dionex ion chromatograph equipped with an AS4A column and conductivity detector (Dionex Instruments, CA). Ba, Ca, Fe, Si, and Sr concentrations were determined by flame atomic absorbance spectroscopy using Perkin-Elmer models 2380 and 5000 AA spectrophotometers (Perkin-Elmer, Inc., Shelton, CT). Samples for sulfide analysis were collected with a 10 ml pipette and added directly in equal volumes to anoxic zinc acetate (10% solution). Sulfide was quantified by the methylene blue assay (Cline et al., 1969). Alkalinity of the stream was determined by titration. Methane was determined in samples collected by completely filling 120 ml serum bottles and stoppering them directly in the field with no headspace. Samples were transported to the laboratory on ice and methane was analyzed within four hours of collection. A N_2 bubble was added to

each bottle, and the methane concentration in the bubble was quantified by gas chromatography. Oxygen concentrations in the stream were measured in the field by YSI 52 dissolved oxygen meter and YSI 5739 field probe. Stable isotope ratios were determined by mass spectroscopy (Coastal Science Laboratories, Austin, TX).

To quantify zero-valent sulfur, 1 l of spring water was collected, acidified with HCl to pH 1-2, and bubbled with oxygen free N_2 gas to volatilize sulfide. Acidification causes the precipitation of sulfane-S (polysulfides, polythionates) thiosulfate, and soluble S^0 (Burton and Machmer, 1968, Meyer, 1977, Fossing and Jørgensen, 1989), which were removed by filtration. Since no thiosulfate was detected in the spring water, precipitated sulfur was considered sulfane-S and soluble elemental sulfur, and will be referred to as zero-valent sulfur throughout the text (Van Gemerden and Mas, 1995). A known area of the filter was then placed in a serum bottle with a 12 x 75 mm test tube containing 2.5 ml anoxic zinc acetate solution (10%). Sulfur was converted to sulfide using a Cr(II) extraction procedure (Ulrich et al., 1997) modified to include dimethylformamide in the extraction mix (Hsieh and Chang, 1989). Volatilized sulfide was then trapped in the zinc acetate-containing test tube and measured spectrophotometrically as described above. The total amount of zero-valent sulfur present in 1 l could be extrapolated by calculating the zero-valent sulfur per area of filter, and the total zero-valent sulfur on the filter could be determined as the total zero-valent sulfur per l of water. Sulfide was extracted by purging it from the acidified spring water directly into traps containing 2 M $AgNO_3$ for $\delta^{34}S$ analysis. The AgS precipitate was allowed to settle in the test tubes and dried before shipment to Coastal Science Laboratories (Austin, Texas) for δ^{34} S analysis by mass

spectroscopy. Sulfate was precipitated from acidified spring water with excess $BaCl_2$ before $\delta^{34}S$ analysis.

Submerged cores were collected from a pool in the stream approximately 15 m from the source that contained extensive microbial mats (Fig. 1). The cores (including approximately 20 ml of stream water) were collected using an inverted 60 cc syringe modified by cutting off the flange at the plunger end. The open-ended syringes were pushed into the soft streambed sediment, and the top end was sealed with syringe needles plugged with rubber stoppers. The syringes were withdrawn from the streambed and the plunger was immediately replaced while the syringe was still submerged in the spring water. No headspace was allowed in the syringes. Cores were stored on ice for transport back to the lab before further processing. Samples for electron microscopic analysis were collected in 15 ml plastic tubes using a sterile spatula from mats in the spring near the confluence of the spring and the Creek (Fig. 1). These samples were stored on ice for transport back to the laboratory for further processing.

Electron microscopy and XRD. Mineral samples were dried, mounted on aluminum stubs, carbon coated, and observed in both a JEOL JSM-880 and ETEC Autoscan scanning electron microscopes for analysis. Biological samples were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer for 1 hour at room temperature. They were subsequently dehydrated in ethanol, critical point dried, mounted on aluminum stubs and coated with gold/ palladium for SEM analysis.

X-ray diffraction of powdered mineral samples was determined in an automated Rigaku diffractometer.

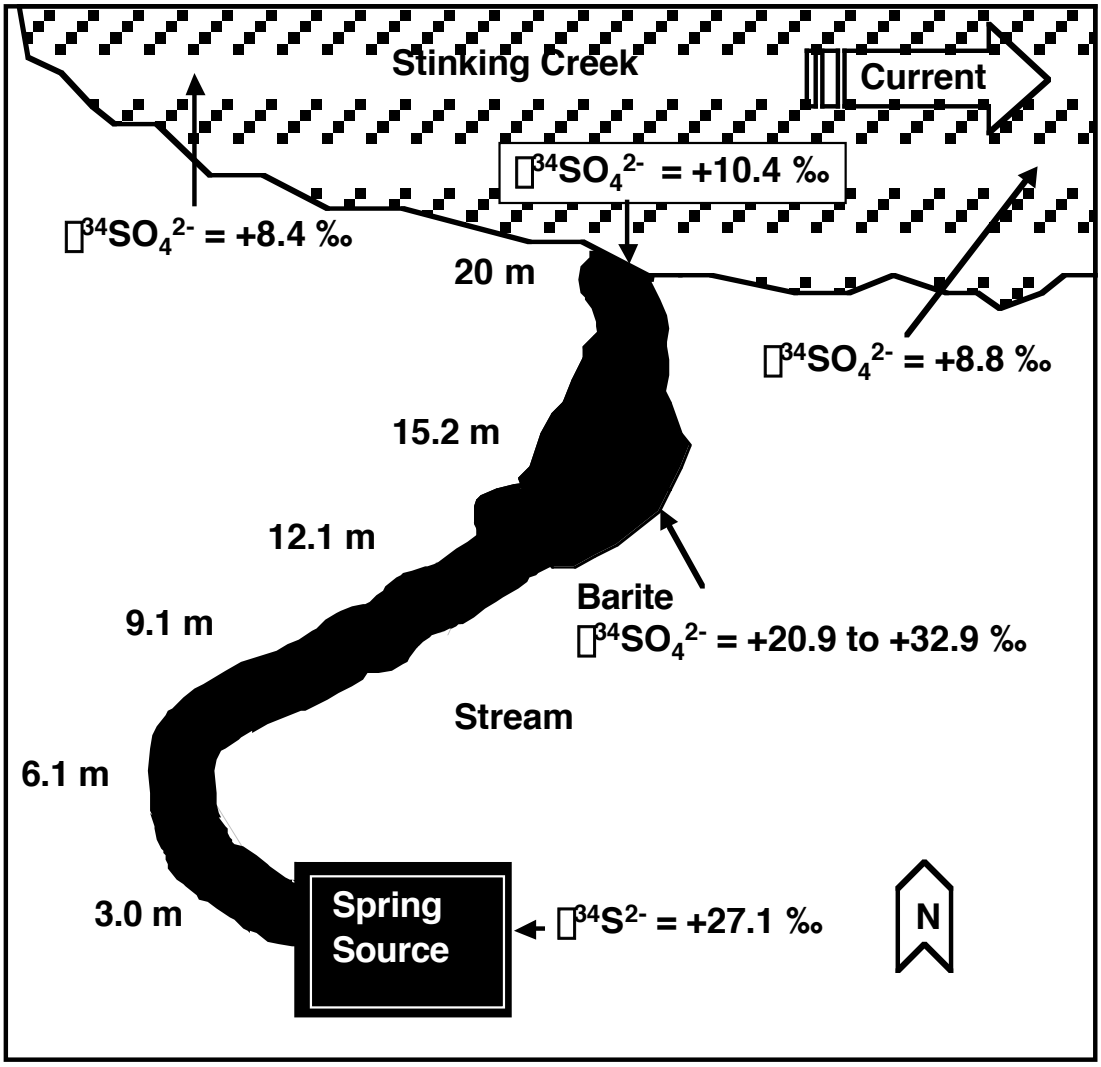

Fig. 1. Schematic diagram of study site illustrating the spring source, stream, and Stinking Creek with $\delta^{34}S$ values for sulfide or sulfate in the system.

Sulfide oxidation activity experiments. For sulfide oxidation experiments, $^{35}S^{2-}$ was produced by incubating $^{35}SO_4^{2-}$ with *Desulfovibrio desulfuricans* in SRB medium (Tanner, 1989) for 2 days. $^{35}S^{2-}$ was extracted by acidifying the culture with 6 N HCl and recovering the volatilized H_2S in a 0.5 M NaOH trap (Ulrich et al., 1997). Trapped sulfide was added directly to the cores and caused no change in pH. Triplicate core incubations were performed in the dark (by wrapping core-containing syringes in foil) or in the light, under grow lamps (100 μmoles quanta/sec/cm^2) at room temperature. Anaerobic incubations were performed with no headspace in the modified syringes, while aerobic incubations were performed with approximately 20 ml of air in the headspace. To determine evolution of $^{35}SO_4^{2-}$ in the core incubations, sulfide was removed by precipitation as ZnS (with 10% zinc acetate), and the supernatant was removed. Sulfate was separated from this supernatant by addition of 30 mM $BaCl_2$ and cold sulfate (as Na_2SO_4). Pellets were washed with 30 mM $BaCl_2$ and analyzed by liquid scintillation counting. Conversion of sulfide to sulfate was calculated by multiplying the ratio (μCi/ml $^{35}SO_4^{2-}$ as $BaSO_4$/ initial $^{35}S^{2-}$ [approx. 0.3 μCi/ml]) by the initial sulfide concentration (approx. 10 mM). While isotopic exchange of ^{35}S may have occurred between the sulfide added and zero-valent sulfur species, it is unlikely that isotopic exchange occurred between sulfide and the sulfate produced in the incubations (Fossing and Jørgensen, 1990a; Fossing and Jørgensen, 1990b). Therefore, the production of $^{35}SO_4^{2-}$ can be attributed to the oxidation of reduced inorganic sulfur species, whether sulfide, polysulfides, polythionates, or elemental sulfur.

