FINAL REPORT

Factors Controlling *In Situ* Uranium and Technetium Bio-Reduction and Reoxidation at the NABIR Field Research Center

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RESEARCH GOAL AND HYPOTHESES

The overall goal of this project was to better understand factors and processes controlling microbially-mediated reduction and reoxidation of U and Tc in the unconsolidated residuum overlying the Nolichucky shale at the Field Research Center (FRC) at Oak Ridge National Laboratory. Project activities were designed to test the following hypotheses:

1. The small rates of denitrification and U bio-reduction observed in laboratory incubations of sediments from FRC Area 1 at low pH (< 5) are due to the presence of high concentrations of toxic metals (especially Al and Ni). Rates of Tc reduction will also be small at low pH in the presence of high concentrations of toxic metals.

2. In situ rates of U and perhaps Tc bio-reduction can be increased by increasing system pH and thus precipitating toxic metals from solution.

3. In situ rates of U and Tc bio-reduction can be increased by the addition of humic substances, which complex toxic metals such as Al and Ni, buffer pH, and serve as electron shuttles to facilitate U and Tc reduction.

4. Microbially-reduced U and Tc are rapidly oxidized in the presence of high concentrations of NO_3^- and the denitrification intermediates NO_2^-, N_2O, and NO.

5. An electron-donor-addition strategy (type and form of donor, with or without pH adjustment and with or without the co-addition of humic substances) can be devised to reduce U and Tc concentrations for an extended period of time in low pH groundwater in the presence of high concentrations of NO_3^-, Al, and Ni. This strategy operates by removing or complexing these components of FRC groundwater to allow the subsequent reduction of U(VI) and Tc(VII).

APPROACH

Hypotheses were addressed by an integrated series of experiments including (1) over 200 small-scale (~ 1 m3, 4-6 weeks duration) in situ (push-pull) tests conducted in over 40 monitoring wells in Areas 1 and 2; (2) four long-term (~ 20 months), intermediate-scale (~ 2 m long) column experiments; and (3) many small-scale (~ 50 g) laboratory microcosm experiments with site sediment and groundwater. Experimental data included contaminant and related geochemical measurements on sediment and groundwater as well as microbiological measurements of community composition and activity. Additional laboratory experiments were conducted at the Environmental Molecular Sciences Laboratory (EMSL) and additional specialized analyses of field and laboratory samples were conducted at EMSL, Pacific Northwest National Laboratory, Oak Ridge National Laboratory, and Argonne National Laboratory. Samples from field and laboratory experiments conducted for this project were also shared with several ERSP collaborators for additional analyses and for use in additional experiments.

SUMMARY OF RESULTS FROM FIELD TESTING

The push-pull test method proved to be extremely useful for rapidly detecting and quantifying the effects of electron donor addition on subsurface microbial activity at the FRC. Results demonstrated that indigenous microorganisms in the shallow (< 8 m) aquifer in Areas 1 and 2 are capable of coupling the oxidation of injected ethanol, acetate, or glucose to the reduction of U(VI) and Tc(VII). The identification of ethanol as a suitable electron donor for sites with high initial nitrate and sulfate concentrations was particularly important and ethanol has been used by many other researchers in field experiments at the FRC and in laboratory experiments using site sediments and/or groundwater. Despite highly variable conditions and contaminant concentrations (pH: 3.3-7.2; Nitrate: 0.1-140 mM; U(VI): 1-12 μM; Tc(VII): 200-15000 pM), sequential donor additions resulted in increased rates of microbial activity (Denitrification: .01-4.0 mM/hr; sulfate reduction: 0- 0.03 mM/hr; U(VI) reduction: 10^{-4} to 10^{-3} μM/hr; Tc(VII) reduction: 4-150 pM/hr) in all wells tested. **Push-pull tests conducted for this project provided the first in situ measurements of microbial activity, including rates of U(VI) and Tc(VII) reduction at the FRC or any other DOE site.**

Reoxidation of U(IV) but not Tc(IV) was also identified as an important biogeochemical process, especially at sites with high nitrate co-contamination. Field testing (confirmed by laboratory studies described below) suggest that U(IV) is likely oxidized by Fe(III) produced as microorganisms reduce nitrate. U(IV) reoxidation occurred during denitrification at rates (10^{-3} to 10^{-2} μM/hr) somewhat larger than subsequent U(VI) reduction rates indicating that continuous nitrate removal will be necessary to maintain the stability of U(IV) in this environment. **Push-pull tests provided the first in situ measurements of U(IV) reoxidation at the FRC.** The production of metal sulfides following addition of sulfate was shown to reduce U(IV) reoxidation rates in field tests conducted in Area 2.

