

**DOE-NABIR
PI WORKSHOP:
Abstracts**

March 18–20, 2002
Warrenton, Virginia

**Natural and Accelerated Bioremediation Research Program
(NABIR)**

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Introduction
DOE–NABIR PI Workshop
March 18–20, 2002

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 place 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 is implemented through four interrelated scientific research elements (Biogeochemistry, Biomolecular Science and Engineering, Biotransformation, and Community Dynamics/Microbial Ecology); and through an element called Bioremediation and its Societal Implications and Concerns (BASIC), which addresses societal issues and potential concerns of stakeholders. The material presented at this year's workshop focuses on approximately 60 research projects funded in FY 2000–2002 by DOE's Office of Biological and Environmental Research (BER). Abstracts of NABIR research projects are provided in this book.

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Agenda
NABIR PI Meeting
Airlie Conference Center
Warrenton, VA
March 18–20, 2002

Monday, March 18

- 8:30–8:45 a.m. Welcome—Anna Palmisano
- 8:45–9:00 a.m. Ari Patrinos, Associate Director, Biological and Environmental Research
- 9:00–9:15 a.m. Teresa Fryberger, Deputy Director, Science and Technology, Environmental Management
- Field Research Overviews
- 9:15–9:45 a.m. NABIR Field Research Center (Dave Watson, ORNL)
- 9:45–10:15 a.m. Research at UMTRA Sites (Phil Long, PNNL)
- 10:15–10:45 a.m. BREAK
- 10:45–11:15 a.m. Push-Pull experiments at FRC, UMTRA (Jack Istok, Oregon State)
- 11:15–11:45 a.m. Biotransformation (Joel Kostka, Florida State)
- 11:45–12:15 p.m. Biogeochemistry (Jiamin Wan, LBNL)
- 12:15–2:00 p.m. LUNCH

<i>Afternoon Session</i>			
2:00–5:00 p.m.	Posters	Breakout: Factors affecting radionuclide/metal mobility (Criddle)	Breakout: Molecular tools for characterizing communities and processes (Chandler)
5:00–6:00 p.m.	Free time		
6:00–7:00 p.m.	Dinner		
7:00–9:00 p.m.	Poster Session: Community Dynamics/Microbial Ecology, BASIC, Biomolecular Science and Engineering. Authors must be at posters.		

Tuesday, March 19

- 8:30–9:00 a.m. Biogeochemistry (Mary Neu, LANL)
- 9:00–9:30 a.m. Biogeochemistry (Colleen Hansen, Stanford)
- 9:30–10:00 a.m. Community Dynamics (Jizhong Zhou, ORNL)
- 10:00–10:30 a.m. BREAK
- 10:30–10:50 a.m. INEEL Mesocosm (Gil Geesey, INEEL)
- 10:50–12:00 a.m. EM Roundtable: Addressing EM Needs
- 12:00–2:00 p.m. LUNCH

<i>Afternoon Session</i>			
2:00–5:00 p.m.	Posters	Breakout: Novel technologies at EMSL and other DOE sites (Zachara, Fendorf)	Breakout: Microbiology and geochemistry of low pH (Lovley, Banfield)
5:00–6:00 p.m.	Free time		
6:00–7:00 p.m.	Dinner		
7:00–9:00 p.m.	Poster Session: Biogeochemistry, Biotransformation, FRC projects. Authors must be at posters.		

Wednesday, March 20

- 8:30–9:00 a.m. The Ribosomal Database Project (Jim Cole, Michigan State)
- 9:00–9:30 a.m. Biomolecular Science and Engineering (A.C. Matin, Stanford)
- 9:30–10:00 a.m. Biomolecular Science and Engineering (Jon Lloyd, U. Manchester)
- 10:00–10:30 a.m. BREAK
- 10:30–11:00 a.m. Structural Biology (Woody Woodruff, LANL)
- 11:00–11:30 a.m. BASIC (Denise Lach, Oregon State)
- 11:30–11:45 a.m. Wrap-up; Meeting adjourns
- 12:00–1:00 p.m. LUNCH

Note: UMTRA Working Group will meet on Sunday, March 17, from 1 p.m.–6 p.m.

ABSTRACTS

PROGRAM ELEMENT 1

Biotransformation

The Kinetics of Direct Enzymatic Reduction of Uranium (VI): Effects of Ligand Complexation and U(VI) Speciation

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Calculations based on literature values for reduction of the U(VI)- (poly)maleic acid complex (an analog for fulvic acid) suggest that dissolved humic substances will decrease U(VI) reduction rates to an extent that would require an *in situ* bioreduction zone 32 m thick. Interdisciplinary laboratory research will be performed to investigate U(VI)-ligand species reduction rates by anthraquinone-2,6-disulfonate (AQDS), hematin (a core structure in cytochrome c_3), cytochrome c_3 (from *D. vulgaris*), and intact cells of *D. vulgaris* (a DMRB organism) as a function of ligand type and structure.

The central tenets of this research are that: (1) reduction of U(VI) follows the electron-transfer (ET) mechanism developed by Marcus; (2) it is this transfer reaction that dominates the mechanism of U(VI) reduction, and (3) transfer reaction is the step most affected by complexation, and hence it is rate-limiting in cell-free systems. Research is based on the following hypotheses: (a) only those ligands that exhibit complexation and configuration constraints toward the ET process will be significant; (b) the ET step in these reactions will follow Marcus theory; and (c) only those ligands exhibiting both complexation and conformational constraints will decrease the ET rates to such an extent that they will govern whole cell U(VI) reduction. The reduction of complexed U(VI) will be investigated via stopped-flow kinetics with fluorescence, UV/Vis, and electron spin resonance spectroscopy detection. Experimental rate data will be collected in a systematic manner, through the selection of specific ligands under specific conditions (pH, pCO₂, temperature, etc.), for AQDS, Hematin, Cytochrome c_3 , and whole cells to understand the variations in U(VI) reduction observed in the environment. Marcus theory will be used to develop a fundamental basis and understanding of electron-transfer reaction in these systems and to develop a predictive structure-reactivity relationship based on theory and experimental data.

While this research was initiated this year, we have investigated the homogeneous kinetics of electron transfer from bacterially reduced AQDS to Fe(III) as a function of speciation and complexation by various organic acids. The reduction of aqueous Fe(III) depends on its speciation. For instance, the $\text{Fe}(\text{H}_2\text{O})_6^{+3}$ exhibits almost no reduction in the presence of AH₂DS, but, depending on the ligand, the rate of complexed Fe(III) reduction varies widely. The homogeneous reduction kinetics will be discussed as a function of ligand, pH, and ionic strength. Rate controls were modeled using density functional calculations and will be presented in the framework of Marcus theory.

The Uranium Immunosensor: Functional Assessment and Reagents to Enhance Performance

Diane A. Blake (PI) and Haini Yu

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A better understanding of *in situ* bioremediation processes and the development of strategies to enhance bacterial remediation of contaminated sites depend either directly or indirectly upon accurate detection and measurement of organics, metals, and other toxic elements prior to, during, and following the remediation process. The portable immunosensor-based assays being developed and refined in this project could yield reliable data in real time (i.e., <1 hour), be field-ready (i.e., simple, durable, and accurate), be inexpensive (i.e., <<\$100/test and <\$5,000 for the initial equipment investment), and exhibit minimal invasiveness. Thus, this project will provide basic scientific knowledge that could revolutionize clean-up technologies and significantly reduce future costs.

The aims for the present project period are: (1) to test and validate the present uranium immunosensor-based assay and develop protocols for its use at the NABIR Field Research Center in Oak Ridge, Tennessee; (2) to develop new protein reagents that will provide superior performance in the hand-held immunosensor; and (3) to develop new monoclonal antibodies for DTPA and Cr(III) or Hg(II). A portable sensor for U(VI) that is under development employs a monoclonal antibody with specificity for chelated U(VI) and an instrument built by Sapidyne Instruments, Inc. (Boise, ID). In this method, the fluorescently labeled antibody is permitted to equilibrate with a soluble metal-chelate complex derived from the environmental sample. Then the equilibrium mixture is rapidly passed through a disposable microcolumn that contains an immobilized capture reagent. When the fluorescent signal is plotted versus time, the slope of the line is inversely proportional to the concentration of UO_2^{+2} in the original solution. The prototype immunosensor could detect soluble UO_2^{+2} at concentrations from 10 to 100 nM (2.5 to 24 ppb). The maximum coefficient of variation in the linear portion of the assay was 6.5%. New capture reagents have been developed from chemically functionalized particles of poly(methyl methacrylate). This chemical modification procedure enhanced by ~30-fold the capture of the fluorescently labeled antibody. Studies are underway to incorporate these new supports into the handheld sensor.

Biodegradation of Pu EDTA and Impacts on Pu Mobility

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The contamination of many DOE sites by plutonium (Pu) is a long-term problem because of Pu's long half-life (240,000 years) and the low drinking water standard ($<10^{-12}$ M). Ethylenediaminetetra-acetate (EDTA) was co-disposed with radionuclides (e.g., Pu, ⁶⁰Co), formed strong complexes, and enhanced radionuclide transport at several DOE sites. Biodegradation of EDTA should decrease Pu mobility. One objective of this project was to determine the PuEDTA aqueous species present under environmentally relevant conditions and determine how the biodegradation of EDTA by bacterium BNC1 influenced Pu solubility and mobility. The Pu(IV) formed strong Pu(OH)_xEDTA^{x-} complexes (x = 1, 2, or 3), which enhanced Pu solubility. Pu(IV) solubility increased with decreasing pH and increasing EDTA. At high concentrations of EDTA (i.e., 10^{-4} M), the presence of Pu enhanced the biodegradation rate of the EDTA. As the concentration of EDTA decreased to 10^{-5} M, the presence of Pu decreased the biodegradation rate of the EDTA. It is currently unclear why the concentration of Pu directly affects the rate of EDTA biodegradation. The soluble Pu concentration decreased as the EDTA was biodegraded.

A second objective was to investigate the genes and enzymes involved in EDTA biodegradation. BNC1 can use EDTA and another synthetic chelating agent nitrilotriacetate (NTA) as sole carbon and nitrogen sources. The same catabolic enzymes are responsible for both EDTA and NTA degradation, except that additional enzymes are required for EDTA degradation. When the catabolic genes were cloned and sequenced, the gene cluster also contained genes encoding a hypothetical ABC-type transporter. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the transporter genes and EDTA monooxygenase gene (emoA) are co-transcribed. EppA is one of the transporter genes that codes for a periplasmic binding protein, responsible for binding to the substrate before transport across the membrane can occur. EppA was cloned, expressed, and purified in *Escherichia coli* and found to bind MgNTA, CaNTA, and Fe(III)NTA, but not free NTA. Our data also suggest that BNC1 uses the same ABC-type transporter for both EDTA and NTA uptake. Results from these studies can provide mechanistic understanding and approaches to assist in the bioremediate PuEDTA and other radionuclide-EDTA complexes at DOE sites.

Iron Reduction and Radionuclide Immobilization: Influence of Natural Organic Matter and Reaction-Based Modeling

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The proposed research evaluates the potential for humic substance addition to stimulate *in situ* Fe(III)-reducing activity and U(VI) bioimmobilization, and to construct and validate a comprehensive reaction-based model to simulate these processes. Humic substances are known to stimulate solid-phase metal oxide reduction by their ability to shuttle electrons between dissimilatory metal-reducing bacteria (DMRB) and oxide surfaces. In addition, our recent research has demonstrated that humics can also enhance solid-phase ferric oxide reduction through complexation of biogenic Fe(II). The presence of humic substances is therefore likely to increase the abundance and activity of DMRB. The addition of a natural, native material (e.g., humic substances at the DOE Field Research Center [FRC]) may be more acceptable to the general public and key stakeholders compared to other biostimulants such as quinones or complexants. The proposed research is based on a series of hypotheses regarding the impact of free oxide surface site concentration, chemical speciation of surface-associated Fe(II), natural organic matter (NOM), and hydrologic transport on the kinetics and thermodynamics of ferric oxide bioreduction, uranium reduction, and DMRB growth.

The objectives of our proposed research are to elucidate the factors controlling the rate and extent of biological ferric oxide reduction in subsurface sediments, the mechanisms of U(VI) immobilization under Fe(III)-reducing conditions, and the influence of NOM on these processes. The emphasis of our proposed research is to independently describe important reactions using reaction-based model formulations/parameters. More complex systems will be examined by incremental addition of other reactive species (e.g., U(VI) and NOM), reactive processes (e.g., DMRB growth), or hydrologic transport.

Our approach involves the following experimental and concurrent modeling efforts using synthetic hematite-coated sand, an FRC reference sediment, and the FRC reference humic substances: (1) kinetic measurements of Fe(III) bioreduction in subsurface sediments and associated DMRB growth; (2) kinetic measurements of the bioreduction of U(VI) and FRC humics; (3) kinetic and equilibrium measurements of Fe(II) and U(VI) complexation by solid sediments and dissolved FRC humics; (4) kinetic and equilibrium measurements of the abiotic reduction of U(VI) by biogenic Fe(II) and bioreduced FRC humics; (5) experiments examining the above suite of reactions occurring in concert in batch and column reactors; and (6) construction and validation of a series of reaction-based models (incorporating both kinetic and equilibrium speciation formulations) of uranium immobilization under Fe(III)-reducing conditions.

Bio-oxidation of Fe(II) and Radionuclide Immobilization by *D. suillum*

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Previous studies on microbial attenuation of heavy-metal and radionuclide (HMR) contamination have focused on the ability of some anaerobic respiratory microorganisms to utilize the HMR as alternative electron acceptors and precipitate them in the reduced state from solution. However, this is only a short-term solution, because once the environment becomes reoxidized, reduced and immobilized metals such as U(IV) will abiotically react with dissolved oxygen and will rapidly remobilize. Previously, we proposed an alternative microbial-based remediative strategy based on microbial nitrate-dependent Fe(II) oxidation. Our studies focused on *Dechlorosoma* species, which we previously demonstrated to be a ubiquitous group of Fe(II)-oxidizers in the environment that can couple anaerobic oxidation of Fe(II) to the reduction of either nitrate or (per)chlorate. We demonstrated that precipitation of Fe(III)-oxides by these organisms resulted in the rapid adsorption and removal of soluble HMR from solution.

A more in-depth analysis of this metabolism demonstrated that, similarly to most known nitrate-dependent Fe(II)-oxidizers, *D. suillum* did not grow by anaerobic Fe(II) oxidation. In the absence of a suitable organic carbon source, cells rapidly lysed even though nitrate-dependent Fe(II) oxidation was still occurring. In the presence of a utilizable carbon source such as acetate, Fe(II) oxidation was inhibited until the acetate was completely oxidized. The coupling of Fe(II) oxidation to a particular electron acceptor was dependent on the growth conditions of cells of *D. suillum*. As such, anaerobically grown cultures of *D. suillum* did not mediate Fe(II) oxidation with oxygen as the electron acceptor, while conversely, aerobically grown cultures did not mediate Fe(II) oxidation with nitrate as the electron acceptor. The endproduct of Fe(II) oxidation was determined by the rate of oxidation. Rapid bio-oxidation resulted in the production of amorphous ferrihydrite, while slow oxidation resulted in the production of green-rust and the subsequent production of various crystalline endproducts.

D. suillum oxidation was specific for Fe(II), and the organism did not enzymatically oxidize U(IV) or Co(II). Small amounts (less than 2.5 μM) of Cr(III) were reoxidized by *D. suillum*. However, this appeared to be inversely dependent on the initial Cr(III) concentration. Abiotic oxidation of insoluble U(IV) to soluble U(VI) by biogenically produced Fe(III) did not result in remobilizing the U(VI). X-ray absorption spectroscopy analysis indicated that the uranium was bound to the biogenically produced Fe(III)-oxides and was present as U(VI), and that the U(VI) formed bidentate and tridentate inner-sphere complexes on the Fe(III)-oxide surfaces.