Results

Site Description. The spring emerges near Zodletone Mountain in southwestern Oklahoma, at the boundary between the Cambro-Ordovician Slick Hills, a sequence of folded carbonate rocks uplifted and exposed in association with late Paleozoic compressional tectonics, and flat-lying Permian clastic and evaporite rocks (Younger, 1986; Campbell et al., 2000). At this site, barite is present along with calcite as abundant cement within Pleistocene stream alluvium, as well as in seasonal whitish streambed sediments. Spring source water is saturated with respect to methane (Table 1), and flow rates of water emanating from the spring have been maintained at 8 l/min since first described by Havens (1983). Spring water flows for about 20 m and discharges into a nearby creek (Fig. 1). It has been suggested (Younger, 1986) that the spring chemistry represents a mixture of deeper basinal brine and shallow groundwater. The brine is ejected from deep within the Anadarko basin along with petroleum, which occurs in seeps in the general vicinity. Spring water chemistry is anomalous compared to surrounding waters (Havens, 1983), containing 0.2 M NaCl and minor amounts of fluorine and bromide as well as boron, strontium, barium (390 □□), sulfate (60 □□), and sulfide (8-10 mM). Results of chemical modeling using PHREEQC (Parkhurst, 1995) show that the emergent spring water is saturated with respect to quartz, fluorite, and aragonite, slightly supersaturated with respect to calcite, dolomite, and barite, and undersaturated with respect to witherite (barium carbonate) (Table 1). Dissolved oxygen was not detected in the spring, as the high sulfide concentration likely maintains anoxic conditions. Neither nitrate nor thiosulfate was detected in the spring. Sulfide concentration in the spring decreases from 8 mM to approximately 0.1 mM with distance

Table 1. Results of chemical modeling using PHREEQC for various minerals and gasses in Zodletone spring.

Phase	Saturation Index	Log(IAP)	Log(K)	Formula
Anhydrite	-3.14	-7.5	-4.36	$CaSO_4$
Aragonite	0.09	-8.24	-8.34	$CaCO_3$
Barite	1.23	-8.74	-9.97	$BaSO_4$
Calcite	0.24	-8.24	-8.48	$CaCO_3$
Celestite	-2.44	-9.07	-6.63	$SrSO_4$
CH_4(g)	0.1	-2.76	-2.86	CH_4
CO_2(g)	-1.5	-19.65	-18.15	CO_2
Dolomite	0.52	-16.57	-17.09	$CaMg(CO_3)_2$
FeS(ppt)	-2.13	-6.04	-3.92	FeS
Fluorite	0.16	-10.44	-10.6	CaF_2
Gypsum	-2.93	-7.51	-4.58	$CaSO_4{:}2H_2O$
O_2(g)	-3.22	-6.18	-2.96	O_2
Pyrite	8.82	-18.07	-26.89	FeS_2
Quartz	0.01	-3.97	-3.98	SiO_2
Strontianite	-0.54	-9.81	-9.27	$SrCO_3$
Sulfur	-1.56	-12.03	-10.47	S
Witherite	-0.92	-9.48	-8.56	$BaCO_3$

from the source, while zero-valent sulfur concentration increases from 0.1 mM at the source to 1 mM at 9 m (Fig. 2). At approximately 15 m, sulfate concentration begins to increase, eventually reaching 2.2 mM shortly before confluence with the creek (Fig. 2). At the same time, Ba^{2+} concentration decreases dramatically (Fig. 2), presumably as a result of precipitation with sulfate as barite. Microbial mats are abundant throughout the spring. Creek water chemistry upstream of the confluence with the spring is typical of oxic surface water in the area.

Some green and purple sulfur bacteria have the ability to anaerobically oxidize sulfide to sulfate and to fix carbon dioxide phototrophically (Pfennig and Widdel, 1982, Van Gemerden, 1983, Trüper, 1984, Van Gemerden, 1993, Brune, 1995, Van Gemerden and Mas, 1995, Friedrich, 1998). Given the extensive mats of phototrophic organisms present at the site, we hypothesized that anaerobic, phototrophic oxidation of sulfide to sulfate is occurring in the spring.

Characterization of minerals associated with microbial mats. Electron microscopy of encrusted mat samples revealed the presence of filamentous (Fig. 3a) and rod-shaped (Fig. 3b) microorganisms attached to mineral surfaces. Minerals associated with the mats were identified as calcite and barite. Calcite was identified by X-ray mapping and confirmed by powder X-ray diffraction analysis (data not shown). Barite was identified by powder X-ray diffraction. The calcite associated with the microbial mats was predominantly of the scalenohedral, or "nails-head" form (Fig. 3c).

Stable isotope analysis. $\delta^{34}S$ was determined for sulfide in the spring, sulfate in the creek and in barite mineral crusts (Fig. 1). Sulfide at the spring source had a $\delta^{34}S$ of +27.1‰. Barite-SO_4^{2-} was similarly heavy with $\delta^{34}S$ ranging from +20.9 to +32.9‰,

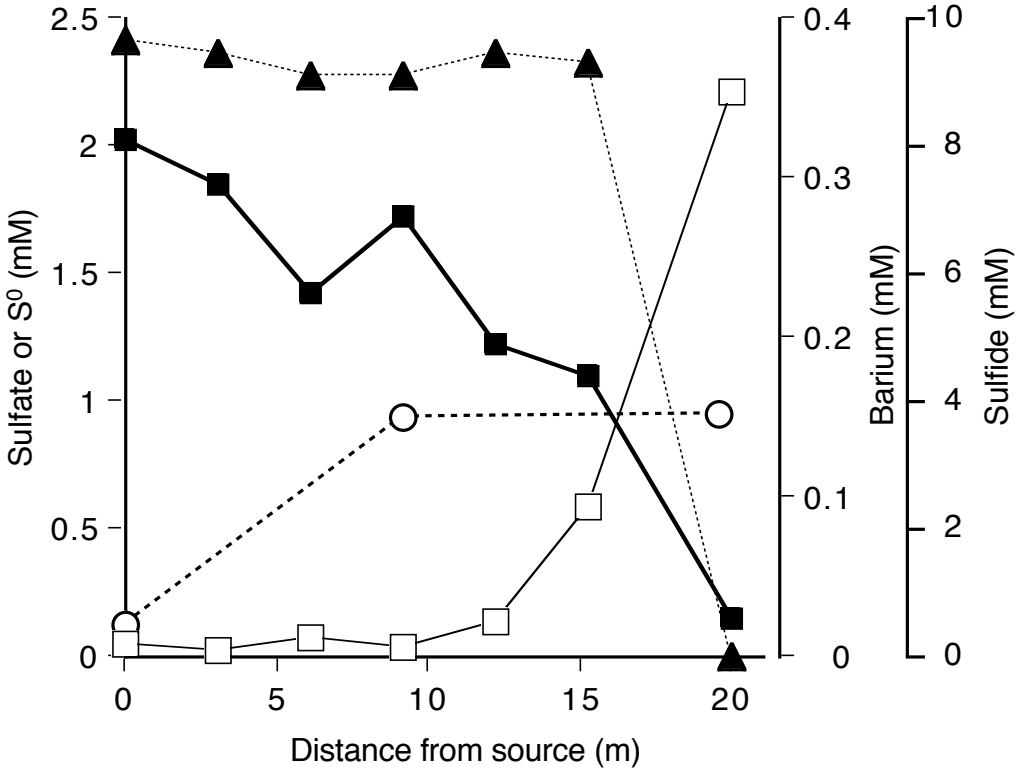

Fig. 2. Profile of dissolved ions over the length of Zodletone spring. Sulfate (□), barium (▲), sulfide (■), and zero-valent sulfur (○) concentrations are expressed with respect to distance from the source.

