DOE-NABIR PI WORKSHOP:
Abstracts

April 18–20, 2005
Warrenton, Virginia

Natural and Accelerated Bioremediation Research Program

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Introduction
DOE–NABIR PI Workshop

April 18–20, 2005

The mission of the NABIR program is to provide the fundamental science that will serve as the basis for the development of cost-effective bioremediation and long-term stewardship of radionuclides and metals in the subsurface at DOE sites. The focus of the program is on strategies leading to long-term immobilization of contaminants in situ to reduce the risk to humans and the environment. Contaminants of special interest are uranium, technetium, plutonium, chromium, and mercury. The focus of the NABIR program is on the bioremediation of these contaminants in the subsurface below the root zone, including both vadose and saturated zones.

The program consists of four interrelated Science Elements (Biotransformation, Community Dynamics/Microbial Ecology, Biomolecular Science and Engineering, and Biogeochemistry). NABIR also has several elements that support, integrate, and cut across the science elements. The Integrative Studies area supports development of innovative approaches and technologies by integrating research from more than one NABIR program element. Assessment identifies areas in which the science is currently limited by lack of appropriate analytical or field-usable technologies, and provides a support for innovation and new methods. The program also funds field studies at the NABIR Field Research Center at Oak Ridge. An element called Bioremediation and its Societal Implications and Concerns (BASIC) addresses potential societal issues of implementing NABIR scientific findings. Also included in this book are abstracts on research in related fields in the DOE environmental science community, including critical research at DOE synchrotron facilities. The material presented at this year's workshop focuses on approximately 65 research projects funded in FY 2002–2005 by the Environmental Remediation Sciences Division in DOE's Office of Biological and Environmental Research (BER) in the Office of Science. Abstracts of NABIR research projects are provided in this book.

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Agenda
Annual NABIR PI Meeting
Warrenton, VA
April 17–20, 2005

Objective: To provide an annual update of research results, discuss significant research issues, and identify opportunities to interact with other research efforts and make use of new capabilities.

Sunday, April 17

All day Arrival of NABIR PIs, Co-PIs, NABIR program staff and guest speakers

Monday, April 18

7:00 AM **Breakfast** (all meals served at the Airlie Conference Center)
8:00 AM Welcome and Opening Remarks (Paul Bayer, NABIR Program Manager)
8:10 AM BER Programs (Ari Patrinos, Director, BER)
8:20 AM Environmental Remediation Sciences Division (ERSD) Update (Mike Kuperberg, Acting Director, ERSD)

Latest Findings on Anaerobic Metal/Radionuclide Reoxidation

8:50 AM Biogeochemical Coupling of Fe and Tc Speciation in Subsurface Sediments: Implications to Long-Term Tc Immobilization (John Zachara, PNNL)
9:15 AM Stabilization of Plutonium in Subsurface Environments via Microbial Reduction and Biofilm Formation (Mary Neu, LANL)
9:40 AM Reoxidation of Bioreduced Uranium under Reducing Conditions (Tetsu Tokunaga, LBNL)
10:05 AM Acceleration of Field-scale Bioreduction of U(VI) in a Shallow Alluvial Aquifer: Temporal and Spatial Evolution of Biogeochemistry (Phil Long, PNNL)
10:30 **Break**

Microbial Communities at Metal/Radionuclide Contaminated Field Sites

10:45 AM Linking Biodiversity to the Assessment of Bioremediation Potential in the Subsurface at DOE Sites (Joel Kostka, Florida State University)
11:10 AM Development and Use of Integrated Microarray-Based Genomic Technologies for Assessing Microbial Community Composition and Dynamics (Jizhong Zhou, ORNL)
11:35 AM Microbial Communities in the Vadose Zone (Fred Brockman, PNNL)
12:00 PM **Lunch**
2:00 PM ***Breakout Sessions***

1. What are the major research needs and approaches for evaluating reoxidation processes? (John Zachara and Jon Lloyd)
2. How distinct are microbial communities at different field sites? (Tony Palumbo and Fred Brockman)

5:00 PM **Dinner**
6:30 PM ***Poster Session: Biogeochemistry, Biotransformation, Assessment, Field Studies, Students***
8:30 PM **Adjourn**

Tuesday, April 19

7:00 AM **Breakfast**

8:00 AM Announcements and Other Logistics (Paul Bayer, ERSD)

DOE User Facility Resources

8:10 AM Overview of Synchrotron Resources for ERSD scientists (Roland Hirsch, BER)

8:15 AM Synchrotron-Based Research at the ALS, APS, NSLS, and SSRL (Ken Kemner, ANL)

8:45 AM Overview of Plans for Genomics: GTL Facilities (Dan Drell, BER)

Molecular and Genomics Techniques for Tracking Microbial Community Changes

9:00 AM Integrated Nucleic Acid System for In-Field Monitoring of Microbial Community Dynamics and Metabolic Activity (Darrell Chandler, ANL)

9:25 AM U(VI) Reduction Experiments and Environmental Genomics at the Old Rifle UMTRA Site (Derek Lovely, University of Massachusetts)

9:50 AM In Situ Community Control of the Stability of Bioreduced Uranium (David White, University of Tennessee)

10:15 AM **Break**

New Directions for Field-Based Research at DOE Sites

10:30 AM Current Opportunities and Future Research Directions at the FRC (Dave Watson, ORNL)

10:55 AM Characterization of Coupled Hydrologic-Biogeochemical Processes using Geophysical Data (Ken Williams, LBNL)

11:20 AM Coprecipitation of Trace Metals in Groundwater and Vadose Zone Calcite: In Situ Containment and Stabilization of Strontium-90 and Other Divalent Metals and Radionuclides at Arid Western DOE Sites (Robert Smith, University of Idaho)

11:45 AM Subsurface Flow and Transport Modeling Research: Incorporating Biologically Mediated Processes (Steve Yabusaki, PNNL)

12:10 PM **Lunch**

2:00 PM ***Breakout Sessions***

1. What changes in microbial community structure can be expected during and after biostimulation? (Terry Hazen and Lee Krumholz)
2. Building on NABIR research: What are the next field research challenges? (Derek Lovley and Susan Hubbard)

5:00 PM **Dinner**

6:30 PM ***Poster Session: Community Dynamics, Biomolecular, Integrative Studies, BASIC, Others***

8:30 PM **Adjourn**

Wednesday, April 20

7:00 AM	**Breakfast**
8:00 AM	Announcements and Other Logistics (Paul Bayer, ERSD)

Electron Transfer to Microbial Reduction

8:10 AM	Composition, Reactivity, and Regulation of Extracellular Metal-Reducing Structures Produced by Dissimilatory Metal-Reducing Bacteria (Yuri Gorby, PNNL)
8:35 AM	*Geobacter* Electron Transfer Mechanisms (Gemma Reguera, University of Massachusetts)
9:00 AM	Update on the Biogeochemistry Grand Challenge at the Environmental Molecular Sciences Laboratory (EMSL) (Jim Fredrickson, PNNL)
9:25 AM	Dissimilatory Metal Reduction by *Anaeromyxobacter* Species (Frank Löffler, Georgia Tech)
9:50 AM	Parallel Proteomic Identification of Metal Reductases and Determination of their Relative Abundance in a Series of Metal-Reducing Bacteria (Mary Lipton, PNNL)
10:15 AM	**Break**
10:30 AM	Comparative Biochemistry and Physiology of Iron-Respiring Bacteria from Acidic and Neutral pH Environments (Tim Magnuson, Idaho State University)
10:55 AM	Field-Integrated Studies of Long-Term Sustainability of Chromium Bioreduction at the Hanford 100H Site (Terry Hazen, LBNL)
11:20 AM	ERSD Programs—Questions and Answers (Mike Kuperberg, ERSD)
11:50 PM	Breakout Session Summary Presentations (Breakout group leads)
12:30 PM	**Adjourn**
1:30 PM	UMTRA Group and FRRP Meetings
5:00 PM	**All meetings adjourn**

ABSTRACTS

PROGRAM ELEMENT 1
Biotransformation

Anaerobic Biotransformation and Mobility of Pu and of Pu-EDTA

Harvey Bolton Jr.[1] (PI), Dhanpat Rai[1], and Luying Xun[2]

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The complexation of radionuclides—e.g., plutonium (Pu) and ^{60}Co—by co-disposed ethylenediaminetetraacetate (EDTA) has enhanced their transport in sediments at DOE sites. Our previous NABIR research investigated the aerobic biodegradation and biogeochemistry of Pu(IV)-EDTA. Plutonium(IV) forms stable complexes with EDTA under aerobic conditions and an aerobic EDTA-degrading bacterium can degrade EDTA in the presence of Pu and decrease Pu mobility. However, our recent studies indicate that while Pu(IV)-EDTA is stable in simple aqueous systems, it is not stable in the presence of relatively soluble Fe(III) compounds—i.e., $Fe(OH)_3(s)$, a 2-line ferrihydrite. Since most DOE sites have Fe(III)-containing sediments, it is likely that Pu(IV) is not the mobile form of Pu-EDTA in groundwater. The only other Pu-EDTA complex stable in groundwater relevant to DOE sites would be Pu(III)-EDTA, which only forms under anaerobic conditions. Research is therefore needed in this brand new project to investigate the biotransformation of Pu and Pu-EDTA under anaerobic conditions. The biotransformation of Pu and Pu-EDTA under various anaerobic regimes is poorly understood. Areas needing better understanding include the reduction kinetics of Pu(IV) to Pu(III) from soluble Pu(IV) [Pu(IV)-EDTA] and insoluble Pu(IV) [PuO_2(am)] by metal-reducing bacteria; the redox conditions required for this reduction; the strength of the Pu(III)-EDTA complex; how the Pu(III)-EDTA complex competes with other dominant anoxic soluble metals [e.g., Fe(II)]; and the oxidation kinetics of Pu(III)-EDTA. Finally, the formation of a stable soluble Pu(III)-EDTA complex under anaerobic conditions would require degradation of the EDTA complex to limit Pu(III) transport in geologic environments. Anaerobic EDTA-degrading microorganisms have not been isolated. These knowledge gaps preclude the development of a mechanistic understanding of how anaerobic conditions will influence Pu and Pu-EDTA fate and transport to assess, model, and design approaches to stop Pu transport in groundwater at DOE sites.

Completed Genome Sequence of *Dechloromonas aromatica*: Analysis of a Microbe with Diverse Bioremediative Capability in Anaerobic Environments

J.D. Coates, K. V. Kellaris, W. S. Feil, H. Feil, L.A. Achenbach, R. Chakraborty, C. Shang, E. Alm, K. Huang, M. Price, K. Keller, S. Trong, G. Di Bartolo, A. Arkin, A. Lapidus, P. M. Richardson, F. W. Larimer, and S.M. O'Connor

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Our previous NABIR-funded studies using pure culture members of the *Dechloromonas* and closely related *Dechlorosoma* genera have demonstrated that radionuclides such as uranium and cobalt are rapidly removed (as much as 80% of the initial 100 μM within 5 days) from solution during the biogenic formation of Fe(III)-oxides resulting from anaerobic nitrate-dependent microbial Fe(II) oxidation. In the case of uranium, x-ray diffraction analysis indicated that the uranium was in the hexavalent form (normally soluble) and was bound to the precipitated Fe(III)-oxides, thus demonstrating the potential of this process. These studies indicate great promise for providing a long-term solution to heavy metal and radionuclide contamination in the environment. However, to date, nothing is known of the underlying molecular mechanisms or regulatory processes involved in the anaerobic oxidation of Fe(II) by microorganisms. *Dechloromonas aromatica* strain RCB is a β-Proteobacterium found ubiquitously in soil and sediment environments. It is a motile facultative anaerobe, capable of nitrate-dependent Fe(II) oxidation, anaerobic aromatic hydrocarbon degradation, and dissimilatory perchlorate reduction. It was first isolated from Potomac River sediments based on its ability to anaerobically metabolize 4-chlorobenzoate coupled to perchlorate reduction. With its metabolic versatility, *D. aromatica* strain RCB was selected for complete genome sequencing at the DOE Joint Genome Institute, Walnut Creek, CA. The completed sequence consists of a single circular chromosomal DNA structure containing a total of 4.5 Mb of DNA with a G+C content of 60%. Initial draft annotation was conducted at Oak Ridge National Laboratory and the Virtual Institute for Microbial Stress and Survival (http://escalante.lbl.gov), identifying approximately 4,000 open reading frames (ORFs). The closest relative with a completed genome sequence is *Ralstonia solanacearum*, also a gram-negative β-Proteobacterium. *D. aromatica* and *Ralstonia* possess very similar genomic content, duplicative 23S and 16S RNA regions, and identical operon structures for many families of genes. In *Ralstonia*, many catabolic capabilities are thought to be concentrated within plasmid DNA structures. However, *D. aromatica* lacks a large genomic plasmid element (the *Ralstonia* pGMI1000MP plasmid is 2.09 Mb in size), which indicates its metabolic versatility is not dependent on a highly transmissible and mutable plasmid structure. *D. aromatica* genes have been annotated for InterPro domains, EC assignments, TIGRfams, KEGGS pathways, and GO ontologies. Genes that likely reflect the versatility of *D. aromatica* within the environment include a remarkably high number of two-component sensors and regulators (11% of the annotated ORFs), placing it in the top 5% of microbial genomes in this capability. The *D. aromatica* genome has been found to contain RuBisCo, indicating the ability to fix carbon dioxide; however, to date the environmental conditions required for the expression of this phenotype have not been identified.

The availability of the completed and partially annotated genome sequence provides us the opportunity to design and utilize a custom *D. aromatica* microarray to identify genes that are up-regulated under Fe(II)-oxidizing conditions and, thus, presumably involved in this anaerobic metabolism.

Anaerobic Nitrate-Dependent Metal Bio-Oxidation Associated with Freshwater Surface and Subsurface Sediments

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Our previous studies demonstrated that nitrate-dependent bio-oxidation of Fe(II) by *Azospira suillium* strain PS results in the formation of crystalline mixed Fe(II)/Fe(III) mineral phases that subsequently immobilize heavy metals and radionuclides. Greater than 80% of the added 100 μM U(VI) was sequestered by the most dense, crystalline Fe(II)/Fe(III) mineral phases, which are not readily reduced by Fe(III)-reducing bacteria. Similarly to *A. suillium* strain PS, a novel autotrophic, nitrate-dependent Fe(II)-oxidizing bacterium, *Cosmobacter millennium* strain 2002, yields a mixed phase Fe(II)/Fe(III) mineral phase, identified as green rust by Mössbauer spectroscopy and x-ray diffraction. Green rust (GR), a mixed valence, layered Fe(II)/Fe(III) hydroxide with anion interlayers, is abundant in hydropmorphic soils and sediments. Recent studies suggested that both Fe(II)-oxidizing and Fe(III)-reducing bacteria play a role in GR formation; however, the extent of either process is unknown. In the case of Fe(III)-reduction, GR is transiently produced and subsequently transformed into magnetite. Anaerobic nitrate-dependent oxidation of Fe(II) provides an alternative mechanism of GR formation. In contrast to the GR biogenically formed by Fe(III) reduction, the biogenic GR product formed via nitrate-dependent Fe(II) oxidation by *C. millennium* strain 2002 did not yield transformation products, i.e., magnetite. X-ray fluorescence spectroscopy identified chloride and phosphate in association with the GR product. Furthermore, anion analysis of the GR by ion chromatography indicated that 6 mg SO_4^{2-}, 32 mg Cl^-, and 66 mg PO_4^{3-} was associated with the oxidation of 111 mg Fe(II). These results suggest that GR(Cl^-) was predominantly formed. It is currently unknown whether phosphate is adsorbed to the GR surface or intercalated into the interlayer. Given that nitrate-dependent Fe(II)-oxidizing bacteria are ubiquitous—identified by the most probable number enumeration as being high as 1.47×10^4 cells (g sediment)$^{-1}$ in freshwater lake sediment, 2.04×10^3 cells mL^{-1} in groundwater (NABIR FRC site), and 1.17×10^3 cells (g sediment)$^{-1}$ in subsurface sediment—it is likely that this metabolism is active in soils and sedimentary environments. These results represent the first demonstration of the biogenic formation of green rust in significant quantities, providing strong evidence for the biological mechanism for the production of GR(Cl^-) in soils and sediments.

Display and Retraction of Outer Membrane Cytochromes by *Shewanella oneidensis* in Response to Electron Acceptor Availability

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Morphological and compositional analyses of the surface of Shewanella oneidensis strain MR-1 were conducted and compared under conditions of electron acceptor limitation and electron acceptor repletion. Atomic force microscopy (AFM) reveals that cells display nanoscale domains that protrude through the outer membrane and bristle the bacterial surface when availability of electron acceptor is limited. These protrusions disappear within seconds from cell surfaces after electron acceptor limitation is relieved by adding oxygen, nitrate, fumarate, or chelated forms of ferric iron. Mutants of MR-1 that do not produce outer membrane secretin-forming protein (GspD) associated with the type II secreton (T2S) system do not produce protrusions under either electron acceptor-limited or excess conditions. Surface-enhanced Raman spectroscopy (SERS), a technique that selectively amplifies Raman vibrational signals from molecules located within a few angstroms of thin films composed of elemental silver or gold, revealed that heme-containing proteins (cytochromes) are selectively exposed on the surface of wild type MR-1 cells in response to acceptor limitation. Surface-exposed heme was not detected under either electron-acceptor-limited or excess conditions for a mutant lacking the GspD secretin. Correlation of data from AFM and SERS supports our hypothesis that cytochromes, possibly those previously implicated in solid phase iron and manganese oxide reduction, are substrates of the T2S and may form a functional protein complex that extends and retracts through the outer membrane in direct response to electron acceptor availability. A physiology-based model describing the control and distribution of redox-reactive heme proteins on the MR-1 cell surface is presented.

Effect of Microbial Exopolymers on the Spatial Distributions and Transformations of Cr and U at the Bacteria-Geosurface Interface

Ken Kemner[1], Shelly Kelly[1] , Ed O'Loughlin[1], Max Boyanov[1], Ken Nealson[2], Susan Glasauer[3], Terry Beveridge[3], Barry Lai[1], Joerg Mase[1], Zhonghou Cai[1]

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Bacteria and the extracellular material associated with them are thought to play a key role in determining a contaminant's speciation and thus its mobility in the environment. In addition, the metabolism and surface properties of bacteria can be quite different when the bacteria exhibit a planktonic (free-floating) versus a biofilm (surface-adhered) habit. The microenvironment at and adjacent to actively metabolizing cells also can be significantly different from the bulk environment. Thus, to understand the microscopic physical, geological, chemical, and biological interfaces that contribute to a contaminant's macroscopic fate, we must characterize, at micron and submicron length scales, the spatial distribution and chemical speciation of contaminants and elements that are key to biological processes. Hard x-ray microimaging and microspectroscopy are powerful techniques for highly sensitive element-specific investigations of complex environmental samples at the needed micron and submicron resolutions. Moreover, the greater spatial resolution of electron microscopy enables investigations of geomicrobial systems beyond those afforded by x-ray microprobes.

The objectives of the studies presented here are to (1) determine the spatial distribution, concentration, and chemical speciation of metals at, in, and near bacteria; (2) use this information to identify the metabolic and/or chemical processes occurring within the cells; and (3) identify the interactions occurring near the interfaces containing the metals, bacteria, minerals, and bacterially produced extracellular materials, under a variety of conditions.

We have used x-ray fluorescence micro(spectro)scopy to investigate the spatial distribution of elements in *Pseudomonas fluorescens* in free-floating (i.e., planktonic) and surface-adhered states and the valence state of Cr associated with these cells after exposure to two different levels of Cr(VI) (25 ppm and 1,000 ppm for ~6 h) [1]. We also have used electron microscopy and x-ray micro(spectro)scopy to investigate previously identified [2] internal precipitates rich in biomineralized Fe in *Shewanella putrefaciens* CN32 cells. Results from x-ray fluorescence imaging experiments with *P. fluorescens* indicate that the distribution of P, S, Cl, Ca, Fe, Ni, Cu, and Zn can define the location of cells. Quantitative analysis of the elemental concentrations within cells can indicate their viability and metabolic state. X-ray absorption near edge spectroscopy (XANES) investigations of the Cr valence state indicated two different modes of abiotic chemical reduction of the element (depending on the microbe's physiological state). Fe K-edge XANES measurements of internal and external precipitates in the *S. putrefaciens* CN32 cells identified different valence states of the precipitates. These results will be presented, with a discussion of the use of combined x-ray and electron micro(spectro)scopies.

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1. *Science* **306** 686–687, 2004.

2. *Science* **295** 117–119, 2002.

Influence of Mass Transfer on Bioavailability and Kinetic Rate of Uranium(VI) Biotransformation

Chongxuan Liu, Zheming Wang, John M. Zachara, and James K. Fredrickson

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The bioavailability and bioreduction rate of metals and radionuclides associated with intraparticle regions of porous media will be constrained by mass transfer processes in long-term contaminated sediments. Recent characterization of U(VI) speciation and physical location in 30-year contaminated Hanford Site sediments demonstrated that U(VI) primarily resides as a U(VI) microprecipitate in small fractures, cleavages, or dead-end voids within sediment particle grains exhibiting pore sizes of a few microns or less. The U(VI) microprecipitates dissolved into undersaturated pore water, but the rate of dissolution and transport out of intraparticle regions was limited by diffusive processes. These results indicated that most of the sorbed U(VI) pool was not physically accessible to metal-reducing bacteria due to size restrictions of the grain porosity, and the overall kinetic rates and extent of microbial U(VI) reduction under such conditions would be limited by the bioavailability and mass transfer rates of U(VI) out of intraparticle regions. The objectives of this project are to: (1) develop approaches to characterize microscopic properties of mass transfer processes; (2) identify and characterize biogeochemical strategies for accessing intraparticle U(VI) by metal-reducing bacteria; (3) evaluate the influence of mass transfer on U(VI) bioavailability and microbiologic reduction rates; and (4) develop coupled kinetic models of the U(VI) dissolution, mass transfer processes, and microbially mediated U(VI) reduction.

To characterize microscopic properties of mass transfer processes, we have developed a nuclear magnetic resonance (NMR) imaging and spectroscopic technique for measurements of intraparticle ion diffusivity and tortuosity. By applying the technique to the particle grains representative of U(VI)-bearing particles from the Hanford vadose zone sediment, we have identified at least two ion diffusion regions having diffusivity values that differ by about two orders of magnitude. The results indicated that a dual porosity model is needed to describe U(VI) mass transfer processes, and a triple porosity model is required to describe U(VI) transport in subsurface sediments that couple the intraparticle mass transfer with reactive transport in intergranular regions at this site.

A coupled theory of nonequilibrium thermodynamics and electrodynamics has been developed that allows us to calculate ion diffusivity in pores at the micron scale. This theory allows us, for the first time, to explicitly incorporate fixed charges and electrostatic double layers into ion diffusion models. We can therefore predict that ion diffusivity is a function of aqueous ion composition, ionic strength, and solid surface electrostatic properties. Some of these findings have now been confirmed by experiments.

To better understand the influence of mass transfer processes on the bioavailability and biotransformation rate, we have synthesized porous alginated beads containing intraparticle solid phase U(VI) (Na-boltwoodite, which has the same U(VI) properties observed in the contaminated Hanford sediments). Studies with the U(VI)-containing particles indicated that U(VI) diffusion limits the rates of intraparticle U(VI) dissolution and microbial reduction. In the case of *Shewanella putrefaciens* strain CN32, U(VI) has to be first dissolved and diffused out of intraparticle regions before it can be assessed and reduced by the bacteria.

Dissimilatory Metal Reduction by *Anaeromyxobacter* Species

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Anaeromyxobacter populations grow by coupling acetate and hydrogen oxidation to the reduction of a variety of electron acceptors, including halophenols, nitrate, and oxygen, as well as soluble and amorphous forms of ferric iron. In addition, evidence from 16S rRNA gene-based analyses from uranium-contaminated sites implicated *Anaeromyxobacter* species in U(VI) reduction and immobilization. To verify and further explore metal and U(VI) reduction in *Anaeromyxobacter* species, pure culture studies with *Anaeromyxobacter dehalogenans* strain 2CP-C were performed in a defined, reduced basal salts medium with acetate and hydrogen as electron donors. Resting cell experiments confirmed previous experiments where we have shown that this organism reduces soluble U(VI) to insoluble U(IV). In growth experiments, U(VI) was completely reduced to uranium (IV) after 60 days. Quantitative real-time PCR targeting the *Anaeromyxobacter* 16S rRNA gene is being done, and the preliminary data analysis indicates that U(VI) reduction is a growth-linked process. Strain 2CP-C also grew with MnO_2, with 1.3 mM MnO_2 completely reduced within 4 days of incubation. Metal reduction continued after repeated feedings of additional MnO_2. In contrast, no reduction of 1 mM chromate ($K_2Cr_2O_7$) was observed, even after one month of incubation. These results distinguish *Anaeromyxobacter* from other metal-reducing species such as *Geobacter metalreducens* and *Geobacter sulfurreducens*. Current efforts explore the effects of alternate electron acceptors on U(VI) reduction by strain 2CP-C. Further, comparative studies will investigate metal and radionuclide reduction in different *Anaeromyxobacter* strains and compare rates and yields with other metal- and radionuclide-reducing bacterial isolates.