The results of this study demonstrate the potential for this novel approach to the stabilization and immobilization of radionuclides and heavy metals in the environment. This strategy may be applied in two ways: (1) by precipitating Fe(III)-oxides over previously immobilized HMR contamination *in situ*, forming an insoluble barrier that will crystallize with time, inhibiting future bio-reduction and adsorbing any leached metal contaminants; or (2) by engineering a semi-permeable Fe(III)-oxide wall *in situ*, downstream of the immobilized HMR. This Fe(III)-oxide wall will “catch” and adsorb HMR that may be solubilized and remobilized because of environmental fluxes such as re-oxidation (biotically or abiotically) or ligation.

Reductive Precipitation and Stabilization of Uranium Complexed with Organic Ligands by Anaerobic Bacteria

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This research addresses the principal microbial-alteration mechanisms of organic-radionuclide complexes and the resultant impacts on radionuclide solubility and stability under anaerobic conditions. We investigated the mechanism of biotransformation and fate of U complexed with organic ligands under anaerobic conditions by Fe- and U- reducing microorganisms *Shewanella putrefaciens* CN32, *Clostridium sphenoides* (ATCC 19403), capable of utilizing citric acid as the sole carbon source, and *Clostridium sp.* (ATCC 53464), capable of fermenting glucose but not citrate. Equimolar and excess ligand complexes of U-ketogluconate, U-malate, U-citrate, and U-catechol were prepared at pH 3.5 (FRC groundwater) and 6.0, and characterized by advanced spectroscopic techniques (x-ray photoelectron [XPS], x-ray absorption near edge structure [XANES], extended x-ray-absorption fine structure [EXAFS], and Fourier Transform infrared [FTIR]). All the complexes examined exhibited di- μ -OH bonding of U.

The 1:1 U: citric acid complex was not metabolized by *S. putrefaciens*, *C. sphenoides*, or *Clostridium sp.* However, in the presence of excess citrate *S. putrefaciens*, *C. sphenoides*, and glucose, *Clostridium sp.* reduced U(VI) bound to citric acid to U(IV), and the reduced uranium remained in solution complexed to citric acid as the U(IV)-citrate complex. These results show that complexed uranium is readily accessible for microorganisms as an electron acceptor, despite their inability to metabolize the organic ligand complexed to the actinide.

The molecular structure for 1:1 U: citric acid complex consists of a binuclear di- μ -OH core with five-fold coordination of oxygen in the equatorial plane. There is tridentate bonding of citric acid to each of the uranium atoms involving the carboxylate and hydroxyl groups of the ligand. In the presence of an electron donor, uranyl ion (U^{+6}) bound to citric acid was reduced to the tetravalent form (U^{+4}). The uranium remained in solution, with citric acid as the U(IV)-citrate complex. There is eight-fold coordination of oxygen to the reduced uranium in the U(IV)-citric acid complex. The presence of axial oxygens or binuclear complex formation was not observed. The increase in total number of oxygens for reduced uranium compared to the U(VI)-citrate complex was most probably the result of hydrolytic reaction. We postulate that the tridentate coordination of citric acid to the uranium does not change. However, the double bonds in the axial oxygen atoms are reduced to single bonds because of a bacterially mediated two-electron transfer reaction.

Analysis of U in the FRC groundwater (freeze-dried) by EXAFS showed that uranium was present as uranyl chloride species. Adjustment of the FRC groundwater pH to 6.8 resulted in precipitation of uranium with aluminum. Incubation of the FRC groundwater sample with and without carbon source and *Clostridium sp.* showed limited reduction of uranium to uranous form, with solubilization of a portion of aluminum and the precipitated uranium.

Heterogeneous Charge Distribution on the Surface of the Iron-Reducing Bacterium *Shewanella oneidensis* Strain MR-1: An Electron Microscopic Study

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Dissimilatory metal-reducing bacteria (DMRB) help control the biogeochemistry of anoxic, non-sulfidogenic subsurface and sedimentary environments, principally through the enzymatic reduction of iron and manganese. Both of these electron acceptors are highly insoluble at circumneutral pH values that are common in nature. Hence, in the absence of soluble organic compounds that can serve as electron shuttles between bacteria and mineral surfaces, iron-reducing bacteria must directly contact mineral surfaces for reduction to occur. Notably, little is known about the interaction between DMRB and oxide surfaces, or about the bacterial surface itself. Recently published results using electrostatic-force microscopy demonstrated that the surface of iron-reducing bacteria is characterized by a heterogeneous distribution of positively and negatively charged sites. At neutral pH, negatively charged “patches” measuring about 20–50 nm in diameter were distributed over the entire cell surface. The patches corresponded with raised areas or blebs that are believed to develop into membrane vesicles.

In the present study, negatively charged areas of the cell surface were labeled using colloidal gold particles (1.4 nm) that were coated with polyamines, which impart a positive charge to the gold at neutral pH. Transmission electron microscopy (TEM) revealed that the labeled areas correlated well with both the size and distribution of the negatively charged patches observed by electrostatic force microscopy. However, the TEM images clearly demonstrated that the patches were actually annular rings of negatively charged groups surrounding an area of neutral or positive charge.

In summary, results obtained using charged colloidal gold particles and TEM confirm and extend results obtained by electrostatic-force microscopy, which revealed heterogeneous charge distribution over the surface of iron-reducing bacteria. Colloidal gold labels may also serve as amplifiers of Raman signals through a technique called “surface-enhanced Raman spectroscopy” and may reveal the chemical nature of charged areas on the cell surface.

The Role of Natural Organic Matter in Microbial Reduction of Metals

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The overall goal of this project is to provide a molecular-level understanding of the roles and mechanisms of heterogeneous natural organic matter (NOM) in facilitating the reductive immobilization of metal and radionuclide contaminants by anaerobic metal-reducing bacteria. Our specific objectives are to (1) isolate and characterize NOM subcomponents or fractions with varying chemical and structural properties, (2) investigate the redox-active functional groups and their reaction kinetics of NOM fractions with metal contaminants such as chromate and uranium (CrO_4^{-2} and UO_2^{+2}), and (3) determine the electron-shuttling capabilities of different NOM components in the microbial reduction and immobilization of metal contaminants, in both batch and soil column flow-through systems.

The NOM and NOM fractions that we have isolated contain both electron-rich and electron-deficient sites. These sites are responsible for its electron-donating and electron-accepting properties. Related structural and functional properties of this NOM have been characterized by both wet-chemical methods (such as potentiometric titration and cyclic voltammetry) and a range of spectroscopic techniques, including nuclear magnetic resonance (NMR), Fourier Transform infrared (FTIR), electron paramagnetic resonance (EPR), fluorescence, and UV/Vis spectrometry. The results have indicated that the different NOM components vary greatly in structural features and functional groups, such as the contents of aromatic moieties, carboxylic and heteroaliphatic hydroxyl functional groups, free radicals as measured by electron spin counts, and molecular weight. Cyclic voltammetry shows that NOM (particularly the polyphenol fraction) gives electrode response similar to that of model quinones such as anthraquinone disulfonate (AQDS), juglone, and lawsone.

Different NOM components were found to directly reduce metals or metal oxides, such as CrO_4^{-2} and Fe(III), although they exhibit varying ability to reduce these metals. However, U(VI) was reduced only in the presence of microorganisms and electron donors such as lactate. The polyphenolic-rich NOM fraction appeared to be the most reactive in reducing CrO_4^{-2} and Fe(III) compounds to Cr(III) and Fe(II) compounds in the absence of microorganisms. The reduction of CrO_4^{-2} was confirmed by x-ray near-edge absorption spectroscopic (XNEAS) analysis. In the presence of microorganisms, the humic acid component appeared to be the most effective in shuttling electrons for the microbial reduction of these metals, which was attributed to its high-molecular-weight and polycondensed aromatic structural features. Our study confirms the heterogeneous nature of NOM. Different components of NOM possess different structural and functional properties, and vary in their abilities to react with metals and to shuttle electrons for microbial reduction of these metals.

Investigation of the Spatial Distributions and Concentrations of Biologically and Environmentally Relevant Elements at the Mineral-Microbe Interface

Ken Kemner¹ (PI), Shelly Kelly,¹ Ed O'Loughlin,¹ Ken Nealson,² Barry Lai,¹ Joerg Maser,¹ Zhonghou Cai,¹ Mark Schneegurt,³ and Chuck Kulpa⁴

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Understanding the fate of heavy-metal contaminants in the environment is of fundamental importance in the development and evaluation of effective remediation and sequestration strategies. 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. Additionally, the metabolic and surface properties of bacteria can be quite different, depending upon whether the bacteria exhibit a planktonic (free-floating) or 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 determining a contaminant's macroscopic fate, the spatial distribution and chemical speciation of contaminants and elements essential to biological processes must be characterized at micron- and submicron-length scales, for bacteria in both planktonic and adhered states. Hard x-ray micro-imaging is a powerful technique for element-specific investigation of complex environmental samples at micron and submicron resolution because of the large penetration depth of hard x-rays in water. This minimizes the requirements for sample preparation and allows the detailed study of hydrated samples.

The objectives of the studies presented here are (1) to determine the spatial distribution, concentration, and chemical speciation of metals at and near bacteria-geosurface interfaces, and (2) to use this information to identify interactions occurring near these interfaces among the metals, mineral surfaces, and bacterially produced extracellular materials under a variety of conditions. We have used x-ray fluorescence microscopy to investigate the spatial distribution of 3d elements in single *Shewanella oneidensis* cells grown with oxygen, fumarate, and lepidocrocite as electron acceptors. Cells analyzed were either in a surface-adhered or planktonic state. The zone plate used in these microscopy experiments produced a focused beam with a cross section (and hence spatial resolution) of 0.15–0.40 micron. The samples (both planktonic and biofilm) were all grown in a consistent manner within a defined minimal-salts medium.

Results from x-ray fluorescence imaging experiments indicate that the distribution of P, S, Cl, Ca, Fe, Ni, Cu, and Zn can define the location of the microbe. Additionally, quantitative elemental analysis of individual microbes is possible. Results of the concentrations of the above-mentioned elements in individual *Shewanella oneidensis* cells grown under the above-mentioned conditions will be presented.

Modeling of Microbial Fe(III) Reduction in Soils and Estimation of Soil Fe(III) Bioavailability

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We have formalized a mathematical reactive transport model describing fluid flow through a porous soil, linked to chemical and biological reduction of Fe(III) oxides. In addition to direct contact between Fe(III) oxides and bacteria, the model incorporates contributions to Fe(III) reduction rates and extents from measurable electron shuttles and chelating agents found in soil organic matter. In this model, Fe(III) (solid phase or chelated) can be reduced directly by bacteria or indirectly by reduced electron shuttles. The model accounts for three potential fates for Fe(II) produced: sorption to Fe(III) oxides, precipitation as Fe(II) carbonate (siderite) or advection of the soluble free species. One of the most difficult model parameters to measure is the fraction of total soil Fe(III) that is bioavailable. A method for estimating soil Fe(III) bioavailability by titrating the soil with reduced anthraquinone disulfonic acid (AQHDS) was developed and compared against hydroxylamine-HCl reduction, using four pure iron oxides and six soils. For the crystalline Fe(III) oxides used in this study—high-surface-area goethite, low-surface-area goethite and hematite—AQHDS and hydroxylamine-HCl gave comparable results, and both slightly underestimated the true bioavailability determined using the model bacterium *Shewanella alga* BrY. However, AQHDS titration provided a much better estimate for bioavailability of amorphous Fe(III) oxyhydroxide, which is the predominant soil form, and also showed sensitivity to occlusion of “available” Fe(III) sites by Fe(II) sorption. In natural floodplain soils collected from the DOE Savannah River Site (SRS), AQHDS titration and hydroxylamine reduction both gave accurate Fe(III) bioavailability estimates. The AQHDS titration method has an advantage over chemical reduction methods in that it can estimate Fe(III) bioavailability in environments where Fe(II) adsorption or deposition reduces the amount of total Fe(III) that is bioavailable.

Impacts of Mineralogy and Competing Microbial Respiration Pathways on the Fate of Uranium in Contaminated Groundwater

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This project addresses the role of mineral-bacteria interactions in the migration of uranium (U) in contaminated sediments from the NABIR Field Research Center (FRC). The focus is on the competition between Fe(III)-reducing bacteria (FeRB) and the sulfate-reducing bacteria (SRB), their impact on Fe mineralogy, and how these microbially mediated mineralogical changes will govern U speciation. Our objectives are to (1) characterize the dominant Fe minerals that are likely to limit U speciation, (2) directly quantify reaction rates and pathways of terminal electron-accepting processes, and (3) identify and enumerate the anaerobic organisms mediating U geochemistry.

During the first year, our approach has been to use enrichment culture studies to determine the factors limiting anaerobic microbial activity to direct future rate measurements in sediment mixtures. Using a most probable number (MPN) approach, SRB counts were negligible at both the contaminated and pristine sites, and counts were corroborated by very low sulfate reduction-rate measurements. In contrast, FeRB were enumerated, isolated, and characterized from a variety of subsurface sites. Pristine and contaminated sediments (high in uranium and nitrate, low in pH) were compared from similar depths below surface. MPN counts of FeRB were generally much lower in contaminated sediments (range of 0 to 46 cells ml⁻¹; 66% nondetect) as compared to pristine sediments (range of 0 to 230 cells ml⁻¹; 8% nondetect). A variety of methods were tested for the cultivation of FeRB over a range of pHs, and organisms were identified by cloning and sequencing of the 16S rRNA genes in culture. Screening of clones from terminal positive MPN enrichments of contaminated sediments revealed that the predominant FeRB were deep-branching members of the delta Proteobacteria, showing <80% sequence identity to all 16S rRNA sequences in current databases. Six pure cultures of FeRB isolated from contaminated sediments showed >99% sequence identity to *Clostridium celerecrescens*. FeRB enrichments from contaminated sites were also shown to reduce U(VI). In parallel subsurface sediment samples, Fe minerals have been size-fractionated, and nucleic acids have been extracted for molecular characterization. Mossbauer spectra of contaminated sediment and the clay mineral fractions of pristine sediment have been obtained and will be presented. From this, we will begin to characterize the anaerobic bacteria likely to catalyze uranium transformation in the subsurface at the FRC. Nitrate concentration and pH appear to be important variables controlling the composition and activity of FeRB populations in contaminated subsurface sediments.

Microbial Influences on Uranium Mobility in Uranium-Contaminated Subsurface Sediments

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Biotransformations influencing the mobility of uranium were investigated in a variety of uranium-contaminated subsurface environments. In studies with sediments from nine different sites at three different Uranium Mill Tailings Remedial Action Project (UMTRA) locations, stimulating microbial reduction of Fe(III) and U(VI) with the addition of acetate was an effective strategy for removing uranium contamination from the groundwater. In all instances, Fe(III) and U(VI) were reduced simultaneously. When nitrate was present, nitrate was reduced prior to Fe(III) and U(VI) reduction. U(VI) reduction was complete prior to sulfate reduction, demonstrating that sulfate reduction was not an important mechanism for U(VI) reduction.

The influence of added nitrate on uranium mobility was examined in detail. The addition of nitrate inhibited Fe(III) and U(VI) reduction in cells of nitrate-grown *Geobacter metallireducens*, suggesting that, in sediments, the inhibition of Fe(III) and U(VI) reduction in the presence of nitrate may be explained, in part, by preferential reduction of nitrate over Fe(III) and U(VI). However, the fact that nitrate had no impact on Fe(III) and U(VI) reduction in *G. sulfurreducens*, which cannot use nitrate as an electron acceptor, and the fact that nitrate inhibited Fe(III) and U(VI) reduction (even when there were high concentrations of acetate in the sediments), suggested that some other factor must also contribute to the inhibition of Fe(III) and U(VI) reduction in the presence of nitrate. Further evaluation demonstrated (for the first time) that when nitrate is available as an electron acceptor, *G. metallireducens* can oxidize Fe(II) and U(IV) with the reduction of nitrate. The addition of Fe(II) stimulated U(IV) oxidation, presumably because the Fe(III) produced could abiotically oxidize U(IV). These results suggest that any Fe(II) or U(IV) produced by nitrate-insensitive dissimilatory metal-reducing microorganisms in sediments will, in the presence of nitrate, be oxidized back to Fe(III) and U(VI), eliminating any net Fe(III) and U(VI) reduction.