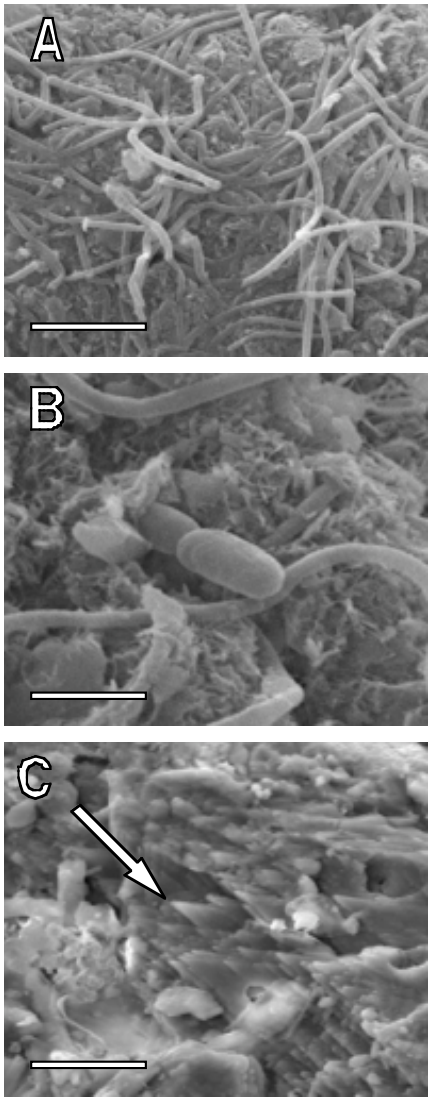

Fig. 3. (A and B) Scanning electron micrograph (3,000x) of bacteria in mats found near spring source at 3000x (A) and 10000x (B) magnification. Note the abundance of filamentous organisms (A) as well as the presence of short rod-shaped organisms on a solid substrate (B). (C)Scanning electron micrograph (2,000x) of nail-head calcite (arrow). Calcium is the major constituent in this region based by X-ray analysis, but sulfur and barium are also present. Samples collected from microbial mats and associated mineral crusts along the bank of Stinking Creek. (Bars = 8 □m (A), 2.7 □m (B), or 12.7 □m (C)).

compared to +8.4‰ for sulfate upstream of the confluence of the spring with the creek (Fig. 1). This suggested that barite-SO_4^{2-} originated as the heavy sulfide in the spring and is not derived from the much lighter sulfate in surrounding surface waters. Furthermore, the soluble sulfate at the confluence of the spring and creek and downstream was heavier than that upstream (Fig. 1), suggesting mixing of heavy sulfate from the spring and lighter sulfate in creek water.

Sulfide oxidizing activity in core incubations. Cores were collected from the spring and incubated with $^{35}S^{2-}$, anaerobically or with a headspace of ambient air under light and dark conditions to determine the role of phototrophic bacteria in sulfide oxidation. Aqueous sub-samples were analyzed for the production of $^{35}SO_4^{2-}$. Minimal oxidation of sulfide occurred in both oxic and anoxic heat-killed controls (Fig. 4), indicating that oxidation of sulfide to sulfate is not due primarily to abiotic processes, most notably the reaction of sulfide with oxygen. Accumulation of 0.5-1 mM sulfate from sulfide was observed in aerobic or anaerobic, dark incubations (Fig. 4). More importantly, in light incubations, 2.5 and 4 mM sulfate was produced from sulfide in aerobic and anaerobic incubations, respectively. These results suggest that oxidation of spring sulfide to sulfate is mediated primarily by phototrophic bacterial activity and that aerobic chemolithotrophic sulfur oxidizing bacteria do not play a major role in sulfate production. The cores incubated in the light, which were black at the beginning of the incubation, were extensively bleached, indicating oxidation of solid-phase sulfide.

Diel fluctuations in sulfate concentration. To determine the role of phototrophic bacterial activity in sulfate production in-situ, diel variations in sulfate concentration were monitored in the spring over a 24 hr period at the same pool from which the cores

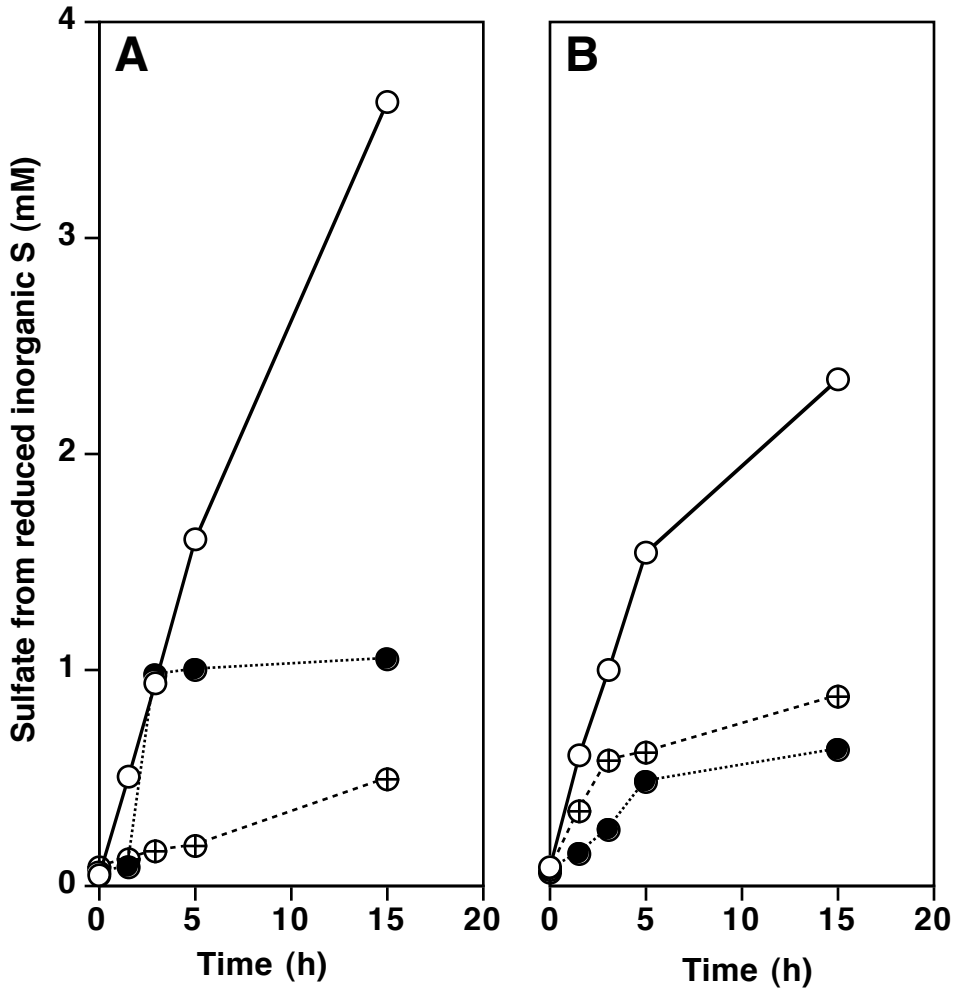

Fig. 4. Production of sulfate from reduced inorganic sulfur in sediment cores collected from a pool approximately 15 m downstream from the spring source. Graphs represent the oxidation of reduced inorganic S to SO_4^{2-} (as indicated by the production of $^{35}SO_4^{2-}$ upon addition of a $^{35}S^{2-}$ tracer) in light-incubated cores (O), dark-incubated cores (●), or heat-inactivated sediments (⊕). Panel A represents anaerobic incubations, while panel B represents aerobic incubations.

were collected. Sulfate concentration increased from 48 μM to 144 μM during daylight hours, peaking at 3:00 PM before decreasing to 48 μM after sunset (Fig. 5). These results confirm our laboratory experiments showing that sulfate is generated primarily as a result of anoxygenic, phototrophic bacterial activity.

Discussion

Extensive mineral deposits identified as primarily barite and calcite along with microbial mats were observed at Zodletone spring. After sulfide-rich water emerges from the spring source, sulfate concentration increases as sulfide and soluble barium concentrations decrease. Stable isotope analysis suggested that the barite-SO_4^{2-} was derived from isotopically heavy sulfide which emerges at the spring source. The heavy sulfur in spring sulfide can be attributed to extensive depletion of lighter sulfur from the subsurface sulfur source by an earlier microbial reduction, followed by removal of lighter sulfide. Alternatively, the heavy sulfur may be derived from closed-system reduction of local Cambro-Ordovician evaporite beds known to be enriched in ^{34}S (+25 to +29 ‰) (Claypool et al., 1980). The higher $\delta^{34}SO_4^{2-}$ (+10.4 ‰) at the spring-creek confluence than upcreek (+8.4 ‰) also suggested that isotopically heavy sulfide in the spring was being oxidized to sulfate which mixed with creek water and lead to isotopically heavier sulfate. The isotopic signature of upcreek sulfate is consistent with sulfate in nearby Permian evaporite beds (Denison et al., 1998), the likely source for creek sulfate. These data suggest that sulfide emerging from the spring is oxidized to sulfate over the course of the spring leading to precipitation of barium as barite.