Biotransformations Involved in Sustained Reductive Removal of Uranium in Contaminated Aquifers

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Previous field studies on in situ uranium bioremediation have focused on the addition of organic electron donors to stimulate the activity of dissimilatory metal reducers. A field study at the NABIR study site in Rifle, CO, further confirmed that this approach can successfully precipitate uranium from contaminated groundwater and that this process can result in long-term immobilization of uranium. However, when acetate is added as the electron donor, a substantial portion of the acetate is wasted as Fe(III) becomes depleted near the injection gallery. This is because sulfate reducers can then compete for the electron donor and, unlike *Geobacter* species, acetate-oxidizing sulfate reducers are ineffective in reducing U(VI). In an effort to overcome this limitation, additional laboratory studies were conducted with sediments from the Rifle site. Previous studies have demonstrated that these laboratory studies, which are carried out under in situ conditions, can accurately predict the outcome of field experiments. Lactate was added as an electron donor with the idea that this might stimulate the growth of lactate-oxidizing, sulfate reducers such as *Desulfovibrio* species, which our previous studies have shown are effective U(VI) reducers. However, the addition of lactate was a less effective stimulant of U(VI) reduction than acetate during the Fe(III) reduction phase of the experiment when *Geobacter* species were prevalent, and no *Desulfovibrio* could be detected during the sulfate reduction phase when there was little or no U(VI) reduction. These results suggest that, although *Desulfovibrio* species can readily reduce U(VI) in laboratory cultures, it is difficult to enrich for *Desulfovibrio* in field experiments. Thus, the potential for using *Desulfovibrio* species as an agent for in situ uranium bioremediation seems limited.

In order to alleviate the waste of electron donor consumption by sulfate reducers and possibly avoid other potential problems, such as sulfide production associated with sulfate reduction, a search was undertaken for an electron donor that might be exclusively used by *Geobacter* species but not sulfate reducers. Our previous studies have demonstrated that *Geobacter* species can accept electrons from electrodes poised at the appropriate potential, but it is unlikely that sulfate reducers can use an electrode as an electron donor. In sterile systems, electrodes poised at –500 mV (vs. Ag/AgCl reference) rapidly removed U(VI) from solution. However, when these poised electrodes were removed, all U(VI) returned to solution, demonstrating that the electrodes did not reduce the uranium. However, if *G. sulfurreducens* was present on the electrodes, U(VI) did not return to solution until the uranium was oxidized by bubbling with air. This finding suggests that *G. sulfurreducens* respiring at the electrode surface reduced the U(VI). Batch studies with contaminated sediment and groundwater demonstrated that poised electrodes removed U(VI) from solution and that the removal was dependent on the poised potential at the working electrode. Column studies demonstrated that electrodes placed in a reactive barrier setting removed uranium from contaminated groundwater and this uranium could be recovered by extracting the electrodes. These results suggest an alternative strategy for providing an electron donor for in situ uranium bioremediation, which will be evaluated in the field in 2005.

Investigating In Situ Bioremediation Approaches for Sustained Uranium Immobilization Independent of Nitrate Reduction

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Uranium and radionuclide contamination at DOE facilities frequently occurs with nitrate. Current bioremediation strategies reduce nitrate/nitrite before reducing U and metals. While consistent with traditional thinking, this approach is problematic within the geochemical environment of DOE wastes, where nitrate may persist for centuries. The goal of this research is to investigate bioremediation strategies involving the addition of specific energy sources and nutrients, such as propane, hydrogen, methanol and triethylphosphate, that can effectively immobilize metals and radionuclides without requiring complete nitrate removal. Such strategies would be compatible with the current FRC Area 1 environmental constraints of high nitrate, low permeability, and low pH. We hypothesize that if even a fraction of the microbial reducing potential can be directed towards the reduction of U and metals, the reduced species can be stable in anaerobic waters, making nitrate/nitrite removal unnecessary. Our initial objective is to demonstrate that the stringent removal of oxygen from groundwater is a key for U(IV) stability rather than removal of nitrate/nitrite. Experiments to date suggest that stringently deoxygenated waters containing uranium plus mM concentrations of nitrate are stable for a month or longer, whereas aerobic or moderately anaerobic suspensions of nitrate result in uranium oxidation within hours to days. Accordingly, transient influxes of nitrate may exhibit little change in the status of reduced metals so long as oxygen is rapidly eliminated and metal-reducing conditions reestablished. A second objective is to stimulate extant microorganisms to reduce metals in microcosm experiments with high nitrate and low pH. Enrichments are in progress, and growth is evidenced. Our final objective is to develop supporting approaches minimizing impacts of nitrate reduction that might facilitate U(IV) reoxidation and mobilization. Evidence that a small percentage of the potential bioreduction capacity within microcosms can be directed toward U reduction and its sustained immobilization would constitute success. Through this microcosm-based laboratory research, we will investigate innovative biotransformation strategies that can provide long-term stability of immobilized metals and radionuclides without requiring nitrate removal. This research will also enhance our understanding of interrelationships between biological nitrate and metal reduction in low pH environments.

Promoting Uranium Immobilization by the Activities of Microbial Phosphatases

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The goal of this project is to examine the role of nonspecific phosphohydrolases in naturally occurring subsurface microorganisms for the purpose of promoting the immobilization of radionuclides through the production of phosphate precipitates. This project will focus on uranium (U), a radionuclide that poses significant risk to human health and the environment. Nonspecific acid phosphohydrolases are a broad group of secreted microbial phosphatases that function in acidic-to-neutral pH ranges and utilize a wide variety of organophosphoester substrates. We hypothesize that subsurface microorganisms that exhibit (acid) phosphatase activity and are resistant to heavy metals have the potential to immobilize U via a biomineralization process. Biomineralization, defined as the immobilization of an element by nonredox microbial precipitation, could prove to be a feasible alternative or complementing remediation approach to U bioreduction and adsorption processes. The primary objective is to demonstrate that the intrinsic phosphatase activities of indigenous subsurface microbes result in the release/accumulation of sufficient PO_4^{3-} to cause the formation and precipitation of low solubility U-phosphate minerals in oxygenated groundwater and soil. We will examine three critical hypotheses: (1) acid phosphatases of subsurface microbes provide resistance to heavy metals, and this phosphatase-mediated resistance trait has been disseminated in subsurface populations by lateral gene transfer; (2) phosphatase activity of these subsurface microbes will promote U immobilization by formation of insoluble U-phosphate precipitates; and (3) subsurface geochemical parameters (pH, nitrate) will affect phosphate mineral formation by altering microbial phosphatase activity and/or affecting the stability of the metal phosphate precipitates. A three-step experimental approach is being conducted to examine the relationship between the occurrence of microbial phosphatases and metal resistance among the DOE Field Research Center (FRC) strains we are currently studying, along with the contribution of lateral gene transfer in the dissemination of these beneficial phosphatase genes. Our preliminary testing of a subset of isolates cultured from contaminated FRC soils and background (reference) soils indicate a higher percentage of FRC isolates exhibiting phosphatase phenotypes (i.e., in particular those we surmise to be PO_4^{3-}-irrepressible) when compared to isolates from the background reference site. A high percentage of strains exhibiting such putatively PO_4^{3-}-irrepressible phosphatase phenotypes was also shown to be resistant to lead as well as cadmium. We have designed PCR primer sets for amplification of classes A, B, and C acid phosphatases from the FRC subsurface isolates.

PROGRAM ELEMENT 2

Community Dynamics/ Microbial Ecology

Microbial Pathways for the Reduction of Mercury in Saturated Subsurface Sediments

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Mercury in contaminated subsurface soils may be leached to the saturated zone where its reduction to the elemental form, Hg(0), may enhance its environmental mobilization. Therefore, microbial transformations that reduce the mercuric ion, Hg(II), to Hg(0) are of key importance to the remediation of mercury in contaminated subsurface sediments. Microbes may reduce Hg(II) by the activity of the inducible mercuric reductase (MR), which is the gene product of *merA*. This process is well understood in a broad range of aerobic bacteria and environments. However, little is known about Hg(II) reduction in anoxic environments. We therefore have initiated a study on the presence of *merA* in microbial communities of anoxic environments and the effect of anaerobic respiratory pathways on MR expression and activities. PCR primers were designed to span the known phylogenetic range of *merA* genes of gram-negative bacteria. In control experiments, these primers successfully amplified a 288 bp region at the 3′ end of previously characterized *merA* genes from *Shewanella putrefaciens* pMERPH, *Acidithiobacillus ferrooxidans*, *Pseudomonas stutzeri* pPB, Tn5041, *Pseudomonas* sp. K-62, and *Serratia marcescens* pDU1358. The abundance and diversity of *merA* were assessed in anaerobic enrichments from Berry's Creek, a highly industrially contaminated site in the Meadowlands, NJ, by sequencing a *merA* clone library obtained by PCR from sediment DNA extracts. Anaerobic sediment slurries were supplemented with two additions of 10 µg Hg(II)/g and incubated for three weeks, at which time the DNA was isolated and PCR amplified. The amplicons were TOPO cloned into pCR2.1 (Invitrogen) and sequenced. A total of 79 clones were sequenced, of which 69 represented unique amplicons. The sequences were aligned and a phylogenetic tree was built. While many sequences aligned with previously described *merA* genes from both gram-positive and gram-negative isolates, five novel lineages (I through V), were identified. These results reveal a previously unrecognized diversity of *merA* and suggest that bacterial activities may play a role in mercury reduction in anaerobic environments.

Anaerobic enrichments of Meadowlands sediments were set up for the purpose of isolating pure cultures carrying novel *merA* genes. Five oligoprobes specific for each of the novel *merA* clusters were designed, tested, and hybridized with genomic DNA of Hg(II)-resistant isolates from the enrichments. Three strains, a *Bacillus* sp. and a *Streptomyces* sp. with *merA* of cluster V, and a *Pseudomonas* sp. with *merA* of cluster II, were isolated from the fermentative enrichment. One denitrifying isolate, a *Paenibacillus* sp., carried a cluster III *merA*. The entire mercury-resistant system in these strains is currently being examined genetically and biochemically to fully characterize the mechanisms by which anaerobic bacteria interact with mercury. Similar studies are being set up with subsurface sediments from mercury contaminated and control aquifers.

Natural Gene Transfer to Develop Resistance to Metal Toxicity in Microbial Communities at the Oak Ridge Reservation Field Research Center

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Our research addresses the need to understand how natural gene transfer can be used to help naturally occurring microbial communities adopt resistance to specific environmental stresses such as heavy metals that inhibit their ability to reduce and immobilize metals and radionuclides. Nickel is being used as a model system to demonstrate how a metal resistance marker can be introduced into both single species and naturally occurring microbial communities in contaminated sediments collected from the Oak Ridge Reservation Field Research Center (ORR FRC). The overall objective of this work is to demonstrate the feasibility of applying natural gene transfer to improve the performance of natural microbial communities under conditions imposed by metal stress, using Ni toxicity and resistance as a model system.

Using natural gene transfer, the nickel resistance operon (*ncc-nre*) was introduced in the following nitrate-reducing bacteria, which were obtained from the fluidized bed reactor that is being used at the ORR FRC to condition groundwater: *Enterobacter* M-53, *Enterobacter* DM-S, *Klebsiella* DM-C3, *Pseudomonas* DM-Y2, *Janthinobacterium* M-A11, *Idobacter* DM-K3, *Stenotrophomonas* M-A15, *Chromobacterium* DM-N3, and *Shewanella oneidensis* MR-1 as a reference strain. The *ncc-nre* operon was introduced either on the broad host-range IncQ plasmid pMOL222 or on a single hopper mini transposon. We had also planned to introduce the *ncc-nre* operon into *Pseudomonas* DM-H2 and *Pseudomonas* M-16, but these strains were found to have a natural high resistance to Ni (MIC of 6 mM).

The integrity of the Ni-resistant transconjugants is being tested by BOX-PCR in comparison with the recipients to check their genetic background and by PCR with *ncc-nre* specific primers to confirm the presence of the *ncc-nre* operon. We are also testing the stability of the Ni-resistance phenotypes by growing the transconjugants for 100 generations under nonselective conditions and then verifying the presence of *ncc-nre.*

Once the Ni-resistance transconjugants have been characterized, we will test them in batch experiments to examine their efficiency in reducing nitrate in the presence of increasing concentrations of Ni. We will also use these strains in column mesocosm experiments to evaluate the possibilities of natural gene transfer to improve the activity of a nitrate reducing microbial community when it is being challenged with high levels of Ni-contamination.

This work is being supported by the U.S. Department of Energy, Natural and Accelerated Bioremediation Research (NABIR) Program, under Contract DE-AC02-98CH10886.

Ecological Interactions between Metals and Microbes that Impact Bioremediation

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Objectives

A. Determine the distribution of phylotypes and metal-resistance genes at the scale of spatial heterogeneity observed in microbial community activity.
B. Determine the effects of environmental factors on community responses to Cr(VI) contamination. These effects may be mediated by physiological responses, species selection, or gene transfer.
C. Determine the role of mobile genetic elements that confer Cr resistance. Microbes with these elements might function as either "bioprotectants" through their physiological activity, or reservoirs of transferable resistance genes.
D. Identify the novel physiological and genetic bases for bacterial resistance to Cr(VI) at concentrations of 15 to >50 mM.

Results

Chromium(VI) can be detoxified in soil if it is reduced to Cr(III), however, the Cr(VI)-reduction potential in anaerobic zones of terrestrial soils has not been extensively explored. Anaerobic microcosms were created with soil having (1) an organic energy source (glucose or protein); (2) a terminal electron acceptor [Fe (III) or NO_3^-]; and (3) no, low, medium, or high levels of Cr(VI) (concentrations that produced reductions of acute microbial activity of 0, 33, 50, or 75%, respectively). Microbial activity monitored by the evolution of CO_2 was stimulated by the addition of glucose in the microcosms without an apparent lag. However, higher doses of Cr(VI) had an inhibitory effect on the rate of CO_2 production. In addition, Cr(VI) was not detected in any microcosm 48 hours after setup, suggesting that detoxification of Cr(VI) is needed before the added carbon source can be used. The addition of protein to microcosms caused a different response by the community. The CO_2 concentration increased in two phases—an initial phase of slow CO_2 accumulation and a second phase of faster accumulation. The length of the initial slow phase increased with increasing concentration of added Cr(VI) and Cr(VI) was not detected when the fast phase commenced. Changes in bacterial community composition were monitored using PCR-DGGE of the 16S rDNA gene. In glucose-amended microcosms, the number of intense bands in DGGE profiles increased (1) with time across all treatments and (2) in treatments in which Cr(VI) was added. Although communities in microcosms receiving Cr differed from those in which no Cr was added, there was no effect by the amount of Cr added. In contrast, there were no significant differences in the bacterial community profiles in protein-amended microcosms with and without Cr additions. To confirm there was a Cr(VI)-tolerant population present, we isolated a total of 101 and 70 Cr^R strains, respectively, from glucose and protein microcosms amended with Fe(III) and medium Cr levels. Nearly all of the isolates were also able to grow at 0.5 mM Cr(VI), and a substantial number were able to grow in up to 10 mM Cr(VI). Real-time PCR was used to follow the *Geobacteraceae* populations in some of the Fe(III)0amended microcosms. In microcosms that received a carbon source but no Cr(VI), copy numbers of the *Geobacteraceae* 16S rRNA gene increased during maximum CO_2 production and then decreased back down to initial levels. In contrast, in microcosms amended with high concentrations of Cr, the *Geobacteraceae* 16S rRNA gene copy number remained constant throughout the entire experiment, suggesting that the iron-reducing bacteria in these soils were adversely affected by Cr(VI) addition. Our results show that Cr(VI) can be detoxified anaerobically in soils when appropriate organic substrates and electronic acceptors are provided.

Subsurface Bacterial Community Dynamics in the Presence of Plutonium and Uranium

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The goals of this project are to (1) determine the effects of different forms and concentrations of Pu and U on the abundance and composition of bacterial communities in soils, and (2) to identify bacterial species that are active in the presence of actinide, and that may be responsible for the reduction of actinide in anaerobic subsurface environments. We are conducting replicated laboratory time course experiments where soil is exposed to different forms of Pu(V/VI) or U(VI) under aerobic or anaerobic conditions. Using a suite of DNA- and RNA-based methods we are monitoring changes in the bacterial community in response to actinides at different concentrations. In preliminary experiments using T-RFLP community fingerprints and clone/sequence libraries, we observed major shifts in the soil bacterial community in anaerobic conditions, in response to U(VI) and Pu (VI) exposure as well as to the $NaNO_3$ and NaCl controls. The major bacterial genera detected in clone libraries after Pu or U exposure were completely different than the major species in the parent soil. Ongoing experiments on a LANL soil include more in-depth comparisons of community changes in the presence of Pu(VI), U(VI), and the Pu(IV)EDTA complex, under anaerobic and aerobic conditions. Nucleic acids have been extracted from 28 different treatments (4 reps/trt) and DNA- and RNA-based comparisons are underway. These include documenting community shifts under the different conditions using 16S rRNA T-RFLP fingerprinting, clone/sequence libraries, and comparisons of DNA- and RNA-based measurements. Measurement of bacterial abundance of Fe(III)- and sulfate-reducing bacterial groups known to reduce U(VI) will be conducted using real-time quantitative PCR assays, followed by analysis of species composition in those groups found to be active and/or correlated with actinide presence. Experiments planned for February 2005 will incorporate additional soils/sediments from the Savannah River site. This study will contribute to the NABIR program by providing information on the dynamics of natural soil and sediment communities in the presence of Pu(V/VI) and U(VI) that complements ongoing pure culture and field studies.

Molecular Analysis of Rates of Metal Reduction and Metabolic State of *Geobacter* Species during In Situ Uranium Bioremediation

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Previous studies have demonstrated that *Geobacter* species may account for over 90% of the microorganisms in the groundwater during active in situ uranium bioremediation. In order to optimize this uranium bioremediation strategy and broaden its application to other sites, it is important to have information on the rates of metabolism and metabolic state of the *Geobacter* species responsible for the bioremediation.

A search for the most suitable gene to monitor the rates of metabolism in *Geobacter* species found that in chemostat cultures there was direct correspondence between levels of transcripts for *gltA*, which encodes for a novel eukaryotic-like citrate synthase, and rates of Fe(III) reduction. In contrast, transcript levels for other genes were not directly related to Fe(III) reduction rates, and/or their levels varied considerably, depending upon whether metabolism was limited for electron donors or electron acceptors.

In order to better understand the physiology of *Geobacteraceae* during in situ uranium bioremediation, levels of mRNA for key *Geobacteraceae* genes were measured every other day during a study at the NABIR study site in Rifle, CO, in which acetate was injected into the subsurface to promote in situ bioremediation of uranium. As expected from previous studies at this site, the addition of acetate-stimulated removal of U(VI) from the groundwater and this was associated with a bloom of *Geobacteraceae*. In fact, *Geobacteraceae* accounted for more than 99% of the microbial population at the height of U(VI) reduction, and analysis of highly conserved *Geobacteraceae* genes demonstrated that the number of *Geobacteraceae* increased by more than 3 orders of magnitude. There was a remarkable correspondence between acetate levels in the groundwater and levels of transcripts for *gltA*. As acetate rose, *gltA* transcript levels increased. Both acetate and *gltA* transcript levels dropped during a rain event that diluted the acetate with rainwater recharge, and then *gltA* levels increased concurrent with a renewed increase in acetate over time. This contrasted with the constant expression, relative to total RNA, of *Geobacteraceae* housekeeping genes such as *recA*, *rpoD*, and *proC*. Numbers of transcripts of *ompB*, which encodes for a novel outer-membrane protein involved in Fe(III) reduction, tracked with numbers of *Geobacteraceae*, but transcripts of the homolog, *mofA*, thought to encode for a metal oxidation protein, were not detected. These results are consistent with the finding that metal reduction, not oxidation, is the predominant terminal electron-accepting process. Expression of *Geobacteraceae nifD* followed a pattern similar to that of *gltA*. This provided further evidence that the metabolism of *Geobacteraceae* was controlled by the availability of acetate and also demonstrated that the growth of *Geobacteraceae* during bioremediation was limited by the availability of fixed nitrogen. These results demonstrate that quantifying levels of mRNA in subsurface environments can provide insight into the in situ metabolism of microorganisms to aid in modeling and optimizing bioremediation.

Significance of Mobile Genetic Elements for Microbial Community Adaptation to Pollutant Stress

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The overall goal of this project is to investigate the effect of mobile genetic elements and conjugal gene transfer on subsurface microbial community adaptation and biotransformation of mercury. Special emphasis is given to the contribution of the "nonculturable" fraction of the microbial communities.

Our studies examine the interaction between the fate of these metals in the subsurface and the microbial community structure and activity. Mercury-contaminated soil samples were achieved from the Lower East Fork Poplar Creek Floodplain at the FRC site. The mercury concentration was determined in the contaminated soils. The results showed that the concentrations decreased with depth. These findings were also reflected in the results of a mercury tolerance assay where the soil was exposed to increasing concentrations of mercury. The top soil showed a clear adaptation towards mercury since soil respiration only was reduced slightly even at the highest mercury concentrations (100 ppm), whereas deeper soils (at subsurface depths of 20 in. and 40 in.) ← **OK?** only showed minor adaptation. None of the soils showed any adaptation/resistance towards chromate.

The number of CFUs resistant to mercury was determined in the mercury-contaminated and noncontaminated soils. The number of mercury-resistant bacteria was highest in the contaminated soils. Adaptation experiments were also performed, including different microbiological analyses, e.g., plate counts, respiration, and BIOLOG mt2 and EcoMicroPlate readings. In these experiments, it was found that the mercury-contaminated soils were more adapted to mercury than the noncontaminated soils. However, pre-exposure of the noncontaminated soils to mercury demonstrated that these soils also had an indigenous mercury-resistant population, although at a lower level than in the contaminated soils.

The potential detoxification of mercury by bacterial mercuric reductases (*merA* encoded) was determined in a culture-independent manner. *merA* specific PCR reactions were performed, and *merA* amplicons were detected from mercury-contaminated topsoil. We are currently performing quantitative PCR to assess *merA* occurrence in subsurface soil prior to and after $HgCl_2$ stress. We will compare the culture independent qPCR with culture-dependent methods to assess the applicability of qPCR for evaluation of mercuric reductase potential of (subsurface) soils. Finally, methods are being developed for the cultivation of the "nonculturable" mercury-resistant bacteria.

Understanding the Bioremediative Potential of FRC Microbial Communities

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We are using stable isotope probing (SIP), supporting ecophysiological studies and metagenomic approaches, to understand the ecology and physiology of microorganisms and communities important for the reduction of U, Cr, Tc and nitrate at the NABIR-FRC, and to identify those that respond under field implementation of bioremediation.

Stable-isotope probing methods are being applied to identify denitrifying microorganisms that incorporate the biostimulatory substrate, ethanol, in the Criddle-Jardine denitrifying reactor. Sequencing of a 16S rRNA clone library from the reactor indicated that the community was dominated by β-Proteobacteria (primarily *Thauera* and *Hydrogenophaga*) and Bacteriodes (*Flavobacterium)*. SIP results indicate that *Thauera, Acidovorax, Dechloromonas, Hydrogenophaga,* and *Sterolibacterium* rapidly incorporated carbon from ethanol. SIP is currently being combined with functional gene analyses to detect a variety of denitrification genes in biostimulated organisms using targeted PCR primers and a functional gene array. Future SIP experiments will be conducted on FRC sediment samples to identify bacteria that respond to the provision of biostimulatory substrates (ethanol, lactate, acetate).

The metal-reducing bacterium, *Desulfitobacterium hafniense* (DCB-2), was screened for reduction of Fe(III), Cu(II), U(VI), and Se(VI). Bacterial growth and metal reduction under metallorespiratory conditions were observed for Fe(III), Cu(II), and U(VI), but not for Se(VI), although Se(VI) was reduced when grown fermentatively with Se(VI) as an electron sink. SEM of fermentatively grown *D. hafniense* revealed multiple small polyps on the surface of cells when grown in the presence of Se(VI). Backscatter imaging and EDS analysis suggested that polyps were of high density and contained selenium concentrations above background. Growth and biofilm formation of *D. hafniense* were observed under both fermentation and respiration conditions using two different surfaces (Dupont and Siran™ beads), although the biofilm consistency and abundance as well as cell morphology varied with media and substrata.