Since these studies with *G. metallireducens* suggested that the addition of nitrate would solubilize rather than precipitate uranium in contaminated subsurface environments (as other NABIR projects have proposed), the effect of nitrate additions on uranium solubility was examined directly in sediments. Addition of nitrate to UMTRA sediments in which all of the uranium had been precipitated as U(IV) resulted in a rapid oxidation of U(IV) to U(VI) and recovery of 85–100% of the uranium as U(VI) in solution in the groundwater. Thus, oxidation of Fe(II) to Fe(III) by adding nitrate is not likely to be a good strategy for immobilizing uranium in subsurface environments, but could be a good approach for recovering precipitated uranium following bioremediation of a site.

In low pH sediments from the Field Research Center, the addition of acetate also stimulated the removal of U(VI) from the groundwater. The surprising result was that U(VI) was removed when only about 10% of the ca. 50 mM nitrate in the groundwater was reduced. These results suggest that increasing the pH by stimulating organic matter oxidation, coupled to nitrate reduction, may be an effective strategy for removing uranium from contaminated groundwater in some low-pH, uranium-contaminated subsurface environments.

New Catalytic DNA Biosensors for Radionuclides and Metal Ions

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We propose to develop 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. The sensors will be highly sensitive and selective, not only for different metal ions, but also for different oxidation states of the same metal ion. They will be applied to the on-site, real-time assessment of concentration, speciation, and stability of radionuclides and metal contaminants during and after bioremediation. To achieve this goal, we will employ a method called “*in vitro* selection” to search for catalytic DNA molecules that are highly specific for radionuclides or other metal ions. Comprehensive biochemical and biophysical studies will be performed on the selected DNA molecules. The findings from these studies will elucidate the structure/function relationship in catalytic DNA and thus facilitate the design of improved sensors. The DNA will be labeled with fluorescent donor/acceptor pairs to investigate, and to signal, the structural changes upon metal ion binding. Once a collection of individual DNA sensors is identified, each specific for a particular metal ion at a particular concentration range, they will be assembled into a DNA microarray for the simultaneous detection and quantification of radionuclides and metal contaminants.

We have successfully used the methodology mentioned above to develop a highly sensitive and selective DNA biosensor for Pb^{+2} . The biosensor consists of a catalytic DNA capable of base-pairing to a DNA substrate containing a single ribonucleotide residue. When a fluorophore (carboxytetramethylrhodamine [TAMRA]) is attached to the 5' end of the DNA substrate, the fluorescence signal at 580 nm is quenched by a fluorescence quencher (Dabcyl) at the nearby 3' end of the catalytic DNA molecule. In the presence of Pb^{+2} , the fluorescence emission of TAMRA increased by 400%, caused by the cleavage of the substrate followed by product release. This DNA biosensor is highly sensitive for Pb^{+2} , with a quantifiable detection range from 10 nM to 4 μM . Even in the presence of other metal ions (Mg^{+2} , Ca^{+2} , Mn^{+2} , Co^{+2} , Ni^{+2} , Zn^{+2} , Cd^{+2} , and Cu^{+2}) and under simulated physiological conditions, this biosensor displays a remarkable sensitivity and selectivity. The principles demonstrated in this work can be used to obtain catalytic DNA biosensors for other metal ions as well. Progress made in (1) understanding and optimizing the Pb^{+2} DNA sensor system, (2) improving metal ion selectivity of the methodology, and (3) applying the methodology to sensing other metal ions such as strontium will be presented.

Calcite Surface Recognition and Attachment of *Shewanella oneidensis*: Initial Results and Environmental Implications

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We are engaged in mechanistic studies of the microbe-mineral interface, particularly with respect to the role of microbial surface recognition and attachment in reduction and oxidation of mineral phases and of related dissolution and precipitation. In our first set of experiments, we have investigated the effect of microbial attachment on the dissolution kinetics of calcite, using vertical scanning interferometry, which possesses an internal reference that allows us to quantify reaction progress over time. *Shewanella* is the model organism for our long-term study for several reasons—its capability for dissimilatory metal reduction, its ability to exist in both aerobic and anaerobic conditions, and its sequenced genome. We have begun the study with calcite because Lüttge and Lasaga (2001) have previously used this technique to study the dissolution kinetics of calcite, dolomite, and feldspar in abiotic conditions, so well-quantified control data exists on these environmentally important substrates. Moreover, calcite possesses excellent cleavage faces, which are essential to the experimental method we have developed.

Shewanella oneidensis (MR-1) was cultured under aerobic conditions and applied to a fresh surface of a calcite crystal exposed by cleavage along a (10-11) face. The mirror-equivalent plane of the cleaved crystal was exposed to the growth medium only, a 10% LB broth with added 10 mM Na lactate at neutral pH. After only 20 minutes, the abiotic control crystal exhibited a large number of etch pits, whereas the formation of etch pits was almost entirely inhibited by the microbes on the crystal with the *Shewanella*. In addition, the rare etch pits present were quite shallow compared with those developed on the control crystal.

The microbes quickly (<20 minutes) settled on the crystal surface, creating negative features, as though moving into the crystal. They formed chains and, ultimately, networks as they attached to the crystal, appearing to control dissolution of the calcite, a very different behavior from the dissolution of the control crystal. At 45 minutes, dissolution pits were ubiquitous and coalescing with each other on the control crystal, whereas the attachment of the microbial biomass still almost totally prevented formation of these etch pits. By 90 minutes, the crystal surface was almost entirely covered with a mat-like film of organisms and exo-polysaccharide filaments. Etch pits were shallow and undeveloped with respect to those that formed and coalesced on the control surface.

These preliminary results imply that *Shewanella* is able to detect the high-energy sites at the mineral surface (i.e., the outcrops of dislocations), settle, and take control over the dissolution process. Moreover, such results further imply that microbes might play a significant role in the stabilization of carbonates under conditions that might otherwise favor their dissolution. We are presently conducting the anaerobic experiment and will report this data as well in March.

Environmental Actinide Mobility: Plutonium and Uranium Interactions with Exopolysaccharides and Siderophores of Naturally Occurring Microorganisms

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Our overall goal is to understand the interactions of actinides and bacteria that can stabilize or destabilize actinides in the subsurface environment with respect to mobility. Our objectives toward achieving this goal are to: (1) characterize the chemical toxicity of actinides to bacteria that could stabilize actinides using biostimulation or bioaugmentation; (2) determine and characterize the binding of actinides to extracellular polymers and whole cells and show how that binding will affect environmental speciation and distribution; (3) examine and quantify the siderophore-mediated redox, speciation, and membrane translocation of actinides; and (4) investigate the redox influence of bacterial reductants on actinides.

Complete results for all of the objectives will be presented at the workshop, a sample of which are as follows: First, we have studied the toxicity of the metals As, Ba, Co, Cd, Pb, Cr, ^{55}Fe (a beta-emitter), natural Fe, ^{239}Pu (an alpha emitter), and depleted U, as well as the chelators tiron, nitrilotriacetic acid (NTA), desferrioxamine B (DFB), and citrate to *D. radiodurans*, *B. licheniformis*, and *P. putida*. For *D. radiodurans*, tolerance to all the first six metals is relatively high, with the exception of Cd, which showed growth inhibition at 0.1 ppm. Tolerance to Fe was very high. The DFB complexes of both ^{55}Fe and natural Fe inhibited growth at 1680 ppm or 30 mM (0.1 mCi of ^{55}Fe), showing that the toxicity was chemical rather than radiological. The DFB complex of U(VI) inhibited growth of *D. radiodurans* at 600 ppm (2.5 mM), and the DFB complex of ^{239}Pu (IV) inhibited growth at 1100–1200 ppm (5.0 mM, ~0.4 mCi alpha). These concentrations correspond to radiation-dose equivalents orders of magnitude lower than what has been reported to be toxic, based on gamma irradiation studies.

Second, we have studied the Fe(III), U(VI), and Pu(IV) binding of the polyglutamate exopolymer (PGA) of *B. licheniformis*, the polysaccharide (EPS) exopolymer of *R. erythropolis*, and the whole cells of *D. radiodurans*, *B. licheniformis*, and *Microbacterium flavescens* (JG-9). PGA bound 0.12, 0.18, and 0.24 mmol/mg for U, Fe, Pu, respectively; whereas EPS bound approximately 40% of those amounts for Fe and U, and was not a sufficiently strong chelator to outcompete hydrolysis for Pu(IV). Interestingly, *D. radiodurans* shows very little capacity for whole cell sorption of Pu and Fe, but has higher sorption of U.

Third, hydroxamate siderophores readily reduce Pu(VI and V) and oxidize Pu(III) for form Pu(IV) complexes. The redox potential of Pu(IV/III)-siderophore couples at neutral pH range from $E_{1/2} = -225$ to -527 mV (versus NHE), showing that siderophores will stabilize Pu(IV) over Pu(III) and will also catalyze the oxidation of Pu(III) to Pu(IV). We have shown that a Pu(IV)-siderophore complex can be recognized and partially taken up by a microbe in a process similar to the analogous Fe(III)-siderophore complex, via protein-mediated, metabolically dependent uptake. The corresponding U(VI) complex is not recognized or taken up.

Bridging the Batch-Field Gap: Chromium Biogeochemistry in Diffusion-Limited Domains

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Linking laboratory-based understanding of biogeochemical transformations of contaminants to field-based measurements remains a formidable challenge. The broad range of transport times and reaction rates encountered in the subsurface often makes it unrealistic to characterize on the basis of well-mixed, laboratory batch experiments, because coupling between different microenvironments is not accounted for. Multi-region flow and transport models are often useful for describing fast advective transport through fractures and macropores, and slower diffusion-dominated transport within sediment blocks and soil aggregates. However, the nature of reactive transport within diffusion-controlled domains is commonly only inferred or assumed. This is potentially a major weakness in models, because diffusion-controlled domains can make up most of the subsurface. Direct, spatially resolved measurements within poorly mixed domains of soil aggregates and sediment blocks are needed to understand how subsurface biogeochemical processes are coupled. Redox-sensitive metals constitute an important class of contaminants for which such fine-scale, spatially resolved analyses are necessary. In biologically active sediments, oxygen levels are often diffusion-limited, such that steep gradients in redox-sensitive species are established. Measurements that average across such gradients cannot be correctly interpreted.

In response to this problem, we have conducted a series of laboratory studies on chromium contamination of sediments, and subsequent *in situ* remediation—reduction of Cr(VI) to Cr(III)—by organic carbon infusion. Spatially resolved determination of Cr concentrations and oxidation states using micro-XANES, and spatially resolved microbial community analyses, were done on synthetic and natural soil aggregates. During the diffusion-limited contamination process, more Cr(VI) was transported, but to shorter distances, in more microbially active aggregates. Sharply terminated diffusion fronts, within 2 to 10 mm of the aggregate surface, result from increasing Cr(VI) reduction rates with depth. Infusion of organic carbon into previously Cr(VI)-contaminated aggregates resulted in more rapid reduction to Cr(III) with higher organic carbon concentrations, as well as lower reduction rates in more highly contaminated sediments. These results show that intra-aggregate Cr dynamics are strongly diffusion-limited in more microbially active systems, and that bulk soil chemical and microbial characterization can obscure relevant biogeochemical processes.

PROGRAM ELEMENT 2
**Community Dynamics/
Microbial Ecology**

Artificial Neural Network Tools for the Analysis of Microbial Biomarker Data

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New data analysis tools are needed to help understand biomolecular community characterization data. In our project, we are developing artificial neural network (ANN) tools for relating changes in microbial biomarkers to the concentration of heavy metals and radionuclides. ANNs are nonlinear pattern-recognition methods that can learn from experience to improve their performance. We have successfully applied these techniques to the analysis of membrane lipids and nucleic acid biomarker data from both laboratory and field studies. This poster will summarize the results of our analyses, outline some refinements to standard ANN methods that help with the analysis of small-sample-size data sets, and outline the Web-based tool kit we are developing for use by other NABIR investigators.

We have used ANNs to analyze microbial-biomarker data from several field and laboratory studies. The results from several of these analyses will be summarized in our poster. In general, ANNs perform better than multiple linear regression when used for feature prediction. Also, ANNs are typically superior to linear techniques (such as principal-components analysis) in reducing the dimensionality of a data set.

Overfitting is a potential problem when using ANNs to analyze biomolecular data in which the ratio of the number of biomarkers to samples is large. The result of overfitting is that the ANN generalizes poorly to new data that were not used during training. We have implemented two techniques to help reduce the problem of overfitting: (1) a sensitivity-based pruning algorithm to help identify efficient ANN models for nonlinear prediction and (2) a global optimization (simulated annealing) technique to select parsimonious ANNs that generalize well to new data sets. We have also implemented an input training (IP) technique that can be used to reduce the dimensionality of the input variables for visualization. This IP method is more efficient than a popular autoassociative ANN technique used for the same purpose.

We are also developing a Web-based tool kit that incorporates several innovative analyses for applying ANNs to biological data sets with a small number of samples. A variety of tools will be provided, including input data transformations, selecting network architecture, various learning algorithms, regularization techniques, and cross-validation methodologies. In addition, several standard linear statistical tools will be available, such as principal-components analysis and multiple linear regression. A Web-based interface will be an integral component of the system that links all of the tools.

Impact of Biostimulation Conditions on Diversity and Dynamics of Key Genes Involved in Metal Reduction

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The relationships between diversity, ecosystem function, and stability of community structure over time remains a major challenge in microbial ecology. However, this knowledge is needed to anticipate the effects of perturbations (e.g., bioremediation operating parameters) on the performance of the system. For example, different types, rates, or timing of nutrient additions may impact intra-species competition and generate different community structure end-states, which may profoundly impact the performance of a bioremediative process such as uranium reduction. We propose to collaborate with and assist four currently funded NABIR investigators in the analysis of selected Field Research Center (FRC) samples from both laboratory and field studies investigating biological uranium reduction. Samples will come from batch and dynamic column experiments, and from groundwater and core samples before and during *in situ* biostimulation to promote uranium reduction. Conserved functional genes for metal reduction and sulfite reduction will be amplified from the samples and approximately 10,000 clones sequenced, without a prior restriction enzyme screening step, at the DOE Joint Genome Institute. Three hypotheses will address diversity in three key metal and uranium-reducing genes, how diversity in these genes changes in response to different conditions imposed in laboratory studies and in response to field bioremediation phases, and the utility of the diversity information for microarray analysis at the population and expression levels. The resulting robust data sets will (1) characterize macrodiversity and microdiversity within the functional genes at the FRC site, (2) describe how nutritional and hydrogeochemical experimental factors impact the diversity and dynamics of the functional genes, and (3) be exploited to construct and utilize a high-resolution DNA array at the FRC site.

Integrated Particle Handling Methods for Multiplexed Microbial Identification and Characterization in Sediments

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The objective of this project is to develop integrated microbial and nucleic acid detection methods and instrumentation for monitoring metal-reducing microbial communities in subsurface sediments, before and after bio-stimulation. This objective is being met by coalescing recent developments in 16S rRNA microarrays, microfluidic systems, microparticle chemistries, renewable surface techniques, and suspension array technology to address fundamental scientific and technical gaps associated with *in-field* purification and detection of 16S rRNA from sediments.

In the first quarter of the project, we coupled 16S rRNA-targeted DNA and peptide nucleic acid (PNA) oligonucleotides to avidin-coated and carboxylated 5 µm microspheres, developed direct and indirect labeling methods for target nucleic acids, and established baseline target DNA capture and detection limits in a simplified flow-cytometer instrument. Peptide nucleic-acid probes resulted in 10–100 times greater signal intensity and shorter hybridization times in batch capture experiments than identical manipulations with DNA probes. One nanogram of *Geobacter* 16S rDNA could be captured and detected in as little as 15 minutes in the presence of 1 µg nontarget DNA. Hybridization specificity could be improved through the use of heat or formamide during hybridization.