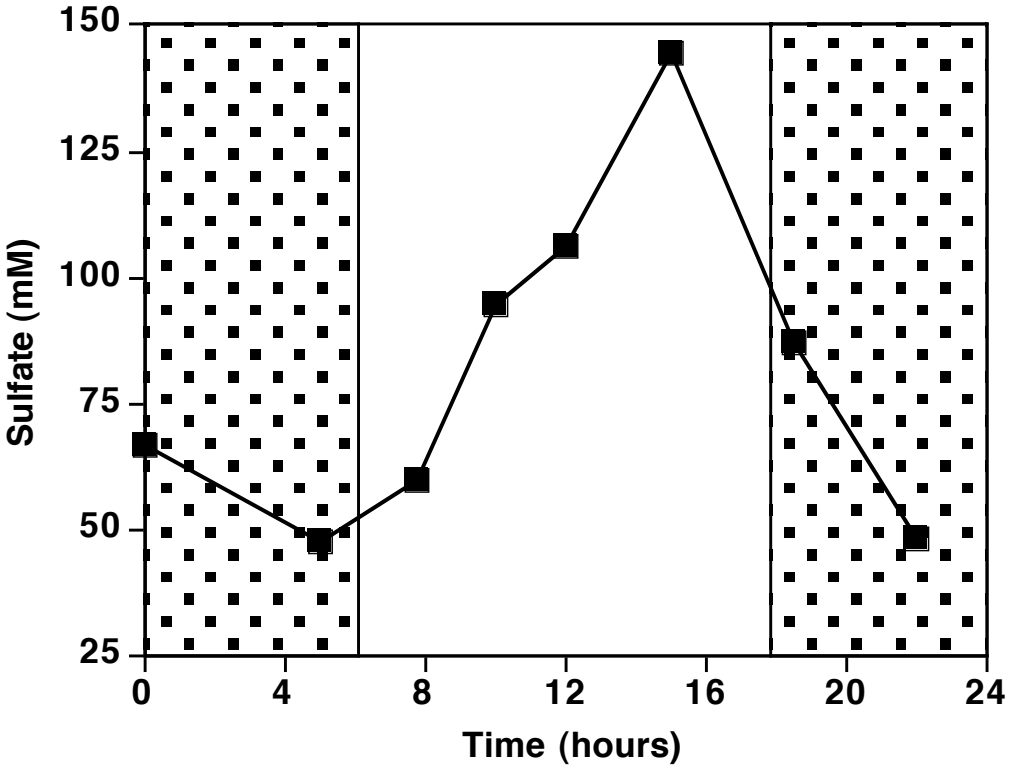

Fig. 5. Diel variation in dissolved sulfate concentration (■) over a 24 hour period. Unshaded regions correspond to daylight hours.

Authigenesis of minerals including carbonates, sulfides, and phosphates is often associated with phototrophic bacterial activity (Goncharova et al., 1993, Zavarzin, 1994, Reid et al., 2000, Stolz, 2000). The presence of microbial mats and microorganisms associated with barite and calcite minerals in Zodletone spring lead us to hypothesize that bacterial activity was responsible for the oxidation of sulfide to sulfate and subsequent deposition of barite. Greater amounts of sulfide were converted to sulfate in anaerobic light sediment core incubations than in aerobic, light incubations, dark incubations (aerobic or anaerobic) or heat killed incubations (aerobic or anaerobic). These results suggest that the oxidation of sulfide to sulfate in the spring is not likely due to abiotic oxidation of sulfide by oxygen or by aerobic, chemotrophic oxidation of sulfide. Sulfide oxidation to sulfate in this system is due to the activity of phototrophic microorganisms, and occurs under the anoxic conditions that are maintained in the spring. Further evidence that the production of sulfate is mainly due to phototrophic bacterial activity is shown by the diel fluctuations of sulfate concentration in-situ.

While the evidence we present suggests that sulfate is a result of phototrophic activity, based on these results, we are unable to determine if anoxygenic phototrophic bacteria are exclusively responsible for the production of sulfate. All bacterial activities in microbial mats occur at greater rates during daylight hours (Van Gemerden, 1993). While we did not detect dissolved oxygen in the spring water, Cyanobacteria may produce oxygen within the mats which could be used as an electron acceptor for chemotrophic sulfide or sulfur oxidation to sulfate. This is unlikely to be occurring, as sulfide inhibits oxygenic, cyanobacterial photosynthesis, sometimes at concentrations as low as 0.1-0.2 mM (Van Gemerden, 1993), and many Cyanobacteria switch to

anoxygenic photosynthesis (sulfide oxidation) at high sulfide concentrations (Van Gemerden, 1993). With the high sulfide concentrations present in Zodletone spring, it is unlikely that Cyanobacteria would be performing oxygenic photosynthesis.

A geochemically similar spring to Zodletone spring was microscopically characterized by Douglas and Douglas (2001). Like Zodletone spring, this spring was anoxic, contained abundant sulfide (3.8 mM), and was not thermal (9ºC). Interestingly, they measured low concentrations of barium (3.2 □M-6.2 □M) in spring water and in microbial mat pore water, but did not indicate the presence of barite minerals. Cyanobacteria which were believed to be carrying out anoxygenic photosynthesis, and purple and green sulfur bacteria were found in this spring (Douglas and Douglas, 2001). Indeed, work performed at Zodletone spring by Elshahed et al. (2003) revealed the presence of green and purple sulfur bacteria, green non-sulfur bacteria, and Cyanobacteria, all of which are capable of phototrophic sulfide oxidation to zero-valent sulfur and/or sulfate. (Pfennig and Widdel, 1982, Van Gemerden, 1986, Brune, 1995, Van Gemerden and Mas, 1995)

The increase in spring water zero-valent sulfur concentration with distance from the spring source suggests that zero-valent sulfur is an intermediate in sulfide oxidation to sulfate at Zodletone spring. Zero-valent sulfur may be further oxidized to sulfate in spring water by anoxygenic phototrophic bacteria (Van Gemerden and Mas, 1995), or may be disproportionated to sulfate and sulfide (Bak and Pfennig, 1987, Bak and Cypionka, 1987, Jørgensen, 1990, Janssen et al., 1996, Finster et al., 1998). In either case, sulfate is a product of the activity of these organisms, and the production of the

partially oxidized sulfur species is a result of anoxygenic phototrophic bacterial activity. In the latter scenario barite-SO_4^{2-} is also ultimately derived from phototrophic activity.

Sulfate-reduction activity has been detected at the site and is associated with the phototropic mats (Elshahed et al., 2003). It is well known that sulfate reducing bacteria are able to grow on solid phase sulfates such as barite albeit at lower rates (Bolze et al., 1974, Phillips et al., 2001; Karnachuk et al., 2002) and are therefore often responsible for Ba^{2+} mobilization from barite (Greinert et al., 2002, Torres et al., 1996). While sulfate reduction is occurring in Zodletone spring, the observed increase in sulfate concentration in the spring suggests that sulfide oxidation to sulfate is occurring at a greater rate than sulfate reduction, and therefore, much of the barite that may be mobilized by sulfate reducing activity may have subsequently precipitated in the presence of high sulfate concentrations.

This study underscores the importance of sulfur cycling microorganisms in modulating the solubility of barite. Figure 6 illustrates the proposed role for microbial sulfur cycling in modulating barium solubility in Zodletone spring. Once barium is mobilized (often as a result of sulfate reduction activity (Bolze et al., 1974, Torres et al., 1996, Phillips et al., 2001, Karnachuk et al., 2002)), anoxygenic phototrophic sulfide oxidizing bacteria (i.e. purple and green sulfur bacteria) in surficial systems may produce conditions for barite precipitation. While the precipitation of soluble barium with sulfate is ultimately an abiotic reaction, it is the result of bacterial sulfate production. Further, sulfur-disproportionating bacteria may also catalyze the precipitation of barite, by producing sulfate and sulfide from zero-valent sulfur or thiosulfate. While the site we

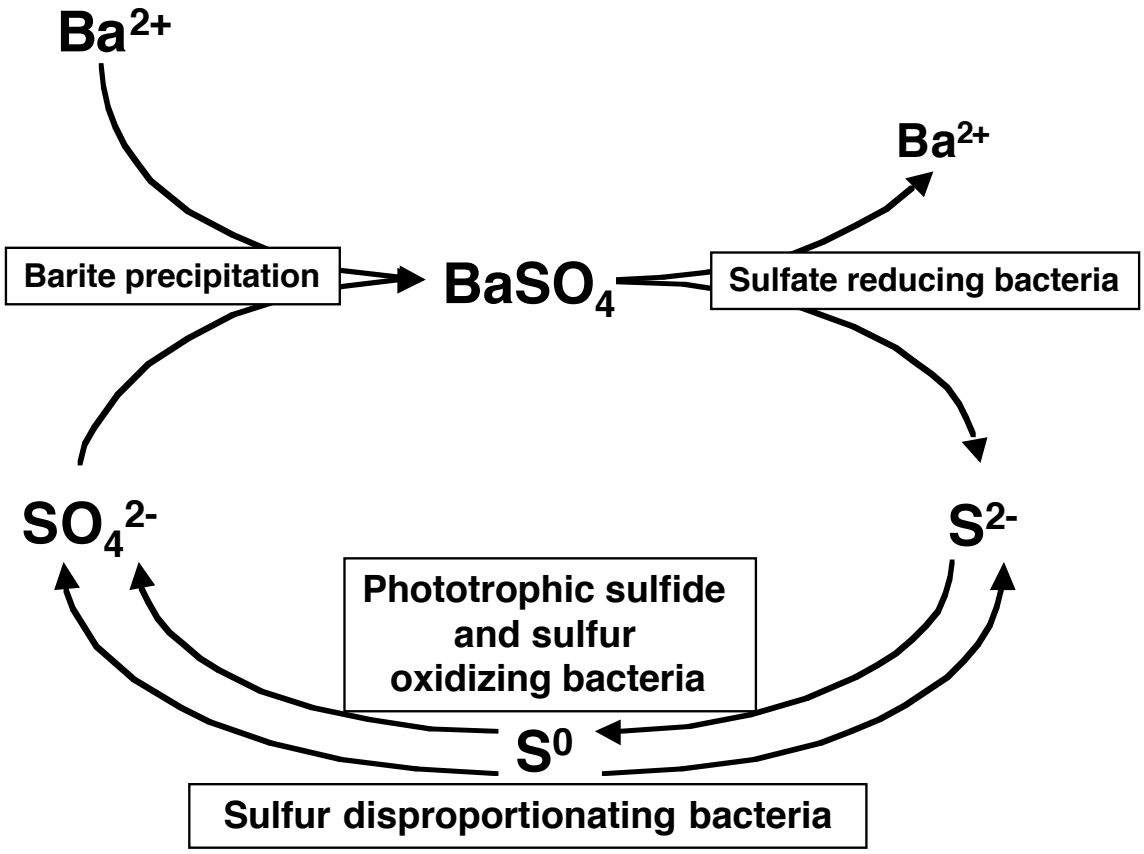

Fig. 6. Bacterially mediated sulfur cycling reactions modulate the solubility of barite in Zodletone spring. Soluble barium can be released from barite by sulfate reducing bacteria and precipitated as barite by bacterial processes that generate sulfate: sulfide and sulfur oxidation by phototrophic bacteria, or by the disproportionation of zero-valent sulfur or thiosulfate to sulfide and sulfate. (Modified from Trüper (1984) to reflect the interaction of biological sulfur cycling and barium solubility in Zodletone spring).