Direct and indirect DNA extraction methods have been evaluated and improved for the recovery of high-quality, large-fragment DNA from FRC sediments and groundwater for use in metagenomic library construction. The direct DNA extraction yielded up to 200 kb DNA fragments that were successfully used to generate metagenomic libraries with an average insert size of 30–40 kb. A new indirect DNA extraction was also developed, in which soil particles were flocculated and precipitated with Ca(II) or Mg(II) and suspended bacterial cells extracted, yielding DNA fragments larger than 300 kb. This DNA is currently being used to construct a metagenomic library, and preliminary results suggest that it can generate libraries with >50 kb insert size. Approximately 120 L of groundwater has been collected from FRC well FW111 (up to 500 L will ultimately be collected) and extracted for a JGI community sequencing project.

Development and Use of Integrated Microarray-Based Genomic Technologies for Assessing Microbial Community Composition and Dynamics

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The recent development of microarray technology provides a great opportunity for the simultaneous identification of thousands of microbial genes/populations. However, low microbial biomass often prevents application of this technology to microbial communities in natural settings. We have developed a whole community genome amplification (WCGA)-assisted microarray-based detection approach for analysis of microbial communities whose members can not be studied using conventional technology. With optimized buffer systems, as few as two bacterial cells could be detected. Whole genome microarray hybridization showed that representative detection of individual genes or genomes was obtained within the DNA concentrations of 1 to 100 ng from individual or mixed genomes. Significant linear relationships were observed between signal intensity and initial DNA concentration ranging from: (1) 40 pg to 125 ng for the majority of *Shewanella* genes ($r^2 = 0.65$–0.99) and other organisms as detected by whole genome arrays; (2) genomes in constructed communities from 0.1 to 1,000 ng ($r^2 = 0.91$) using community genome arrays; and (3) community DNAs diluted from a stimulated natural groundwater ranging from 0.01 ng to 250 ng ($r^2 = 0.96$–0.98) using functional gene arrays. We applied this technology to investigate microbial communities in six groundwater samples contaminated with uranium and other metals using functional gene arrays (~2,000 probes). The results indicated that microbial populations containing important genes involved in contaminant degradation and immobilization have locally heterogeneous distributions and that microbial diversity is greatly decreased in contaminated environments. This is the first time that microarrays have been successfully used to analyze low biomass communities, such as those commonly found in settings important to human health, industry, and environmental management.

We have also developed a software program, CommOligo, for designing probes from whole-genomes, meta-genomes, or a group of sequences. The program uses a new global alignment algorithm to design single or multiple unique probes for each gene with default settings for a maximal similarity of 85%, a maximal number of continuous match of 15 bases, and free energy of –30 kcal/mol. The program is also able to design single- or multiple-group-specific probes for a group of genes with a minimal similarity of 96% within a group and the same parameters as unique probes outside a group. The program was evaluated using both whole-genome and highly homologous sequence data and compared with other probe design software. The results clearly demonstrate that CommOligo performed better and can be used for oligonucleotide probe design from various types of sequence data. With this program, more comprehensive functional gene arrays containing ~23,000 probes for important biogeochemical cycling (C, N, S, and P), metal resistance, and contaminant degradation genes have been designed and constructed. This is the most comprehensive array currently available for environmental studies. We are applying this microarray to the study of several microbial communities and processes at the NABIR-FRC, including a denitrifying fluidized bed reactor and groundwater stimulated with ethanol for uranium reduction.

PROGRAM ELEMENT 3
Biomolecular Sciences and Engineering

Mechanism of Uranium and Technetium Reduction by Metal-Reducing Members of the Genus *Shewanella*

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Metal-reducing members of the genus *Shewanella* respire anaerobically on a wide range of terminal electron acceptors, including oxidized forms of the radionuclides uranium [U(VI)] and technetium [Tc(VII)]. The genes and gene products required for U(VI) and Tc(VII) reduction, however, remain poorly understood. In a previous study, *S. putrefaciens* strain 200 respiratory mutants unable to respire with U(VI) as an electron acceptor were generated by ethyl methane sulfonate (EMS) mutagenesis and subsequently tested for anaerobic growth on nitrate, nitrite, sulfite, thiosulfate, Fe(III), Mn(IV), fumarate or trimethylamine-*N*-oxide as an electron acceptor. All Urr mutants were deficient in anaerobic growth on U(VI) and nitrite, including Urr mutant U14, which retained the ability to grow on all other electron acceptors. In the present study, a 13 kb wild-type DNA fragment from a *S. putrefaciens* gene clone bank was found to restore U(VI) and nitrite reduction activity to U14. Nucleotide sequence analysis of the 13 kb complementing fragment revealed two gene clusters: the first encoding a set of eleven putative RNA polymerase and ribosomal protein subunits (flanking the general secretory protein SecY), and the second encoding a set of five *c*-type cytochrome maturation (Ccm) proteins followed by ScyA, a soluble mono-heme c_5-type cytochrome. An homologous region was identified in the *S. oneidensis* genome (>80% amino acid similarity to *S. putrefaciens* 200) from loci SO0245-to-SO0264. Nucleotide sequence analysis of the smallest complementing fragment will provide information on the *S. putrefaciens* genes and gene products required for anaerobic respiration on U(VI).

Microbial reduction of soluble Tc(VII) results in the formation of Tc(IV) which precipitates as the highly insoluble hydrous oxide TcO_2, a Tc immobilization process forming the basis of alternate remediation strategies. Although the enzymatic reduction of Tc(VII) has been studied in *Escherichia coli* and *Desulfovibrio*, such studies have not been carried out in metal-reducing members of *Shewanella*. Chemical mutagenesis procedures and a newly developed screening technique were combined to identify a bank of putative Tc(VII) reduction-deficient (Tcr) mutants. The Tc(VII) reduction deficiency of each Tcr mutant was confirmed in anaerobic cell suspensions supplemented with H_2, lactate or formate as an electron donor. Putative Tcr mutants were subsequently grown either aerobically or anaerobically in minimal medium with fumarate as an electron acceptor. After aerobic growth, Tcr mutants Tcr-9, Tcr-17 and Tcr-18 were unable to reduce Tc(VII) with either lactate or H_2. Tcr-9, however, displayed the ability to reduce Tc(VII) with formate as electron donor, while Tcr-17, and Tcr-18 did not. After anaerobic growth with fumarate as electron acceptor, Tcr-17 was unable to reduce Tc(VII) with any electron donor. Tcr-18 was unable to reduce Tc(VII) with lactate or formate (yet displayed activity with H_2), while Tcr-9 reduced Tc(VII) with all three electron donors. These results indicate that Tc(VII) reduction in *S. oneidensis* MR-1 proceeds via electron transport pathways that are electron donor-specific and whose expression depends on the electron acceptor used for growth. Genetic complementation analysis is currently underway to identify the genes required for Tc(VII) reduction activity.

Construction of Whole Genome Microarrays, and Expression Analysis of *Desulfovibrio vulgaris* Cells in Metal-Reducing Conditions

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Objectives. (1) Construct whole-genome microarrays for *Desulfovibrio vulgaris*; (2) determine optimal conditions for hybridization conditions; and (3) identify whole-genome expression patterns of *D. vulgaris* cells grown under metal-reducing conditions.

Results. Recent studies have shown that the design characteristics of probes can significantly affect hybridization behavior, and therefore, possibly affect the dataset of genes with altered expression profiles. In order to experimentally establish the criteria for the design of gene-specific probes, an oligonucleotide array was constructed that contained perfect-match and mismatch probes. The effects of probe-target identity, continuous stretch, mismatch position, and hybridization free energy on specificity were examined. Second, in order to determine the effect of probe length on signal intensities, microarrays with different length probes were used to monitor gene expression. Based on the experimental results, a set of criteria was suggested for the design of gene-specific and group-specific probes, and these criteria should provide valuable information for the development of new software and algorithms for microarray-based studies.

The whole-genome microarrays of *D. vulgaris* were constructed using 70mer probes. Currently, growth conditions and medium are being evaluated for transcriptomic characterization with respect to growth phase and heavy-metal-responsive genes. The elucidation of growth-phase-dependent gene expression is essential for a general understanding of growth physiology that is also crucial for data interpretation of stress-responsive genes (e.g., chromium). We recently cultivated *D. vulgaris* in a defined medium and sampled biomass over time for approximately 70 hr to characterize the shifts in gene expression as cells transitioned from logarithmic phase growth to stationary phase. Each respective time point during exponential phase had a similar number of ORFs (7 to 9% of the genome) that were up-expressed (>3-fold) and approximately 5.5% to 6.0% of the ORFs were down-expressed (<3-fold). Also, a majority of the predicted ORFs did not display altered expression patterns when early- and late-exponential phases were compared. As the cells entered into early stationary phase, approximately 7% of the ORFs were up-expressed, whereas 3.5% to 4% of the genome was up-expressed as the cells experienced prolonged stationary-phase. Within the first hours of stationary-phase, up-expressed ORFs included proteases, chaperonins, permeases, and hypothetical proteins. Interestingly, preliminary comparisons suggest that many of the ORFs that were up-expressed in the early stationary phase also had altered expression levels in response to other cellular stresses. Preliminary data suggest that 0.05 mM Cr(VI) but not 0.01 mM Cr(VI) decreased growth in the defined medium with lactate and sulfate. In addition, *D. vulgaris* grew well in a defined, minimal medium designed to be analogous to FRC groundwater. We are presently analyzing microarray data to identify subsets of genes that respond to growth phase changes and/or stresses, as well as the determination of the optimal growth conditions in the presence of Cr(VI).

Identification and Characterization of the Membrane Proteome of *Shewanella oneidensis* MR-1

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Shewanella oneidensis MR-1, a metal-reducing bacterium, can utilize a large number of electron acceptors, including both soluble and insoluble metal oxides. Our previous analysis of the MR-1 whole lysate proteome showed significant changes in the abundance of several housekeeping proteins in response to electron acceptor availability, but few proteins directly involved in the electron transport pathways required for the metal reduction process were found. Since a majority of the electron transport-related proteins are believed to be associated with the membrane structures of this microbe, our current project is focused on characterization of proteins located in the inner and outer membrane compartments of MR-1 and the identification of proteins found to be altered in abundance in response to growth with different electron acceptors. In the first year of this project, we have optimized the fractionation of inner and outer membranes of MR-1 grown under aerobic and anaerobic with fumarate conditions. The presence of flagella protein in the fractions was used as a measure of the enrichment of the outer membrane relative to the inner membrane. Outer membrane proteins were also purified from aerobically and anaerobically grown MR-1 by affinity chromatography. The surface proteins were selectively biotinylated using two different hydrophilic biotinylation reagents (EZ-Link Sulfo-NHS-LC-Biotin and EZ-Link Sulfo-NHS-LC-LC-Biotin) to provide a wide range of labeled membrane proteins. The proteins were then purified using an avidin column. Inner and outer membrane proteins were compared using two-dimensional electrophoresis (2DE) and liquid chromatography with tandem mass spectrometry (LC/MS-MS). By using both 2DE and LC/MS-MS, we have been able to compare the protein complements of the inner and outer membrane preparations and identify proteins unique to one compartment or the other. The surface labeling indicated which proteins isolated with the outer membrane fraction are actually exposed to the cell surface under the growth conditions used. Proteins identified thus far include flagellin, flagellar hook protein FlgE, decaheme cytochrome *c*, TolA, TolB, TolC, TonB-dependent receptor protein, TonB system transport protein ExbD2, pilin protein MshB, OmcA, OmcB, OmpA, OmpH, MtrB, cytochrome *c*, cytochrome *c*552 nitrite reductase, Mol/TolQ/ExbB proton channel family protein, GspD, outer membrane porin, several ABC transporter proteins, and chaperone DnaK and GroEL. Significant differences in the expression of some of the outer membrane proteins were observed in MR-1 grown under aerobic and anaerobic conditions. With the methods for isolation of inner and outer membranes optimized, analysis of inner and outer membrane proteins from MR-1 grown on insoluble metal oxides or solid supports is now in progress.

Characterization of Sediment Survival Mutants and U(VI)-Sensitive Mutants of *Desulfovibrio desulfuricans* G20

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Desulfovibrio desulfuricans G20 is a model subsurface organism for studying genes involving in situ radionuclide transformation and sediment survival. Our research objective for this project has been to develop a signature-tagged mutagenesis (STM) procedure and use it to identify mutants in genes of strain G20 involved in sediment survival and radionuclide reduction. This involved first generating a library of 5760 G20 mutants and screening for potential nonsurvivors in subsurface sediment microcosms. After two rounds of screening, a total of 117 mutants were confirmed to be true nonsurvivors. Among these, 84 transposon insertion regions have been sequenced to date, representing transposition events in 74 different open reading frames. Upon further analysis of these 74 mutations, we classified the sediment survival genes into COG functional categories. These included proteins related to metabolism (29%), cellular processes (29%), and information storage and processing (16%). We also noted 14% of STM mutants identified had insertions in genes for hypothetical proteins or unknown functions. Interestingly, at least 38 interrupted genes encoded cytoplasmic proteins, 28 encoded inner membrane proteins, and at least 5 mutations were within genes encoding periplasmic space and outer membrane associated proteins. Not surprisingly, 85% of all genes required for sediment survival have homologs in *D. vulgar*is, 74% of those genes have homologs in *Geobacter metallireducens*, and 73% of those genes have homologs in *Geobacter sulfurreducens.*

We have also screened the STM library for mutants unable to transform U(VI). To date, nine mutants have been identified that are impaired in the ability to grow in lactate-sulfate medium with 2 mM U(VI)-acetate. Six of these mutants are completely unable to grow. Transposon insertion sites have been amplified, sequenced, and studied at the bioinformatics level, revealing that several genes disrupted may be involved in DNA, RNA, and protein maintenance. We have identified three genes believed to be directly involved in DNA repair, one gene involved in rRNA methylation, which could stabilize rRNA under stress; and one RNA polymerase subunit, which is required for RNA polymerase renaturation. Several other mutated genes may be involved in redox reactions within the cell, as two mutations observed might be involved in molybdopterin synthesis and one mutation was in a hypothetical protein with a thioredoxin domain. Orthologs to this hypothetical protein are present in *D. vulgaris*, *Geobacter metallireducens*, and *G. sulfurreducens*. We also obtained mutants in genes for a phosphotransacetylase, a dnaJ-like gene, and a histidine kinase. The latter two genes are known to be involved in stress responses. Based on the analysis of mutants, uranium appears to exert its toxicity at DNA, RNA, and protein levels. Resistance to the effects of U(VI) involve either detoxification, which likely involves biological reduction, or repair of damaged cell material.

These findings may help us better understand mechanisms used by sediment microorganisms to survive and reduce radionuclides in contaminated environments.

Identification of Metal Reductases and Determination of their Relative Abundance in Subsurface Sedimentary Systems Using Proteomic Analysis

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Heavy metal and radionuclide contamination at U.S. Department of Energy (DOE) sites nationwide constitutes a major environmental problem. Of particular interest are U and Tc, as well as Fe and Mn, due to their potential direct and indirect effects on contaminant biogeochemical behavior. For the past decade bacteria that use metals as terminal electron acceptors have been isolated and identified. These bacteria include members of three major anaerobic groups: the denitrifying, sulfate-reducing, and Fe(III)-reducing bacteria. The electron transfer pathways within these bacteria are still not well understood. Moreover, this lack of information substantially impedes efforts to increase in situ bioremediation efficiency. Hence, identification of metal reductases and determination of their similarity among these bacterial groups are essential for understanding these mechanisms and assessing bioremediative potential at DOE sites.

We have used cell fractionation techniques to resolve subcellular protein fractions and quantify the purity of proteins within each enriched fraction. Additionally, we have applied classical biochemical separations of fractions to enrich for specific proteins responsible for metal reduction activity. The application of advanced proteomics techniques allows for the identification of all the proteins in the enriched fractions eliminating the need for purifying each protein to homogeneity. We are using orthogonal purification approaches in both series and parallel to created fractions containing different complements of proteins. As each fraction exhibits the metal reduction activity, the proteins common to all the fractions are the most likely targets for further study by molecular biological techniques.

Mechanisms for the Reduction of Actinides and Tc(VII) in *Geobacter sulfurreducens*

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Uranium and technetium are the primary radioactive metals contaminating subsurface environments at U.S. Department of Energy (DOE) sites. Dissimilatory Fe(III)-reducing microorganisms can control the mobility of these contaminants through the enzymatic reduction of highly soluble U(VI) and Tc(VII) to insoluble tetravalent forms, which will precipitate from groundwater and be immobilized in the subsurface. The aims of this project are to use the tools of biochemistry and molecular biology to confirm the identities of the genes encoding the relevant U(VI) and Tc(VII) reductases in *G. sulfurreducens* and to elucidate the detailed mechanisms of U(VI) and Tc(VII) reduction by the corresponding enzymes. Furthermore, we aim to explore the range of other metals and radionuclides reduced by *Geobacter sulfurreducens*—including Np(V), Pu(IV), and Hg(II)—and identify the roles of the U(VI) and Tc(VII) reductases in the reduction of these other priority pollutants.

During the first two years of this grant, we have focused on identifying the Tc(VII) reductase of *G. sulfurreducens*, developing the analytical tools for monitoring the reduction of penta and tetravalent actinides in microbial culture, and characterizing the mechanisms of U(VI) and Np(V) reduction in detail. Extensive biochemical and genetic evidence, including studies with a defined mutant from Derek Lovley's group at the Univ. of Massachusetts, have shown that the enzyme responsible for Tc(VII) reduction in *G. sulfurreducens* is a NiFe hydrogenase localized in the periplasm. In collaboration with Mireille Bruschi's group in Marseille, France, we have also partially purified and characterized a suite of hydrogenases in *G. sulfurreducens* as a first step towards studying hydrogenase-mediated radionuclide reduction in vitro.

X-ray absorption spectroscopy (XAS) was also used to show that *G. sulfurreducens* reduces U(VI) by a one-electron reduction, forming an unstable U(V) species, which is subsequently too disproportionate to give insoluble U(IV). This was confirmed by challenging *G. sulfurreducens* with the Np(V) analogue, which is stable with respect to disproportionation and was not reduced by whole cells. Similar results were obtained in vitro using purified cytochrome c_7 from *G. sulfurreducens*, with hydrogenase as the electron donor for actinide reduction. This surprising degree of selectivity for hexavalent actinides illustrates the need for mechanistic understanding and care in devising in situ bioremediation strategies for complex wastes containing other redox-active actinides, including plutonium. In the final year of this program, we will explore the impact of *G. sulfurreducens* on plutonium in a range of oxidation states, confirm the role of *c*-type cytochromes in actinide reduction using in vivo and in vitro techniques (via collaboration with Marianne Schiffer at Argonne National Laboratory and Derek Lovley and colleagues at the Univ. of Massachusetts), and characterize the mechanism of reduction of other contaminant metals, including Cr(VI) and Hg(II). Studies on Cr(VI) reduction by *G. sulfurreducens* are already well advanced in our laboratory.

Biomolecular Mechanisms for Microbe-Fe(III) Oxide Interactions in *Geobacter* species

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Previous studies have indicated that *Geobacteraceae* are the primary agents for metal reduction during in situ uranium bioremediation and that, even when uranium levels are high, electron transfer to Fe(III) reduction accounts for ca. 99% of the growth of the *Geobacteraceae*. Thus, it is important to understand how *Geobacteraceae* interact with the Fe(III) oxides.

Previous studies on the mechanisms for Fe(III) reduction in *Geobacter* species have focused on the role of outer-membrane *c*-type cytochromes, but this approach has ignored the fact that *Pelobacter* species, which are phylogenetically intertwined with *Geobacter* species, lack *c*-type cytochromes but still reduce Fe(III) oxides. Previous studies had demonstrated that pili were required for Fe(III) oxide reduction by *Geobacter sulfurreducens*, and *Pelobacter* species as well as other *Geobacter* species possess pili. Therefore, studies this year focused primarily on the role of pili in Fe(III) oxide reduction. Transmission electron micrographs of cultures grown with poorly crystalline Fe(III) oxide as the electron acceptor revealed that Fe(III) oxides were much smaller than the microorganisms and that the Fe(III) oxide particles were preferentially associated more with the pili of *G. sulfurreducens* than the outer cell surface. These results suggested that the pili might have a direct role in electron transfer to Fe(III) oxides. In order to evaluate this, the electrical conductivity through the pili was measured. Pili and other proteins released from the outer surface of *G. sulfurreducens* were immobilized on a graphite surface and analyzed with an atomic force microscope equipped with a conductive tip and electronics that permitted mapping of the local conductance from the tip to the substrate. Topographic analysis revealed pili as well as other, unidentified, more globular, proteins which were also sheared off the outer cell surface. When a voltage was applied to the tip there was a strong current response along the pilin filaments, which was positive when a positive voltage was applied and negative with a negative voltage.

In contrast, the non-pilin proteins had no detectable conductivity and in instances in which the non-pilin proteins covered the pili filaments, they insulated the pili from the conductive tip. When similar studies were carried out with pili from *Shewanella oneidensis*, no conductance was detected. This is consistent with the concept that this organism produces electron shuttles to transfer electrons onto Fe(III) oxides. Analysis of the sequence of *pilA*, which encodes for the pilin structural protein, suggested that the pilin subunits of *Geobacteraceae* are likely to have significantly different properties than the previously described pili of other microorganisms. The finding that the pili of *G. sulfurreducens* are highly conductive suggests that *G. sulfurreducens* requires pili in order to reduce Fe(III) oxides because pili are the electrical connection between the cell and the surface of the Fe(III) oxides. This contrasts with the nearly universal concept that outer-membrane cytochromes are the proteins that transfer electrons to Fe(III) oxide in Fe(III) reducers. In addition to serving as a conduit for electron transfer to Fe(III) oxides, pili could conceivably be involved in other electron transfer reactions.

Comparative Physiology and Biochemistry of Metal-Reducing Organisms from Acidic and Neutral pH Environments.

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Comparative biochemical and molecular approaches have revealed interesting similarities and differences between neutrophilic and extremophilic mineral transforming bacteria. New methods have been developed to investigate the biochemistry of electron transport to metals and minerals, and new findings challenge the paradigm of electron transfer mechanisms to metal and mineral electron acceptors. Acidophilic dissimilatory iron-reducing bacteria (DIRB) have not been extensively studied, in contrast to their neutrophilic (i.e., *Geobacter*) counterparts. They are important organisms in metal and mineral transformation in acidic environments, and could represent bioremediative agents in metal- and radionuclide-contaminated low pH systems. Our NABIR-funded work is focused on the physiology and biochemistry of a representative acidophilic iron reducer, *Acidiphilium cryptum* JF-5. Preliminary data gathered thus far show *A. cryptum* to be an excellent system for these types of studies. Physiology studies show that *A. cryptum* effectively reduces both Fe(III) and Cr(VI), similar to many neutrophilic DIRB. Analytical and preparative methods for isolating and characterizing the extracellular polymeric substance (EPS) of *Acidiphilium* and *Geobacter* have been developed. Preliminary biochemical analysis of *Acidiphilium* shows the presence of *c*-type cytochromes in cell surface/EPS extracts, including two high-mass (42 and 48 kDa) outer-membrane-associated cytochromes *c*. These cytochromes have been purified by ion exchange and gel filtration techniques, and spectroelectrochemical analysis suggests that these proteins are capable of Cr(VI) and Fe(III) reduction in vitro. Neutrophilic DIRB also contain a suite of *c*-type cytochromes, but in much greater overall abundance. Microscopy studies of *A. cryptum* using lectin probes reveal biofilm structures, and attachment to mineral surfaces, even at low pH. Neutrophilic DIRB have similar biofilm structures when grown with soild-phase electron acceptors. The results indicate that *A. cryptum* may influence the fate of not only Fe, but also Cr in low-pH environments. It thus appears that acidophilic DIRB share certain features and abilities with their neutrophilic counterparts, but also have other traits that allow for this type of metabolism in extreme environments. Future studies will include genome-enabled studies of *A. cryptum* (facilitated by the DOE Joint Genome Institute), analysis of protein-mineral interactions using *A. cryptum c*-type cytochromes, resolution of select redox proteins and protein complexes of *Acidiphilium* by multidimensional PAGE techniques, and further biochemical characterization of redox proteins present in both *Acidiphilium* and in neutrophilic DIRB.