Detailed information on vertical variations on porewater geochemistry are being obtained through the use of Multilevel Samplers (MLS) installed in sets of three wells in Areas 1 and 2. Tracer tests conducted in the MLS wells provided a quantitative description of the portion of the aquifer interrogated during push-pull tests. The MLS is currently being to investigate geochemical and microbial community changes resulting from donor additions in prepared sediments and pure mineral phases deployed in the MLS. Loss of hydraulic conductivity has been observed in field tests conducted with low pH, high nitrate groundwater but not with moderate pH, low nitrate groundwater. This is likely due to a combination of three factors: precipitation of dissolved solids, growth of biomass, generation of N_2 gas, and well-screen clogging. Acid treatment and scrubbing of wells has

partially restored hydraulic conductivity but values are still smaller than pre-test levels. Field tests were conducted in collaboration with Susan Hubbard (LBNL) in Areas 1 and 2 and confirmed that gas and precipitate production could be detected, quantified, and monitored using noninvasive geophysical methods. The effect of biomass and gas production on hydraulic conductivity of site sediments was also investigated in a series of laboratory experiments conducted in collaboration with Mart Oostrom at EMSL. A flow cell was packed with a mixture of FRC background sediment and crushed Maynardsville Limestone and microbial activity was stimulated by several additions of ethanol. Complete removal of ~ 100 mM nitrate was observed, which should have produced substantial quantities (> 1 L N_2/L water) of gas. However, measured gas saturations increase to only about ~10-20 % tiviand hydraulic conductivity was only slightly decreased, suggesting that most nitrogen gas leaves the porespace by flowing to the surface of the sediment pack. These results suggest that porespace clogging by nitrogen gas produced during denitrification may not be a major factor in reducing sediment hydraulic conductivity during field tests.

SUMMARY OF RESULTS FROM LONG-TERM COLUMN EXPERIMENTS

Collectively our project results suggest that bioimmobilization of Tc(VII) and U(VI) should be possible at the FRC using a permeable reactive barrier consisting of three defined zones (1) pH adjustment to precipitate Al and Ni, (2) denitrification and Tc(VII) reduction, and (3) U(VI) reduction. This hypothesis was tested in long-term (20 months) experiments conducted using intermediate-scale (2 m long) columns packed with uncontaminated site sediments deployed above-ground at Areas 1 and 2. The results of these experiments clearly indicate that essentially complete U(VI) and Tc(VII) removal can be achieved by stimulating microbial activity in a permeable reactive barrier.

For example at Area 2, nitrate, sulfate, U and Tc concentrations initially decreased along the length of both columns during the first two weeks of operation and this trend continued in the stimulated column for the remainder of the experiment. Concentrations decreased to a lesser extent in the control; eventually uranium and sulfate concentrations were ~ constant along the control column length. Methane (~1 mM), acetate (~ 2.8 mM) and propionate (~1.6 mM) were detected during a single sampling event, indicating methanogenisis and fermentation also occurred in the stimulated column. Mossbauer spectra collected from stimulated sediment samples indicated that iron reduction occurred and XANES spectra confirmed the presence of U(IV) in stimulated sediment and a single control sediment sample. During the 408 days of the experiment prior to sediment collection from the stimulated column, 1450 L of groundwater were passed through the column and 18.1 mol of EtOH, 0.77 μmol Tc, 6.0 mmol U, 0.94 mol nitrate, and 1.2 mol sulfate were removed. During the 290 days prior to sediment collection from the control column, 694 L of groundwater were passed through the column and 0.16 μmol Tc, 0.83 mmol U, and 0.45 mol nitrate were removed. No sulfate was removed in the control column. Sulfate reduction and nitrate reduction appeared to be the major terminal electron accepting processes in the stimulated and control columns, respectively.