describe here is surficial and anoxic, the mechanism we describe for barite precipitation may be applicable to other systems.

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Appendix 2

Confirmation of Microbiological U(VI) Reduction in Sediment Incubations by X-ray Absorption Near-Edge Spectroscopy (XANES)

Abstract

U(VI) reduction to U(IV) and subsequent immobilization has been previously reported in sediment incubations. In these studies, the reduction of U(VI) to U(IV) was determined based on loss of U(VI) from solution and chemical extraction of U species. We confirmed microbiological U(VI) reduction by X-ray absorption near-edge spectroscopy (XANES).

Introduction

Stimulation of microbiological reduction of soluble U(VI) to insoluble U(IV) has been proposed as a means of preventing the off-site migration of that element with groundwater. Recent studies have suggested that U(VI) is reduced to U(IV) in sediment incubations (Finneran et al., 2002; Senko et al., 2002; Elias et al., 2003a). This conclusion was based on the loss of U(IV) from solution, and the chemical extraction of solids-associated (adsorbed) U(VI) using a bicarbonate solution and U(IV) using concentrated nitric acid under aerobic conditions (Finneran et al., 2002; Senko et al., 2002; Elias et al., 2003a; Elias et al., 2003b). We confirmed the reduction of U(VI) to U(IV) using X-ray absorption near-edge spectroscopy (XANES).

Materials and Methods

Sediments were collected from a shallow, alluvial, landfill leachate-impacted aquifer in Norman, OK as previously described (Senko et al., 2002). Groundwater was collected using a peristaltic pump and transferred to sterile bottles with no headspace. Sediment and groundwater were transported back to the laboratory and stored at 4°C before further processing (≤ 2 weeks). For sediment incubations, 25 g of sediment were placed in 120 ml serum bottles in an anoxic glovebag (Coy Laboratory Products, Inc., Grass Lake, MI). Groundwater was aerated to oxidize dissolved Fe(II) and provide a substrate for Fe(III)-reducing bacteria. This groundwater was then bubbled with 80:20 N_2:CO_2 and 50 ml of was added to the sediments and bottles were sealed with rubber stoppers, removed from the glovebag and flushed with 80:20 N_2:CO_2. U(VI) ($(CH_3COO)_2UO_2$) was amended to incubations from a concentrated, sterile, anoxic stock. Final pH of the sediment slurries was 6.8-7.1. Sediment slurries were autoclaved for heat-inactivated controls. Biogenic U(IV) was produced as described by Elias et al. (2003b).

Analytical methods. Samples for analysis (0.2-0.5 ml) were removed from serum bottles by needle and syringe and placed in microcentrifuge tubes in an anoxic glovebag. Insoluble material was removed by centrifugation, and the supernatant was analyzed. Samples for Fe(II) analysis were preserved in 0.5 M HCl. Sulfate concentration was determined by ion chromatography with conductivity detection (Dionex DX 500 fitted with an AS-4A column and conductivity detector; Dionex Corporation, Sunnyvale, CA). Soluble Fe(II) was quantified by ferrozine assay as described by Lovley and Phillips (1987). Soluble U(VI) was quantified by Kinetic Phosphorescence Analysis (KPA) on a

KPA-11 (ChemChek Instruments, Richland, WA) (Brina and Miller, 1992). For XANES analysis, sediment was dried in an anoxic glovebag, embedded in epoxy and mounted as a thin section on fused silica slides. Samples were analyzed using the PNC-CAT beamline 20-ID at the Advanced Photon Source at Argonne National Laboratory. The thin sections were first mapped using a 5 μm beam to locate regions with high U concentration. The microbeam was then fixed on these regions and XANES spectra collected by varying the x-ray energy and monitoring the x-ray fluorescence using a 13-channel Ge detector. The x-ray beam was monochromated using Si(111) crystals with an estimated energy resolution of 2.5 eV. An online Uranophane powder standard was used to monitor the energy calibration. The uranyl edge location was taken as 17171 eV in the edge plots. The spectrum obtained in sediments was compared to that for biogenic U(IV) (prepared as described above) and uranophane, a U(VI)-containing mineral.

Results and Discussion

To assess rates of U(VI) reduction and U(IV) oxidation, sediments were incubated with groundwater amended with 0.1 mM U(VI). U(VI) was almost completely reduced after 99 days, and U(VI) reduction occurred concurrently with Fe(III) and sulfate reduction (Figure 1). Fe(III) and sulfate were completely reduced after 21 and 46 days, respectively (Figure 1). These results suggest that the concerted action of several groups of organisms (including both Fe(III)- and sulfate-reducing bacteria) results in U(VI) reduction. This confirms recent findings by Elias et al. (2003a) who suggested that the U(VI) reduction occurs under both Fe(III)- and sulfate-reducing conditions, but is

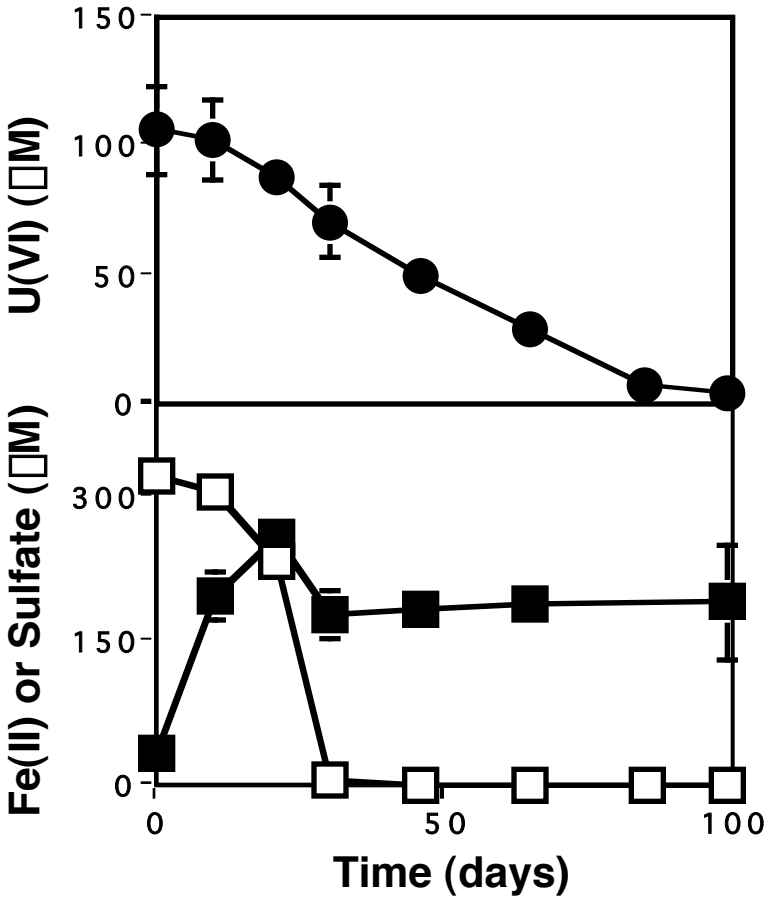

Figure 1. Reduction of Fe(III), sulfate, and U(VI) in sediment incubations. Symbols represent soluble Fe(II) (■), sulfate (□), and U(VI) (●), concentrations in the sediment incubations.

inconsistent with other results suggesting that U(VI) reduction does not occur during sulfate reduction in sediment incubations and in situ tests (Finneran et al., 2002; Anderson et al., 2003). Sulfate reduction is the predominant terminal electron accepting process in the sediments used for our experiments (Cozzarelli et al., 2000; Ulrich et al., 2003), so sulfate reducing bacteria capable of U(VI) reduction may be more abundant in these sediments than sediments used in other experiments that are dominated by Fe(III)-reducing bacteria. U(VI) reduction was confirmed by XANES spectroscopy (Figure 2). The spectroscopic evidence of U(VI) reduction confirms chemical extraction evidence of U(VI) reduction (as opposed to sorption to mineral surfaces) (Senko et al., 2002; Elias et al., 2003b). This also confirms that U(VI) reduction results in the removal of U from solution (Anderson et al., 2003), and that U(IV) does not remain suspended in the liquid phase as U(IV) nanoparticles (Suzuki et al., 2002).