These preliminary results are in keeping with our hypothesis that PNA probes will be more sensitive than identical DNA probes for the automated recovery and direct detection of metal- and sulfate-reducer 16S rRNA and mRNA from sediment extracts. Successful coupling of DNA and PNA probes to two different bead surfaces, and subsequent capture of target DNA in batch capture experiments, provides two different methods to address our tunable surface hypothesis for increasing the direct capture and detection efficiency of metal- and sulfate-reducer 16S rRNA and mRNA. A fluidic system with a custom flow cell was assembled to serve as an automated “front end” for the flow cytometer detector. Methods for automated capture and release of nucleic acids on 5 µm particles in the fluidic system were developed.

By the March 2002 meeting, we anticipate comparing the capture efficiency of DNA and PNA probes in both batch (above) and automated systems, with the expectation that automated capture of DNA targets will be greater than or equal to the efficiency of manual protocols, but in only a fraction of the time. We also anticipate developing direct capture and detection of intact rRNA, utilizing a capture strategy developed under our prior NABIR microarray project.

Ecological Interactions between Metals and Microbes That Impact Bioremediation

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Investigators have found distinct microbial communities in contaminated soils that vary in their concentrations of Pb, Cr, and aromatic compounds. The functional diversity of the indigenous microbial community was determined using substrate-induced respiration (SIR) and community-level physiological profiling (CLPP). Microbial activities determined with SIR were higher on carboxylic acids than on carbohydrates, amino acids, amines and amides, and aromatics, especially in highly contaminated soils. Redundancy analysis of the SIR indicated that microbial functions differed between highly contaminated soils and slightly or noncontaminated soils (as had been found for analyses of community biomarkers), and that the levels of hydrocarbons, Pb, and Cr were correlated to the changes in microbial functions.

Contamination with metals and hydrocarbons is correlated in these soils. Therefore, it is difficult to distinguish between their effects on community structure and activity. Microcosms were constructed in which either Pb^{+2} or CrO_4^{-2} was added at levels that produced a modest or severe effect on community activity. Either glucose or xylene was added as C-source, as substrates broadly used by microbes or restricted in their catabolism. Activity was monitored via carbon dioxide evolution, and total biomass changes were monitored via analyses of phospholipid phosphate. Heavy-metal additions had more severe effects on xylene catabolism than on glucose. Analyses of changes in community structure are in progress, using both PL-FAME analysis and DGGE of 16S rDNA genes.

The correlation between microbial activity, community structure, and metal level is being analyzed on 150 mg aggregates collected at spatial scales of <1, 5, 15, and 50 cm. Preliminary results show that glucose mineralization varies 10-fold, and extractable Pb and Cr levels vary 3 to 7-fold among aggregates located within 1 cm from each other. DGGE profiles have similarities >0.85 at scales <1 cm.

The characteristics of bacteria isolated from these habitats have also been investigated. *Arthrobacter* VN23-1 is Pb-resistant. Lead resistance can be transferred to a Pb-sensitive *Arthrobacter* strain by conjugation, although at a relatively low frequency. Lead resistance appears to be inducible. We have cultivated bacteria resistant to elevated concentrations of Pb and Cr and others that are able to degrade aromatic compounds (e.g., naphthalene), but none has both phenotypes. The majority of the metal-resistant isolates are *Arthrobacter* spp. and the hydrocarbon-degraders are Gram-negative bacteria. However, activity assays indicate that aromatic degradation occurs in these habitats. Detection of functional genes involved in biodegradation would provide a more direct measure of degradation activity. We have identified polymerase chain reaction (PCR) primer sets that allow detection of biphenyl dioxygenase, naphthalene dioxygenase, toluene dioxygenase, and toluene monooxygenase. Multiplex PCR protocols were developed to permit simultaneous detection of aromatic oxygenase genes. A real-time PCR quantification method using these primers was also optimized to determine gene copy number. Gene amplification was tracked using SYBR green I as a marker. Using this approach in the future, we can quantify any metal-resistance genes and additional catabolic genes in our sites.

Molecular Analysis of the Distribution, Genomic Potential, and Activity of Dissimilatory Metal-Reducing Microorganisms in Uranium-Contaminated Subsurface Environments

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Continued evaluation of the microbial communities in sediments from the Uranium Mill Tailings Remedial Action (UMTRA) site in Shiprock, New Mexico, demonstrated that in sediments from sites 853, 857, and 1103, the stimulation of Fe(III) and U(VI) reduction was associated with a remarkable bloom of microorganisms in the family *Geobacteraceae*. This was observed in 16S rDNA clone libraries in which the percentage of *Geobacteraceae* increased from undetectable, prior to the onset of metal reduction, to over 40% of the 16S rDNA sequences during the Fe(III) reduction phase. This enrichment of *Geobacteraceae* was confirmed with quantitative polymerase chain reaction (PCR) methods. Both MPN-PCR and TaqMan analyses demonstrated that the number of *Geobacteraceae* sequences increased by two or more orders of magnitude once metal reduction began in the acetate-amended sediments. There was no increase in *Geobacteraceae* in control, unamended sediments in which there was no metal reduction. It is significant that other well-studied dissimilatory metal-reducing microorganisms, such as *Shewanella* and *Geothrix* species, were not detectable with sensitive PCR methods, even during the height of metal reduction in these sediments.

Potential physiological characteristics that might explain why *Geobacteraceae* are such superior competitors in anoxic subsurface environments were investigated. It was found that when only Fe(III) or Mn(IV) oxides are available, *G. metallireducens* becomes mobile via flagella, chemotactic to Fe(II) and Mn(II), and produces pili that are required for Fe(III) oxide reduction. These and other results suggest that *G. metallireducens* specifically moves toward and attaches to Fe(III) and Mn(IV) oxides to reduce them. In contrast, *Shewanella* and *Geothrix* produced electron-shuttling compounds and Fe(III) chelators, which permitted them to transfer electrons to Fe(III) oxide without the need to establish direct contact with the Fe(III) oxide. Although this may be a beneficial strategy in a culture tube, this is likely not an energetically favorable method for reducing Fe(III) oxide in nutrient-poor subsurface environments.

To evaluate the genetic potential of the *Geobacteraceae* found in high numbers in subsurface environments, genomic DNA was extracted from sediments in which metal reduction was important. It was then cloned into BAC libraries, and BAC clones containing *Geobacteraceae* genomic DNA were sequenced. Preliminary data has indicated that the *Geobacteraceae* in the sediments contain genes whose function can be confidently predicted and that are similar to the genes found in the *Geobacter sulfurreducens* genome. However, some *Geobacteraceae* genes of unknown function do not have a closely related counterpart in the *G. sulfurreducens* genome. To find molecular targets that could be used to measure the activity of *Geobacteraceae* in the subsurface, the expression of genes specifically produced during growth on Fe(III) was quantified in chemostat cultures reducing Fe(III) at different rates. Levels of mRNA for a gene for a 36 kDa, membrane-bound, *c*-type cytochrome increased in direct correlation with rates of Fe(III) reduction. Results from linking the expression level of other genes to rates of anaerobic respiration, as well as other studies on molecular analysis of microbial communities at other sites (including the FRC) will be presented, as well as the results from novel isolates.

Development of a Multiplexed, Bead-based Assessment Tool for Rapid Identification and Quantitation of Microorganisms in Field Samples

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This work is aimed at developing a quantitative, high-throughput molecular assessment tool suitable for field testing. The tool is based upon a new multiplex technology that involves hybridization of nucleic acids on the surface of microscopic, fluorescent, polystyrene beads to identify specific target sequences in complex mixtures of DNA or RNA. Following capture of target sequences obtained from environmental samples, the fluorescent beads are analyzed by flow cytometry.

During the past year, our primary accomplishments are as follows: (1) We have developed a multiplex method involving a Scatchard-type model for quantifying individual sequences in polymerase chain reaction (PCR) products generated from community DNA. Experiments were conducted on 16S rRNA genes amplified from microorganisms in contaminated groundwater. Our findings will be published in *Applied Environmental Microbiology*. (2) We have improved the lower detection limit by a factor of five, using novel labels known as dendrimers, and are currently refining the assay. (3) We have developed software that greatly speeds up the determination of the mean reporter signal for a multiplexed assay and can process raw flow cytometry data at the rate of 10 tubes (each containing, for example, a different environmental sample) in a few seconds. Now we need to evaluate the bead method by testing a greater variety of groundwater and sediment samples than before. We are currently focusing on uranium-contaminated sites. In collaboration with several investigators, we are developing a set of 16S rRNA gene-capture probes targeting iron-reducing and sulfate-reducing bacteria. These will be applied to laboratory and field samples originating from Lawrence Livermore National Laboratory, Uranium Mill Tailings Remedial Action (UMTRA), the NABIR Field Research Center, and the Aberdeen Proving Grounds (APG) in Maryland. From APG, a series of microcosms are currently being constructed to explore the effect of nutrient addition (e.g., ethanol, lactate, acetate) on the chemical speciation of depleted uranium. Split-samples of these experiments will be processed by the bead method to elucidate microbial-community responses. A similar analysis will be performed on groundwater samples already obtained from the Istok/Krumholz FRC site. Our aim is to determine the limits of detection and threshold densities of bacteria required for bioremediation to be effective. The suitability of groundwater as a sampling matrix for detecting microbial community changes will also be explored, using samples from column and field studies.

Characterization of Aerobic and Microaerophilic Isolates from Contaminated and Uncontaminated NABIR Field Research Center Sediments

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Six samples from contaminated areas and one sample from the background area were provided for our analyses. From the contaminated regions, the pH ranged from 3.5 to 5.1 in our samples (data supplied by the NABIR Field Research Center). We have isolated aerobic and microaerophilic organisms from three different sediments, two contaminated sediments (pH 3.5 and 5.1) and a background sample (pH 5.1). We were unable to obtain any isolates from the low pH sediments. Contaminated soils with pH 5.1 yielded both aerobes and microaerophiles, as did the control background sediments. The plateable microbial community of the uncontaminated sediments was 5-fold greater than the contaminated sediments (4.1×10^4 CFU/gram versus 8.6×10^3 CFU/gram), but the contaminated sediments had a larger fraction of microaerophiles (59% from contaminated versus 48% from background sediment). Repetitive-sequence-based-polymerase chain reaction (REP-PCR) of the isolates revealed broad taxonomic diversity. Based on similarity indices of the REP-PCR profiles, four clusters were detected that were dominated by isolates derived from the contaminated samples. Representative isolates spanning the REP-PCR measured diversity were selected for phylogenetic analysis. These isolates proved to be dominated by Gram-positive bacteria, including representatives from *Brevibacillus*, *Paenibacillus*, *Microlunatis*, *Arthrobacter*, *Cellulomonas*, *Rathayibacter*, and *Clavibacter*. The sole Gram-negative identified was a *Flavobacterium*.

Spatial and Selective Factors Determining Subsurface Microbial Community Structure and Dynamics

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To experimentally test factors controlling community structure, and to assess the competitiveness of microbial groups of interest to DOE remediation, we have been developing multispecies microcosms and community genome arrays (CGAs) for assaying multiple populations. Prototype glass slide-based microarrays were constructed to evaluate the application of CGAs for assessing microbial community composition. CGAs contained pure genomic DNA from 54 different formally characterized reference organisms and environmental isolates, including Gram-positive bacteria and α -, β -, and γ -*proteobacteria*. Immobilized probe genomic DNA was interrogated with Cy3- or Cy5-labeled target genomic DNA prepared by a random prime labeling method. Optimization studies for microarray fabrication and hybridization indicated that (1) as little as 50 ng/ μ l of probe genomic DNA could be detected with fluorescently labeled target genomic DNA, while no substantial increase in signal intensity was observed for arrayed genomic DNA >200 ng/ μ l; and (2) reasonable specificity was obtained using 50% formamide in the hybridization buffer at 55°C. Genomes of species within the genera of *Pseudomonas* and *Shewanella* were clearly distinguished. Sensitivity experiments demonstrated that as little as 0.2 ng of pure genomic target DNA could be detected by CGA hybridization. We are now selecting strains of DOE interest from the FRC site for developing the experimental array.

We have also developed and tested functional gene arrays for tracking important ecosystem function. A first-generation array allowed us to evaluate specificity, sensitivity, and quantitation. Towards development of a second-generation microarray, we constructed numerous different functional gene clonal libraries from a variety of environmental sources, screened them, and determined their nucleotide sequences. This array included five different functional genes (*nifH*, *amoA*, *nirS*, *nirK*, and *dsrAB*), and 77, 9, 56, 68, and 79 phylogenetically different (\leq 85% sequence identity) cloned representatives of each, respectively. As an example, the majority of the FRC *nirK* and *nirS* clones were not closely related to previously described genes and may have been selected under the unique conditions of the site.

Down-Well Microcosm “Bug Traps” and Subsurface Sediments for Rapid Expanded-Lipid-Biomarker Analysis and DNA Recovery for Monitoring Bioremediation Microbial-Community Ecology within Samples from Uranium-Contaminated Sites

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Microbial activity is of primary importance in the bioremediation of metal-contaminated subsurface environments. Our principal objective is to develop more expedient and cost-effective methods for biomarker recovery and analysis, utilizing a combination of expanded signature lipid biomarkers (SLB), polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) analyses of 16S rDNA, and specific genes. These tools are used to define the viable community composition and provide indications of important specific activities relative to the impact of metals and radionuclides on indigenous microbial communities.

We have initiated the testing emplacement of sterile surfaces with and without nutrient sources in the well fields of impacted sites—the “bug traps.” After a 2–3 week exposure, the traps were recovered and analyzed. Traditionally, methods employed to monitor microorganisms require *ex situ* culture analysis of groundwater membrane retentates and sediments. However, these methods poorly represent *in situ* microbial communities. Phospholipid fatty acid (PLFA) analysis has been utilized to determine shifts in microbial biomass, nutritional/physiological status, and community diversity *in situ*. For greater specificity, we have complemented PLFA analysis with a PCR-DGGE approach, employing primers that recognize the 16S rDNA of almost all known and inferred bacterial species and of specific functional genes. Sequence analysis of individual bands from DGGE gels was used to provide fine-scale biomarkers and loosely infer the identity of the source organisms, using database searches and phylogenetic methods to relate the complexity, band positions, and relative band intensities of DGGE patterns to contaminant load. Unfortunately these monitoring technologies require at least several days of intensive work with highly skilled personnel. To exploit potential economies in sampling realized by the use of the “bug traps,” we are developing methods that are faster and require less specialized analyses, yet provide more comprehensive insight into the *in situ* microbial ecology.

Room-temperature extraction, fractionation, derivatization, and GC/MS analysis for PLFA requires at least three working days. We have shown that high-temperature/high-pressure sequential extraction can be completed in less than an hour. The lipid-extracted residue can be acid-hydrolyzed with “magic” methanol and the ester-linked OH fatty acids readily detected by GC/MS. Utilization of HPLC/APCI/MS/MS, particularly of the ubiquinones (UQ), menaquinones (MK), and intact phospholipids, expands insight into metabolic activities and increases sensitivity several fold. T-RFLP expands the purview of DNA analyses from DGGE without a great increase in time. Applications to the “Bug Traps” show great promise. The LC/MS/MS analyses of UQ/MK allow monitoring manipulations of *in situ* terminal-electron-acceptor concentration that are critical to heavy-metal and radionuclide immobilization.

Development and Use of SSU rRNA Gene-Based Oligonucleotide Microarrays for Assessing Microbial Community Composition and Dynamics

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To understand microbial diversity of the NABIR Field Research Center (FRC), 1870 SSU rRNA clones were analyzed from groundwater samples for six different sites at the FRC. These sites differed with respect to nitrate, organic carbon, uranium, heavy metals, and pH levels. The bacterial community structure was diverse at the background site, and representatives of at least six bacterial groups were detected, including α -, β -, γ -, δ -Proteobacteria, high G+C, and low G+C Gram-positive bacteria. Only one other site was as diverse as the background with the additional observation of Nitrospira, Cytophagales/Bacteroides/Flavobacterium, and Acidobacter/Fibrobacter groups. The nitrate and heavy metal levels at this site were most comparable to background, but moderate levels of uranium were detected. At a site with increased nitrate but comparable pH and heavy-metal levels, the recoverable diversity was dramatically decreased. This site appeared to be dominated by α - and β -Proteobacteria, including Rhizobium, Azoarcus, and Acidovorax species. Three sites had acidic pH values, increased uranium and nickel levels, and decreased diversity compared to background. These sites were predominated by Azoarcus, Psuedomonas, and Ralstonia species. The results indicated that contaminant levels impacted the bacterial community structure at the respective sites.