Phospholipids fatty acid (PLFA) analysis showed both an increase in biomass (~2 orders of magnitude) and decreased ratios of cyclopropane to monoenoic precursor fatty acids in the stimulated column compared to the control, confirming electron donor limitation in the control. Spatial shifts in microbial community structure were confirmed by DGGE analysis as well as quantitative-PCR, which showed that *Geobacteraceae* increased significantly near the stimulated column outlet. Clone libraries of 16S rRNA genes from select flow path locations in the stimulated column showed that Proteobacteria sequences dominated near the inlet (46-52%) while sequences belonging to the candidate division OP11 were dominant near the outlet (67%), where electron acceptors were limited. Redundancy

analysis revealed a highly significant difference in stimulated and control community structure and confirmed correlations with geochemistry.

SUMMARY OF RESULTS FROM LABORATORY MICROCOSM STUDIES

Geochemical Controls on Microbial Nitrate-Dependent U(IV) Oxidation. 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 subsurface sediments. Nitrate-dependent U(IV)-oxidizing bacteria were at least two orders of magnitude less numerous in these sediments than glucose- 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 nitrate-dependent Fe(II)-oxidizing bacteria or by oxidation of Fe(II) by nitrite that accumulates during organotrophic dissimilatory nitrate reduction. 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. On the other hand, 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. 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.

A Role for Fe(III) Minerals in Nitrate-Dependent Microbial U(IV) Oxidation. 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 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 µM U(IV)/day, respectively). In aquifer sediments, Fe(III) may be produced during microbial nitrate-reduction by direct oxidation of Fe(II) by nitrite or by enzymatic Fe(II) oxidation coupled to nitrate reduction.

In order to determine which of these two 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 bioremediated aquifers.

The Effect of Oxidation Rate and Fe(II) State on Microbial Nitrate-Dependent Fe(III) Mineral Formation. A nitrate-dependent Fe(II)-oxidizing bacterium was isolated and used to evaluate whether Fe(II) chemical form or oxidation rate had an effect on the mineralogy of biogenic Fe(III) (hydr)oxides

resulting from nitrate-dependent Fe(II) oxidation. The isolate (designated FW33AN) had 99% 16S rRNA sequence similarity to *Klebsiella oxytoca.* FW33AN produced Fe(III) (hydr)oxides by oxidation of soluble Fe(II) ($Fe(II)_{sol}$) or FeS under nitrate-reducing conditions. Based on X-ray diffraction (XRD) analysis, Fe(III) (hydr)oxide produced by oxidation of FeS was shown to be amorphous, while oxidation of $Fe(II)_{sol}$ yielded goethite. The rate of Fe(II) oxidation was then manipulated by incubating various cell concentrations of FW33AN with $Fe(II)_{sol}$ and nitrate. Characterization of products revealed that as Fe(II) oxidation rates slowed, a stronger goethite signal was observed by XRD and a larger proportion of Fe(III) was in the crystalline fraction. Since the mineralogy of Fe(III) (hydr)oxides may control the extent of subsequent Fe(III) reduction, the variables we identify here may have an effect on the biogeochemical cycling of Fe in anoxic ecosystems.

Several denitrifying organisms have been cultured from Area 1 during biostimulation that are able to tolerate relatively low pH (4.5). These strains have been shown to be dominant denitrifying bacteria in biostimulated Area 1 sediments based on analysis of 16S rRNA and nitrite reductase (nirK) clone libraries. Additional support for these conclusions are provided by the results of microbial sampling by Aaron Peacock and David White at the University of Tennessee; Joel Kostka at the University of Florida, and by Chris Schadt at ORNL, which confirm that donor additions resulted in the creation of anaerobic conditions and the growth of metal reducing organisms. Also, production of Fe(II) in sediments was confirmed using variable temperature Mossbauer spectroscopy by Joe Stucki at the University of Illinois and the presence of U(IV) in biostimulated sediments was confirmed by Shelly Kelly, Ken Kemner, and Steven Heald at Argonne National Laboratory's Advanced Photon Source.

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