Engineering Bacteria for Superior Chromate Bioremediation

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Chromate [Cr(VI)] is a wide-spread environmental contaminant, and is particularly abundant at DOE waste sites. An attractive way to minimize its toxicity is through reduction to Cr(III) using bacteria. The long-term objective of this project is to engineer bacteria with superior capacity for chromate (and certain nuclide) remediation under field conditions. Several improvements are needed both at the enzymatic as well as cellular levels to make bacteria efficient agents of chromate bioremediation [1–4]. A critical one is minimizing chromate toxicity to remediating bacteria. In support of our previous findings that much of this toxicity arises from reactive oxygen species (ROS) generation during chromate reduction, we now show that "tight" [4,5] two-electron chromate reductases can out-compete one-electron reducers of this compound, generating much less ROS and thus constitute a safer pathway for this reduction. Studies with appropriate mutants and cloned genes demonstrate that loss or overproduction of the "tight" enzymes enhances or decreases, respectively, chromate toxicity in *P. putida* and *E. coli*. Thus, initial steps at generating strains more suitable for chromate reduction have been achieved using the principle of maximizing flow of chromate through the "safer" pathways of its reduction. Further improvements are being attempted, using the DNA shuffling approaches and appropriate screens developed in this laboratory. Initial results indicate isolation of shuffled products encoding higher turnover number chromate reductases.

To assess what effects enzyme engineering can have besides the intended improvements, it is important to know the biological role of the enzyme involved. We show [6] that the cellular role of "chromate reductases" is safe disposal of quinonoid compounds, which are abundant in the environment and in metabolic products. The "safe" chromate reductases reduce quinones by simultaneous two-electron transfer, avoiding formation of highly reactive semiquinone intermediates from one-electron reducers of quinones, and producing quinols that promote tolerance of oxidative stress. A *P. putida* strain over-expressing one of these enzymes (ChrR) recovers more quickly than wild-type in H_2O_2-amended media, whereas a *chrR* mutant takes much longer than the wild type. These growth phenotypes correlate with intracellular indicators of oxidative stress: protein carbonylation (oxidation) analyzed by Western analysis; and cellular H_2O_2 stress analyzed by FACS and the H_2O-responsive dye, H_2DCFDA. Thus, enhancing the activity of ChrR will not only increase the *P. putida* chromate reduction rate but also augment its capacity to withstand oxidative stresses of polluted sites.

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Studies of Multi-heme Cytochromes from *Geobacter sulfurreducens*

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The *Geobacteraceae* family predominates in the reduction of uranium in subsurface environments. We are focusing on the model organism, *Geobacter sulfurreducens;* its genome contains a large number (>100) of cytochromes *c* that function in the metal reduction pathways. Intensive functional genomics and physiological studies are in progress in Derek Lovley's laboratory (Univ. of Massachusetts), and the complete genome sequence of this organism has been determined by Methe et al., 2003. We are studying cytochromes from the c_7 family that are required for the reduction of Fe(III).

We have determined the three-dimensional structures of five 3-heme cytochromes c_7 coded by the *G. sulfurreducens* genome. Although these proteins are highly homologous to each other, their structures differ, specifically in their surface characteristics and the arrangement of the hemes. We found that their thermal stabilities are different, and they have different reduction potentials. Laurie DiDonato, of Derek Lovley's group, found that the physiological functions of the above homologs are also different, which might make it possible to determine the functional correlates of the observed structural differences.

PpcA, the most abundant cytochrome c_7 in the periplasm, differs from the other four; it was only possible to crystallize it in the presence of deoxycholate. In the structure of PpcA, there is a binding cavity for the deoxycholate; the structures of the other four homologs did not reveal any such binding sites. This binding site in PpcA might be occupied in vivo by a quinone molecule that plays a role in the function of PpcA.

Since the PpcA and its homologs differ by several residues, it is difficult to pinpoint the residue or residues responsible for their different properties. To understand the roles of the individual residues in PpcA, we have made 25 site-specific mutants and determined the structures of 10 of them. The site-specific mutants were designed to probe the role of residues that are close to the hemes, or to influence the surface charge of the molecule. The change in redox potential of the mutants of the conserved residue F15 buried between the hemes was determined by our collaborator, Carlos Salgueiro (Universidade Nova de Lisboa). Jon Lloyd (Univ. of Manchester) is collaborating with us to study the reduction of metals such as U(VI) and Np(V) by the site-specific mutants of cytochrome c_7 PpcA and its homologs.

The *G. sulfurreducens* genome also codes for two proteins (GSU0592 and GSU1996, also called ORF0901 and ORF3300, respectively), which consist of four c_7-type domains. Previously, we have determined the structures of domain C and domains CD coded by gene GSU1996. We now have crystallized both full-length proteins and collected diffraction data for both.

Structure-Function Analyses of Hg(II) Metalloregulation and Enzymology

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Objectives. Unlike many metals of concern at DOE waste sites, Hg is highly mobile and extremely toxic in all forms (oxidized, reduced, and organic). Its geochemical mobility reflects abiotic and biotic processes; bacteria are key in the latter as they have evolved protection against its toxicity. The widely found bacterial mercury resistance (mer) operon functions in Hg biogeochemistry and bioremediation by converting reactive inorganic [Hg(II)] and organic [RHg(I)] mercurials to relatively inert monoatomic mercury vapor, Hg(0). We study its metalloregulator, MerR, and two enzymes that affect these processes—MerA, the Hg(II) reductase; and MerB, the organo-mercurial lyase—to dissect their basic mechanisms and enhance their in vivo and in vitro performance.

Results: MerR. Having found in vitro MerR binds several thiophilic metals (Song et al, *J. Bacteriol*. 1**85**, 1861), we asked "how can it function in vivo as a metal-specific activator?" We found that even when bound to operator DNA, MerR still binds many metals, suggesting identification of the metal is embodied in distinct allosteric changes in MerR as it binds the metal. With a fluorotyrosine reporter we observed a unique NMR signal only upon Hg(II) binding, not with Cd(II) or Zn(II) binding. The residue involved lies in the DNA binding domain ~8 Å through the protein from the metal-binding center (by homology modelling on ZntR/CueR crystal structures), so the trigonal coordination preferred by Hg(II) provokes a unique conformational change in MerR propagated from the metal center to the DNA recognition helix. This is the first evidence of an inducer-specific allosteric change in any MerR-type regulator. Isothermal titration calorimetry shows all 3 metals bind spontaneously with similar free energies, but Hg(II) binding is driven by ΔH, and Cd(II) and Zn(II) binding are driven by ΔS, consistent with the latter 2 provoking very different changes in MerR. We are now examining key gain- and loss-of-function MerR mutants to pinpoint structural links between the "input" (metal-binding) protein end and "output" (DNA binding) end.

MerB With Jim Omichinski, we reported an NMR structure of DTT-inhibited MerB (DiLello et al., *Biochem.* **43**, 8322), revealing a novel fold with an N-terminal winged helix surmounting an alpha-beta-alpha domain bearing the active site. A higher resolution crystal structure of the Hg(II)-product-bound form of MerB completed with UGA colleague Cory Momany (near submission) reveals Cys96 and Cys159 are the key ligands to Hg(II) as predicted by our earlier biochemical and genetic work (Pitts et al., *Biochem.* **41**, 10287). This more compact structure identifies candidate proton donors and a pocket for the substrate organic group. Crystal structures of mutants C96S and C159S are in progress.

MerA. With Sue Miller, we focused first on defining the N-terminal domain's (NmerA) role in catalysis. In vitro studies (Ledwidge et al., near submission) show NmerA provides a significant kinetic advantage by removing Hg(II) bound to cell proteins when small thiols are low, suggesting this MerA domain is most needed by cells under redox stress, as occurs during Hg(II) exposure. In vivo studies confirm NmerA domain significantly increases growth in the presence of Hg(II) for *E. coli* cells lacking glutathione (gsh^-). Structure/function studies of NmerA to identify key residues for Hg(II) transfer to the active site are in progress. Second, we evaluated MerA's ability to reduce other metal ions. Initial work showed no in vitro reduction of CrO_4^{2-} by MerA, so we focused on Au(III) as in vivo work shows *mer* operon expression stimulates Au(III) reduction. Indeed, pure MerA catalyzes several turnovers of Au(III) reduction to Au(I), but at rates much slower than of Hg(II). Au(III)'s partial reduction by MerA's $2e^-$ catalytic cycle leaves MerA in a product(Au(I))-inhibited state that dissociates slowly with Au(0) forming by disproportionation. Being both substrate and inhibitor, Au(III) is a unique model for unnatural MerA substrates, so we are using it to dissect the enzyme cycle and as a tool in directed evolution to extend MerA's catalytic range.

Transcriptome and Proteome Dynamics of the Cellular Response of *Shewanella oneidensis* to Chromium Stress

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Shewanella oneidensis strain MR-1 is a metal-reducing bacterium that can respire a wide array of organic and inorganic substrates, including Cr(VI), U(VI), and Tc(VII). *S. oneidensis* can potentially be used to immobilize metals/radionuclides by reducing soluble and more mobile forms to sparingly soluble, less bioavailable forms. However, the prediction and assessment of bioremediation performance is compounded by the lack of fundamental knowledge on microbe-metal interactions and on a microorganism's ability to survive and function in relevant contaminated environments.

The overall goal of this DOE NABIR project is to characterize the molecular basis and regulation of hexavalent chromium [Cr(VI)] stress response and reduction by *S. oneidensis* MR-1. Here we describe transcriptome and proteome analyses of the molecular stress response and adaptation of MR-1 to toxic levels of Cr(VI). The acute stress response of aerobic, mid-exponential phase cultures shocked to a final concentration of 1 mM K_2CrO_7 was examined after 5, 30, 60, and 90 min and compared to untreated cultures. The transcriptome of mid-exponential cultures was also examined 30 min after shock doses of 0.3 mM, 0.5 mM, or 1 mM K_2CrO_7. The *tonB1-exbB1-exbD1* genes comprising the TonB1 iron transport system were some of the most highly induced genes after 90 min (up to ~240 fold), followed by other genes involved in iron acquisition and sulfate transport and metabolism. Transcripts predicted to encode hydrogenases (e.g., HydA, HydB), iron-sulfur cluster binding proteins (e.g., SO4404), and cytochromes (e.g., OmcA, OmcB) were some of the most highly repressed genes after 90 min. Transcripts for DNA repair and detoxification genes such as *recA, kat2*, and a putative azoreductase-encoding gene were also more abundant in cells exposed to chromium. Transcriptome profiles generated from MR-1 cells adapted to 0.3 mM Cr(VI) differed significantly from those characterizing cells exposed to acute Cr(VI) stress without adaptation.

Parallel proteomic characterization of soluble protein and membrane protein fractions extracted from Cr-shocked and Cr-adapted MR-1 cells was performed using multidimensional HPLC-ESI-MS/MS. For a 45 min shock sample, ~720 proteins were confidently identified, which compared favorably with the ~800 identifications from a control cell culture (i.e., cells grown in the absence of Cr). A total of 12 proteins were found to be up-regulated and 7 proteins were found to be down-regulated in the Cr-shocked *S. oneidensis* samples, relative to the untreated control. The up-regulated proteins included a putative azoreductase and proteins involved in iron binding and transport. The 90-min shock sample revealed 14 proteins that were up-regulated, including two subunits of sulfate adenylyltransferase (CysN and CysD) and a sulfate ABC transporter (CysA-2) that were not found to be up-regulated in the 45 min shock sample. These studies provide important insights into cellular chromium tolerance. Future research will focus on the structural and regulatory genes implicated in Cr(VI) reduction and detoxification.

The Role and Regulation of Melanin Production by *Shewanella oneidensis* MR-1 in Relation to Metal and Radionuclide Reduction and Immobilization

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Bacteria of the genus *Shewanella* grow by transferring electrons to soluble and insoluble metals for energy production. When *Shewanella* transfer electrons to toxic metals and radionuclides, these compounds become less mobile and toxic in the environment. A better understanding of the biomolecular mechanisms involved in the electron transfer to insoluble metals by *Shewanella* offers potential in developing new strategies for bioremediation of contaminated sites.

The pigment pyomelanin is particularly important in this process, and is produced by *Shewanella oneidensis* MR-1. In the presence of excess pyomelanin, *S. oneidensis* MR-1 reduces the insoluble Fe(III)-oxide, hydrous ferric oxide (HFO), at a greater rate than without additional pyomelanin. This is accomplished because, under anaerobic conditions, pyomelanin serves as a soluble electron shuttle to insoluble minerals.

The overall hypothesis of this work is that pyomelanin production in the genus *Shewanella* plays a significant role as a mechanism of metal and radionuclide reduction and immobilization, and its production can be manipulated with the addition of proper nutrients. By understanding the role and regulation of pyomelanin production in microorganisms, remediation of metal- and radionuclide-contaminated environments may be accelerated. We are concentrating our studies on *S. oneidensis* MR-1, the type organism of this genus.

To date we have demonstrated the inhibition of pyomelanin production by *S. oneidensis* MR-1 using sulcotrione [2-(2-chloro-4-methane sulfonylbenzoyl)-1,3-cyclohexanedione], a competitive inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4HPPD) that is responsible for pyomelanin production in MR-1. Using the suicide vector system (pDS3.1) developed in our lab we generated a 700 bp deletion in the gene *melA*, which encodes for 4PHPPD. The *melA*$^{-}$ mutant was unable to produce pyomelanin. In addition, we have generated a pyomelanin-overproducing mutant through a deletion in the gene *hmgA,* which encodes for homogentisic acid oxidase. Both homogentisic acid oxidase and 4PHPPD are involved in tyrosine catabolism. Our studies demonstrated that HFO reduction decreased but was not completely halted with sulcotrione-treated cells or in the absence of *melA*. The amount of HFO reduction increased with the pyomelanin overproducing mutant. The addition of pyomelanin to the pyomelanin deficient mutant increased HFO reduction. Our results also show that pyomelanin production is a function of tyrosine concentration in the growth medium and plays a significant role in HFO reduction but is not the only mechanism involved in this process.

Genes for Uranium Bioremediation in the Anaerobic Sulfate-Reducing Bacteria: *Desulfovibrio* mutants with Altered Sensitivity to Oxidative Stress

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Objective. Sulfate-reducing bacteria of the genus *Desulfovibrio* are ubiquitous in anaerobic environments such as groundwater, sediments, and the gastrointestinal tract of animals. Because of the ability of *Desulfovibrio* to reduce radionuclides and metals through both enzymatic and chemical means, they have been proposed as a means to bioremediate heavy metal contaminated sites. Although classically thought of as strict anaerobes, *Desulfovibrio* species are surprisingly aerotolerant. Our objective is to understand the response of *Desulfovibrio* to oxidative stress so that we may more effectively utilize them in bioremediation of heavy metals in mixed aerobic-anaerobic environments.

Experimental approach and results. The enzymes superoxide dismutase, superoxide reductase, catalase, and rubrerythrin have been shown by others to be involved in the detoxification of reactive oxygen species in *Desulfovibrio*. Some members of the genus *Desulfovibrio* can even reduce molecular oxygen to water via a membrane bound electron transport chain with the concomitant production of ATP, although their ability to grow with oxygen as the sole electron acceptor is still questioned. We are taking a genetic approach to identify those genes involved in the oxidative response in *Desulfovibrio*. We have recently generated a library of mini Tn5 transposon mutants in *Desulfovibrio desulfuricans* G20, and we have begun screening this library for mutants with altered sensitivity to oxygen. Examination of one of the mutants with an increased resistance to oxygen revealed that the transposon had inserted into a gene encoding a putative uracil DNA *N*-glycosylase (*ung*). In *Escherichia coli*, Ung is the first enzyme in the pathway of uracil excision and repair in DNA, and *E. coli ung* mutants have previously been shown to be more resistant to some types of DNA damage. We hypothesize that exposure of *Desulfovibri*o to molecular oxygen results in DNA damage, and that the excision and repair of this DNA damage by Ung has a greater potential to create a lethal double stranded break in the chromosome. Therefore *ung* mutant cells of *Desulfovibrio*, which are impaired in uracil excision and repair in DNA, appear to be more resistant to killing by molecular oxygen. *E. coli ung* mutants have previously been shown to accumulate A:T-to-C:G transversions. As expected, the *ung* mutant of *D. desulfuricans* G20 also shows a greater frequency of spontaneous mutations than the wild-type.

A Novel Mechanism for Reduction of Mercury(II) by Mercury-Sensitive Dissimilatory Metal-Reducing bacteria.

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Shewanella oneidensis MR-1 is a facultative anaerobe that is of great interest for bioremediation in the subsurface due to its ability to reduce toxic metals and radionuclides. We present evidence that MR-1 can reduce ionic mercury [Hg(II)] to elemental mercury [Hg(0)] by a novel pathway that is not related to the well-characterized *mer* system. Additionally, MR-1 does not display Hg(II) resistance typical of an organism with a *mer* operon. In the presence of 200 nM Hg(II), in 24 hours live cells grown aerobically reduced 83±6.3% of the Hg(II), whereas autoclaved cells reduced 21±0.9%. Hg(II) reduction by MR-1 also occurred with iron oxyhydroxide and fumarate as terminal electron acceptors. Reduction of Hg(II) showed a strong dependence on the presence of an electron donor and an electron acceptor, as incubation of cells in media that lacked either resulted in activity that is not significantly different from that of autoclaved cells ($p > 0.01$). Unlike *mer*-mediated Hg(II) reduction, this activity is not inducible, as exposed cells and unexposed cells had a specific activity for reduction of Hg(II) of 3.14±0.25 and 3.07±0.35 nmol min^{-1} mg $protein^{-1}$ respectively. This activity is not restricted to MR-1, as two other dissimilatory metal-reducing bacteria (DMRB) that lack a *mer* system (*Geobacter sulfurreducens* PCA and *Geobacter metallireducens* GS-15) also are able to reduce Hg(II). Live and autoclaved cells of strain PCA had specific activities of 2.8±1.3 nmol Hg(II) min^{-1} mg $protein^{-1}$ and 0.34±0.5 nmol Hg(II) min^{-1} mg $protein^{-1}$, respectively. For strain GS-15, live and autoclaved cells had specific activities of 6.05±1.4 nmol Hg(II) min^{-1} mg $protein^{-1}$ and 1.65±0.9 nmol Hg(II) min^{-1} mg $protein^{-1}$, respectively. However, Hg(II) reduction is not universal among DMRB or anaerobes, as it was absent in *Anaeromyxobacter dehalogenans* strain 2CP-C, which can reduce iron, and the nitrate reducer *Pseudomonas stutzeri* OX1. The discovery of constitutive reduction of Hg(II) at low concentrations by DMRB may have future applications in the remediation of mercury in subsurface environments.

PROGRAM ELEMENT 4
Biogeochemistry

The Biogeochemistry of Pu and U: Distribution of Radionuclides Affected by Microorganisms and their Siderophores, Reductants, and Exopolymers

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Radionuclide0contaminated environments are often oxic, including the Rocky Flats Environmental Technology Site (RFETS), and the contaminated groundwater at the NABIR Field Research Center (FRC). Radionuclide distribution within such environments is affected by indigenous biogeochemical processes, including the metabolic activities of aerobic microorganisms, key members being the ubiquitous *Pseudomonas* and *Bacillus* genera. Because of the chemical similarities between the actinides, uranium (U) and plutonium (Pu), and iron (Fe), the metabolic processes of these microorganisms that affect the biogeochemistry of Fe could also significantly affect Pu and U distribution. We propose to determine the extent to which metabolic processes involved in Fe acquisition and exopolymer production affect the distribution of Pu and U between the aqueous and solid phases. We will determine the equilibrium distribution of Pu and U between these phases, in the presence and absence of microorganisms, and in relation to Fe bioavailability.

We have continued our investigations of the redistribution of actinide species during aerobic dissolution of Fe=bearing minerals. For example, *Pseudomonas putida* and *P. mendocina* are common siderophore producing bacteria in soil and subsurface environments. Our research has produced unequivocal evidence that, driven by the process of aerobic Fe acquisition from minerals of high crystalline order (e.g., hematite), these species desorb and solubilize U(VI) that was sorbed previously to the mineral surface. Most striking about our results is not only that all of the U(VI) was removed from the solid phase but, more importantly, U(VI) remained in solution following centrifugation, under both oxic and circumneutral pH conditions. It did not adsorb onto the cells or surface of the reaction vessel, precipitate, or re-adsorb onto the mineral surface. The importance of this result to NABIR is that under specific nutrient conditions U can be mobilized (i.e., chelated) and thus becomes less likely to adsorb to solid phases. Such knowledge is essential for management and treatment strategies.

The results of the research program will contribute to NABIR's stated needs to understand both "the principal biogeochemical reactions that govern the concentration, chemical speciation, and distribution of metals and radionuclides between the aqueous and solid phases" and "what alterations to the environment would increase the long-term stability of radionuclides in the subsurface."

Quantification of Iron Reoxidation in Microbially Reduced FRC Sediments

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We are reporting preliminary results of a new project initiated in December of 2004 entitled "Reduction and Reoxidation of Soils during and After uranium Bioremediation; Implications for Long-Term Uraninite Stability and Bioremediation Scheme Implementation." The proposed research focuses on the conditions and rates under which uranium will be remobilized after it has been precipitated biologically, and what alterations can be implemented to increase its long-term stability in groundwater after the injection of an electron donor has been discontinued. Furthermore, the proposed research also addresses short-term iron reoxidation as a mechanism to enhance/extend uranium bioremediation under iron reduction, without its remobilization.

Microbial reduction of iron has been shown to be important in the transformation and remediation of contaminated sediments. Reoxidation of microbially reduced iron may occur in sediments that experience cycles of oxidation and reduction and can thus impact the fate and extent of contaminant remediation, yet little research has been performed to quantitatively measure the rate of iron re-oxidation and changes in iron phases. The purpose of this initial phase of this research was to measure rates and extent of iron oxidation in a flow-through column filled with previously reduced sediment. In addition, the iron phases in the reoxidized sediment were compared to the original sediment as well as the biologically reduced sediment.

An unsaturated background FRC sediment containing both Fe(III)-oxides and silicate Fe(II)/Fe(III) was biologically reduced in phosphate buffered (PB) medium at circumneutral pH utilizing the indigenous microbial population of the sediment in a long-term (500-day) column experiment in which acetate was supplied as the electron donor. Long-term iron reduction resulted in partial reduction of silicate Fe(III) and some goethite biotransformation, based on Mössbauer spectroscopy study. There was no evidence of vivianite formation, a ferrous phosphate. This reduced sediment was treated with an oxygenated PB solution in a flow-through column, resulting in the reoxidation of 38% of the biogenic Fe(II), mainly silicate Fe(II). Additional batch experiments show that the Fe(III) in the reoxidized sediment was more quickly reduced compared to the initial sediment, indicating that reoxidation of sediment not only regenerated Fe(III) but also enhanced iron reduction compared to the initial sediment and may be a viable method to extend iron-reducing conditions during in situ bioremediation scenarios.

Novel Imaging Techniques, Integrated with Mineralogical, Geochemical, and Microbiological Characterizations to Determine the Biogeochemical Controls on Technetium Mobility in FRC Sediments

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Technetium-99 is a priority pollutant at numerous DOE sites, due to a combination of its long half-life (2.1×10^5 yr), high mobility as Tc(VII) (TcO_4^-, the pertechnetate anion) in oxic waters, and bioavailability as a sulfate analog. Under anaerobic conditions, however, the radionuclide is far less mobile, forming insoluble Tc(IV) precipitates. As anaerobic microorganisms can reduce soluble Tc(VII) to insoluble Tc(IV), microbial metabolism may have the potential to treat sediments and waters contaminated with Tc. In previous studies we have focused on the fundamental mechanisms of Tc(VII) bioreduction and precipitation, identifying direct enzymatic (hydrogenase-mediated) mechanisms, and a range of potentially important indirect transformations catalyzed by biogenic Fe(II), U(IV), or sulfide. These baseline studies have generally used pure cultures of metal-reducing bacteria, in order to develop conceptual models for the biogeochemical cycling of Tc. There is, however, comparatively little known about interactions of metal-reducing bacteria with environmentally relevant trace concentrations of Tc, against a more complex biogeochemical background provided by mixed microbial communities in the subsurface. This information must be available if in situ remediation of Tc(VII) contamination is to be successful at DOE sites.

The aim of this project is to use a highly multidisciplinary approach to identify the biogeochemical factors that control the mobility of environmentally relevant concentrations of Tc(VII) in FRC sediments, and to assess the effectiveness of strategies proposed to stimulate Tc(VII) reduction and precipitation in the subsurface. Initial experiments have focused on obtaining baseline data from FRC "background" sediments. Progressive microcosms incubated with and without added electron donor (20 mM acetate) have shown that Tc(VII) reduction occurs concomitant with Fe(III)-reduction. The addition of 10 mM nitrate and 20mM acetate had little impact on metal reduction, but 100mM nitrate (with acetate) completely inhibited the reduction of both Tc(VII) and Fe(III). Molecular analyses of the microbial communities present in the sediments confirmed the presence of Fe(III)-reducing bacteria known to reduce both Fe(III) and Tc(VII) in axenic cultures (*Geobacter* and *Geothrix* species), while nitrate-reducing bacteria were also detected (including *Azoarcus* species), and were present at higher concentrations than Fe(III)-reducing bacteria in MPN dilution series. X-ray absorption spectroscopy confirmed that the dominant form of Tc in the post reduction sediments is TcO_2. Reoxidation of immobilized, reduced Tc was also assessed using nitrate and air exposure as oxidants. Addition of nitrate (to 25 mM) had no effect on Tc solubility in reduced sediments, while air exposure resulted in approximately 80% solubilization of reduced technetium, presumably as reoxidized Tc(VII). Current experiments are focusing on the biogeochemical controls on Tc solubility in low pH/nigh nitrate sediments from Area 3 of the FRC, and in developing a sediment column system for analysis using γ-camera imaging techniques, which will allow real-time monitoring of Tc mobility in sediments.