As a part of this project, we have determined whether single mismatch discrimination can be achieved with microarray hybridization for SSU rRNA genes. Our results indicate that the position of the mismatch, the type of mismatch, and the concentration of hybridization additives, such as formamide and tetramethylammonium chloride (TAMCl), significantly affected discrimination power and signal intensity. The hybridization signal intensity of the probes with a single-base mismatch was about 10–30% of the signal intensity with the perfect-match probes, depending on the type of nucleotides. The signal intensity of the probes with two-base mismatches was about 5–25% compared to the perfect-match probes. Probes with three or four base-pair mismatches gave very low signal intensity, which was less than 5% of the perfect-match probes. These results indicated that single-base discrimination for SSU rRNA genes can be achieved with array-based hybridization.

We have also developed new software for designing gene-specific oligonucleotide probes. For a given gene, the program automatically identifies gene-specific probes by heuristic basic local alignment search tool (BLAST) search for each gene (query) against all other genes. The optimal sequence alignment between the query and each of the homologous sequences was obtained using dynamic programming technique. Based on the alignment, the program selects gene-specific fragments. All the parameters (e.g., probe length, similarity threshold, and melting temperature) can be adjusted by the user.

PROGRAM ELEMENT 3
Biomolecular Sciences and Engineering

The Effect of Heavy-Metal Pollution on the Microbial Community in Subsurface Soil

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The aim of this study is to investigate the adaptation of subsurface microbial populations to heavy metals. The capability of the microbial community to adapt to different environmental perturbations is a well-known phenomenon. This has been demonstrated by the development of resistant subpopulations and an increase in degradation capabilities of specific xenobiotic compounds. However, most methods used to show adaptation obtain limited data (e.g., resistance frequency and degradation rate). Most of these methods are very laborious and usually focus, therefore, on measurements of a single function. Moreover, few studies have focused on tolerance assays in which the community's capability to perform a function in the presence of a stressor is evaluated. We have evolved an adaptation assay based on a new multifunction tolerance test, in which the entire bacterial community is evaluated with regard to the sole-carbon utilization pattern of 31 different carbon sources (EcoPlates) in the presence of heavy metals (as compared to metal-free controls). Finally, we have applied this approach to microcosm experiments designed to investigate the role of the horizontal transfer of resistance gene in the adaptation of subsurface microbial populations to heavy-metal contamination.

Engineering *Deinococcus radiodurans* and *Deinococcus geothermalis* for Bioremediation of Radioactive Waste Environments

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Seventy million cubic meters of ground and three trillion liters of groundwater have been contaminated by leaking radioactive waste generated in the United States during the Cold War. A cleanup technology is being developed based on the extremely radiation-resistant bacterium *Deinococcus radiodurans* that is being engineered to express bioremediating functions. We have demonstrated that *D. radiodurans* can be genetically engineered for metal remediation and organic toxin degradation, using expression systems that are functional during growth of the bacteria at 60 Gy/hour. With respect to DOE facilities, there has been no adequate method for microbiological treatment of radioactive contaminant waste sites containing both hazardous organic and metallic components, since other more conventional bioremediating organisms like *Pseudomonas* spp. are very radiation sensitive. A variety of organic toxin-degrading genes has been successfully introduced in *D. radiodurans*. For example, an engineered strain expressing cloned *todABCDE xylJFK* efficiently mineralizes toluene, yielding CO₂ and water, and preliminary analyses support that this strain can use toluene to support growth under certain conditions.

Deinococcus geothermalis is closely related to *D. radiodurans*, but is thermophilic and is able to grow at temperatures as high as 52°C. It is also the only known member of the deinococcal family that is endowed with a complete biosynthetic repertoire, and has superior growth capabilities in nutritionally restricted environments exposed to chronic radiation. It is possible, therefore, that *D. geothermalis* may be a better candidate than *D. radiodurans* for bioremediation of radioactive wastes, particularly in thermally insulated environments (e.g., within or beneath leaking tanks) where temperatures can be elevated because of radioactive decay. We have shown that the genetic technology developed for *D. radiodurans* is suitable for *D. geothermalis* and have transferred both autonomous plasmids and chromosome integration vectors to this thermophile. Engineered *D. geothermalis* appears to be as proficient as *D. radiodurans* at expressing cloned functions applicable to the treatment of radioactive wastes.

Comparative Analysis of Gene and Protein Expression in *Shewanella oneidensis* MR-1

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Shewanella oneidensis MR-1 is a facultative anaerobe capable of using a variety of metals as electron acceptors. Because of this anaerobe's potential usefulness in bioremediation protocols, DOE is interested in the biochemical reactions used by this microbe for metal reduction and the regulatory mechanisms controlling the activation or inactivation of these reactions. The availability of a genome sequence for MR-1 provides the opportunity to analyze patterns of both gene and protein expression in this microbe after growth with different electron acceptors. Regulation of gene expression occurs at the DNA level, whereas protein expression can be altered at the DNA, mRNA, or even the protein level (i.e., changes in the rate of protein degradation). Therefore, parallel analysis of both gene and protein expression is necessary to provide a complete picture of cellular response to the environment at the molecular level.

In our project, MR-1 and mutant *S. oneidensis* strains are grown under a variety of experimental conditions. At specific time points, the mRNA and proteins are isolated. A microarray representing a subset of the MR-1 genome has been used to screen the mRNA population, and two-dimensional gel electrophoresis, coupled with peptide mass spectrometry, has been used to monitor the protein component. A close correlation has been found between quantitative changes in some mRNAs and the corresponding proteins under varying growth conditions (e.g., DNA topoisomerase II, AlcC protein, electron transfer flavoprotein, dihydrolipoamide succinyltransferase). However, a significant lack of correlation has been observed between others (e.g., dihydrolipoamide succinyltransferase, prismane, phosphomannomutase, fumarate reductase). The differences in correlation between mRNA levels and protein levels are indicative of differences in regulatory mechanisms at the level of protein expression.

Metabolic Engineering of Microorganisms for Actinide and Heavy-Metal Precipitation

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Heavy metals, actinides, and organics are significant problems at a number of DOE sites and industrial locations in the U.S. Because of the costs associated with excavating, transporting, and remediating contaminated sediments at remote locations, an economically viable solution is to mineralize the organic contaminants *in situ* and immobilize the metals and actinides to prevent movement to other locations. There are few reports in the literature of organisms capable of all of these functions. Besides their potential use *in situ*, these organisms should find use in treating waste tanks at such sites as Hanford that contain mixed organics, metals, and actinides.

During the previous grant period, we isolated and characterized a novel strain of *Pseudomonas aeruginosa* from a deep-sea hydrothermal vent. This strain was capable of removing high levels of cadmium from solution by reducing thiosulfate to sulfide and precipitating cadmium as cadmium sulfide on the cell wall. To improve upon this system, we successfully engineered *Escherichia coli*, *P. aeruginosa*, and *P. putida* to remove heavy metals and actinides from solution and immobilize them on the cell wall. For precipitation of cadmium, zinc, lead, and other metals that form strong sulfide complexes, we developed two systems for aerobic sulfide production: (1) expression of serine acetyl transferase and cysteine desulphydrase in *E. coli* for overproduction of cysteine and subsequent conversion to sulfide, and (2) expression of thiosulfate reductase in *E. coli* and *P. putida* for reduction of thiosulfate to sulfide. The *P. putida* system was shown to allow simultaneous heavy-metal precipitation and organics degradation. For precipitation of actinides as complexes of phosphate, we overexpressed polyphosphate kinase in *E. coli* and *P. aeruginosa*. This enabled these organisms to accumulate high levels of polyphosphate during phosphate excess and exopolyphosphatase for polyphosphate degradation and concomitant secretion of phosphate from the cell. All of these systems were shown to be capable of removing relatively high levels of metals from solution. They all have potential for metal and actinide removal from contaminated waste streams or immobilizing these elements *in situ*.

The goal of our work is to engineer heavy-metal and actinide precipitation in two microorganisms that will be relevant for treatment of DOE sites contaminated with heavy metals, actinides, and/or organics: *P. aeruginosa* and *D. radiodurans*. Specifically, we propose to (1) engineer polyphosphate synthesis and degradation into *D. radiodurans* and *P. putida* for removal of uranium(VI) and plutonium(VI, V); (2) engineer aerobic sulfide production into *D. radiodurans* for removal of cadmium, zinc, and lead; and (3) test removal of actinides, actinides and heavy metals, and actinides, heavy metals, and organics using the engineered organisms.

Differential Expression of Environmentally Relevant Genes in *Desulfovibrio* sp.

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In recent years, microorganisms living in environments contaminated by human activities have been recognized as contributing to the degradation of organic pollutants and immobilization of metals. As growth conditions and habitats change with the introduction of a pollutant, microorganisms respond by repressing or derepressing the expression of proteins that are no longer essential or are critical to survival. To better understand gene expression of sulfate-reducing bacteria growing under different environmental conditions and pressures, signature-tagged mutagenesis, random arbitrarily primed-polymerase chain reaction (RAP-PCR), can be employed.

Signature-tagged mutagenesis is a genetic technique used to identify genes needed to survive in a particular ecosystem. Our study is aimed at the identification of genes within sulfate-reducing bacteria that are needed for survival in anaerobic subsurface systems. To date, we transformed several strains of *Desulfovibrio* with a Tn5-containing plasmid. We also transformed strain *D. desulfuricans* G20 with a Tn7 derivative containing plasmid with a resultant efficiency of 10^{-4} – 10^{-5} . We performed a southern blot on randomly selected mutants and showed that transposon insertion into SRB genomic DNA was not random. We have also examined the ability of mutants generated by pRL1058a transposition to survive in subsurface sediments. For both *D. vulgaris* and *D. desulfuricans*, cell numbers increased during incubation in sediments, with a greater increase occurring when H₂ was added to sediment incubations. However, after growth peaked at around 8–12 days, numbers declined and remained at initial levels.

To examine differential expression, we analyzed RNA extracts of *D. desulfuricans* subsp. *aestuarii* and *D. vulgaris* using RAP-PCR. Following the sequencing of differentially expressed mRNA-derived bands, basic local alignment search tool (BLAST) sequence comparisons were made. One sequence from RAP-PCR bands of hydrogen-grown *D. desulfuricans* was identified as dissimilatory sulfite reductase, an enzyme involved in respiration. Northern blotting analysis verified that increased expression of the gene occurred in hydrogen-grown cells. Subsequently, Northern blot analysis was used to show an increase in expression of several respiratory proteins during growth on H₂. Sulfate respiration was also shown to occur at a higher rate in H₂-grown cells by measuring sulfide production of H₂ or lactate-grown cells during growth. RAP-PCR appears to be an effective tool in assessing differential gene expression of *Desulfovibrio* species when grown with various electron donors, and this technique will be applied to other questions of environmental significance, including identification of genes that are up-regulated in the presence of contaminants.

AdnA-Regulated Genes in *Pseudomonas fluorescens* Associated with Soil Colonization, Motility, and Persistence

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Pseudomonas fluorescens is a soil bacterium with potential applications in biocontrol and bioremediation. Motility and adhesion appear as key elements in both uses. AdnA is a transcriptional regulator that controls these functions in *P. fluorescens*. Its closest matches are FleQ (*Pseudomonas aeruginosa*), FlaK (*Vibrio parahaemolyticus*) and FlrA (*Vibrio cholerae*), which play an important role in early stages of the regulatory cascade in the polar flagella synthesis. To identify the genes regulated by adnA in *P. fluorescens*, we developed a genetic screen using a knockout mutant (Pf0-2x) of the wild type (Pf0-1). We inserted an omega cassette in adnA by marker exchange using a suicide vector. Pf0-2x was then mutagenized with Tn5-B22 (lacZ), generating random transcriptional fusions to chromosomal genes. The adnA was introduced into each of the mutants by a mass-mating technique using a broad-host range plasmid (pJB866). Differences in lacZ expression between mutants with and without adnA were screened on a medium with Xgal. Quantitative b-galactosidase assays confirmed these differences. Among 12,000 mutants screened, 12 mutants contained insertions in 8 genes positively regulated by adnA. The sequences of the transposon-chromosome junctions were obtained using an arbitrary-primed polymerase chain reaction (PCR) method. The genes corresponded to flagellar structural components, perosamine synthetase, subunit II of the cytochrome oxidase, and methyl-accepting chemotaxis proteins (MCPs). Two of the genes showed no significant match with sequences in the databases. Northern analysis confirmed the differences in the expression of these genes between the wild type and the deletion mutant. Electron microscopy provided information about the presence of flagella. All mutants were nonmotile, but only eight showed defects in adhesion to surfaces (plastic and sand). The results in this work indicate the existence of different pathways involved in motility and adhesion, with some aspects common to both processes. The finding of genes involved in flagella synthesis and chemotaxis validate our screen, while the other genes may give new ideas about other pathways controlled by AdnA involved in soil persistence and survival.

Mechanisms for Uranium and Technetium Reduction in *Geobacter sulfurreducens*

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The aim of this project is to characterize the mechanisms of electron transfer to U(VI) and Tc(VII) in a model dissimilatory Fe(III)-reducing bacterium. *Geobacter sulfurreducens* has been chosen for initial studies because: (1) this organism is closely related to the predominant organisms that emerge when dissimilatory metal reduction is stimulated in subsurface environments by the addition of various electron donors and/or electron shuttling compounds; (2) the genome sequence of this organism will soon be completed; and (3) a genetic system for this organism is available.

The protein hypothesized to reduce U(VI) *in vivo*, a periplasmic tri-heme cytochrome *c*₇ designated CycA, was purified to homogeneity and characterized in detail. In common with an analogous 13,000 kDa c-type cytochrome of closely related *Desulfovibrio* species, cytochrome *c*₇ reduced U(VI) *in vitro*. A mutant, prepared by polymerase-chain-reaction (PCR) mediated disruption of the *cycA* gene, was constructed, and was compromised in its ability to couple acetate oxidation to the reduction of U(VI) and other metals. When *cycA* was expressed *in trans*, the full capacity for metal reduction with acetate was restored. However, the mutant was able to couple hydrogen oxidation to U(VI) reduction at the same rate as the wild type strain. This suggests that the 9.6 kDa cytochrome, although involved in electron flow from acetate to U(VI), may not be the terminal reductase for the radionuclide. An outer membrane cytochrome of molecular mass 41 kDa was also studied in detail. This protein was active against U(VI) *in vitro* and was localized to the surface of cells (using a range of biochemical techniques), behavior consistent with a role in reductive precipitation of extracellular insoluble U(IV). Reduction of Fe(III) oxide was abolished by protease treatment, suggesting a role for the 41 kDa cytochrome in Fe(III) reduction and confirming that Fe(III) and U(VI) are reduced via different mechanisms in *G. sulfurreducens*.

Several potentially important mechanisms of Tc(VII) reduction have also been characterized. Whole cells of *G. sulfurreducens* coupled the oxidation of hydrogen to Tc(VII) reduction, resulting in precipitation of Tc(IV) in the periplasm. A periplasmic Ni/Fe hydrogenase was implicated as the Tc(VII) reductase by CO profiling. An alternative, indirect mechanism for Tc(VII) reduction was also demonstrated. Fe(II)-bearing magnetite formed during the reduction of insoluble ferric oxide by *G. sulfurreducens* was able to abiotically transfer electrons to Tc(VII), leading to rapid and efficient precipitation of Tc(IV) on the surface of the mineral. Technetium was removed to below the limit of detection by scintillation counting. Environmental relevance of this indirect mechanism was confirmed in enrichment cultures and sediment experiments. Uranium was also shown to function as an electron shuttle to Tc(VII), resulting in the capture of insoluble Tc(IV) by the extracellular U(IV) mineral phase formed. These results suggest the possible immobilization of Tc(VII) through a direct enzymatic route or through an indirect route via optimization of Fe(III) or U(VI) reduction in the subsurface.