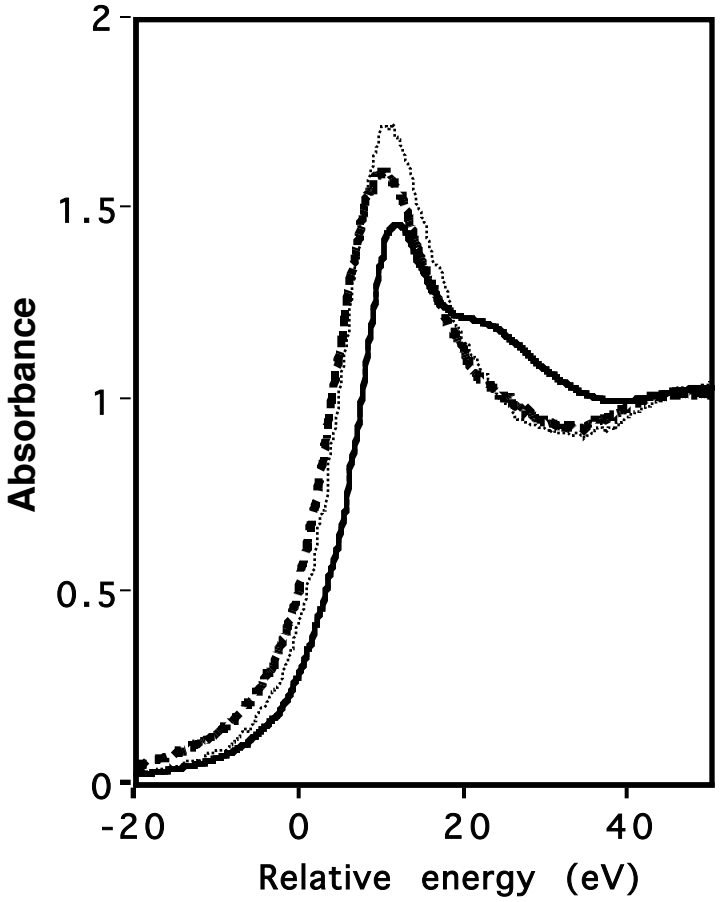

Figure 2. XANES spectra for uranium in sediments recovered from sediment incubations after 99 days of incubation. Lines represent XANES spectra for biogenic U(IV) (**- - -**), uranophane (U(VI) ( ), and sediment-associated U (·····).

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Appendix 3

Attempted Isolation of Nitrate-dependent Fe(II)- and U(IV)-Oxidizing Microorganisms

Abstract

Nitrate-dependent lithotrophic (Fe(II)-, FeS-, and U(IV)-oxidizing) enrichment cultures were obtained from landfill leachate impacted sediments. Roll-tubes were prepared to isolate these different lithotrophic organisms. While distinct colonies were obtained in all cases transfers to fresh media were unsuccessful.

Introduction

Nitrate-dependent Fe(II) and U(IV) oxidizing activity has been reported in landfill leachate-impacted sediments in Norman, OK. We established enrichment cultures for nitrate-dependent soluble Fe(II)-, FeS-, and U(IV)-oxidizing bacteria (Senko et al., in preparation). These enrichment cultures were maintained for at least 10 transfers. Soluble Fe(II)-oxidizing enrichments required 0.5 g/l yeast extract for growth, while FeS- and U(IV)-oxidizing enrichments could be maintained autotrophically, where the sole C source was CO_2/bicarbonate. We attempted to isolate organisms from these enrichments using the roll-tube technique described by Hungate (1969).

Materials and Methods

Microbial enrichments were maintained using 5 ml of a bicarbonate-buffered (3.5 g/l) mineral medium containing 0.8 g/l NaCl, 1 g/l NH_4Cl, 0.1 g/l KCl, 0.03 g/l, $MgSO_4.7H_2O$, and 0.04 g/l $CaCl_2.2H_2O$, vitamins, and trace metals (Tanner, 1997). Phosphate (KH_2PO_4) was included at a lower concentration (30 μM) to minimize precipitation of U(VI) as uranyl phosphate. Nitrate (3 mM $NaNO_3$) was added as a terminal electron acceptor and yeast extract (0.5 g/l) was provided for soluble Fe(II)-oxidizing enrichments. The medium was boiled under a stream of 80% N_2 and 20% CO_2 to remove oxygen and dispensed into serum tubes under a headspace of 80% N_2 and 20% CO_2 (Balch et al., 1979). FeS (1.5 mM), biogenic U(IV) (2 mM), or $FeCl_2$ (4 mM) were added as electron donors from sterile, anoxic, concentrated stock solutions or suspensions.

Solid medium (5 ml/serum tube) for isolation was prepared as describe by Senko et al. (in preparation) with 20 g/l agar and 20 mM $NaNO_3$. FeS, $FeCl_2$, and biogenic U(IV) were added to final concentrations of 3 mM, 10 mM, and 10 mM, respectively, while the media were still molten. Enrichment cultures were serially diluted and used to inoculate molten media before rolling (Hungate, 1969).

Results

Nitrate-dependent lithotrophic colonies were obtained for all substrates. U(IV) oxidation was indicated by clearing of the black U(IV) precipitate (Figure 1). Similarly, FeS oxidation was indicated by clearing of the black FeS precipitate and formation of red colonies, while soluble Fe(II) oxidation was indicated by the formation of red colonies.

U(IV)-oxidizing colonies were obtained from 10^{-4} dilution, FeS-oxidizing colonies were obtained from 10^{-8} dilution, and soluble Fe(II)-oxidizing colonies were obtained from 10^{-7} dilution. Attempts to transfer these colonies to fresh media were unsuccessful.



Figure 1. Roll tube containing nitrate-dependent biogenic U(IV)-oxidizing bacterial colonies. Zones of clearing U(IV) oxidation. Similar zones of clearing were observed for nitrate-dependent FeS-oxidizing bacterial colonies.

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Appendix 4

Isolation and Characterization of Nitrate-Reducing Bacteria from U(VI)- and Nitrate-Contaminated Groundwater

Abstract

Acetate- and glucose-oxidizing bacteria were isolated from a nitrate and radionuclide contaminated aquifer and partially characterized. Several isolated bacteria were incubated in FRC groundwater at various pHs. Nitrate reduction by glucose-oxidizing bacteria was inhibited in groundwater at pH 5.5 and below, while nitrate reduction by acetate-oxidizing organisms was inhibited in groundwater at pH 3.9 and below. An acetate-oxidizing, nitrate-reducing bacterial isolate was chosen for further study, and found to be most closely related to *Klebsiella* species. The survival of this organism in acidic FRC groundwater was enhanced by the addition of humic acids recovered from the FRC. This bacterial isolate was more tolerant of high nitrite concentrations than *Pseudomonas stutzeri*.

Introduction

Uranium and nitrate contaminate groundwater at the Field Research Center (FRC) in Oak Ridge, TN. Groundwater chemistry at the site is heterogeneous in regards to pH (3.3-6.8), and nitrate concentration (0.7-150 mM) (Istok et al., 2004). The reduction of soluble U(VI) to insoluble U(IV) has been proposed as a means to preventing the off-site migration of that element, but nitrate must be completely reduced before U(VI) reduction will commence (Istok et al., 2004). In order to determine controls on nitrate reduction in this relatively inhospitable environment, we isolated nitrate-reducing bacteria from FRC

groundwater and examined their tolerance for the inhospitable conditions of the contaminated FRC groundwater.

Materials and Methods

Isolation of nitrate-reducing bacteria from FRC groundwater. During push-pull tests to stimulate nitrate and U(VI) reduction (Istok et al., 2004), biomass that had accumulated in injection wells FW032 and FW033 was collected, refrigerated, and shipped to the laboratory. Samples were serially diluted and spread on agar plates (described below) with glucose or acetate as the electron donor and nitrate as the electron acceptor. Colonies were transferred to fresh liquid medium (described below). The purity of the cultures was routinely assessed microscopically.

Culture media. Organisms were isolated on the minimal medium described above with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered (50 mM, pH 7) minimal medium containing 0.8 g/l NaCl, 1 g/l NH_4Cl, 0.1 g/l KCl, 0.03 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7H_2O$, and 0.04 g/l $CaCl_2.2H_2O$, vitamins, and trace metals (Tanner et al., 1987). Acetate (30 mM) or glucose (10 mM) were added as electron donors and nitrate (20 mM) was the sole terminal electron acceptor. Where appropriate, agar (20 g/l) was used as a solidifying agent. Plates were prepared under oxic conditions before transfer to an anoxic glovebag (Coy Laboratory Products, Grass Lake, MI) where they equilibrated overnight under anoxic conditions. Colonies exhibiting distinct morphologies were picked and transferred to anoxic HEPES-buffered acetate-nitrate or glucose-nitrate liquid medium (as above but with no agar) in serum tubes with a N_2 headspace. In experiments

to determine nitrite tolerance, colony-forming units were determined using aerobic Luria-Bertani solid medium (Eisenstadt et al., 1994).