Investigation of the Transformation of Uranium under Iron-Reducing Conditions: Reduction of UVI by Biogenic Fe^{II}/Fe^{III} Hydroxide (Green Rust)

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The recent identification of green rusts as products of the reduction of Fe^{III} oxyhydroxides by dissimilatory iron-reducing bacteria (DIRB), coupled with the ability of synthetic green rust to reduce U^{VI} species to insoluble UO_2, suggests that biogenic green rusts may play an important role in the speciation (and thus mobility) of U in Fe^{III}-reducing environments. The objective of our research is to examine the potential for biogenic green rusts to affect the speciation of U^{VI} under Fe^{III}-reducing conditions. To meet this objective, we will test the following hypotheses: (1) the formation of green rusts from dissimilatory Fe^{III} reduction is controlled by Fe^{III} speciation, solution composition, and microbial physiology; (2) the chemical composition and structural properties of biogenic green rusts are variable and depend on the conditions under which they were formed; (3) the rate of U^{VI} reduction by biogenic green rusts varies depending on their chemical composition and structure; (4) the rate of U^{VI} reduction by a given biogenic green rust is affected by the solution composition, which affects both the speciation of U^{VI} and U^{IV} and the stability of the green rust; and (5) the reduction of U^{VI} to U^{IV} can be coupled to dissimilatory Fe^{III} reduction under conditions that promote the formation of biogenic green rust and other reactive Fe^{II} species. The research we are proposing will examine the effects of growth conditions on the formation of biogenic green rusts resulting from the reduction of Fe^{III} oxyhydroxides by DIRB and the effects of U concentration, carbonate concentration, pH, and the presence of reducible co-contaminants on both the kinetics of U^{VI} reduction by biogenic green rusts and the identity of the resulting U-bearing mineral phases. The results of this research will significantly increase our understanding of the coupling of biotic and abiotic processes with respect to the speciation of U in iron-reducing environments. In particular, the reduction of U^{VI} to U^{IV} by biogenic green rust with the subsequent formation of U-bearing mineral phases may be effective for immobilizing U in suboxic subsurface environments.

Detoxification of Uranium under Sulfate-Reducing Conditions: Reactivity of Biologically Reduced Uraninite with Fe(III)-(hydr)oxides

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Uranium (U) is a known carcinogen, and the high solubility of its hexavalent form can result in U transport to sensitive receptors such as drinking water sources. One potential method of treating U contamination is by using natural dissimilatory metal-reducing bacteria such as sulfate-reducing bacteria (SRB) to reduce soluble U(VI) to insoluble U(IV) (as uraninite, UO_2), which can lead to in situ U immobilization and plume stabilization. Before SRB can be applied for bioremediation, however, it is important to understand U toxicity and the reactivity and stability of SRB-mediated uraninite. The effects of U(VI) on SRB were studied using *Desulfovibrio desulfuricans* G20 in a metal toxicity medium containing bicarbonate or PIPES pH buffer (each at 30 mM, pH 7). In batch experiments, U(VI) toxicity was dependent on the medium buffer and was observed in terms of longer lag times and, in some cases, no measurable growth. The minimum inhibiting concentration (MIC) that caused a complete inhibition of growth was 140 μM U(VI) in PIPES buffered medium. This is 36 times lower than previously reported for *D. desulfuricans*. The solubility of U(VI) was significantly lower in the PIPES buffer than in the bicarbonate buffer; however, U(VI) in the PIPES buffer was much more toxic than in the bicarbonate buffer. Analysis of thin sections of G20 treated with 90 μM U(VI) using HR-TEM revealed that reduced U was precipitated not only in the periplasmic spaces, but also in the cytoplasmic spaces. These results suggest that U(VI) toxicity and detoxification mechanisms of G20 greatly depend on the speciation of U(VI) present.

To examine the reactivity of reduced U in batch experiments, using *Desulfovibrio desulfuricans* G20, the effects of hematite, goethite, and ferrihydrite on the microbial reduction of U(VI) were evaluated under lactate-limited sulfate-reducing conditions. Our results suggested that once lactate was depleted, microbially reduced U served as an electron donor to reduce Fe(III) present in Fe(III)-(hydr)oxides. Subsequent addition of hematite [15 mmol Fe(III)/L] to stationary phase cultures containing microbially reduced U(IV) also resulted in rapid reoxidation to U(VI). Analysis by U L_3-edge XANES spectroscopy of microbially reduced U particles yielded spectra similar to those of natural uraninite. Observations by HR-TEM, SAED, and EDS analysis confirmed that precipitated U associated with cells was uraninite with particle diameters of 3–5 nm. These aqueous uraninite particles were separated from the bacterial cells using a 0.2 μm membrane filter, and treated with hematite and ferrihydrite. Both hematite and ferrihydrite oxidized uraninite; however, in these systems, the reactivity of uraninite was greater with ferrihydrite than with the more crystalline structure of hematite. The results have strong implications for field application of in situ biological reduction of U(VI). Care should be taken to maintain the organic substrate in excess throughout the system, such that the aquifer is electron-acceptor limited rather than electron-donor limited, at least until available Fe(III) is depleted from the system. Failing to maintain sufficient substrate concentrations until available Fe(III) is reduced could lead to unfavorable consequences for the long-term stability of immobilized uranium.

Subsurface Bio-Immobilization of Plutonium: Experiment and Model Validation Study

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A concurrent experimental and modeling study centered on the interactions of *Shewanella alga BrY* with plutonium species and phases is proposed. The goal of this research is to investigate the long-term stability of bioprecipitated "immobilized" plutonium phases under changing redox conditions in biologically active systems. The longevity of the subsurface immobilization of plutonium (e.g., by bioreduction) is a key consideration in the effectiveness of remediation/containment approaches used, affects the design/choice of immobilization approaches, and defines issues regarding the closure of contaminated sites (e.g., natural attenuation). Plutonium is the key contaminant of concern at several DOE sites that are being addressed by the overall NABIR program.

This is a new project, which is being done in collaboration with Professor Bruce Rittmann at Arizona State University. Key elements of the research planned for this year include the following:

- Establish the radiotoxicity of plutonium complexes towards *S. alga*.
- Perform growth studies for *S. alga* under a range of environmental conditions in the absence and presence of plutonium, and as a function of plutonium speciation.
- Update the CCBATCH model to incorporate plutonium data relevant to these *S. alga* studies to model the experimental results.
- Confirm and establish the conditions under which bioreduction of plutonium by *S. alga* in the +5 and +6 oxidation states (i.e., as PuO_2^+ and PuO_2^{2+} aqueous species).
- Initiate studies to investigate the bioassociation of plutonium with *S. alga* at environmentally relevant concentrations.
- Initiate a CCBATCH model upgrade to include transport experiments.

The overall hypothesis for the proposed research is that stable recalcitrant plutonium phases will prevail in biologically active systems where bioreduction occurs. Understanding the relationship between aqueous speciation and composition, biological effects and interactions, and the fate and immobilization of the plutonium is the longer-term goal of this research.

Mesoscale Biotransformation of Uranium

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Two outstanding issues central to the success of reduction based in situ uranium bioremediation are the long-term stability of bioreduced U(IV), and the extent of U reduction achievable in transport-limited regions that comprise most of the subsurface. These critical concerns are being addressed through series of long-term column experiments.

Long-term Stability of In situ Microbial Reduced U(IV). Although previous short-term experiments on microbially mediated U(VI) reduction have supported the prospect of immobilizing the toxic metal through formation of insoluble U(IV) minerals, our longer-term (17 months) laboratory study showed that microbial reduction of U can be transient, even under sustained reducing (methanogenic) conditions. FRC Area 2 sediments containing 200 mg kg^{-1} U, at first primarily as U(VI), were packed into columns and infused with lactate solutions to accelerate reduction. Uranium was reduced during the first 60 days, but later (100 to 500 days) reoxidized and mobilized, even though a microbial community capable of reducing U(VI) remained active. Microbial respiration caused increases in (bi)carbonate concentrations and formation of very stable uranyl carbonate complexes, thereby shifting U(IV)/U(VI) equilibrium to much more reducing potentials. Fe(III) and Mn(IV) are likely terminal electron acceptors for U reoxidation. Thus, U remediation by organic carbon-based reductive precipitation can be problematic in sediments and groundwaters with neutral to alkaline pH, where uranyl carbonates are most stable. Our research is now focusing on biogeochemical conditions that can sustain U(IV) solid phases in the presence of carbonate.

Uranium Reduction in Sediments under Diffusion-Limited Transport of Organic Carbon. Delivery of organic carbon (OC) into contaminated sediments for stimulating U reduction is diffusion-limited in less permeable regions that comprise most of the subsurface. In order to study OC-based U reduction in diffusion-limited domains in sediments, Oak Ridge and Altamont soils were treated with $UO_2(NO_3)_2$ [U(VI) concentration ≈ 320 mg kg^{-1}], packed into columns, then hydrostatically contacted with tryptic soy broth solutions. Thus, OC was mineralized while diffusing into initially oxidizing soils. Even at average OC supply rates of 1 μmol OC (g soil)$^{-1}$ day^{-1}, the U reduction zone extended only about 40 mm into the soil columns. Spatial distributions of redox potentials, U oxidation state, and microbial communities were well correlated. Stratification of a reduced U(IV) zone over an oxidizing U(VI) zone persisted over 600 days because the OC supply was diffusion-limited and completely oxidized within the thin zone immediately adjacent to its point of application. These results show that low U concentrations in groundwater samples from OC-treated sites are not necessarily indicative of pervasive U reduction.

Integrated Investigation on the Production and Fate of Organo-Cr(III) Complexes from Microbial Reduction of Chromate

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Chromium contamination exists at several Department of Energy facilities; microbial reduction to form insoluble trivalent Cr [Cr(III)] is a potential treatment for such sites. We have discovered that some soluble organo-Cr(III) complexes are likely formed and then further transformed to insoluble Cr(III) precipitates. The production of organo-Cr(III) complexes from chromate reduction is well documented in Cr toxicity studies in humans. However, the formation of soluble organo-Cr(III) complexes from microbial reduction of chromate has only recently been discovered. Here we present more evidence on the formation and mineralization of organo-Cr(III) complexes. First, production of soluble organo-Cr(III) complexes by selected microorganisms has been observed. Second, we have observed the production of organo-Cr(III) complexes during chromate reduction in the presence of microbial cellular components. Third, a bacterium capable of mineralizing an organo-Cr(III) complex has been isolated. Fourth, the transport and fate of organo-Cr(III) complexes in soils have been investigated. Some results have been published. Our data thus far point to a likely extensive formation of various organo-Cr(III) complexes during bioreduction of chromate. Further studies will help establish a more complete biogeochemical cycle for Cr, including organo-Cr(III) complexes as an integral link. The information should also provide guidance on whether organo-Cr(III) complex formation should be considered during application of Cr bioremediation.

Integrative Studies

Aqueous Complexation Reactions Governing the Rate and Extent of Biogeochemical U(VI) Reduction

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Laboratory research has shown that dissimilatory metal-reducing bacteria (DMRB) can effectively reduce oxidized uranium [U(VI)] to the sparingly soluble U(IV) with the concomitant precipitation of UO_2 phases. Despite the promise of bioreduction as a remediation strategy, the factors that enhance or inhibit the rate and extent of biogeochemical U(VI) reduction under representative environmental conditions are not well defined. Before effective bioimmobilization can be realized, the factors governing contaminant reactivity in multicomponent systems must be better understood. Only recently has the quantification of a few key interactions been established. For example, we recently reported the inhibition of bacterial U(VI) reduction by DMRB in the presence of environmentally realistic concentrations of soluble calcium (Ca) (Brooks et al., *Environ. Sci. Tech.*, 37:1850, 2003). This finding has significant implications for field applications of bioreduction because Ca^{2+} is a dominant soluble and cation-exchangeable species in soils and aquifers. We propose to identify and quantify the important biogeochemical reactions that also equilibrate with the U-carbonate solution species and may inhibit or enhance U(VI) reduction. Initially, cation exchange resins, with well-defined Ca^{2+} selectivities, will be employed to establish the distribution of Ca-U-carbonate species in the presence of varying amounts of cation-exchangeable forms of Ca^{2+}; other potentially important competing cations in the exchange equilibria (e.g., Mg^{2+}, Sr^{2+}) will be examined in later phases of the proposed research. Concurrent with the measurement of the competing equilibria among soluble and cation exchangeable phases, the reduction of the major cation-U-carbonate species will be studied using both abiotic and microbial agents. Our state-of-the-art measurement techniques (XAS, XRFS, EDX, TEM, radioisotopes, ICPMS, and KPA) will be applied to quantify these soluble complexes and precipitated phases. By understanding these important key equilibria, more predictable and effective approaches can be established for in situ bioremediation of U under realistic field conditions.

Reaction-Based Reactive Transport Modeling of Iron Reduction and Uranium Immobilization at Area 2 of the NABIR Field Research Center

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The proposed research is focused on developing mechanistic descriptions of important reactions and mathematical formulations for modeling the in situ immobilization of uranium promoted via microbial iron(III) reduction. Our goal is to develop and validate a mathematical model that can simulate all important chemical reactions and physical processes associated with in situ U(IV) immobilization. Experimental conditions will be designed to match those in saturated zone sediments at Area 2 of the DOE-NABIR Field Research Center (FRC).

The research will pursue three major objectives: (1) elucidate the mechanisms of reduction of solid-associated U(VI) in Area 2 sediment; (2) evaluate the potential for long-term sustained U(IV) reductive immobilization coupled to dissimilatory metal-reducing bacterial (DMRB) activity in Area 2 sediments; (3) numerically simulate the suite of hydrobiogeochemical processes occurring in experimental systems so as to facilitate modeling of in situ U(IV) immobilization at the field scale. The proposed research is based on the following five hypotheses: (1) the biological and chemical reduction of sediment-associated U(VI) is fundamentally controlled by its mineralogic and coordination environment; (2) the addition of humic substances can stimulate the reduction of solid-associated U(VI); (3) coupled Fe(III)/U(VI) reduction can be sustained in long-term flow-through reactor experiments with hydrologic residence times (week-to-month) comparable to those expected in pore domains likely to be colonized by DMRB in Area 2 sediments; (4) modest levels of nitrate input will not significantly inhibit coupled Fe(III)/U(VI) reduction in Area 2 sediments; and (5) the kinetics and thermodynamics of simultaneous biogeochemical reactions can be described by a series of reaction-based kinetic and equilibrium formulations derived independently from batch experiments.

To address the first hypothesis, we propose to use surface complexation modeling and spectroscopic techniques (XAS, LIFS) in a complementary fashion. Uranium(VI) sorption experiments will use Area 2 sediments and specimen minerals presumed to control U(VI) sorption and reducibility in these sediments (illite, goethite, and goethite-coated illite). The second hypothesis will be tested using a series of biotic (*Geobacter sulfurreducens*) and abiotic [excess Fe(II)] U(VI) reduction experiments with Area 2 sediments and specimen mineral assemblages in the presence/absence of FRC humic substances in batch reactors, semicontinuous culture reactors (SCRs), and constructed column reactors (CCRs). SCR experiments will provide suspension samples over time for solid-phase analyses (XAS, XPS, Mössbauer spectroscopy, ESEM, and wet chemistry). The third hypothesis will be tested in long-term SCR and CCR experiments containing Area 2 sediment inoculated with *Geobacter sulfurreducens*. The fourth hypothesis will be tested in long-term SCR and CCR experiments with *Geobacter metallireducens* (capable of both nitrate and metal reduction) and the presence/absence of 0.5 mM nitrate in the reactor feed solution. The fifth hypothesis will be addressed via continued development and calibration/validation of the BIOGEOCHEM–HYDROBIOGEOCHEM series of reaction-based computer models.

Integrated Nucleic Acid System for In-Field Monitoring of Microbial Community Dynamics and Metabolic Activity

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Molecular analysis of subsurface microbial communities requires some combination of sample collection, concentration, cell lysis, nucleic acid purification, polymerase chain reaction (PCR) amplification, and specific detection in order to address fundamental questions of microbial community dynamics, activity, and function in the environment. As a result of our prior NABIR grant, we developed a suite of integrated microparticle chemistries, Fe- and SO_4-reducer phylogenetic probes and suspension arrays, and combined sample purification/bead array fluidic devices for the automated and direct analysis of 16S rRNA and microbial community dynamics in environmental samples. However, changes in microbial community composition and/or abundance are still insufficient to detect or make conclusions regarding specific microbial activity. Thus, fieldable methods for the direct analysis of functional genes and messenger RNA (mRNA) in the environment are still required. The objective of this project is therefore twofold. First, we seek to verify the 16S rRNA methods, Fe- and SO_4-reducer array and instrumentation on sediment and groundwater samples taken from the Schiebe/Roden FRC site, and continue to expand the probe suite for microbial community dynamics as new sequences are obtained from DOE-relevant sites. Second, we propose to address the fundamental molecular biology and analytical chemistry associated with the direct analysis of functional genes and mRNA (hence, microbial activity) in environmental samples. These studies will investigate the behavior of oligonucleotide and cDNA capture and detection probes for direct mRNA purification and detection on microparticle surfaces; "tunable surface" chemistries to increase mRNA capture and detection efficiency; new mRNA reporter and detection chemistries required for the development of in-field monitoring methods and devices; and fluidic strategies for integrating complex biochemistry for direct detection of mRNA in sediments and groundwater, without using PCR.

Recognizing that not all field sites contain enough RNA for direct hybridization and detection, and as a prelude to cDNA versus mRNA detection, efforts in the first two quarters of the project have focused on investigating the behavior of DNA (e.g., PCR amplicon) versus RNA hybridization and applying both DNA and RNA methods and model array to DOE sites. The testable hypothesis is that hybridization specificity on the 85-bead array will differ between rRNA and equivalent PCR amplicons. At the spring meeting, we will present comparative DNA and RNA hybridization data for a validation set of Fe- and SO_4-reducing microorganisms, preliminary hybridization data for a time course microcosm study initiated from the Schiebe/Roden FRC field site, and tunable bead array rRNA profiles from Phil Long's UMTRA field site before and after biostimulation. The team's latest advances (and challenges) in autonomous, single-particle RNA purification, fragmentation, and labeling chemistry will likewise be presented, with a discussion of the implications for developing fully autonomous mRNA analysis systems.

Characterizing the Catalytic Potential of *Deinococcus, Arthrobacter*, and other Robust Bacteria in Contaminated Subsurface Environments of the Hanford Site

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We have recently isolated several distinct species of *Deinococcus* and *Arthrobacter* from pristine and contaminated soils of DOE's Hanford Site in south-central Washington state. Generally, *Deinococcus* bacteria are exceptionally robust, not only surviving exposure to radiation, but also to other DNA-damaging conditions typical of DOE environments. The ability to reduce a variety of toxic metals also appears to be a characteristic shared by these organisms, and we have shown that *D. radiodurans* and *D. geothermalis* are proficient at reducing U(VI), Tc(VII) (in the presence of an electron shuttle), and Cr(VI), and have engineered both species for Hg(II) reduction. Recent experimental advances in the genetic management of *D. radiodurans* and *D. geothermalis* will facilitate our efforts to characterize the metal-reducing mechanisms of both organisms. These advances include our comprehensive analysis of the *D. radiodurans* genome, and for *D. geothermalis*, the development of a system for genetic transformation and ongoing genome sequencing, annotation, and analysis of this organism. *Arthrobacter* species are capable of free-living growth in many extreme environments, such as under chronic radiation. *Arthrobacter* spp. also have recently been reported to efficiently resist and reduce high concentrations of hexavalent chromium. These and other cultured isolates from the Hanford Site will be examined for their metal-reducing capabilities. Furthermore, we have shown that the ionizing radiation doses that yield 17% survival of *Escherichia coli* and *D. radiodurans* are higher by factors of 20 and 200, respectively, than those for *Shewanella oneidensis* MR-1, a dissimilatory metal-reducing gamma-proteobacterium that has been proposed for bioremediation of DOE waste sites. However, *S. oneidensis* is extremely sensitive to ionizing radiation, desiccation, and other oxidative stress conditions. Recent advances in understanding how *D. radiodurans* survives ionizing radiation support the possibility that the resistance characteristics of sensitive organisms such as *S. oneidensis* might be increased significantly with antioxidants, which could benefit applications in environmental biotechnology. We are examining if antioxidants can increase the efficacy of *S. oneidensis* in genotoxic environments, without the need for genetic engineering. The specific aims of this project are: (1) characterizing the genetic basis of metal reduction in *D. radiodurans* and *D. geothermalis*, and novel deinococcal isolates from the Hanford Site; (2) characterizing the microbial ecology of contaminated waste sites and the metal-reducing potential of these communities; (3) defining environmental factors that govern in situ growth, environmental robustness, metabolism, and Cr(VI)-reduction of *Deinococcus* and *Arthrobacter* spp.; (4) characterizing the genetic basis of metal reduction in *Arthrobacter aurescens* (TC1) and novel *Arthrobacter* isolates from the Hanford Site; and (5) testing the ability of antioxidants to support the growth of *S. oneidensis* under chronic ionizing radiation.

Heterogeneity in Bioreduction and Resulting Impacts on Contaminant and Microbial Dynamics

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Soil and sediments are complex mineral assemblages hosting a diverse microbial community, all within a convoluted physically heterogeneous setting. As a consequence, biogeochemical processes exhibit high spatial variability due to chemical conditions dictated by local mineralogical and physical proximity. Bioreductive stabilization of contaminants is dependent on convoluted coupling of biological, chemical, and hydrologic processes that will vary spatially from the micro- to macro-scale. We have therefore been conducting a series of studies that encompass increased chemical and physical heterogeneity on bioreductive processes. A principal repository of contaminants within soils and sediments are iron (hydr)oxides, which have a high affinity for numerous metal contaminants, including uranium. As such, the transformation of iron (hydr)oxides, whether by dissolution or recrystallization, will have profound impacts on contaminant fate and transport. Various iron phases are present within most soils and sediments; one of the most reactive forms common to such systems is ferrihydrite, a short-range order ferric hydroxide. Given the surface reactivity of ferrihydrite, most 'natural' phases will not possess pristine surfaces but will instead host numerous anions. Accordingly, we investigated the role of surface modifiers such as phosphate, silicate, arsenate, and a series of organic acids on the reductive dissolution of ferrihydrite. The impact of phosphate is particularly illustrative of surface alterations on microbially induced transformation of iron oxides. Previously, we and others observed the rapid and near complete conversion of ferrihydrite to goethite and magnetite during active metabolism of *Shewanella putrefaciens*. However, modifying the ferrihydrite surface through the addition of phosphate alters the extent of reduction and the reaction products. At low coverage (<50% of the adsorption maximum), phosphate has little impact on ferrihydrite reduction and, in fact, will help sustained reduction by providing a source of necessary and often limiting nutrients. As surface coverage increases to near the adsorption maximum, reduction becomes appreciably retarded and the appearance of green rust becomes pronounced. Thus, despite the high reactivity of synthetic ferrihydrite, most natural forms will likely have a greatly diminished reactivity due to the retention of both common cations and anions.

In addition to examining the impact of chemical complexity on bioreductive processes, we have been conducting a series of experiments having increased physical complexity to examine microbial distribution and biogeochemical consequences. Using a constructed cell containing domains representative of diffusive and advective flow, we have examined bioreductive processes of iron (hydr)oxides using micro-focused x-ray fluorescence mapping and x-ray absorption spectroscopy. To help resolve the underlying phenomena, we conducted simulations of biomineralization processes using rate parameters derived for transformation of ferrihydrite to goethite and magnetite in conjunction with reduction rates and secondary reactions of goethite and hematite. Gradients in dissolved organic carbon and resulting metabolic products occur along diffusive zones emanating from advective flow-paths and lead to large variations in biological activity and resulting (bio)mineralization processes. Metabolic rates are greatest along advective flow paths and diminish with progressive distance into the diffusive domains. As a consequence, bioreduction of both contaminants such as uranium and mineral constituents such as ferric (hydr)oxides are localized along advective flow paths with a progressive decrease upon entering diffusive pore domains. Thus, appreciable spatial heterogeneity will result in contaminant sequestration, both in degree and mechanism, within soils and sediments.