Bacterial Genes and Proteins Involved in Chromate Reduction

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Chromate [Cr(VI)], a wide-spread pollutant at DOE sites, is amenable to bioremediation, since Cr(III) [the product of bacterial reduction of Cr(VI)] is less toxic, less soluble, and less prone to spread. Biomolecular engineering can enhance bacterial effectiveness in this respect by decreasing the sensitivity of these enzymes to other toxic waste present in chromate-contaminated sites, increasing their substrate affinity and V_{\max} , and broadening their substrate range, enabling an individual enzyme to remediate multiple heavy-metal pollutants. We have therefore cloned six genes of hitherto unknown function from different bacteria and obtained in pure form the soluble proteins they encode. These proteins, which contain bound FMN and conserved FMN binding sites, fall into two homology classes whose putative structural homologues appear to be widely distributed among bacteria. They are not involved in chromate respiration. All possess a broad electron-acceptor range, and can reduce chromate and quinones; the Class II proteins can also reduce nitroaromatic compounds—another class of important environmental pollutants. The K_m of class II enzymes for chromate reduction is lower than that of Class I. Knockout mutants of these proteins exhibit impaired ability for chromate and/or nitrocompound reduction. Some of these proteins reduce chromate by a combination of one- and two-electron transfer, others by successive single-electron transfers. Because the former appear to generate fewer reactive oxygen species when reducing chromate or quinone, they are a better agent for bioremediation.

The biology of the knockout mutants and the regulatory pattern of these proteins suggest that they are involved in cellular redox homeostasis. Some may be functional homologues of the mammalian enzyme, DT-diaphorase. Preliminary findings indicate changed redox cellular status of the mutants as monitored by the use of appropriate probes and fluorescent microscopy. These genes and proteins are directly useful in chromate bioremediation. Ongoing further study will suggest optimal strategies for enhancing their heavy-metal remediation capacity and assist in generating bacteria capable of sustained high-level activity under stressful field conditions. Because the genes we have cloned are related, the DNA shuffling approach is optimally applicable for improving these enzymes; screening protocols for such selection have already been developed.

Molecular Approaches to Studying Transcription, Protein Expression, and Protein Localization in *Shewanella oneidensis* MR-1

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Data will be presented on uses of the genome sequence of *Shewanella oneidensis* MR-1 to construct and characterize (1) transcriptional reporters through fusion of MR-1 promoters to GFP and (2) targeted knock-out mutations. We are currently using both a targeted and random approach for cloning promoters upstream to GFP encoded by promoter-less cloning vectors, pProbe NT and pProbe NT². For targeted cloning, DNA fragments of approximately 600–1000 bp upstream to genes predicted to be important in respiratory processes were produced by polymerase-chain-reaction (PCR) amplification. For random cloning of promoters, restriction fragments of approximately 5 Kb were generated by partial *Sau3A* restriction digestion. Results from monitoring GFP fluorescence from various constructs expressed in MR-1 under different growth conditions will be presented.

In addition, results from proteomic analysis of a MR-1 mutant, in which the twin arginine protein translocation gene, *tatC*, was deleted, will be presented. Mutant and wild type MR-1 cells were fractionated into membrane, cytoplasmic, and periplasmic pools and analyzed by both high-resolution separation and high mass accuracy and sensitivity Fourier Transform ion-cyclotron-resonance (FTICR) mass spectrometry and by two dimensional gel electrophoresis. A dramatic difference in protein content was observed between the wild type and mutant cellular fractions. In addition, results suggest that it may be possible to utilize proteome data to predict members of multiprotein complexes associated with the membrane. For example, consistent with the adenosine triphosphate (ATP) synthase model, integral components of the membrane associated ATPase F₀ unit were identified only in membrane fractions, while the soluble F₁ unit components were observed in both membrane and cytoplasmic fractions.

Global Analysis of *Shewanella oneidensis* Strain MR-1 Proteome Using Accurate Mass Tags

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Bacterial strains such as *Shewanella oneidensis* strain MR-1 are key organisms in the bioremediation of metals, on account of their ability to enzymatically reduce and precipitate a diverse range of heavy metals and radionuclides. Important in these processes is the need to develop improved enzymatic pathways in these organisms. As a first step, the proteome of the organism, defined as the entire protein complement of the cell expressed under a given set of conditions, must be completely characterized. A single genome can exhibit many different proteomes, depending on stage in cell cycle, cell differentiation, response to environmental conditions (nutrients, temperature, stress, etc.), or the manifestation of disease states. Therefore, the study of proteomes under well-defined conditions can provide a better understanding of complex biological processes, which requires faster and more sensitive capabilities for the characterization of cellular constituents.

We have developed a new technology based on the combination of global tryptic digestion, high-resolution liquid chromatography, and tandem mass spectrometry and high-field Fourier Transform ion-cyclotron-resonance (FTICR) mass spectrometry to define a proteome of an organism, thereby bypassing the 2-D polyacrylamide gel electrophoresis technologies currently used. Protein identification is based upon global approaches for protein digestion and accurate mass analysis for the generation of “accurate mass tags” (AMTs), and this approach is being applied to the recently sequenced organism *Shewanella oneidensis*. Additionally, we have developed technologies that allow the visualization of the protein complement by obtaining comparative displays for the expression of many proteins simultaneously, based upon stable-isotope labeling. Two versions of each protein are generated and analyzed simultaneously, to precisely establish changes in expression. Using a combination of these two technologies, we have evaluated the change in the protein expression patterns of the organism for cell culture on aerobic versus anaerobic growth.

Protein Engineering the Metalloregulator, MerR, and the Organomercurial Degrading Enzyme, MerB

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We are studying two proteins of the bacterial mercury (Hg) detoxification system: the metalloregulator MerR and the organomercurial lyase MerB. MerR is a 144 aa metalloregulatory protein that controls expression of the ubiquitous Hg resistance operon, whose gene products biotransform inorganic or organic Hg compounds to volatile, less toxic Hg vapor. MerR has very high affinity and specificity for Hg(II), and we are re-engineering a portion of it to serve in metal sequestration and also as a metal biosensor. These studies illuminate two major control points in the biotic mercury cycle and will provide specialized tools for monitoring *in situ* remediation and effecting *ex situ* remediation.

With respect to MerR, we have generated a derivative consisting of two tandem repeats of the coiled-coil metal-binding domain (MBD). This small (107 residue) MBD protein, produced in substantial amounts in *E. coli*, allows cells to bind two-fold more Hg than cells containing only the vector without any enhanced toxicity to the cell. *In vitro*, the Hg-binding properties of the MBD protein are equivalent to those of the parent protein, full-length MerR. When attached to a solid bead substrate, the MBD is 2.5-fold more effective at binding Hg(II) than is full-length MerR tethered to beads as single monomers. Current work focuses on biophysical-techniques characterization of the affinity and specificity of the MBD protein (in intact cells and in pure form) for metals of interest to DOE. To re-engineer the metal specificity of the MBD protein, we have inserted it into both T7 and modified M13 phage display systems. The protein was only weakly displayed in the T7 system, and we are presently focussing on optimizing the modified M13 system, which can handle protein insertions of larger size better than the T7 system.

With respect to MerB, the organomercurial lyase is a unique enzyme with no presently known homologues. Previous genetic work by others had shown that each of the four cysteine residues of MerB plays some role in resistance to phenylmercuric acetate, but the nature of this role was unknown. Our work has shown that:

- MerB is cytosolic, not periplasmic.
- None of its cysteines forms disulfide bonds.
- Cys96 and Cys159 are essential for catalysis, but Cys160 is partially dispensible.
- Cys117 plays a structural role and may also play a catalytic role.
- Without exogenous thiols, MerB can release benzoic acid from p-hydroxymercuri-benzoate, but it retains Hg(II), strongly suggesting that the proton comes from a MerB residue, not the solvent.

Our observations suggest the following reaction course: bis-coordination of the Hg atom of RHg compounds by C96 and C159, protein-based proton donation to the R- group, entrapment of the Hg(II) by bis-coordination at C159-C160, followed by release of Hg(II) to buffer thiols such as cysteine or glutathione.

In collaboration with Jim Omichinski (UGA Biochemistry Dept.), we have a preliminary NMR fold for MerB as an Hg-DTT adduct. Also, in collaboration with Cory Momany (UGA School of Pharmacy) we have established conditions for growing large crystals of MerB in two distinct habits, one of which should soon be large enough for data collection.

Genes for Uranium Bioremediation in the Anaerobic Sulfate-Reducing Bacteria

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The ubiquitous sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, reduces soluble U(VI) to the less soluble U(IV). Because this process has potential for application in bioremediation efforts of contaminated environments, the parameters that delimit its functioning are of great interest. *In vitro* analyses and mutant studies of *Desulfovibrio* support the involvement of the tetraheme cytochrome c_3 in the reduction of U(VI). Surprisingly, when *D. desulfuricans* strain G20 was grown in the presence of a nonlethal concentration of uranyl acetate (1 mM), its ability to reduce U(VI) was greatly decreased. In contrast to extracts from control cultures, cytochrome c_3 was not detected in the periplasm or whole-cell extracts from these cultures when examined by Western analyses. Reverse transcription-polymerase chain reaction (RT-PCR) and Northern experiments showed that transcripts for *cycA* encoding this cytochrome were still present under these growth conditions. Further proof that the effect of the uranyl acetate was not at the level of transcription came from studies with a fusion of the promoter region of *cycA* with the *lacZ* gene. An alteration in the ability to process heme-containing proteins under these conditions was considered unlikely, since the synthesis of the high molecular weight *c*-type cytochrome appeared normal. Finally, neither uranyl acetate nor the reduced product, uraninite, appeared to destabilize the outer membrane in such a way that cytochrome c_3 was released into the growth medium. The possibility of rapid turnover of the tetraheme cytochrome c_3 following its interaction with U(VI) or U(IV) complexes is being investigated, as is the role of alternative tetraheme cytochromes.

Single-Molecular and Conventional Characterization of the Interaction between Flavin Reductases and Reduced Flavin-Utilizing Monooxygenases

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The objectives of the project were to investigate the potential metabolic channeling of reduced flavins between flavin reductases and reduced flavin-utilizing monooxygenases. The primary systems were nitrilotriacetate monooxygenase (NmoA) with its partner flavin reductase (NmoB), and ethylenediaminetetra-acetate (EDTA) monooxygenase (EmoA) and its partner flavin reductase (EmoB). Both NmoA and EmoA are FMNH₂-utilizing monooxygenases that initiate the metabolism of chelating agents, such as nitrilotriacetate and EDTA.

Single-molecule microscopy techniques were used to detect the protein-protein interaction of NmoB and NmoA. Both proteins were labeled separately with fluorescent dyes. If the two proteins came together during catalysis, FRET would be observed under the microscope. Unfortunately, the interactions were not detected by the microscopy method. To demonstrate that the two proteins do not interact during catalysis, a genetic approach was used, as was a homologous system in *Escherichia coli* to take advantage of the *E. coli* genetic system. HpaC is a flavin reductase that is homologous with NmoB and EmoB, and it supplies FADH₂ to 4-hydroxyphenylacetate (4HPA) 3-monooxygenase (HpaB), a flavin-adenosine-dinucleotide (FAD)-H₂-utilizing monooxygenase. The potential coupling of HpaC and HpaB was investigated *in vivo*, which showed that *E. coli* W cells growing on 4HPA had 13 times higher FAD reductase activities than cells growing on glycerol. The increase likely resulted from the expression of hpaC. When hpaC was inactivated, the cells lost the ability to grow on 4HPA, indicating that the background level of FAD reductase activity was not sufficient to support adequate HpaB activity for growth on 4HPA.

When four different flavin reductase genes were supplied on plasmids, the mutant recovered the ability to grow on 4HPA, with different growth rates depending on the level of expression and catalytic properties of the reductase. Excessive FAD reductase activities were not as good as in the wild type, but more than sufficient. In addition, kinetic analysis ruled out apparent protein-protein interaction between HpaB and HpaC. The findings were consistent with the single-molecule experiments indicating that protein-protein interaction is not a function of reduced flavin-utilizing monooxygenases *in vivo*. This information can be used to guide the construction of genetically engineered microorganisms employing reduced flavin-utilizing monooxygenases for bioremediation or biosynthesis.

PROGRAM ELEMENT 4
Biogeochemistry

Evolutionary Mechanism of Biogenic Solids Derived from Microbial Reduction of Ferrihydrite under Dynamic Flow Conditions

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Iron cycling and the associated changes in solid phase have dramatic implications for trace-element mobility and bioavailability. Here we explore the formation of secondary iron phases during reductive dissolution of ferrihydrite-coated sand under dynamic flow and static conditions. We also explore the contributions of biological and abiological (chemical) reaction mechanisms in the biomineralization process. Additionally, reactive transport modeling was used to quantitatively link observed trends in aqueous chemistry and solid-phase mineralogy with time, distance along flow path, and flow velocities. Solids were characterized using x-ray absorption spectroscopy; spatial relationships between minerals and microbes were resolved using transmission electron and scanning electron microscopes in combination with epifluorescence optical microscopy.

In experiments having moderate flow velocities (three pore-volumes per day), an initial period (10 days) of rapid reduction, indicated by consumption of lactate and production of acetate and Fe(II) to the pore water in association with a darkening of the column material, is followed by a much lower rate of reduction to the termination of the experiment after 48 days. A small fraction of the total Fe (<25%) is lost to the effluent pore water; the majority remains within the column as ferrihydrite and the secondary mineral phases magnetite and goethite. Ferrihydrite converts to goethite in the influent-end of the column, where dissolved Fe(II) concentrations are low, and converts to magnetite toward the effluent end, where Fe(II) concentrations are elevated. Greater than 80% of the residual Fe remains in the ferric state. A dramatic decline in the rate of Fe(II) production occurs concurrent with the formation of goethite and magnetite; at the termination of the experiment, the rate of reduction is <5% the initial rate. These results highlight the importance of coupled flow and water chemistry in controlling the rate and solid-phase products of Fe (hydr)oxide reduction.

The degree of secondary mineral-phase accumulation is influenced by dissolved Fe(II) concentration and ferrihydrite reactivity. Abiotic experiments (static and batch) reveal products similar to those having biological activity, provided that all other conditions are comparable. Biomineralization pathways proceed via abiotic transformations (dissolution/precipitation or solid-state conversion), and bacteria appear to act solely as the sources and controls of Fe(II) concentration. Ferrous iron concentration has the dominant control on solid-phase products (magnetite and goethite), leading to interesting and complex reaction zones within columns having low flow velocities. When flow velocities decline, the system becomes electron-donor-limited, resulting in the development of reaction fronts within the column. The most dramatic impact of these reaction fronts is the conversion of ferrihydrite to goethite at the expense of magnetite formation. Thus, reductive dissolution of minor amounts of ferrihydrite may catalyze the conversion of large amounts of ferrihydrite to goethite. High flow velocities lead to magnetite formation. The reaction products of ferrihydrite reduction are variable, leading to appreciably different reactivities that are coupled to the flow velocity and rate of Fe(II) supply.

Oxidation of Biotically and Abiotically Reduced Technetium and Uranium in Fe and Mn Oxide-Containing Sediments

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Technetium-99 and uranium are subsurface contaminants at multiple U.S. Department of Energy sites. These contaminants are major risk-drivers at these locations because of their relative mobility in the subsurface. TcO_4^{-2} and soluble U complexes can potentially be immobilized in soil or sediment via reduction to poorly soluble $^{99}\text{Tc(IV)}$ or U(IV) oxides. Reduction may be catalyzed either via direct microbial reduction or via reduction by Fe(II) resulting from the microbial reduction of sediment Fe(III). Both direct microbial and abiotic reduction mechanisms have been proposed for immobilizing Tc and U *in situ* in the subsurface. However, for such strategies to be effective for long-term immobilization, the radionuclides must be maintained in the reduced form.