DNA isolation, PCR amplification, cloning, sequencing, and phylogenetic analysis of isolates. Cells were disrupted by bead-beating in sodium dodecyl sulfate lysis buffer, and DNA was isolated from other cellular material by extraction with phenol:chloroform:isoamyl alcohol (Steger et al., 2002). 16S rRNA genes were amplified by PCR as described by Elshahed et al. (2004) using the universal forward primer 8f (5' AGAGTTTGAGCCTGGCTCAG 3') and the universal reverse primer 804r (5' GACTACCAGGGTATCTAATCC 3'). PCR products were cloned directly into a TOPO-TA vector (Invitrogen) according to the manufacturer's instructions. Inserts were sequenced as described by Elshahed et al. (2004).

Phylogenetic affiliations of isolates were roughly determined using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Nucleotide sequences were aligned using ClustalX software (Thompson et al., 1997). An evolutionary distance tree (neighbor-joining algorithm with Jukes-Cantor corrections) was constructed for the 549 base pairs from position 127 through 676 using PAUP 4.0beta10 (Sinauer Associates, Sunderland, MA).

Resting cell incubations. For incubations, cells were grown to stationary phase (15-20 hours) and harvested by centrifugation, washed three times in anoxic bicarbonate buffer (3.5 g/l, pH 6.8), resuspended in the same buffer, and added to incubations at a concentration of approximately 0.15 mg protein/ml. For incubations in FRC groundwater, water produced form FW021 (pH approximately 3.5) was used (Istok et al., 2004). The pH of the water was adjusted with the addition of sodium bicarbonate under

aerobic conditions. To remove oxygen, groundwater was boiled under a stream of 80:20 N_2:CO_2 and dispensed into serum bottles with an 80:20 N_2:CO_2 headspace. Bottles were sealed with rubber stoppers, and autoclaved. FRC humic acids were provided by Bahoua Gu at the Oak Ridge National Laboratory. To determine the tolerance of organisms to nitrite, cells were incubated in bicarbonate buffer with various concentrations of nitrite.

Analytical methods. Samples were removed from incubations by syringe in an anaerobic glovebag (Coy Laboratory Products, Grass Lake, MI) and solids were removed by centrifugation. Nitrate and nitrite were quantified by ion chromatography with conductivity detection (Dionex DX 500 fitted with an AS-4A column; Dionex Corporation, Sunnyvale, CA). Acetate was quantified by ion chromatography using the same system fitted with an AS-11 column. Ammonium was quantified by the alkaline phenolate assay (Daniels et al., 1994). Protein was quantified using the bicinchoninic assay (Pierce Biotechnology, Inc., Rockford, IL).

Results and Discussion

Isolation and putative identification of nitrate-reducing bacteria from FRC groundwater. Several nitrate-reducing bacteria were isolated from FRC groundwater. Isolates were named by the well from which they were isolated (i.e. FW32 or FW33) and the substrate on which they were isolated (i.e. acetate or glucose). The putative identities of these organisms are shown in Table 1.

The effect of pH on nitrate-reducing activity in FRC groundwater. Since sediments at the FRC contain low pH groundwater, it may be necessary to stimulate nitrate-reducing activity at low pH. We tested the activity of isolates FW32AN1, FW33AN, FW32GN1,

Table 1. Putative identification of nitrate-reducing bacteria isolated from FRC groundwater based on 16S rDNA sequencing.

Isolate ID	Affiliation*	GenBank Accession number	Identity (%)
FW33AN	*Klebsiella pneumoniae*	AY043391	99
FW32AN1	*Pseudomonas* sp.	AJ387903	99
FW32AN2	*Alcaligenes defragans*	AJ005450	96
FW33GN1	*Pseudomonas* sp.	AJ387903	99
FW33GN2	*Citrobacter* sp.	AF025367	99
FW33GN3	*Sinorhizobium saheli*	X68390	95
FW32GN1	*Agrobacterium* sp.	AF508099	95
FW32GN2	*Agrobacterium* sp.	AF508099	98
FW32GN3	*Agrobacterium* sp.	AF508099	98

*Affiliations were determined based on closest GenBank match by BLAST search

and FW33GN2 in FW021 groundwater at various pHs. The acetate-oxidizing isolates FW32AN1 and FW33AN completely reduced nitrate at pH as low as 5.5 (Figure 1). Glucose-oxidizing isolates FW32GN1 and FW33GN2 would not reduce nitrate at pH below 6.2 (Figure 1). Due to its robust nitrate-reducing activity in FRC groundwater, FW33AN was chosen for further study.

Characterization of FW33AN. FW33AN was most closely related to Gly302-3B, a sequence recovered from an Fe(III)-reducing enrichment culture obtained from FRC sediments (Figure 2). These organisms were most closely related organisms to *Klebsiella* species (Figure 2). FW33AN coupled acetate oxidation to nitrate reduction with transient nitrite accumulation (Figure 3). No ammonium accumulation was observed during growth of FW33AN, suggesting that nitrate was denitrified, nitrite produced during nitrate reduction was reduced to N_2O, or the ammonium produced during nitrate reduction was assimilated. The hypothesis that nitrate was denitrified is unlikely, since denitrification has not been previously reported in enterobacteria (Guynot et al., 1998). Production of N_2O during nitrate reduction by a *Klebsiella* species has been previously reported (Bleakley and Tiedje, 1982), but only 30% of the nitrate in these experiments was converted to N_2O, with the remainder of the nitrate reduced to ammonium, making this hypothesis also appear unlikely to be occurring in our experiments. Pinar and Ramos (1998) showed that *Klebsiella oxytoca* may completely assimilate 40 mM NO_3^- provided as a terminal electron acceptor. This latter scenario is the most likely explanation for why ammonium accumulation was not observed in nitrate-reducing cultures of FW33AN, where the organism accumulates the ammonium produced by dissimilatory nitrate reduction to ammonium.

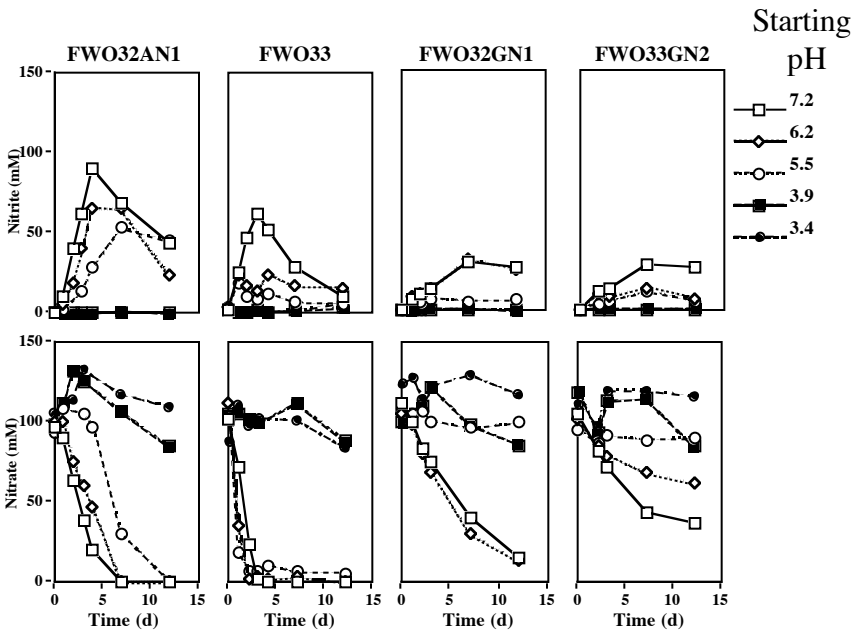

Figure 1. Nitrate reduction and nitrite accumulation by bacteria isolated from FRC groundwater at various pH in FW021 groundwater.

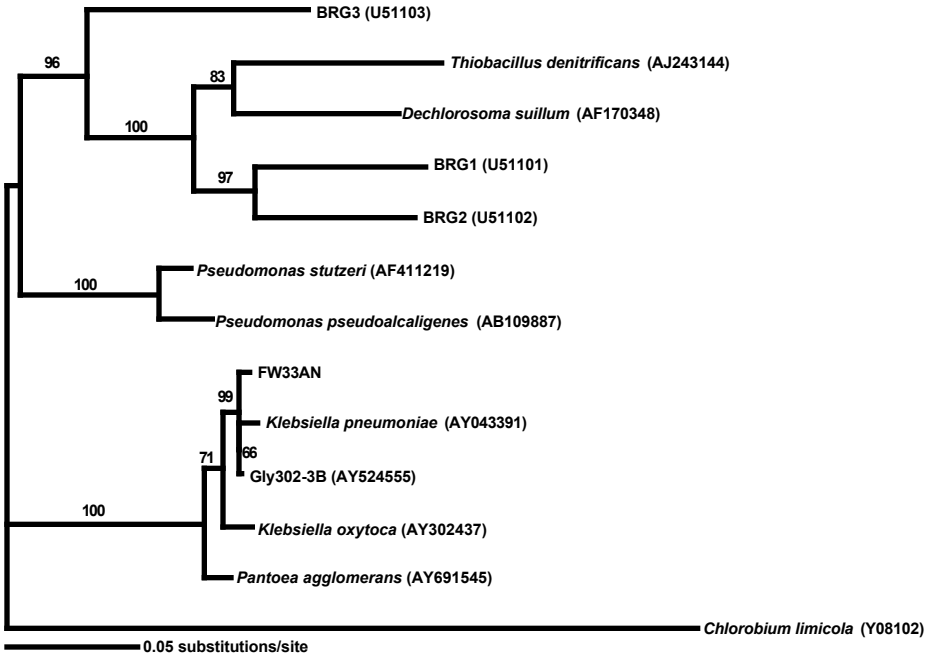

Figure 2. Distance dendrogram constructed using 16S sequence of FW33AN. Bootstrap values were determined on the basis of results for 1000 replicates and are shown for branches with more than 50% bootstrap support. Accession numbers are provided in parentheses.