The Role of *Shewanella oneidensis* MR-1 *c*-type Cytochromes and Type II Secretion System in Uranium Reduction and Localization of Nanoparticles

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Due to its relatively high mobility in oxidizing subsurface environments, uranium(VI) is a primary contaminant of concern at multiple U.S. Department of Energy sites, including Hanford. The reduction of U(VI) generally results in the formation of poorly soluble $U(IV)O_{2(s)}$ particles that are considerably less mobile in the environment. In spite of extensive research efforts, little is known regarding the reduction and precipitation mechanisms and the subsequent reactivity and transport of biogenic $UO_{2(s)}$ particles. *Shewanella oneidensis* MR-1 effectively reduces a wide range of metals and radionuclides and produces a cadre of periplasmic and outer membrane associated *c*-type cytochromes that are believed to facilitate the transfer of electrons to solid metal oxides. Additionally, genomic analysis of *S. oneidensis* MR-1 has revealed that this organism possess a functional type II (T2S) secretion pathway that appears to be involved in the proper localization of certain cytochromes and possibly in the localization of other proteins involved in the reduction or translocation of $UO_{2(s)}$ nanoparticles. To characterize the role of these *c*-type cytochromes and the T2S in the reduction of U(VI), a series of cytochrome gene deletion mutants and insertional mutants interrupting critical genes in the T2S pathway (*gspD*, *gspG*, and *pilD*) were constructed and characterized for phenotypic differences when using uranium as the terminal electron acceptor. MR-1 quantitatively reduced 250 μm U(VI) to U(IV) within 24 hr, whereas mutations in genes encoding three decaheme cytochromes (OmcA, MtrA, and MtrC) and a related outer membrane protein (MtrB) reduced only 50% of the U(VI) in the same time period. The rate of uranium reduction by the T2S mutants was also initially slower than MR-1; however, the mutants eventually reduced all U(VI), including the cytochrome mutants. Together, these data suggested that the T2S system is not essential for U(VI) reduction but may be involved in the localization of cytochromes or other proteins involved in reduction of uranium or, alternatively, the translocation of U(IV) nanoparticles from the cell periplasm.

Transmission electron microscopic (TEM) images of one T2S mutant (*gspD*$^{-}$) revealed nm-size U precipitates confined to the periplasm while UO_2 was predominantly present as 1–5 nm fine-grained particles associated with fiber-like or sheath-like structure external to the cell in MR-1. TEM of mutants deficient in OmcA or MtrC revealed an accumulation of $UO_{2(s)}$ exclusively in the periplasmic space, whereas accumulation in MtrA- or MtrB-deficient mutants was predominantly in the periplasmic space and to a lesser degree in association with some of the structures external to the cell, as observed in the wild type. Using density gradient centrifugation, the structures associated with solid-phase extracellular UO_2 were isolated and additional microscopic, biochemical, and compositional analysis studies are under way to characterize the structures and cytochrome content associated with solid phase, extracellular UO_2. These results are anticipated to have important implications for the long-term fate and transport of biogenic UO_2 in subsurface environments.

Biogeochemical Cycling and Environmental Stability of Pu Relevant to Long-Term Stewardship of DOE Sites

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Plutonium contamination is widespread in surface soils and subsurface sediments throughout the DOE complex. Until the last decade or so, Pu was generally considered to be relatively immobile in the terrestrial environment, with the exception of transport via aeolian and erosional mechanisms. More recently, however, the transport of colloidal forms of Pu has been invoked as providing a mobilization pathway in low intensity stream environments and the subsurface.

Central to understanding the environmental behavior of Pu in vadose- and saturated-zones, as well as in waste streams, is the contribution of microbial communities to Pu speciation. This research addresses the principal mechanisms by which naturally occurring microbial communities regulate transformations in Pu chemical speciation; such changes may lead to either enhanced Pu immobilization or its release from immobile phases and subsequent transport.

The overall objective of this proposed research is to understand the biogeochemical cycling of Pu in environments of interest to long-term DOE stewardship issues. Central to Pu cycling (transport initiation → immobilization) is the role of microorganisms. The hypothesis underlying this proposal is that microbial activity is the causative agent in initiating the mobilization of Pu in near-surface environments: through the transformation of Pu associated with solid phases, the production of extracellular polymeric substances (EPS) carrier phases, and the creation of microenvironments that foster microbial transformations. In addition, microbial processes are considered to be central to the immobilization of Pu species: through the microbially induced transformation of organically complexed Pu species and Pu associated with extracellular carrier phases (e.g., Pu-EPS complexes) and the creation of environments favorable for Pu transport retardation. In general, the biogeochemical processes that will be addressed in this research are:

1. The release of Pu sorbed to immobile phase substrates through the microbially mediated destruction of that host phase (e.g., reductive dissolution of Fe(III) oxides);
2. 'Scavenging' of sorbed Pu by microbially produced ligands;
3. Biodegradation of Pu-organic ligand complexes; and,
4. Mobilization of Pu under advective flow conditions by microbially produced EPS.

Biostimulation of Iron Reduction and Uranium Immobilization: Microbial and Mineralogical Controls

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The overall objective of our project is to understand the microbial and geochemical mechanisms controlling the reduction and immobilization of U(VI) during biostimulation in acidic subsurface sediments such as those present at the Field Research Center (FRC). The focus is on the activity and community composition of microbial populations (metal- and nitrate-reducing bacteria) and iron minerals which are likely to make strong contributions to the fate of uranium during in situ bioremediation.

We conducted microcosm experiments using near in situ conditions with FRC subsurface materials co-contaminated with high levels of U(VI) and nitrate. Rates of electron acceptor/ donor utilization were measured in acidic subsurface sediments across a range of environmental variables (pH, nitrate) relevant to bioremediation. Microbial activity was minimal at pH 5 or below, indicating that acidity is a master variable controlling microbial metabolism in FRC sediments. In concurrence with previous studies of neutrophilic uranium-contaminated subsurface environments, metal reduction in the acidic subsurface did not occur until after nitrate was depleted to low levels in response to pH neutralization and carbon substrate addition. Nitrate reduction, iron reduction, and electron donor utilization rates in neutralized acidic microcosms were among the most rapid reported for aquatic sedimentary environments. Acidity influenced not only the rates but also the pathways of microbial activity. The majority of C equivalents in neutralized glucose-amended microcosms were recovered as fermentation products, mainly as acetate. Although biostimulation leads to rapid nitrate and metal reduction in acidic subsurface sediments, environmental extremes appear to have selected for microbial communities with different metabolisms, and metal reduction may be substantially catalyzed by fermentative bacteria. By targeting rRNA, we sought to provide a novel assessment of the metabolically-active microbial groups as well as their response to changing environmental conditions in microcosm sediments. Clone libraries, constructed from the 16S rRNA gene and cDNA reverse transcribed from the 16S rRNA, contained representatives from the phyla Planctomycetes, Proteobacteria (α, β, δ, γ), Bacteroides, and Firmicutes. The most abundant phylotypes found in the DNA libraries were members of the Alphaproteobacteria, while Gammaproteobacteria-related clones dominated the RNA libraries. Currently, the response of metal-reducers and other heterotrophic communities is being investigated further in sediment microcosms in which metal reduction was stimulated by carbon substrate addition and pH neutralization.

Three novel iron(III)-reducing organisms were isolated from the contaminated FRC subsurface. Stable enrichment cultures were established and isolations were performed in soft gelrite dilution series using FeOOH and AQDS as the sole electron acceptor, respectively. Using the 16S rRNA gene as a molecular marker, pure cultures shared high sequence identity (96 to 99%) to *Geobacter bremensis*, *Clostridium* sp. CITR8, and *Desulfotomaculum ruminis*, respectively. Isolates were also closely affiliated with phylotypes detected in the FRC subsurface using cultivation-independent methods.

Stabilization of Plutonium in Subsurface Environments via Microbial Reduction and Biofilm Formation

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Contaminant Pu is present at DOE sites that have complicated hydrogeology, and redox environments are affected by direct and indirect microbially mediated processes. The influence of these processes is particularly difficult to predict, due to the complicated redox behavior and rich chemistry of Pu. Our team aims to understand how bacteria can affect Pu speciation and environmental mobility, with a focus on (1) biotransformation and cell internalization by aerobic bacteria, (2) accumulation and immobilization in biofilms, and (3) immobilization via direct enzymatic and indirect biogeochemical reduction of Pu species by metal-reducing bacteria.

Biotransformation of Pu Species. Plutonium is accumulated by *Microbacterium flavescens* (JG-9) via an active hydroxamate siderophore recognition and uptake process. In order to determine if siderophore-mediated bacterial internalization of Pu is a general phenomenon, the pyoverdin/*Pseudomonas putida* system is being studied. The pyoverdin produced by *P. putida* strain ATCC 33015 contains a chromophore group derived from 2,3-diamino-6,7-dihydroxyquinoline with a catechol linked to a six amino acid peptide chain (determined using mass spectroscopy and multinuclear, multidimensional NMR). Pyoverdin binds Pu(IV) and Th(IV) to form 1:1 or 1:2 metal:pyoverdin complexes, which are being characterized. This siderophore mediates the transport of Pu, Fe, and Th into cells at similar accumulation rates and amounts. These studies indicate that under environmental conditions Pu^{IV} will be accumulated by bacteria via siderophore receptors and encourage us to investigate Pu accumulation by bacteria in biofilms.

Stabilization of Pu within Biofilms. Bacteria in nature grow mostly in biofilms, surface associated cells enveloped by hydrated extracellular polymeric substances (EPS). Plutonium may be sequestered within biofilms. Unsaturated biofilms of *P. putida* are being cultivated on membranes overlaying solid media with the individual addition of soluble metal (Cr, U, or Pu), mineral (hematite), and metal + mineral. Biofilms are characterized by imaging techniques, and then separated into cell and EPS fractions for subsequent inorganic speciation and biomolecule analyses. Initial results confirm that biofilm morphology varies by treatment and show how Cr is distributed between cells and EPS.

Bacterial reduction of Pu(VI/V). Pu(V) and Pu(VI) are the most soluble Pu species found under environmental conditions. Their reduction by metal-reducing bacteria to Pu(IV) solids could stabilize Pu in situ with respect to migration. Cell suspensions of *Shewanella oneidensis* MR1 and *Geobacter metallaredceans* GS15 rapidly reduce Pu(V) and Pu(VI) to form a solid that has spectroscopic features consistent with Pu(IV) (hydr)oxides. For example, with an approximate cell density of 5×10^9 cells/mL, *S. oneidensis* removed from solution ~99% of the added Pu within 4 h, and *G. metallireduciens* removed from solution ~97% of the added Pu within 24 h. Preliminary experiments indicate plutonyl may also support growth of these bacteria.

An Integrated Assessment Of Geochemical And Community Structure Determinants Of Metal Reduction Rates In Subsurface Sediments

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Fundamental questions still persist concerning the interactive effects of geochemistry and community structure on metal reduction rates in the subsurface. The overall goal of our project is to provide an improved understanding of the relationships between microbial community structure, geochemistry, and metal reduction rates. Many microorganisms can change the subsurface geochemical conditions (e.g., cause a drop in redox) such that metal reduction becomes an energetically favored reaction. Some microbes can directly catalyze the necessary reactions so that metal reduction occurs at a more rapid rate than without microbial activity. Many microorganisms can accomplish the first role but much fewer can accomplish the second. Physical and geochemical factors such as mass transport, oxygen level, and nitrate concentration will likely dominate the rate of microbial change in the redox potential. Thus, it is possible that the importance of community structure at this stage of metal reduction may be minimal. However, the effect of community composition on the rate of metal reduction may be important. We are using controlled laboratory experiments with sediments and groundwater from the NABIR Field Research Center (FRC) to compare the effects of manipulations designed to influence community structure (differences in electron donors) to those designed to influence geochemistry (the presence of humics as electron shuttles) on uranium reduction. Also, the effect of carbon:phosphate ratios on community structure and uranium reduction rates will be examined in the context of resource-ratio theory to help predict the nutrient supply rates and ratios that maximize uranium reduction at the FRC site. This theory predicts that the structure of a biological community depends on the supply rates and ratios of potentially growth-limiting resources. A preliminary scoping experiment is underway using methanol (20 mM), ethanol (10 mM), and glucose (5 mM) as electron donors at equivalent electron transfer potential. The experiment is being run at pH 7.0 with archived site material and a groundwater inoculation. At two weeks, there was evidence of metabolic activity (increased headspace pressure compared to the controls) for all three electron donors. Total U, organic C, pH, and nitrate are being measured. The next experiment will use six electron donors and will be run at several pH levels. These combinations should lead to even greater differences in community structure. This study will help assess the applicability of NABIR research to other sites with diverse community structures.

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Biogeochemical Coupling of Fe and Tc Speciation in Subsurface Sediments: Implications to Long-Term Tc Immobilization

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Technetium-99 is an important DOE subsurface contaminant. It is long-lived ($t^{1/2} = 2.\times 10^5$ yr), and exists in groundwater as the mobile pertechnetate anion [$Tc(VII)O_4^-$]. Pertechnetate can be immobilized by biotic and abiotic reduction to insoluble $Tc(IV)O_2 \bullet nH_2O$ that exhibits a solubility of 10^{-8} mol/L or less. The half-cell potential for this reaction is "intermediate" in environmental redox space.

Our past NABIR research demonstrated that Fe(II) resulting from the activity of dissimilatory iron-reducing bacteria (DIRB) can reduce and immobilize pertechnetate from high nitrate waters in Hanford and Oak Ridge sediment. The reduction kinetics depend on the biogenic Fe(II) concentration and its molecular and mineralogic environment in the sediment. Ongoing laboratory research is utilizing batch and column experimental systems along with various forms of x-ray, electron, and gamma-ray spectroscopies/microscopies to: (1) quantify the kinetic reactivity of different biotic and abiotic Fe(II) forms to allow rigorous kinetic modeling of parallel reaction/reduction paths; and (2) investigate the oxidation/remobilization reaction with oxygen, and determine the mineralogic, biogeochemical, and microbiologic factors that control it during extended in-ground residence times.

At the 2005 NABIR meeting we will: present quantitative results on the homogeneous reduction rate of Tc(VII) by $Fe(II)_{aq}$ (which is surprisingly significant); identify the nature of mineral products formed using isotopic enrichment with ^{57}Fe and Mössbauer spectroscopy, and high-resolution transmission electron microscopy; and compare these rates to heterogeneous ones with insoluble Fe(II) in different mineralogic states. These results will be complemented with oxidation rate studies of precipitated Tc(IV) resulting from both homogeneous and heterogeneous reduction reactions. It is shown that the molecular/mineralogic environment of Fe(II) has a major influence on both Tc reduction and oxidation rates, albeit for different reasons.

Field Studies

Field-Scale Demonstration of In Situ Biological Uranium(VI) Reduction at Area 3, NABIR Field Research Center, Oak Ridge, TN

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Mixed wastes and extreme conditions pose a significant challenge for remediation of uranium-contaminated soils at many contaminated sites. We have evaluated the potential for field-scale in situ biological reduction of uranium(VI) in Area 3 of the DOE NABIR Field Research Center in Oak Ridge, TN. At this site, high levels of uranium are present in the groundwater (~60 mg/L) and soil (up to 700–1,000 mg/kg). Many other contaminants are also present. Notably, the groundwater contains high levels of nitrate (8–10 g/L), calcium (0.9 g/L), and aluminum (0.5 g/L) at a pH of 3.4. To evaluate options for site remediation, we carried out slurry and column experiments. These studies suggested the feasibility of a three-step approach: (1) flushing with a low pH water to remove aluminum and bulk nitrate; (2) adjustment of pH to a range favorable for microbial activity; and (3) ethanol addition to stimulate U(VI) reduction. This strategy was then implemented at the field site, using an aboveground treatment system to remove nitrate, calcium, and aluminum, and to adjust pH, and belowground recirculation wells for ethanol addition.

The field experiment began with a clean-water flush. Water from the subsurface was extracted and treated to remove Al, Ca, and nitrate, supplemented with tap water and injected into the well system. The pH of the injected water was then gradually increased. After three months, nitrate concentrations had fallen to less than 1.0 mM. In situ bioremediation was then initiated by intermittent ethanol addition. A vacuum stripper was installed between the innerloop extraction well and injection wells to remove nitrogen gas created by denitrification of residual nitrate. Immediately after ethanol injection, nitrate levels dropped to 0.3 mM or lower. After three months of ethanol addition, ethanol addition was accompanied by a reduction in aqueous U(VI). Sulfate consumption and sulfide formation also occurred concomitantly. A tracer test with bromide and ethanol confirmed that the removal of ethanol was due to biological activity. Most Probable Number (MPN) analyses of the groundwater from extraction well and multiple level sampling (MLS) wells indicated the growth of denitrifying bacteria, sulfate-reducing bacteria (SRB) and iron-reducing bacteria (FeRB) in the subsurface. In the extraction well, recent cell numbers (cell/mL) are: denitrifiers, 107; SRB, 104; and FeRB, 105. More SRB and FeRB were detected in MLS wells. During biostimulation operations, we generally maintained the pH of the well system at 5.8–6.0 to prevent growth of methanogens. The growth of these organisms is of concern because they compete for ethanol with U(VI)-reducing bacteria and they produce methane, a gas that could clog the aquifer. To date, we have not detected methanogenesis.

Chemical clogging by aluminum precipitates was observed in the injection and extraction wells during the initial phases of operation. Biological clogging was observed during biostimulation. To restore hydraulic conductivity, we moved surge blocks up and down within the clogged wells. This operation was repeated as needed, with a frequency of about once every 1.5 months. This cleaning operation released biomass and sediment from around the well screens and into the well itself. The resulting slurry was removed by pumping. X-ray absorption near edge structure spectroscopy of the slurry solids confirmed that U(VI) was reduced to U(IV). After 9 months of operation, up to 42% of the uranium in injection-well slurry samples was present as U(IV); uranium in extraction well slurry samples remained as U (VI).

Field-Integrated Studies of Long-Term Sustainability of Chromium Bioreduction at Hanford 100H Site

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The focus of these project is to understand the coupled hydraulic, geochemical, and microbial conditions necessary to maximize Cr(VI) bioreduction and minimize Cr(III) reoxidation in groundwater. To demonstrate the feasibility of a cost-effective remediation technology for bioimmobilization of Cr(VI) in contaminated groundwater, using a slow release polylactate Compound, HRC™, we have conducted a series of bench-scale and field-scale integrated treatability studies.

Using bench-scale studies, we have shown the presence of several types of bacteria in the sediments from the Hanford 100H site, including *Bacillus/Arthrobacter* and *Geobacter* species, which are known to reduce or sorb hexavalent chromium. Under background conditions, the total microbial population in Hanford sediments is $<10^5$ cells g^{-1}, which is likely insufficient for direct enzymatic Cr(VI) reduction. We have shown that different types of HRC and metal remediation compound (MRC™) products could stimulate an increase in biomass to $>10^8$ cells g^{-1}, generate highly reducing conditions, and enhance Cr(VI) removal from the pore solution.

At the Hanford 100H field site, we drilled and equipped two new wells—injection Well 699-96-45, located 15 ft downgradient from the existing monitoring well 699-96-43, and a monitoring and pumping Well 699-96-44, located 15 ft downgradient from the injection well. To assess the background hydraulic properties of the Hanford formation, three Br-tracer injection tests and two pumping tests (concurrently with the Br-tracer tests) were performed before the HRC injection. We also performed a series of geophysical (seismic and radar) cross-borehole measurements.

A pilot field-scale test was conducted, injecting 40 lb of ^{13}C-labeled HRC via injection Well 699-96-45 into the Hanford formation (over 44 ft to 50 ft depth intervals). Redox potential, pH, dissolved oxygen (DO), nitrate, Cr(VI), and sulfate concentrations in groundwater were monitored. Following HRC injection (27 days), redox potential dropped to –130 mV, and DO and nitrate practically disappeared. Cr(VI) concentrations declined steadily over 6 weeks. Analysis of $\delta^{13}C$ ratios in dissolved inorganic carbon confirmed microbial metabolism of HRC. Hydrogen sulfide production was first observed after about 20 days post-injection, which corresponds with the enrichment of a *Desulfovibrio* species identified using fluorescent antibodies. DO and nitrate began to return to background concentrations two months after HRC injection, despite bacterial densities remaining high ($>10^7$ cells/mL). Cr(VI) concentrations in the monitoring and pumping wells remained below up-gradient concentrations. Thus, microbial and geochemical analysis of groundwater coupled with stable isotope monitoring allowed for accurate tracking of microbial processes during this field treatability study, and confirmed that Cr(VI) was successfully removed from groundwater at a contaminated site using HRC as an electron donor and a carbon source.

Directions of ongoing research include the determination of whether dissolved oxygen and manganese oxides could reoxidize Cr(III) to Cr(VI), and the development of a 3D reactive transport code, TOUGHREACT-BIO, to simulate coupled biological and geochemical processes.

Factors Controlling In Situ Uranium and Technetium Bioreduction and Reoxidation at the NABIR Field Research Center

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Summary of Recent Field Testing. Extensive in situ field testing using the push-pull method has demonstrated that indigenous microorganisms in the shallow (<8 m) aquifer in FRC Areas 1 and 2 are capable of coupling the oxidation of injected ethanol to the reduction of U(VI) and Tc(VII). Despite highly variable conditions and contaminant concentrations (pH: 3.3–7.2; nitrate: 0.1–140 mM; U(VI): 1–12 μM; Tc(VII): 200–15,000 pM), sequential donor additions resulted in increased rates of microbial activity (denitrification: 0.01–4.0 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. Reoxidation of U(IV) was observed when injected test solutions contained initial nitrate concentrations > ~20 mM. Field data and laboratory studies suggest U(IV) is likely oxidized by Fe(III). 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. Production of metal sulfides following addition of sulfate was shown to reduce U(IV) reoxidation rates in field tests conducted in Area 2. Several denitrifying organisms were cultured from Area 1 during biostimulation that can 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 is provided by the results of microbial sampling by Aaron Peacock and David White at the Univ. of Tennessee; Joel Kostka at the Univ. of Florida, and Chris Schadt at ORNL, and confirms 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 Univ. 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.

Ongoing Field Activities. 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 four factors: precipitation of dissolved solids, growth of biomass, generation of N_2 gas, and well-screen clogging. Field tests are in progress in collaboration with Susan Hubbard (LBNL) in Areas 1 and 2 to see if precipitate, biomass, or gas production can be detected and quantified using noninvasive geophysical methods. The effects of biomass and gas production on hydraulic conductivity of site sediments are also being investigated in laboratory experiments conducted in collaboration with Mart Oostrom and Tom Wietsma at EMSL.

Collectively, our project results suggest 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, (2) denitrification and Tc(VII) reduction, and (3) U(VI) reduction. This hypothesis is being tested in intermediate (~2 m) scale physical models at Areas 1 and 2. The physical models have been in operation for approximately 16 months, and results indicate that essentially complete U(VI) and Tc(VII) removal can be achieved. Characterization of the microbial community is in progress in collaboration with Aaron Peacock and David White at the Univ. of Tennessee. This detailed information should provide us a nearly complete description of the biogeochemical processes that will occur as this complex, contaminated groundwater interacts with a biologically active treatment zone in the subsurface.

Stability of U(VI) and Tc(VII) Reducing Microbial Communities to Environmental Perturbation: Development and Testing of a Thermodynamic Network Model

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In situ field experiments at the NABIR Field Research Center (FRC) have shown that cooperative metabolism of denitrifiers and Fe(III)/sulfate reducers is essential for creating subsurface conditions favorable for U(VI) and Tc(VII) bioreduction. Although much has been learned about the physiology and metabolic potential of specific microorganisms with these capabilities that have been isolated from the FRC and other sites using pure cultures of microorganisms, major gaps exist in our understanding of the functioning of these organisms when they are present in intact microbial communities. For example, although ongoing NABIR studies have demonstrated the large genetic diversity of subsurface microorganisms at the FRC, many of these have never been isolated in pure culture. However, it is the collective metabolic capability of these largely uncharacterized microorganisms that must be relied on for effective U(VI) and Tc(VII) bioimmobilization.

The overall goal of this project is to develop and test a thermodynamic network model for predicting the effects of substrate additions and environmental perturbations on the composition and functional stability of subsurface microbial communities. The overall scientific hypothesis is that a thermodynamic analysis of the energy-yielding reactions performed by broadly defined groups of microorganisms can be used to make quantitative and testable predictions of the change in microbial community composition that will occur when a substrate is added to the subsurface or when environmental conditions change.

The proposed research will be conducted at the FRC using four intermediate-scale (~ 2 m) bioreactor models currently deployed in Areas 1 and 2. The network model will be used to predict the effects of substrate additions on the microbial community composition in the bioreactors by predicting the growth of major metabolic groups of organisms (aerobes, fermenters, denitrifiers, Fe(III)/sulfate/metal reducers). Model predictions will be tested by quantifying changes in the abundance of each of these groups using a combination of functional genes and lipid analysis. The network model will also be used to examine the stability of the U(VI) and Tc(VII) reducing microbial communities to changing environmental conditions. These predictions will be tested by challenging the microbial community in the bioreactors with a series of perturbations representative of those likely to occur (due to heterogeneity in groundwater geochemistry) in a full-scale bioreactor at the FRC. These will include variations in pH, nitrate, and sulfate concentrations in the bioreactor influent.