To this end, we investigated the susceptibility of reduced Tc and U to oxidation in air and in subsoil from the Oak Ridge Field Research Center (FRC) background site and in subsurface Ringold Formation sediment collected from the Hanford Site. We investigated the solubilization of biogenically reduced TcO_2 and UO_2 in the presence of air-saturated buffer or unreduced sediment, and the solubilization of abiotically reduced (by Fe^{+2}) sediment-associated TcO_2 after exposure of biologically reduced sediment suspensions to air. Oxidation of biogenic TcO_2 in aerated bicarbonate buffer was initially rapid (50% in ~48 hours) followed by a slower oxidation that continued over a period of 28 days. Biogenic TcO_2 was also oxidized in deoxygenated, unreduced Hanford Ringold sediment and FRC subsoil, with the rates being linear. Oxidation of biogenic TcO_2 was more rapid in Hanford Ringold sediment (100% in 28 days) than in the FRC soil (<75% in 42 days), probably because of differences in Mn oxide mineralogy and content: Mn(IV) is believed to be the main oxidant in both of these materials. The hydroxylamine HCl extractable Mn(IV) was 6-fold lower in the FRC subsoil compared to the Hanford Ringold sediment. Oxidation of abiotically reduced (by biogenic Fe^{+2}) TcO_2 after aeration of bioreduced FRC soil suspensions was much slower relative to the oxidation of biogenic TcO_2 added to unreduced FRC soil, with approximately 30% of the added TcO_2 oxidized to a soluble form over a 14-day period. These differences are hypothesized to result from redox buffering afforded by reduced Fe and Mn and/or to physical protection mechanisms.

Our results indicate that biogenic TcO_2 and UO_2 are susceptible to oxidation by O_2 and soil Mn(IV) oxides, but that Tc reduced via biogenic Fe(II) may be more stable and less susceptible to air oxidation.

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

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Radionuclide-contaminated environments are often oxidic, including the Rocky Flats Environmental Technology Site (RFETS), and the contaminated groundwater at the NABIR Field Research Center (FRC) in Oak Ridge, Tennessee. Radionuclide distribution within such environments is effected 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 effect the biogeochemistry of Fe could also significantly effect Pu and U distribution. We propose to determine the extent to which metabolic processes involved in Fe acquisition and in exopolymer production affect the distribution of Pu and U between the aqueous and solid phases. First, 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. Second, using transposon mutagenesis, we will determine to what extent microbial processes (including siderophore production and metabolism as well as exopolymer and reductant production) directly or indirectly influence aqueous/solid phase distribution of Pu and U.

Uranium interaction with iron oxides has been studied extensively, and to date we have studied the interactions of some Pu(IV) and Pu(V) species with the Fe(III)(hydr)oxide goethite. We have also studied actinide-exopolymer and actinide-whole cell binding for *Bacillus licheniformis*, which produces a polyglutamate exopolymer. Our initial result from ternary, actinide-mineral-bacteria interactions showed that uranyl was sorbed onto goethite under conditions previously reported by Bargar et al. ([U] = 10⁻⁵ M, 0.1 M NH₄NO₃, pH 6.5). The resultant solid was then added to a growth medium, and the mixture was inoculated with *Pseudomonas putida*. After 60 hours, the aqueous fraction of this biogeochemical mixture was analyzed for dissolved Fe and U liberated by metabolic activity of the bacteria. We found that 60% of the uranyl originally sorbed to the mineral (~0.12 micromoles of U) was desorbed and in a soluble (molecular) form, versus 16% in the uninoculated control. In this same experiment, ~0.16 millimoles of Fe were determined to be solubilized, versus ~0.06 millimoles in the control. Finally, following sorption of ²³⁹Pu(IV)/(V) onto goethite, *P. mendocina* removed approximately 20% of the Pu. We estimate that if conditions were optimized, it would be possible to remove greater than 90% of the sorbed Pu.

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."

Microbial Stabilization of Plutonium in the Subsurface Environment

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Plutonium (Pu) contamination is widespread in the surface soils and subsurface sediments throughout the DOE complex. Plutonium is generally considered to be relatively immobile; however, transport of Pu, albeit at very low concentrations, has been observed at many DOE sites.

The focus of this recently awarded grant (September 2001) is to elucidate the processes that can lead to the enhanced stabilization (i.e., immobilization) of soluble (organic- and inorganic-Pu complexes) and colloidal forms of Pu by naturally occurring microbial communities. While several processes responsible for mobilizing and transporting Pu have been hypothesized, the potential for the stabilization of Pu by microorganisms present in the contaminated environment has not been fully evaluated. Results of this basic research should lead to: (1) a better understanding of environmental conditions likely to foster Pu immobility, and (2) strategies for engineering the long-term *in situ* immobilization of Pu in soils and sediments. At most Pu-contaminated sites, removal of contaminated media is financially prohibitive; the development of methods for *in situ* stabilization of Pu is crucial for the long-term, cost-effective stewardship of Pu contaminated sites.

This is a multidisciplinary, integrated research project involving expertise in actinide microbiology (BNL), surface and coordination chemistry/radiochemistry (Colorado School of Mines) and environmental radiochemistry/biogeochemistry and radiocolloids (Texas A&M). This presentation will provide the NABIR community with an overview of the project.

Hydrogen as an Indicator to Assess Biological Activity during Trace-Metal Bioremediation

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The design and operation of a trace-metal or radionuclide bioremediation scheme requires that specific redox conditions be achieved at given zones of an aquifer for a predetermined duration. Tools are therefore needed to identify and quantify the terminal electron acceptor processes (TEAPs) that are being achieved during bioremediation in an aquifer, and that this is done at a high spatial resolution.

Hydrogen holds the promise of being a key parameter that may be used to identify TEAPs. Theoretical analysis have shown that steady-state hydrogen levels in the subsurface are solely dependent upon the physiological parameters of the hydrogen-consuming microorganisms, and that hydrogen concentrations increase as each successive TEAP yields less energy for bacterial growth. The assumptions for this statement may not hold during a bioremediation scheme in which an organic substrate is injected into the subsurface and where organisms may consume hydrogen and carbon simultaneously.

The objective of this research is to gain a basic understanding of the hydrogen dynamics in an aquifer during a trace metal/radionuclide bioremediation scheme. For this purpose, a series of batch studies have been conducted during the first year of this project. In these studies, the use of acetate and hydrogen by *Geobacter sulfurreducens* were studied. In all cases, Fe⁺³ was the electron acceptor. Microcosms were set up to investigate the utilization of hydrogen and acetate when either of them is the sole electron donor and when both are present and utilized simultaneously as electron donor. These experiments were conducted for varying initial conditions of the hydrogen and acetate concentration, and the disappearance of these compounds, plus the evolution of Fe⁺² (as well as biomass), was monitored over time.

The results of these batch studies indicate that the rate of hydrogen utilization is not affected by the simultaneous utilization of acetate. While there is no difference in acetate and/or hydrogen utilization when they are the sole electron donor or used simultaneously, evolution of Fe⁺² and biomass over time is increased by the simultaneous utilization of hydrogen and acetate, compared to the case in which either of them is the sole electron donor. The biokinetic constants for the degradation process are currently being determined by obtaining a best fit of the experimental results to a model of the degradation kinetics. Once these biokinetic coefficients are established, we can determine the theoretical hydrogen levels for a specific TEAP as a function of the acetate concentration. These results will be tested against hydrogen profiles in continuous-flow column experiments utilizing media from the NABIR Field Research Center in Oak Ridge, Tennessee. These column experiments are under way, and hydrogen levels will be monitored until a steady-state profile is reached.

The Role of Biogeochemical Dynamics in the Alteration of U Solid Phases under Oxidic Conditions

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To assist the U.S. Department of Energy (DOE) with long-term stewardship issues associated with bioremediation of uranium (U), we have as an overall goal to define the mechanisms by which microorganisms facilitate the formation of hexavalent U solid phases. Under anoxic conditions, microbial reduction of U(VI) to U(IV) can potentially decrease groundwater U contamination by lowering solubility and by slowing migration through the soil. However, such biological alteration must be considered temporary unless long-term anoxia can be maintained. When oxidic conditions return, U(IV) will likely oxidize to U(VI), which is generally more soluble and potentially more mobile. For example, in U ore deposits in which uraninite (consisting of reduced, tetravalent U as UO_{2+x}) is the parent material, exposure to oxidizing conditions results in alteration to U^{+6} minerals, with the U^6 phosphates frequently defining the boundaries of the ore body. Of the U(VI)-containing minerals, these U(VI) phosphates are of interest because they are the least soluble of the U(VI) solids found in nature.

Microorganisms present in soils may play a role in the formation of U(VI)-phosphate solid phases. However, the role of microorganisms in transformation and, ultimately, precipitation of actinide-containing minerals is not well understood. We are investigating the role of some model microorganisms (including *Bacillus sphaericus* and *Shewanella putrefaciens*) commonly found in soils as well as microbial consortia isolated from the NABIR Field Research Center at Oak Ridge National Laboratory on U(VI) sorption, solid phase formation, and transformation. Data will be presented on the relative abilities of these bacteria to sorb U over a wide pH range, with special attention to the sorption behavior at low concentrations of U ($\leq 10^{-6}$ M), such as would be commonly found in the environment. Microbial cell surface active sites responsible for U interaction are identified with time-resolved laser-induced fluorescence spectroscopy (TRLIFS). We are also investigating the impact of these bacteria on the transformation of the U(VI) oxide hydrates (e.g., metaschoepite, $[(\text{UO}_2)_8\text{O}_2(\text{OH})_{12}] \cdot 12\text{H}_2\text{O}$), to meta-autunite, $\text{Ca}[(\text{UO}_2)(\text{PO}_4)]_2 \cdot 6\text{H}_2\text{O}$. Previous studies on abiotic transformation had indicated kinetic barriers in the nucleation and formation of meta-autunite. Our current biotic studies suggest that the bacterial surfaces may serve as nucleation sites, possibly via the surface-sorption sites identified by TRLIFS. In addition, other phosphate solid phases form to which any dissolved U(VI) sorbs, further reducing the solution concentration of U(VI).

Bacteria are believed to influence actinide geochemistry through various mechanisms that are a part of the biogeochemical cycle of U. Our research links important geochemical and microbiological aspects of this problem, providing a fundamental basis for predicting the complex and dynamic interplay of biological treatment strategies.

Biogeochemistry of Uranium Under Reducing and Re-oxidizing Conditions: An Integrated Laboratory and Field Study

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In prior work, we evaluated the effects of goethite, hematite, ferrihydrite, and quartz on reduction of U(VI) under sulfate-reducing conditions. Growth of *Desulfovibrio desulfuricans* G20 and simultaneous reduction of U(VI) to U(IV) was observed. After depletion of all lactate under anaerobic conditions, re-oxidation of U(IV) to U(VI) was measured, with the extent of U(IV) re-oxidation higher with hematite than goethite. With ferrihydrite, no re-oxidation was observed even after four months. Uranium L3-edge XANES spectra for abiotic controls showed that iron minerals adsorbed U(VI) to yield a typical U(VI) XANES spectrum. In contrast, the goethite/quartz and hematite/quartz samples incubated with SRB yielded a spectrum similar to that of uraninite, but having distinct features, notably a much more intense and slightly broader white line. Flow cells were also used to examine uranium reduction/precipitation processes in the presence of SRB. X-ray photoelectron spectroscopy (XPS) binding-energy determinations suggest that a mixed valence U-phase was present at the hematite surface. Microelectrode measurements in U-free SRB accumulations show that the hematite or quartz surface influences H₂S concentration profiles. SRB accumulations on quartz tended to have higher H₂S concentrations than those on hematite. In a two-species system containing *D. desulfuricans* and *Pseudomonas fluorescens* (non-SRB), bacterial accumulations had a heterogeneous structure consisting of cell clusters separated by voids. Profiles of local H₂S concentration, pH, mass transport coefficient, flow velocity, and relative effective diffusivity in the clusters and voids were measured using microelectrodes.

Work in this newly funded project contains four major tasks that will build on our prior research and integrate laboratory and field studies focused on the NABIR Field Research Center. The first task will characterize field cores from the fracture-flow region of Nolichucky shale for chemical, mineralogical, microbial, and physical properties, and conduct *in situ* coupon experiments, in which mineral surfaces will be exposed to groundwater and indigenous flora for periods of time ranging from days to months. Coupon surfaces will be examined by spectroscopic and microscopic techniques to determine changes resulting from exposure to aquifer conditions. The second task will explore the dynamics of DIRB and SRB microbial communities with respect to the relative effects of biotic and abiotic (Fe(II) associated) reductive U precipitation on hematite surfaces. Kinetic experiments will determine rate laws governing U immobilization reactions for calibration with channel-flow-reactor (CFR) and fracture-flow reactor (FFR) studies. The third task will determine rates of U(VI) immobilization/remobilization in accumulations of SRB and DIRB attached to hematite and quartz. Microelectrodes will be used to probe boundary-layer chemical heterogeneity to determine local factors affecting stability of uranium deposits. The final task will evaluate the effects of fracture flow hydrodynamics and field-oriented nutrient addition strategies on the spatial and temporal development of SRB/DIRB/mineral surface accumulations resulting from combined U(VI), Fe(III), and SO₄⁻² reduction under fracture flow conditions.

Transformation and Stabilization of Heavy-Metal Contaminants in Subsurface Environments

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Under anoxic and suboxic conditions, heavy-metal contaminants are immobilized by microbial processes through three major pathways: (1) precipitation as sulfides, (2) reduction to an insoluble lower oxidation state, and (3) sorption on microbial biomass and biopolymers. An important prerequisite for precipitating metal sulfides is the formation of hydrogen sulfide through bacterial sulfate reduction. Although hydrogen sulfide is the primary product in sulfate-reducing systems, thiols are probably generated as well, from either biochemical processes or abiotic reactions involving the reaction of the bisulfide ion with functionalized organic molecules. Sulfur nucleophiles, including the bisulfide ion and thiols, are highly labile molecules and readily react with many transition metal contaminants, forming metal sulfides or some complexes. In addition, they play a role in reducing the metal contaminants.

To better understand the pathways of microbially mediated transformations of metal contaminants in the presence of sulfur nucleophiles, we conducted mechanistic studies of the reactions of two contaminants, mercuric ion [Hg(II)] and hexavalent chromium [Cr(VI)], with the bisulfide ion and some low-molecular-weight thiolates. X-ray absorption spectroscopy provided information on the changes of speciation in both sulfur species and the metal. The Hg(II) ion reacted rapidly with the bisulfide, precipitating HgS. Likewise, thiols reacted to form mercury-thiol complexes. Remarkably, however, disulfides that do not have an active nucleophilic sulfur center also reacted with Hg(II). Analysis of the reaction products by sulfur K-edge XANES spectroscopy revealed the presence of mercury-thiol complexes and oxidized forms of sulfur, including sulfinate, sulfonate, and/or sulfate. Thus, it seems that Hg(II) induces a disproportionate reaction of the disulfide, causing reduction of one sulfur atom to a thiol functionality, with concomitant oxidation of the other one to a sulfinate, sulfonate, or sulfate sulfur through an intramolecular electron transfer. This strong reactivity may explain why mercury is invariably associated with sulfur in natural organic matter.

We also investigated the reaction of Cr(VI) with hydrogen sulfide and a common biological thiol, glutathione. The Cr K-edge analysis indicated the dramatic effect of glutathione in reducing Cr(VI) to Cr(III). However, adding the bisulfide did not have a strong effect in reducing Cr(VI) to Cr(III), as evidenced by the continued presence of the characteristic pre-edge peak of Cr(VI). These results suggest that organic sulfhydryl groups may be involved in the biological reduction of Cr(VI) to Cr(III).

Influence of Reactive Transport on the Reduction of U(VI) in the Presence of Fe(III) and Nitrate: Implications for U(VI) Immobilization by Bioremediation/Biobarriers

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The coupling of biogeochemical and transport processes is important to the bioremediation of metals and radionuclides in the field. However, experimental research systems with transport and biologically mediated redox reactions are severely lacking. We propose to examine the reduction of U(VI) in the presence of nitrate and Fe(III)-containing minerals under conditions representative of biostimulation. This research will establish: (1) mechanisms by which the fluxes of electron acceptors, electron donors, and other species can be controlled to maximize the transfer of reductive equivalents to the aqueous and solid phases; and (2) associated process models that describe the transport and reaction of U(VI) and iron species under conditions relevant to bioremediation.