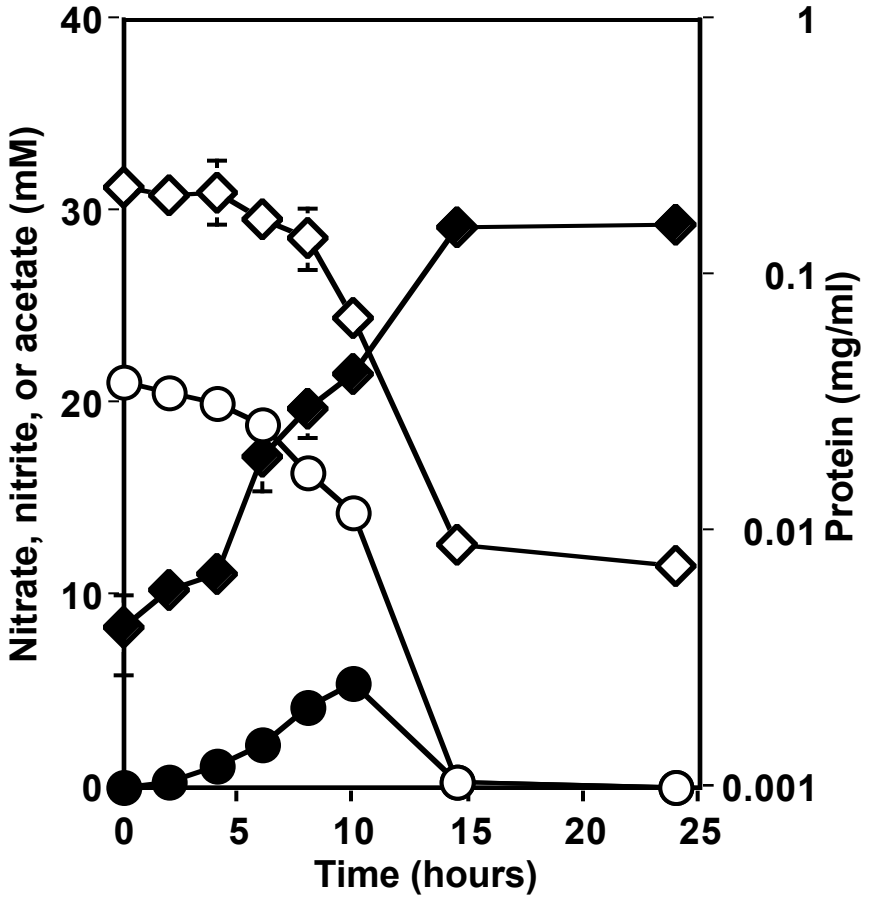

Figure 3. Growth (as indicated by protein concentration (◆)) of FW33AN with acetate (◇) consumption, nitrate (○) reduction, and transient nitrite (●) accumulation.

FW33AN has also been shown to be capable of nitrate-dependent Fe(II) oxidation, but is not closely related to other groups of organisms capable of nitrate-dependent Fe(II) oxidation (Figure 3), providing further evidence that the ability to couple Fe(II) oxidation to nitrate reduction is widespread among nitrate-reducing bacteria (Straub et al., 1996; Straub et al., 1998).

Protection of FW33AN from FRC groundwater toxicity by humic acids. FW33AN was incubated in unneutralized FW021 groundwater (pH 3.5) with various concentrations of FRC humic acids for 15 days. Nitrate reduction was only observed in neutralized groundwater but not in acidic groundwater, regardless of humic acid addition. However, live cells could be recovered from acidic groundwater amended with 500 and 1000 ppm humic acids (Figure 4). These results suggest that while low pH FRC groundwater is toxic to microorganisms, the presence of high amounts of humic acids protect organisms from the toxic effects of this groundwater. The reason for this is unclear, but it is possible that toxic metals (e.g. Ni or Al) are bound by the humic acids, thus limiting their bioavailability and mitigating their toxicity.

Nitrite tolerance of FW33AN. The high level of transient nitrite accumulation by FW33AN lead us to hypothesize that this organism has an unusually high tolerance for nitrite. We incubated FW33AN and *Pseudomonas stutzeri* with various concentrations of nitrite, and assessed the survival of these organisms after 7 days. While *P. stutzeri* was killed by nitrite concentrations of 50 mM or greater, FW33AN survived at nitrite concentrations as high as 100 mM (Figure 4). High levels of nitrite accumulation have been observed during nitrate reduction in FRC sediments, and organisms in these sediments may have unusually high tolerances for nitrite.

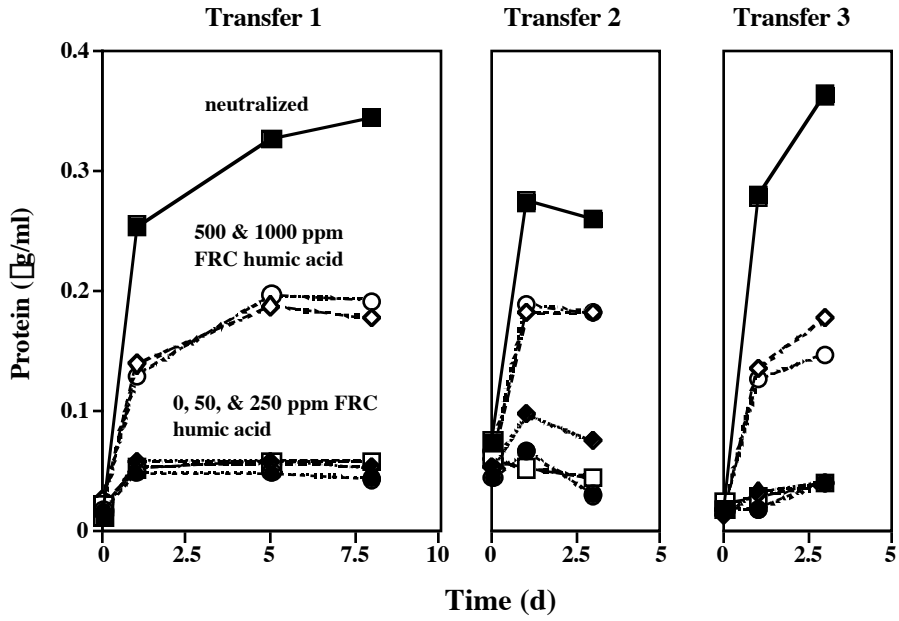

Figure 4. Growth of FW33AN through three transfers after incubation in neutralized FW021 groundwater (■), or acidic FW021 groundwater amended with 0 (□), 50 (●), 250 (◆), 500 (○), or 1000 (◇) ppm FRC humic acids.

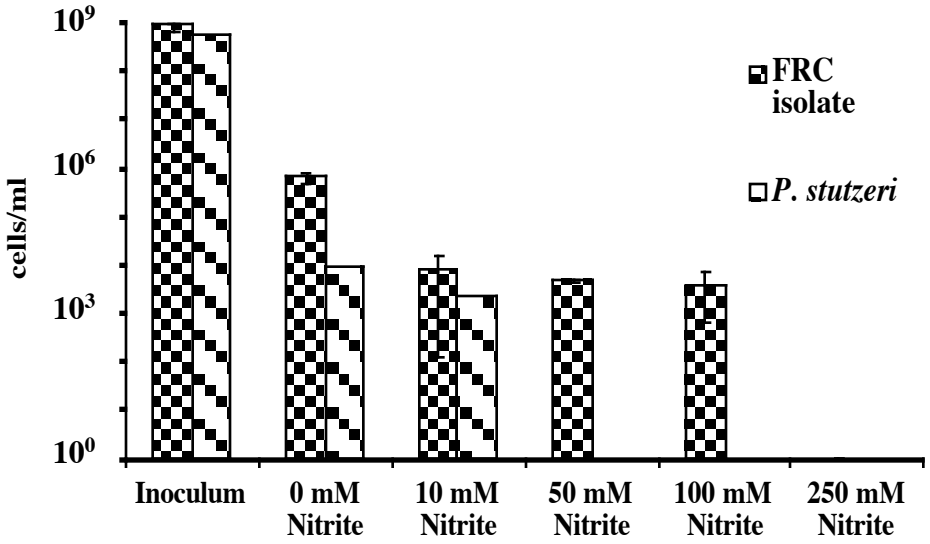

Figure 5. Nitrite tolerance of FW33AN and *Pseudomonas stutzeri* after incubation in bicarbonate buffer with various concentrations of nitrite.

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