The ability to predict the effects of donor addition on change in microbial community composition is essential for creating conditions that favor the long-term stability of bioreduced U and Tc. Moreover, the ability of a microbial community to maintain functional stability [i.e., maintain high rates of U(VI) and Tc(VII) reduction] when subjected to various environmental perturbations (e.g., fluctuating pH and concentrations of electron acceptors) is of critical importance for the ultimate use of bioimmobilization at DOE legacy waste sites. The principal immediate significance of this project will the establishment and testing pf a theoretical framework for designing and interpreting complex field experiments and aiding in "bridging-the-gap" between basic NABIR laboratory and field research.

Acceleration of Field-scale Bioreduction of U(VI) in a Shallow Alluvial Aquifer: Temporal and Spatial Evolution of Biogeochemistry

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Uranium contamination in a shallow aquifer at Rifle, CO provides an opportunity for repeated field-scale electron donor amendment experiments allowing investigation of rates of U(VI) reduction under varying terminal electron0accepting processes. In the first two experiments conducted in the same experimental plot during the 2002 and 2003 field seasons, U(VI) loss from groundwater occurred synchronously with growth of *Geobacter* after amendment of the subsurface with acetate at concentrations of either ~3 mM (2002) or ~10 mM (2003). These two experiments demonstrated the importance of (1) maintaining Fe(III)-reducing conditions for optimal U(VI) bioreduction and (2) controlling the spatial location of sulfate reduction to facilitate down-gradient Fe(III) reduction. Monitoring of U(VI) concentrations and other geochemical parameters after the 2003 experiment suggests that loss of U(VI) as groundwater flows through the test plot can be maintained for >1 yr after the last addition of acetate, particularly in zones where high levels of bioreduction occurred during acetate amendment. Mechanisms for removal of U(VI) from groundwater in the absence of acetate amendment are not known and will be a focus of future field-scale experiments.

During the 2004 field season, acetate was amended to the subsurface in a new, smaller experimental plot under conditions similar to that of the 2002 experiment. The objectives of the 2004 experiment included included: (1) determine if the U(VI) loss and *Geobacter* growth of the 2002 experiment can be replicated at a second location; (2) define the growth of *Geobacter* with high-frequency sampling during the first month of acetate amendment; (3) collect and filter groundwater samples for mRNA and genomic analysis under biostimulated conditions; and (4) obtain preliminary geophysical measurements (complex resistivity) to assess the possibility of using noninvasive techniques to monitor microbially mediated changes in subsurface mineralogy. Analysis of data from the 2004 experiment is in progress, but preliminary results indicate that all objectives of the experiment were met. Uranium concentrations in groundwater down gradient decreased in a fashion similar to the 2002 experiment except that the experiment was terminated prior to development of extensive sulfate reduction. The decrease in U(VI) paralleled dominance of *Geobacter* in groundwater samples, strongly indicating that *Geobacter* is responsible for enzymatic reduction of U(VI) in situ (see Lovley et al., abstract, p.__, for clone library data and mRNA results). Inversion of complex resistivity data show a marked anomaly below the water table in the vicinity of the injection gallery, which is interpreted as a response to the changed redox status of Fe-bearing mineral coatings on detrital grains. This promising result suggests that more extensive resistivity measurements may provide detailed tracking of Fe redox status in response to in situ bioreduction. Future research at the Rifle site will include detailed reactive transport modeling and additional in situ experiments that will elucidate controls on U(VI) reduction and reoxidation rates under both Fe-reducing and sulfate-reducing conditions.

In Situ Immobilization of Uranium in Structured Porous Media via Biomineralization at the Fracture/Matrix Interface

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We are preparing for an in situ field-scale biostimulation experiment at Area 2 of the NABIR Field Research Center (FRC). The field experiment will evaluate the feasibility of stimulating microbial U(VI) reduction activity at the interfaces between different pore regions within a heterogeneous aquifer and thereby decreasing mass transfer of U from immobile or slow-moving regions to actively flowing groundwater. We have focused our attention on a shallow layer of disturbed saprolite (fill), including a gravelly layer at the base of the disturbed zone. Locally distributed microbarriers can form at interfaces at multiple scales including (1) grain scale (if intragranular porosity serves as a source/sink for contaminants); (2) local scale, where multiple porosity domains exist within a single porous media (e.g., fractures and matrix); and (3) field scale, where large heterogeneous features establish preferential flow (e.g., the gravelly layer in Area 2). We conducted a bromide tracer test in August 2004, which provided evidence of significant inter-region mass transfer. We will compare pre- and post-biostimulation flush experiments using donor-free water in order to directly measure changes in the rate of uranium transfer into the advective groundwater domain associated with the stimulation of microbial activity. Sediment samples (core material) collected pre- and poststimulation will also be compared using high-resolution microscopic methods to determine changes in uranium and other metal speciation and identify associations with pore domain interfaces at the grain and pore scales. Column experiments using three large intact cores from the background area are being prepared for concurrent lab-scale evaluation of the project hypothesis. Early data for nonreactive (bromide) and reactive (U) tracers from the intact cores are consistent with multidomain solute transfer and transport. Smaller intact and repacked cores have been used for a variety of geochemical and microbiological characterization activities. Uranium breakthrough curves from the repacked cores are well described by a reactive transport model that incorporates a surface complexation model to account for U sorption onto the saprolite. A numerical model of terminal electron acceptor processes in laboratory batch (slurry) experiments using Area 2 sediments has been developed and applied to estimate reaction parameters for use in field-scale numerical models. The field-scale models are being used to evaluate alternative biostimulation scenarios in order to optimize the experimental design.

NABIR Field Research Center—Oak Ridge, Tennessee

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NABIR Research Objectives and Results. To encourage hypothesis-based field research and process-level understanding, the NABIR program has established a Field Research Center (FRC). The FRC provides a site for investigators to conduct field-scale research and to obtain DOE-relevant subsurface samples for laboratory-based studies. The FRC is located on the U.S. Department of Energy's Oak Ridge Reservation (ORR) in Oak Ridge, Tennessee. Staff from Oak Ridge National Laboratory's Environmental Sciences Division has operated the FRC since April 2000. Both contaminated and background (uncontaminated control) areas are located on the ORR's Y-12 National Security Complex in Bear Creek Valley. The initial focus of research at the FRC has been on in situ biostimulation experiments to promote the immobilization of uranium and technetium, but future research will be expanded to include EMSP investigators and other relevant DOE contaminants (e.g., organics, Hg, and other metals) and other processes.

The FRC is used by investigators for various purposes, including:

- Laboratory studies on FRC groundwater and sediment samples and humic material by dozens of national laboratories and universities
- Evaluation of new field and laboratory characterization and monitoring methods
- Multidisciplinary in situ accelerated bioremediation research projects
    - In situ Uranium Reduction Experiments Using Push-Pull Techniques (Jack Istok, Oregon State; located in Areas 1 and 2)
    - Field-scale Bioreduction of Uranium (Craig Criddle, Stanford; located in Area 3)
    - In situ Immobilization of Uranium in Structured Porous Media via Biomineralization at the Fracture/Matrix Interface (Tim Scheibe, PNNL; located in Area 2)

FRC Working Groups were established to coordinate technical efforts across FRC field and laboratory projects, identify key technical issues that need to be investigated, and stimulate cross-disciplinary evaluation and integration of data and findings.

The Working Groups are currently organized around four topics (Overall lead—Phillip Jardine, ORNL):

- Microbial community analysis (Lead—Joel Kostka, Florida State)
- Geochemical/geophysical characterization (Lead—Phillip Jardine, ORNL)
- Rates and mechanisms of microbially mediated metal reduction (Lead—Bill Burgos, Penn State)
- Numerical modeling (Lead—Jack Parker, ORNL)

Additional information and data can be obtained at the FRC website: http://www.esd.ornl.gov/nabirfrc/.

In situ Community Control of the Stability of Bioreduced Uranium

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The long-term stability of microbially reduced uranium in the subsurface is a pivotal issue for the eventual application of biostimulation (e.g., electron donor amendment) as a means of bioremediation of uranium contaminated groundwater. Recent field experiments (Anderson et al. 2003, and unpublished data) demonstrate that U(VI) can be removed from groundwater by electron donor amendment at the field scale. It was also observed that several months after electron-donor (acetate) amendment, some U(VI) reduction was maintained. We hypothesize that, while *Geobacter* dominates the microbial population during the metal reduction phase, the ongoing U(VI) reduction may be related to metabolically versatile sulfate reducers. Furthermore, precipitated $FeS_{0.9}$ is not sufficient, without microbial mediation, to reduce U(VI) under subsurface conditions of flowing groundwater.

However, direct estimation of reoxidation rates is difficult under field conditions. We are initiating a series of in situ experiments using in-well sediment incubators that will enable direct measurement of U(IV) removal rates from prereduced sediments with specific microbial and mineralogic amendments. By comparing U(IV) loss rates with different DIRB and SRB populations we will be able to clearly determine the relative impact of sulfate reducers vs. Fe reducers. This approach also makes it possible to assess actual in situ conditions during the experiment and to directly observe reoxidation (or bioreduction) end points after the field experiment is completed without drilling. Finally, in-well sediment incubators are relatively inexpensive and could ultimately displace both field-scale electron donor amendment experiments and push-pull tests as the preferred means of assessing site response to bioremediation and long-term stability of both biostimulated and naturally bioattenuated sites.

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Anderson, R.T., Vrionis, H.A., Ortiz-Bernad, I., Resch, C.T., Long, P.E., Dayvault, R., Karp, K., Marutzky, S., Metzler, D.R., Peacock, A.D., White, D.C., Lowe, M., and Lovley, D. R. (2003), Stimulating the in situ activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer, *Appl. Env. Microbiol.* **69**, 5884–5891.

Physical, Chemical, and Microbial Processes Controlling Uranium Mobility at the Old Rifle UMTRA Site

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Previous and ongoing laboratory and field studies at the Old Rifle UMTRA site in western Colorado indicate that the biogeochemical transformation of uranium to less mobile forms is a dynamic function of specific microbial populations (i.e., iron reducers and sulfate reducers) responding to biostimulation (amendment with acetate) in the context of site-specific hydrologic and geochemical conditions. Using data that are now available from these experimental studies, we are creating a quantitative model of the mechanistic contribution by individual subsurface processes to observed uranium behavior. Specific issues to be addressed include: (1) the impact of hydrologic conditions on bromide transport and oxygen stratification; (2) the mechanisms controlling the rate of microbially mediated uranium reduction during and after biostimulation; and (3) multicomponent surface complexation controlling the sorption of U(VI) species. Multicomponent, biogeochemical reactive transport simulations based on coupling of these conceptual process models will then be used to interpret and unravel interactions between processes under the dynamic and spatially variable hydrologic and geochemical conditions at the Old Rifle UMTRA field site. The goal is to enhance understanding of the scale-up of fundamental mechanisms, which is critical to the engineering of in situ conditions favorable to bioremediation.

Analysis of the hydrologic conditions for the 2002 and 2003 field experiments, including the transport conditions governing the migration of the bromide tracer and the delivery of the electron donor, acetate, is in progress. The resulting velocity fields will be used to investigate the stratified distribution of dissolved oxygen and U(VI) found at the site. In conjunction with the velocity field identified for the 2002 field study at Old Rifle, we have implemented a biogeochemical reaction network using a dual Monod kinetics formulation that includes response to the acetate injection by iron reducers that also use U(VI) as a terminal acceptor, and sulfate reducers that succeed the iron reducers after depletion of available Fe(III) terminal electron acceptors. Analyses of mechanisms controlling microbially mediated uranium reduction during the biostimulation events in 2002 and 2003 will be the basis for the collaborative analysis of the Princeton (Jaffe et al.) laboratory studies and refinement of the conceptual model for all terminal electron acceptors. This will include the determination of rate laws for the transformation reactions involving oxygen, nitrate, uranium, Fe(III) minerals, and sulfate with potential dependencies on biomass generation and decay. The ongoing development and testing of a preliminary multicomponent, generalized composite, surface complexation model will focus on characterizing uranium sorption as a function of solution chemistry. The final task will be to integrate the updated hydrologic, biogeochemical, and surface complexation process models to investigate site-specific hydrologic and biogeochemical conditions affecting the development of chemically reducing conditions, including the dissolution of Fe(III) minerals and the liberation of U(VI) adsorbed to those minerals; and the identification of mechanistic linkages between biostimulation in the Old Rifle aquifer and subsequent secondary mineral reactions, associated mineral volume changes, and the impact of these changes on accessibility to Fe(III) and U(IV) minerals.

Assessment

Development of Recombinant Antibodies with Specificity for Chelated Uranyl Ions

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The goal of our project is to continue the development of new techniques for rapid, automated identification of radionuclides, metals, and chelators that may contaminant surface and groundwater at DOE sites. One of the four specific aims of the present project is to develop new technologies in antibody engineering that will enhance our immunosensor program. Recombinant antibodies have potential advantages over monoclonal antibodies produced by standard hybridoma technology. The cloned genes represent a stable, recoverable source for antibody production. In addition, the recombinant format offers opportunities for protein engineering that enhances antibody performance and for studies that relate antibody sequence to binding activity. In this study, a hybridoma that synthesized an antibody (12F6) that recognized a 1:1 complex between 2,9-dicarboxyl-1,10-phenanthroline (DCP) and UO_2^{2+} was used as a source of RNA for the development of a recombinant $(Fab)'_2$ fragment. RNA was isolated from the 12F6 hybridoma, and the cDNA encoding the entire κ light chain and the linked V_H and C_1 portions of the heavy chain were amplified from total RNA. cDNA sequences were verified by comparison with the N-terminal amino acid sequences of the light and heavy chains of the native 12F6 monoclonal antibody. A leader sequence and appropriate restriction sites were added to each chain, and the fragments were ligated into a commercial dicistronic vector (pBudCE4.1, Invitrogen, Inc.). COS-1 cells were transfected with this vector and the culture supernatant was assayed for activity and the $(Fab)'_2$ protein. Cells transfected with vector containing 12F6 cDNA synthesized and secreted recombinant $(Fab)'_2$ fragments that bound to the UO_2^{2+}-DCP complex with an affinity indistinguishable from that of a $(Fab)'_2$ fragment prepared from the native antibody. Molecular models of the heavy and light chain variable domains were constructed according to the canonical structures method detailed by Morea et al. (*J. Mol. Biol.* **275**, 269), and the participation of specific residues in antigen recognition was assessed using site-directed mutagenesis. Three amino acids in the light chain variable region (H34, Y49, and F98) were particularly important in antigen recognition.

In a separate series of experiments, a recombinant phage-displayed antibody library has been prepared using RNA isolated from the spleens of sheep and rabbits immunized with specific metal-chelate complexes. Phage-display libraries produced from an immunized source are inclined to include variable genes specific for the immunized antigen(s), many of which are already affinity matured. An antibody fragment specific for the UO_2^{2+}-DCP complex was isolated from this combined phage display library. While the binding affinity of this antibody fragment for UO_2^{2+}-DCP was not as high as that of the 12F6 monoclonal antibody, the beauty of antibody phage display technology is that it allows for the potential manipulation and maturation of the antibody's binding affinity, which may drastically improve and ultimately surpass that of monoclonal antibodies.

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Characterization of Coupled Hydrological-Biogeochemical Processes using Geophysical Data

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Because coupled biogeochemical and hydrological (BGH) processes vary over a wide range of scales, application of remediation approaches (e.g., in situ chemical manipulation and biostimulation) induces dynamic spatiotemporal transformations in subsurface systems, including dissolution and precipitation of minerals, gas evolution, redox variations, biofilm generation, and changes in permeability and porosity. Limited understanding of the coupled nature of BGH processes and inadequacy of conventional approaches for characterizing or monitoring the processes at the field scale hinder development of accurate conceptual and numerical reactive transport models to guide effective cleanup at DOE sites. Based on our success using geophysical methods to estimate hydrogeological and geochemical properties under static conditions, with EMSP support we recently investigated the capability of time-lapse geophysical datasets for remotely detecting changes in hydrological-biogeochemical properties as aquifers are perturbed during biostimulation. We performed multiple column-scale experiments to induce a specific reaction (i.e., gas evolution, metal sulfide precipitation, or biofouling). During the experiments, discrete geophysical measurements were collected using seismic, radar, and complex electrical methods; geochemical fluid and soil samples were collected and analyzed using ion chromatography and ICP methods; and microbial biomass was assessed using quantitative living/dead staining and lipid analysis. Post-destructive evaluation included detailed SEM and TEM analysis of column sediments, using microscopy facilities at PNNL's Environmental Molecular Science Laboratory. Results suggest seismic and radar methods are sensitive to gaseous end products of denitrification and methanogenesis, while frequency-dependent electrical measurements are more sensitive to pore-space alterations in mineralogy, including precipitation of insoluble phases, mineral aggregation dynamics, and solid-state transformations of aqueous contaminants that are sequestered as solids (e.g., U, Sr, Cr, and heavy metals).

We tested the potential of geophysical methods for elucidating system transformations. Our field-scale tests were conducted during remediation experiments performed by various ERSD researchers at 3 different sites: the UMTRA U(VI)-contaminated site (with Phil Long, PNNL; Derek Lovely, Univ. Mass.); Area 1 of the U(VI)-contaminated FRC site (with Jack Istok, Oregon State); and a Cr(VI)-contaminated site at the 100H site, Hanford, WA (with Terry Hazen, LBNL). At the UMTRA site, we monitored a biostimulation experiment aimed at reducing elevated concentrations of U in a shallow aquifer using surface-based complex resistivity data. Results suggest variations in complex resistivity are indicative of the effective changes in mineral transformations associated with microbial iron reduction, which are related to decreases in U concentrations and changes in aquifer redox conditions. Because sustainability of low U(VI) concentrations at the site is governed by redox conditions favorable to iron reduction, such surface-based imaging could be very useful for understanding system transformations over distances of 10s–100s m. At the FRC site, we used time-lapse seismic and radar data to image changes that occur in the subsurface during push-pull tests associated with denitrification. At the 100H site, we used time-lapse tomographic methods to image the distribution of an introduced remediation amendment (HRC) as well as transformations associated with stimulation over time. FRC and the 100H experiments suggested heterogeneity greatly influences both distribution of the remediation amendment and system transformations associated with the stimulation. Collectively, lab and field-scale tests suggest minimally invasive, high-resolution geophysical methods hold significant potential for monitoring and elucidating processes that occur during bioremediation. Although more work is needed to enable quantitative use of the remote methods under naturally heterogeneous conditions, our results to date indicate such work is warranted. Geophysical methods will be very useful for long-term, field-scale monitoring of remediation sustainability, and for refining and validating conceptual and reactive transport models needed to predict coupled processes at the field-scale, ultimately guiding DOE cleanup efforts.

Catalytic DNA Biosensors for Radionuclides and Metal Ions

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We are developing new DNA biosensors for simultaneous detection and quantification of bioavailable radionuclides, such as strontium, uranium, technetium, and plutonium, and metal contaminants, such as lead, chromium, and mercury [1,2]. The sensors will be highly sensitive and selective, not only for different metal ions, but also for different oxidation states or speciation states of the same metal ion. They will be applied to on-site, real-time assessment of concentration, speciation, and stability of the individual contaminants before and during bioremediation, and for long-term monitoring of DOE contaminated sites. To achieve this goal, we have employed a combinatorial method called "in vitro selection" to search from a large DNA library ($\sim 10^{15}$ different molecules) for catalytic DNA molecules that are highly specific for radionuclides or other metal ions through intricate 3-dimensional interactions as in metalloproteins [3]. The DNA has been converted to fluorescent or colorimetric sensors by attaching to it fluorescent donor/acceptor pairs [4,5,9] or gold nanoparticles.[10-12] Practical application of the biosensors in the NABIR Field Research Center (FRC) at Oak Ridge will be demonstrated, using colorimetric sensors mainly for qualitative and semi-quantitative detections and using fluorescent sensors for quantitative detections.

We have successfully used the methodology mentioned above to develop a highly sensitive and selective DNA biosensor for Pb^{2+}, with a quantifiable detection range from 10 nM to 4 μM [4]. Even in the presence of other metal ions, this biosensor displays a remarkable sensitivity and selectivity. Recently we have carried out several studies to further improve the sensor's sensitivity and selectivity. For example, by combining both inter- and intra-molecular quenchers, we have improved the signal to noise ratio significantly [5]. To further improve the metal ion selectivity, we have developed a "negative" selection strategy that can significantly improve the metal-binding selectivity in the selection process [6]. By immobilizing the DNA on a solid surface, we have improved detection limit to 1 nM (2 ppb) [7].To provide insight into the metal-binding sites in DNA and to allow the design of metal ion sensors and chelators from the first principle, we have also carried out a detailed biochemical [8] and biophysical [9] study of the DNA lead sensors obtained in the lab. The DNA was further converted into colorimetric metal sensors, making on-site, real-time detection even more affordable and achievable because no equipment is needed in the operation [10]. We accomplished this by taking advantage of recent advance in both biotechnology and nanotechnology. Both the speed and accuracy of the operation were improved through detailed understanding of the DNA biology and nanoparticle technology [11,12]. Finally, we are making progress in applying the strategy developed in the lab toward making sensors for radionuclides such as uranium and chromium ions. The latest results will be presented.

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Bioremediation and Its Societal Implications and Concerns (BASIC)

Using a Consensus Conference to Characterize Regulatory Concerns Regarding Bioremediation of Radionuclides and Heavy Metals in Mixed Wastes at DOE Sites

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Research Objectives, Results, and Plans. This project identifies the range of concerns held by state regulators about using bioremediation strategies for in situ cleanup of radionuclides and heavy metals in mixed wastes at Department of Energy (DOE) sites. This project also explores the process of reaching consensus among state regulators about the use of bioremediation. In January and February 2005, we held a consensus workshop involving ten regulators from seven states. In the course of five days, the regulators learned about bioremediation for radionuclides and metals, identified key questions about this application of bioremediation, met with a panel of experts on the subject, and wrote a consensus report that all ten regulators signed. In the consensus report, the state regulators concluded that to consider bioremediation among remedial options, the states must have: (1) confidence in the technology prior to full-scale application, (2) adequate site characterization, (3) adequate maintenance and performance monitoring, and (4) commitment to long-term stewardship. The report also highlights the need for additional research and recommends that research priorities should focus on real-life problems; situations where no other proven, cost-effective technologies exist; and areas with the most potential for broad application.

In addition to holding the consensus workshop, we administered a preworkshop survey and will follow up with a postworkshop survey to measure changes in knowledge about and attitudes toward bioremediation for radionuclides and metals among the state regulators involved in the consensus process. Finally, six months after the workshop, we will conduct face-to-face interviews with some of the participants to further assess the effectiveness of the consensus workshop for educating state regulators about bioremediation.

Related Environmental Research

Support of Synchrotron-based Research at the Advanced Light Source, the Advanced Photon Source, the National Synchrotron Light Source, and the Stanford Synchrotron Radiation Laboratory

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To develop effective and efficient remediation strategies, a better understanding is needed of:

- How and what microbiological and geochemical species are distributed relative to contaminants and within natural geological material;
- Biogeochemical processes that occur within that system and the influence that they have on hydrological properties that govern flow; and
- The rates at which those processes occur.

Synchrotron radiation (SR) approaches have become integral and powerful tools for investigating the speciation, spatial distribution, reactivity, and chemical transformations of contaminants and their relations to chemical, geological, microbiological, and hydrological properties. SR methods enable a wide range of investigations, including the study of solute-solvent systems, poorly crystalline materials, heavy metal interactions with biogeochemical materials, heavy metal interactions with biogeochemical surfaces, and radioactive materials. SR permits investigations at the scale where these processes occur and often under natural conditions. Such studies, coupled with environmental investigations performed at larger length scales, can increase our fundamental understanding of environmental materials and processes. This improved knowledge provides the basis for improved environmental risk assessment, management, and remediation.

This presentation describes a new Environmental Remediation Science Division (ERSD) Program that is being developed at the Advanced Light Source (ALS) of LBNL, the Advanced Photon Source (APS) of Argonne National Laboratory, the National Synchrotron Light Source (NSLS) of BNL, and the Stanford Synchrotron Radiation Laboratory (SSRL) of SLAC. The program will facilitate the use of synchrotron radiation for environmental researchers and, in particular, NABIR and EMSP researchers. Support includes assisting users in identifying the appropriate light source, navigating the light source proposal process, choosing appropriate SR techniques given the problem at hand, providing assistance in running samples and, in some cases, analyzing data and assessing the results.

This presentation will describe this new program, introduce representatives and points of contact from each light source, and provide examples of recent highlights from each light source that are relevant to NABIR and EMSP research. Additional information on how to gain access to each facility and on upcoming workshops designed to educate the novice user about the use of synchrotron radiation in NABIR-related research (SES-III Conference) will also be provided.

Vadoze Zone Microbiology Research at the Hanford Site Sponsored by the EMSP and Microbial Genome Program

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While numerous techniques exist for remediation of contaminant plumes in groundwater or near the soil surface, remediation methods in the deep vadose zone are less established due to complex transport dynamics and sparse microbial populations. There is a lack of knowledge on how physical and hydrologic features of the vadose zone control microbial growth and colonization in response to nutrient delivery during bioremediation. The overall objective of the projects was to increase knowledge of the feasibility of engineered bioremediation in the deep vadose zone, particularly at arid western sites where microbial populations and activities are low. Accomplishments include the following.

For the first time, numeric values have been defined for microbial colonization under nonflowing unsaturated conditions. How sand particle size and water content controls these values was also determined. Motile bacteria were able to rapidly migrate in response to nutrient addition. The work showed that aqueous nutrient delivery to vadose zones with patchy and low density microbial populations appears to be an effective technology for driving rapid microbial colonization in the field. Microbial characterization of vadose zone sediments beneath the 216-Z-9 Trench at the DOE Hanford Site, where large amounts of carbon tetrachloride and radionuclides were disposed. The analysis showed that the highest carbon tetrachloride concentrations were co-located with the highest microbial populations and highest sediment moisture content. These sediments were further used in laboratory experiments to investigate the potential of the microbial community to utilize gaseous hydrocarbons and potentially transform CT under unsaturated conditions.

Five hydrocarbons (methane, ethane, propylene, propane, and butane) and sources of nitrogen and phosphorus that could be delivered to the vadose zone during engineered bioremediation via vapor-phase transport were tested. Eighty percent of the sediments degraded at least one of the hydrocarbons; 20–45% of the sediment samples were able to biodegrade a specific hydrocarbon; more than 3 hydrocarbons were degraded in 70% of the sediments. Gaseous nitrogen and phosphorus did not stimulate removal of gaseous C sources compared to no addition of N and P. The ability to successfully deliver the gases to the in situ subsurface microsites where microbes exist should be tested in intact Hanford deep vadose zone cores. Sediment samples that displayed the ability to use gaseous hydrocarbons were tested for carbon tetrachloride-degrading ability. CT degradation occurred in only 5% of the bottles and only 50-60% was degraded after a 10 month period. It is likely CT degradation would occur more readily in the subsurface vadose zone where porosity and oxygen diffusion rates are lower and anoxic sites would be more readily formed as a result of aerobic degradation of gaseous hydrocarbons. The microbial community beneath a leaking high-level radioactive waste tank was characterized by 16S rDNA sequencing, and metagenome sequencing was performed on pooled enrichments from the sediments. Protein hits were largely consistent with amplified and sequenced 16S rDNA phylogenies from both pooled enrichments and sediments. The 16S data from pooled enrichments showed 10 genera from the Micrococcineae, Propionibacterineae, and Steptomycineae suborders within the Actinobacteria (high GC gram-positive) phylum with nucleotide identities of 93–97%; and one genera (*Pseudomonas*) within the gamma class of the Proteobacteria phylum. A total of 3,840 metagenome clones were sequenced. After removing higher eukaryote clones (contamination), 8,762 nonredundant prokaryotic protein hits were found, representing 489 different EC classes and 113 different KEGG maps. Of these, 6,053 hits were associated with TIGR role categories. Protein hits were 62% Proteobacteria, 36% Actinobacteria, and 1% Bacilli and Clostridia classes of the Firmicutes (low GC gram-positive) phylum. With the exception of the genera *Pseudomonas*, the community at this contaminated site was very similar to previous studies of the noncontaminated vadose zone at the Hanford Site (i.e., strong dominance by high GC gram-positive microbes).

Co-Precipitation of Trace Metals in Groundwater and Vadose Zone Calcite: In Situ Containment and Stabilization of Strontium-90 and Other Divalent Metals and Radionuclides at Arid Western DOE Sites

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Radionuclide and metal contaminants such as ^{90}Sr are present beneath U.S. Department of Energy (DOE) lands in both the groundwater (e.g., 100N area at Hanford, WA) and vadose zone (e.g., Idaho Nuclear Technology and Engineering Center at the Idaho National Laboratory [INL]). In situ containment and stabilization of these contaminants is a cost-effective treatment strategy. However, implementing in situ containment and stabilization approaches requires definition of the mechanisms that control contaminant sequestration. We are investigating the in situ immobilization of radionuclides or contaminant metals (e.g., ^{90}Sr) by their facilitated coprecipitation with calcium carbonate in groundwater and vadose zone systems. Our facilitated approach relies upon the hydrolysis of introduced urea to cause the acceleration of calcium carbonate precipitation (and trace metal coprecipitation) by increasing pH and alkalinity and liberating cations from the aquifer matrix by exchange reactions:

$$H_2NCONH_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$$
$$>\mathbf{X:}_2Ca + 2NH_4^+ \rightarrow 2>\mathbf{X:}NH_4 + Ca^{2+}$$
$$Ca^{2+} + HCO_3^- + OH^- \rightarrow CaCO_3(s) + H_2O$$

where **>X:** is a cation exchange site on the aquifer matrix. Subsurface urea hydrolysis is catalyzed by the urease enzyme, which is produced in situ by native urea-hydrolyzing microorganisms. Because the precipitation process tends to be irreversible and many western aquifers are saturated with respect to calcite, the co-precipitated metals and radionuclides will be effectively removed from the aqueous phase over the long term.

Our research has shown that (1) urea-hydrolyzing microorganisms are ubiquitous in the Snake River Plain Aquifer underlying the INL and also occur beneath the Hanford 100N area; (2) urea hydrolysis and calcite precipitation are linked in laboratory and field settings; and (3) Sr^{2+} is incorporated into calcite precipitated by urea-hydrolyzing bacteria with higher distribution coefficients than in abiotic systems. These experimental results and observations have been embodied in a geochemical and reactive transport computer code that allows field-scale simulations of the urea hydrolysis-calcite precipitation process. The mixed equilibrium-kinetic model accounts for urea hydrolysis by ureolytic bacteria and calcite precipitation/dissolution using kinetic expressions. Cation exchange reactions and metal partitioning into the precipitated calcite are treated as equilibrium processes. Simulations of hypothetical remediation strategies using mM levels of urea show that almost 1 mmol of calcite is precipitated per mmol of hydrolyzed urea, with most of the precipitated cations being derived from exchange of NH_4^+ with the aquifer matrix. Because of the cation exchange reactions and the near absence of NH_4^+ in the groundwater, the long-term persistence of the precipitated calcite is a function of the applied urea concentration and the total cation exchange capacity of the aquifer matrix. Our simulation results suggest that with appropriate urea introduction strategies calcite precipitation can provide for the long-term in situ sequestration of radionuclides and metals.

Student Presentations

Metabolically Active Microbial Communities in Acidic Uranium-Contaminated Subsurface Sediments

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The overall goal of this project is to determine the activity and community composition of microbial populations (such as metal- and nitrate-reducing bacteria, and other heterotrophs) that are likely to make strong contributions to the fate of uranium during in situ bioremediation. Sediments collected from the acidic (pH 3–4) and near-neutral pH (pH 6–7) zones of the DOE-NABIR Field Research Center (FRC), Oak Ridge, TN, were used in microcosm experiments and for total nucleic acid extraction. In these samples, we selectively targeted and amplified extracted DNA (16S rRNA gene sequence) to determine the composition of the in situ microbial communities and RNA (16S rRNA) to determine the metabolically active fraction of the microbial community.

We initially constructed clone libraries from amplified 16S rRNA gene products and from cDNA reverse transcribed from 16S rRNA. Clones were screened using restriction fragment length polymorphism analysis, followed by sequencing of cloned inserts. Clone libraries constructed from sediments prior to biostimulation contained representatives from the phyla Planctomycetes, Proteobacteria ((α, β, δ, γ),), Bacteroides, and Firmicutes. The diversity and numerical dominance of phylotypes varied between the DNA and RNA libraries. Phylotypes from the DNA clone libraries were more diverse than those found in the RNA-derived libraries. The most abundant phylotypes found in the DNA-derived libraries were members of the Alphaproteobacteria, while the Gammaproteobacteria were more frequently detected in the RNA-derived libraries.

Currently, we are examining the structure-function relationships of microbial communities in microcosms of biostimulated sediments by comparing community composition as determined by clone libraries to rates of electron donor/acceptor utilization (see companion abstract of Kostka, et al., p. __). We plan to utilize cloning and sequencing data to develop more rapid and quantitative approaches such as tRFLP and real-time PCR. By targeting rRNA, we provide a novel assessment of the metabolically active fraction of the microbial populations that is likely to catalyze biogeochemical processes contributing to the bioremediation of uranium contamination in the acidic subsurface.

Stable Isotope Probing of U(VI) Bioreduction in Sediment Microcosms

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The microbial community dynamics of sediment microcosms designed to mimic Rifle in situ biostimulation of uranium-reducing organisms were analyzed quantitatively and qualitatively using PLFA and PCR-DGGE analysis combined with stable isotope probing (SIP). The purpose of the experiment was to examine the ecology and association of microbial community with electron acceptor upon acetate stimulation and to validate the feasibility of SIP in field tests.

The microcosms consisted of sediment and groundwater from Rifle, Colorado, UMTRA, site and activated carbon bead microbial traps. Carbon-13 labeled acetate-amended and non-mended microcosms were compared. PLFA analysis demonstrated a community shift in acetate-amended microcosms, mirroring the observation of DGGE analysis. PLFA recovered from beads and sediment also showed incorporation of the ^{13}C acetate, mainly 14:0, 16:1ω7c, 16:1ω5c, 16:0, cy17:0, and 18:1 ω7c, which supports the ^{13}C DNA results. The bacterial community in nonamended microcosms showed notable differences from those amended, with β-Proteobacteria sequences dominating the community. Lipid analysis showed a biomass increase with acetate and ^{13}C DNA analysis indicated that acetate treatment encouraged the growth of gram-negative microorganisms such as *Pseudomonas*, *Geobacter,* and SRB. *Geobacter* and SRB sequences were not detected until the second (day 20) sampling event, while *Pseudomonas* sequences were prevalent at the first sampling event (5 days) and continued to be one of the dominant sequences retrieved. The dominance of *Geobacter* was more pronounced in bead samples than in sediments; GC-IRMS analysis also demonstrated the ^{13}C enrichment in fatty acids of i15:0, 16:0, 16:1ω7c, and i17:0 extracted from bead samples, which can be indicators of the organism.

In this microcosm study, the SIP technique enabled an evaluation of the taxonomic and metabolic diversity of key groups of microbes actively involved in biostimulation. This work demonstrated that microbial monitoring in microcosms can elucidate the bacterial populations responsible for uranium reduction and may indicate that SIP using ^{13}C acetate added to microbial traps can provide important data on ecosystem function in the field.

Cellular Responses of *Desulfovibrio vulgaris* to Stasis

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Desulfovibrio vulgaris is an anaerobic, δ-Proteobacterium that can reduce toxic heavy metals such as chromium and uranium. *D. vulgaris* has become an important model system for bioremediation by sulfate-reducing bacteria, and much work has focused on the biochemical processes that mediate sulfate and heavy metal reduction. However, less is known about the cellular responses to heavy metal and/or environmental stresses in the *Desulfovibrio* species. We are interested in transcriptomic characterization with respect to stasis-regulated genes and heavy metal responsive genes. The elucidation of growth-phase dependent gene expression is essential for a general understanding of growth physiology that is also crucial for data interpretation of stress-responsive genes (e.g., chromium). During our initial experiments, a spike in the total carbohydrate level as cells entered stationary-phase growth was observed. A similar spike was observed in the *D. vulgaris* strain ATCC29579, but the total carbohydrate was decreased by approximately twofold. The strains are thought to be isogenic, and the only difference between the two strains is the length of cultivation under laboratory conditions. Different methods (e.g., salt/formaldehyde wash, zwittergent wash, and centrifugation) were evaluated for the determination of internal versus external carbohydrate in *D. vulgaris*. The best results were obtained with the centrifugation method. The DVH strain had more internal carbohydrate than the ATCC strain (approximately threefold), and the ATCC strain appeared to have increased levels of carbohydrate in the culture supernatant (approximately twofold). In addition, DVH maintained a higher proportion of total carbohydrate that was localized internally. It is highly possible that *D. vulgaris* changes the carbohydrate levels in response to growth conditions with lactate and sulfate as electron donor and acceptor, respectively. The data suggested that an increase in carbohydrate occurred during transition to stationary phase, which may play a role in a general stress response. This information provides a basis for the general responses that *D. vulgaris* has to a non-specific stress. From here, we can begin to analyze how *D. vulgaris* responds to specific stresses, like chromium. Initial results indicated that growth of DVH and ATCC29579 was inhibited at different concentrations of Cr(VI). The results will help us determine how *D. vulgaris* may be responding to heavy metal stress and to design better experiments to analyze these responses.

We are also analyzing gene expression throughout growth and into stationary phase. Preliminary results suggest that within the first hours of stationary-phase, up-expressed ORFs included proteases, chaperonins, permeases, hypothetical proteins, and an Archaeal membrane protein shared with *Desulfovibrio desulfuricans* G20. Interestingly, comparisons suggest that many of the ORFs that were up-expressed in early stationary phase also had altered expression levels in response to other cellular stresses.

This work is being conducted in collaboration with scientists at the University of Missouri, Oak Ridge National Laboratory, and Lawrence Berkeley National Laboratory.

Spectroscopic Confirmation of In Situ Biological Reduction of Uranium within Fractured Saprolite

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In situ immobilization of heavy metals, such as uranium, through biological reduction is a promising means for stabilizing contaminants within subsurface sediments. Species of U(VI) are highly mobile in groundwater systems, while those of U(IV) are only sparingly soluble. Stimulation of biological uranium reduction at the field scale presents several challenges, including heterogeneous sediment mineralogy, a complex and evolving community of bacteria, and the presence of multiple electron donors and acceptors. The NABIR Field Research Center at Oak Ridge National Laboratory is additionally complex owing to U concentrations of 1,000 ppm, pH values less than 3.4, and exceedlingly high concentrations of nitrate (>0.1 M) and aluminum (>0.01 M).

Here, we present evidence of biological uranium reduction in a series of experiments of increasing complexity. First, uranium reduction was investigated in batch experiments using radionuclide-contaminated sediment from ORNL that were inoculated with a denitrifying bacterial community. Next, a packed column was used to mimic uranium reduction under field conditions. Finally, subsurface biological uranium reduction was stimulated at the field scale.

Successful stimulation of biological uranium reduction was accomplished in all experimental systems. However, even after long-term stimulation of biological activity, approximately 50% of uranium within the solid phase remained oxidized [U(VI)]. We are currently attempting to determine the speciation of the remaining U(VI) through micro-X-ray absorption spectroscopy (μ-XAS). Furthermore, U(IV) appears to be easily oxidized after the cessation of electron donor and exposure to air or other oxidants (NO_3^-). These factors complicate the long-term immobilization of uranium through in-situ stimulation of biological activity.

Characterization of a Sulfate- and U(VI)-Reducing Enrichment from Area 3 of the Oak Ridge Field Research Center

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Field-scale bioremediation projects use reactive transport models for design and interpretation, and these models require stoichiometric parameters and empirically verified kinetic expressions for microbial growth and U(VI) reduction. Uncertainty regarding the ecophysiology of U(VI)-reducing bacteria and the rates of their associated reactions hinder modeling. Accordingly, we characterized kinetics and composition of a U(VI)-reducing community derived from sediment from Area 3 of the Oak Ridge Field Research Center, the location of an ongoing field experiment. Here, we summarize findings for the metabolism of this enrichment, kinetic parameters for its growth and U(VI) reduction, and the results of a 16S rDNA clone library.

Sediment was inoculated into a defined sulfate-reducing medium with ethanol as an electron donor, under electron donor-limiting conditions. Enrichment cultures were maintained anaerobically in serum bottles at 30°C. Ethanol was converted to acetate and hydrogen concurrently with the reduction of sulfate. Hydrogen was subsequently consumed, but acetate persisted. The growth yield was 0.13 g VSS/g COD and the maximum specific rate of ethanol utilization was 2.8 g COD/g VSS·d, values within the range expected for sulfate-reducing bacteria. The half-saturation coefficient for ethanol was too small to be determined from substrate depletion curves. Little activity was observed in control bottles that lacked sulfate, indicating endogenous decay was not significant in the time frame of the experiment. The fermentation of ethanol to acetate and hydrogen was observed in control bottles that lacked sulfate, as were acetogenesis and methanogenesis. Late log-phase cells were harvested, washed, and resuspended in buffer, and their rate of U(VI) reduction was measured under nongrowth conditions. The second-order rate coefficient for U(VI) reduction was 181 L/g VSS·d, which is at the low end of values reported for pure cultures of U(VI)-reducing bacteria. Most clones from the 16S rDNA library (75%) were related to *Bacillus* spp., and a significant number were related to *Bacteroides* and *Desulfovibrio* spp. (14% and 10%, respectively). *Bacteroides* species are known to produce acetate during fermentation, and *Desulfovibrio* species oxidize one mole of ethanol to one mole of acetate during sulfate reduction, the same stoichiometry observed for the enrichment. Kinetic parameters and community composition will be compared with parameters and community composition results for the field. The observed detection of methane is of concern for field applications. Methanogens compete for electron donors with U(VI)-reducing populations, and methane gas can block flow paths, preventing the delivery of chemicals to contaminated regions.

Sequential Biogeochemical Redox Reactions as a Result of Biostimulation at the Oak Ridge Field Research Center

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The bioremediation of radionuclides, such as uranium (U) and technetium (Tc), is challenging because the radionuclides are typically at low but hazardous concentrations relative to other redox sensitive elements in the system. At the Oak Ridge Field Research Center (FRC), uranium concentrations in groundwater are up to µM level and technetium concentrations are within the µM to pM range. However, nitrate and sulfate concentrations are at the mM-M range. Several Fe and Mn oxide minerals in the soils and aquifer complicate matters even further. Successful field scale bioremediation must be accomplished in the presence of these competing terminal electron acceptors in the groundwater systems. To understand where uranium reduction is located in a sequence of microbial activity-induced redox reactions and therefore the biogeochemical reaction pathways of uranium reduction, we have collected a comprehensive dataset of elemental concentrations of cations, anions, trace metals, and stable isotopes of S, C, N, and ^{18}O in sulfate, and we are in the process of collecting Fe and U isotope data. These analyses were performed on groundwater samples collected from Professor Jack Istok's push-pull biostimulation field experiment conducted in Area 2. Our aqueous geochemistry data delineated sequential redox reactions involving C, N, S, Fe, U, Tc, and Mn. The isotopic data capture distinct trends of fractionation. This comprehensive set of aqueous geochemistry and isotopic data provides a unique opportunity to decipher the biogeochemical reaction pathways in a complex remediation system, as well as contributing to the fundamental understanding of redox geochemistry, which is scarcely studied. Our analysis of the groundwater is coupled with high resolution (atomic scale) analysis of U speciation in the solid matrix using field emission gun high-resolution transmission electron microscopy (HRTEM). A previously presented poster at NABIR PI Workshop in October 2004 reported five modes of uranium concurrencies in contaminated soils (Stubbs et al., 2004). The multidisciplinary work will allow us to link the observed aqueous uranium concentrations to modifications of Fe and Mn oxides and the changes of uranium speciation in the solid matrix, thereby firmly identifying the biogeochemical reduction pathways and determining the long-term stability of reduced uranium.

Uranium and Technetium Bioimmobilization in Intermediate-Scale Permeable Reactive Barriers

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Extensive in situ (in ground) field testing using the push-pull method (see abstract by Istok et al., p. __) has demonstrated that indigenous microorganisms in the shallow (<8 m) aquifer in FRC Areas 1 and 2 are capable of coupling the oxidation/fermentation of injected ethanol, glucose, or acetate to the reduction of U(VI) and Tc(VII). Collectively, this research suggests that bioimmobilization of Tc(VII) and U(VI) should be possible using a permeable reactive barrier consisting of three defined zones (1) pH adjustment, (2) denitrification and Tc(VII) reduction, and (3) U(VI) reduction. This hypothesis is being tested in small-scale laboratory studies and in intermediate (~2 m)-scale physical models deployed at FRC Areas 1 and 2. A laboratory column packed with crushed limestone and bicarbonate raised the pH of well FW21 groundwater (~3.4) to above 5 for nearly one hundred pore volumes without significant loss in hydraulic conductivity. The high nitrate (~120 mM) column study provided rates of denitrification (~15.25 mM/day), ethanol utilization (~13 mM/day), and technetium reduction (~120 pM/day) by sediment microorganisms, but no uranium reduction was detected. Results of the low nitrate (3 mM) column study indicate that, once the pH of FRC water is adjusted and nitrate is removed, uranium and technetium reduction occurred with ethanol as the electron donor at rates of ~0.5 μM/day and 57 pM/day, respectively. Intermediate-scale physical aquifer models were constructed to allow the processes of pH adjustment, nitrate removal, and metal reduction to occur sequentially during continuous flow through a single sediment pack, modeling a possible configuration for a full-scale permeable reactive barrier. Data from the physical model deployed in Area 1, which was continuously supplied with high nitrate, low pH groundwater from well FW21, indicate that pH was increased to near 7 without detectable clogging, and nitrate and technetium reduction were occurring. Ethanol concentrations were reduced from ~180 mM to zero in ~10 days during the seventh week of model operation and the maximum pseudo-first order reduction rates were: nitrate at 0.76 day^{-1}, Tc(VII) at 0.28 day^{-1}, and U(VI) at 0.12 day^{-1}. Data from the physical model deployed in Area 2, which was continuously supplied with low nitrate, neutral pH groundwater from well GW835, indicate that nitrate, uranium, and technetium reduction were occurring, although the model had only been operational for ~6 weeks. The results of the laboratory experiments and the performance of the intermediate-scale physical models suggest that in situ bioimmobilization of U(VI) and Tc(VII) in a permeable reactive barrier is a viable treatment alternative for contaminated groundwater at the FRC.

Culture-Dependent and Independent Identification of Denitrifying Bacteria in Areas 1 and 2 at the FRC

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An important strategy for bioremediation at the NABIR Field Research Center (FRC) consists of supplying an organic electron donor in situ to stimulate subsurface microorganisms responsible for nitrate reduction and uranium immobilization. The goals of this study were to characterize the microbial community structure of sediments from Areas 1 and 2 and to identify microorganisms whose growth may be stimulated during bioremediation with ethanol-amended groundwater. Push-pull tests were done in wells in Area 1 with ethanol-amended and unamended FW021 site groundwater (>130 mM nitrate) and in Area 2 with ethanol-amended and unamended GW835 site groundwater (<1mM nitrate). Community DNA was extracted from sediment adjacent to three Area 1 wells (two ethanol-stimulated and one control) and three Area 2 wells (two ethanol-stimulated and one control). Bacterial diversity and composition of samples were analyzed through cloning and sequencing of the 16S rRNA gene, and the denitrifying community composition of these samples was analyzed through phylogenetic analysis of cloned nitrite reductase genes, *nir*K and *nir*S. In Area 2, 16S rRNA clone libraries consisted of sequences related to the sulfate-reducing δ-Proteobacteria genus *Desulfovibrio*; and in Area 1, clones related to β-Proteobacteria genera *Alcaligenes* and *Burkholderia* were prevalent. Sequence analysis of *nir*K and *nir*S (nitrite reductase) clone libraries revealed a higher diversity for *nir*S than *nir*K. In Area 1 samples, one clone sequence (OTU 1K) dominated both ethanol-stimulated *nir*K clone libraries (70% of total clone sequences) but only made up a small portion (10%) of the nonstimulated *nir*K clone library. The sequence of OTU 1K is only 82% similar to the *nir*K gene sequence from *Alcaligenes xylosoxidans* and does not cluster with any previously reported *nir*K sequences. In an attempt to cultivate a representative of the dominant nitrate-reducer in Area 1, enrichment cultures were set up using Area 1 sediments, 100 mM nitrate, and 100 mM ethanol. Pure cultures were obtained by streaking serial dilutions of active enrichment cultures onto plates containing nitrate and ethanol. All isolates screened have the *nir*K gene, and sequences of these genes indicate that all isolates belong to OTU 1K from *nir*K clone libraries. Sequence analysis of the 16S rRNA genes revealed that all isolates are 97% similar to nitrate-reducing β-Proteobacterium *Alcaligenes defragrans*. Overall, both culture-independent and culture-dependent techniques suggest that a novel *Alcaligenes* species is important for in situ nitrate removal with ethanol in areas of the FRC with high nitrate concentrations.

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