This research will utilize DOE subsurface sediments collected from the Hanford, Washington, site and the NABIR Field Research Center in Oak Ridge, Tennessee, and synthetic porous media designed to have specific bioavailable iron mineral phases and contents. The facultative dissimilatory metal-reducing bacterium *Shewanella putrefaciens* (strain CN32) will be adopted as a test organism. Experimental research will be conducted using sediment-packed column systems and will be focused on three main areas: (1) the importance of the abiotic reduction of U(VI) by biogenic Fe(II); (2) the influence of the transport process on Fe(III) reduction and U(VI) immobilization (with an emphasis on methods for controlling the fluxes of aqueous species to maximize uranium reduction); and (3) the reductive capacity of biologically reduced sediments (with respect to re-oxidation by convective fluxes of O₂ and NO₃⁻) and the long-term stability of immobilized uranium mineral phases after bioremediation processes are complete. The proposed research is unique in the NABIR portfolio, and it will provide scientifically based information that will be useful in the design and assessment of bioremediation strategies for U(VI) as well as other metals and radionuclides.

We will present some early results from our research, with a focus on research that has been conducted in collaboration with the Environmental Molecular Sciences Laboratory (EMSL) in Richland, Washington. In particular, we will describe some research in which the unique tools available at EMSL have aided in providing information about fundamental processes involved in the growth and metabolism of *S. putrefaciens* in geologic media.

Reduction of TcO_4^{-2} by Biogenic Fe(II) in Sediments from DOE's Oak Ridge and Hanford Sites

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Technetium-99 is an important subsurface contaminant at such DOE Sites as Hanford, Oak Ridge, and Savannah River. At Hanford, it is the most important risk-driving contaminant, and its subsurface inventory and mobility is key to all long-term site assessments and risk analyses. Technetium-99 is persistent, with a long half-life (2.3×10^5 years), and often exists in the subsurface as the mobile pertechnetate [Tc(VII)O_4^{-2}] anion. Technetium-99 can be immobilized from water by reduction of Tc(VII)O_4^{-2} to insoluble Tc(IV) oxides, and this reductive process may potentially be manipulated for remedial purposes. This research is investigating the role of mineral-bound, biogenic Fe(II) in the heterogeneous reduction of Tc(VII) in the absence and presence of NO_3^- , a key DOE co-contaminant.

Pertechnetate reduction was studied in Hanford and Oak Ridge sediments that were bioreduced by the dissimilatory metal-reducing bacterium (DMRB) *Shewanella putrefaciens* CN32. After measuring Fe(III) and Mn(III/IV) reduction for up to 60 days in inoculated sediments, biological activity was arrested by pasteurization. Ammonium pertechnetate ($20 \mu\text{M}$) was added to sediments with variable reduction extent (both without and with NO_3^-), and Tc(VII) (and NO_3^-) loss from the aqueous phase was measured over time. The rate of Tc(VII) reduction by bioreduced Hanford sediment and Oak Ridge saprolite was dependent on the extent of Fe(III) and Mn(III/IV) reduction. Various concentrations of NO_3^- had little effect on Tc(VII) reduction rate; NO_3^- itself showed little reduction. Both of the subsurface materials contained significant amounts of Mn(III/IV) oxides, and Tc(VII)O_4^{-2} reduction was not observed until all of the "free" Mn oxides were reduced and HCl-extractable Fe(II) began to increase. In Oak Ridge soils, with 27% of the Fe(III)-oxide fraction reduced (0.5 N HCl extractable), $20 \mu\text{M}$ Tc(VII) was reduced to $0.3 \mu\text{M}$ or less in 5 hours, and with 20% of the Fe-oxide reduced, $0.6 \mu\text{M}$ or less in 23 hours. In the Hanford sediment, with 5% of the Fe(III)-oxide fraction reduced, $20 \mu\text{M}$ Tc(VII) was completely reduced in minutes. With lower fractional Fe(III) reduction, Tc(VII) removal was variable and highly dependent on the concentration of biogenic, mineral-bound Fe(II). Transmission electron microscopy (TEM), ^{57}Fe Mössbauer spectroscopy, and synchrotron x-ray absorption spectroscopy (XAS) were applied to follow biologically induced mineralogic changes to the Mn(III/IV) and Fe(III) oxides, and to provide insights on the biogeochemistry of Fe and the mineral association of reduced Tc(IV) . These results indicate that biogenic, solid-associated Fe(II) is an effective reductant for Tc(VII) in the presence of NO_3^- . DMRB may therefore be potentially useful in creating biobarriers for Tc(VII) capture on DOE lands.

BASIC:
**Bioremediation and Its Societal
Implications and Concerns**

Testing a Stakeholder Participation Framework for Fielding Bioremediation Technologies

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This research will investigate stakeholder attitudes about the use of bioremediation technologies with the objective of reducing conflict among stakeholders. The research protocol includes four closely related components. First, we will test a framework for stakeholder participation that prescribes appropriate stakeholder involvement strategies, based on stakeholders' trust of the other parties involved in technology-deployment decision making. Second, we will assess conflict among stakeholders regarding the acceptability of *in situ* bioremediation as a means to reduce risks posed by radionuclides and metals in the environment. Third, we will assess the role that awareness of risk exposure plays in the willingness of stakeholders to engage in problem-solving and making risk tradeoffs. Fourth, we will assess the potential of using the results of these first three components to forge consensus among stakeholders regarding the use and oversight of bioremediation technologies and stakeholder involvement in the decision process. The poster presentation will explain the experimental and data collection techniques being used to achieve these four objectives.

Communicating Effectively with NABIR Stakeholders and the Public— Anticipating the Need for Information

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Fundamental science programs such as NABIR incur three challenges to communication and understanding: (1) the diverse interests and information needs of scientists; (2) the diverse interests and information needs of other publics, including other scientists, regulators, interest groups, and local communities; and (3) accommodating the various frames of reference. Developing lessons learned from our past work and the current research literature, we are focusing on how these communication issues affect NABIR and how they can identify new approaches to communicating scientific knowledge, both internally and externally.

Contemporary scientific programs are often characterized by dispersed groups of scientists who are conducting research on the same (or similar) problems from a variety of disciplinary and methodological perspectives. The research can be of great interest to various other publics when its outcomes could affect environment, health, or economics. However, external publics are often diverse, dispersed, uncentralized, and grassroots in organization. Mapping scientific and social communication networks offers a way to reveal information collectives and points of contact among scientists and external publics. Network analysis, using metrics drawn from analysis of information-sharing and commonalities, is particularly useful in identifying boundary-spanning ideas and people. Internally, boundary-spanners are central figures in the acquiring and conveying of knowledge and can serve as integrators and purveyors of multidisciplinary methods or information. Externally, networks are also vital for understanding collectives of common interests, information, issues, and positions. Also, points of commonality and overlap between internal (programmatic) and external (public) networks of influence can be essential in understanding which communication approaches are most appropriate.

Our current work also involves determining ways to enhance communication among parties with different frames of reference, particularly among experts and between experts and nonexperts. Current plans include meetings intended to explore scientist-engineer interactions and community concerns in areas hosting NABIR-related research. In addition, we are now testing a six-part rule-of-thumb approach for experts who meet the public, which also holds promise for preparing for interactions with a wide variety of others. Taken from the transcripts of expert-nonexpert interactions in our previous NABIR studies, the technique, which is reminiscent of an ancient legal method of questioning, is highly adaptable for contemporary science communication. Current work also includes content and user analysis of the effectiveness of communication products, both those intended for the general public and those used by experts, and examination of appropriate ways of disseminating information about NABIR and fundamental science.

**The authors contributed equally to this work.*

Science-Informed Regulatory Policy: The Case of Bioremediation

David J. Bjornstad (PI) and Amy K. Wolfe

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The NABIR Program carries out research that will lead to new ways of treating and remediating DOE legacy wastes, using a set of technologies referred to as bioremediation. To find use, these technologies must be acceptable to relevant stakeholder groups and must meet the requirements of the regulatory process. We are addressing stakeholder issues in our companion Public Acceptability of Controversial Technologies (PACT) Program. This new activity addresses the requirements of the regulatory process. Its goal is to suggest guidance that would ensure that the scientific information and data collected over the multi-year research period that will precede commercial availability of bioremediation options will be sensitive to the needs of the regulatory process and will support science-informed decision making. The activity will take place through three tasks: (1) development of a regulatory template, (2) gathering of a database describing extant legacy wastes and related bioremediation technologies, and (3) analysis of information needs. In the program's first several months, we have begun Task 1 by preparing a review of development of the regulatory process for topics in recombinant DNA and related technologies. We have begun Task 2 by preparing an initial database, from Superfund records of decision and NABIR program outputs, describing legacy waste targets and likely technical options.

Public Perception of Bioremediation Strategies and Long-Term Stewardship at Department of Energy Sites

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This project is designed to identify the range of concerns held by the public about different bioremediation strategies for *in situ* cleanup at Department of Energy sites. We propose to use an interdisciplinary team of sociologists and environmental engineers to work together with members of the public and other experts to test our hypothesis that “recreancy”—the perceived failure of an organization to exhibit technical competency and fiduciary responsibility—helps explain public perceptions of risks associated with bioremediation. Two primary methods will be used to collect data. First, a “consensus conference” will be conducted with a panel of lay members who will work with panel-identified experts to articulate their concerns about bioremediation in general, and about a specific study site in particular (Idaho National Engineering and Environmental Laboratory [INEEL]). Using information from the consensus conference, a structured survey will be created and administered via computer-aided telephone interview technology and in face-to-face interviews with difficult-to-reach community members. Materials developed as part of the consensus conference process and data gathered from the survey will serve as the basis for designing a protocol for site-specific outreach with regard to bioremediation strategies.

Funding for this project was made available in October 2001. In October 2001, four members of the research team visited INEEL and talked with social scientists employed by INEEL. Based on information gathered during that visit, we currently are in the process of establishing the steering committee that will guide the “consensus conference” process throughout the next nine months. In March 2002, we will be prepared to report on the title and focus of the consensus conference, the recruitment of the lay panel (the group of citizens who will work with the steering committee to design the consensus conference), and, if possible, the outcome of the first of two planned weekend meetings of the lay panel.

Public Acceptability of Controversial Bioremediation Technologies

Amy K. Wolfe (PI) and David J. Bjornstad

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NABIR funds fundamental research that ultimately may support the development and deployment of bioremediation technologies. Our research focuses on the issue of social acceptability of bioremediation through its phases of development, from research through deployment. Experience in other realms has shown that some technologies, such as incineration or those involving things nuclear, frequently are dismissed out of hand as nonoptions. Therefore, the fundamental question underlying our work has been: “Under what circumstances would bioremediation technologies be considered seriously as remediation alternatives.” We have emphasized a site-specific, decision-making context in which involved parties—technology sponsors, regulators, local government, researchers, civic and environmental groups—deliberate about remediation options. The most recent phase of our research focuses on simulation exercises designed to gather empirical data in a relatively controlled way. These exercises involve presenting small groups of role-playing “involved parties” with a series of scenarios, each of which incrementally adds pieces of information that have the potential to alter participants’ willingness to consider bioremediation seriously. Ensuing discussions among participants constitute our data.

Field Research Center Activities

NABIR Field Research Center, Oak Ridge, Tennessee

David Watson (PI)

Oak Ridge National Laboratory, Oak Ridge, Tennessee

The Natural and Accelerated Bioremediation Research Program (NABIR) has established a Field Research Center (FRC) on the Oak Ridge Reservation in Oak Ridge, Tennessee. The FRC provides a site for investigators in the NABIR program to conduct research and obtain samples related to *in situ* bioremediation of metals and radionuclides. Oak Ridge National Laboratory's (ORNL) Environmental Sciences Division (ESD) manages the FRC for the Department of Energy (DOE).

Research activities at the FRC are integrated with the existing and future NABIR laboratory and field research, and provide a means of examining the fundamental biogeochemical processes that influence bioremediation under controlled small-scale field conditions. The FRC includes a contaminated area that can be used for conducting experiments on a plume of contaminated groundwater and sediment, and a background area that provides for comparison studies in an uncontaminated environment. The contaminated and background areas are located on DOE land in Bear Creek Valley (BCV), which lies within the Y-12 Plant area. Contaminants include uranium, Tc-99, strontium, nitrate, barium, cadmium, volatile organic compounds (VOCs), and other inorganics and radionuclides of interest to NABIR investigators.

The FRC was established in April 2000 after an environmental assessment was conducted, which resulted in a Finding of No Significant Impact. Necessary FRC field trailers and laboratories have been established, including a glove-bag for processing up to 5 ft lengths of core. Site-characterization activities have been completed, such as installing wells; microbial, geochemical, and physical analyses of soil and groundwater samples; and conducting geophysics studies. Groundwater and sediment samples (cores and composites) have been collected and shipped from the background and contaminated site for use by over 30 NABIR PI's in laboratory studies at 15 universities and five national laboratories. An FRC library has been established and data management tasks have been implemented, including a data server on the FRC Web page (<http://www.esd.ornl.gov/nabirfrc/>). The types of field research studies that can be conducted at the FRC include:

1. Hydraulic tests (e.g., tracer tests, pumping tests, and flow-meter testing)
2. Community dynamics studies
3. Manipulative tests (e.g., injection of acetate, glucose)
4. Bacterial transport R&D (bioaugmentation of native and non-native microorganisms)

Combined *In Situ/Ex Situ* Strategy for Source Zone Bioremediation at the NABIR Field Research Center

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In preparation for a field-scale demonstration of uranium reduction, we characterized a “source-zone” area adjacent to the S-3 pond cap at the NABIR Field Research Center in Oak Ridge, Tennessee. Within this zone, 90–95% of the flow passes through the aquifer 9–15 m below ground surface at a flux of 0.5 m/d. Over this same depth interval, pH was 3.4 to 3.6. Some important contaminant concentrations are as follows: uranium (35–45 mg/L), nitrate (7–10 g/L), aluminum (0.5 g/L), calcium (0.9 g/L), sulfate (1 g/L), and nickel (12 mg/L). Also present are ppm levels of chlorinated solvents. This suite of contaminants poses several challenges for remediation. Acidic conditions correlated with low microbial diversity, indicating the need for pH adjustment; but groundwater neutralization results in the formation of aluminum hydroxide and calcium carbonate precipitates, which will plug the aquifer if generated *in situ*. Neutralization must therefore be accomplished *ex situ*, where precipitated solids can be removed. The high nitrate levels at the site inhibit U(VI) reduction. Although nitrate is removed by denitrification, its conversion to N₂ gas and biomass *in situ* would also result in aquifer plugging. Accordingly, this step will also be performed *ex situ*.

We have now demonstrated successful long-term denitrification of simulated groundwater in two pilot-scale fluidized bed reactors. One advantage of this process is that biologically generated alkalinity can be used to neutralize acid in the groundwater and sediment. Both solids removal and nitrate removal result in simultaneous removal of uranium, either in the precipitated solids or within wasted biomass. However, residual uranium is still expected in the effluent. To demonstrate the feasibility of controlled U removal *in situ*, the residual will be injected into the aquifer and removed in a downstream treatment zone. The downstream zone will include provisions for removal of residual nitrate by ion exchange and for reduction of U(VI) to U(IV) *in situ*. A two-stage system is therefore envisioned, with an *ex situ* treatment train for removal of chlorinated solvents, acidity, metals, nitrate, and uranium, followed by a downstream *in situ* operation for removal of uranium. Bench scale studies and computer simulations of groundwater flow patterns indicate that each of the proposed steps is technically feasible.

***In Situ* Determination of Uranium Reduction Kinetics at the Bear Creek Valley Field Research Center: Feasibility Studies**

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The overall objective of this project is to assess the feasibility of deploying the single-well, “push-pull” test to determine *in situ* kinetics of microbially mediated uranium reduction at the NABIR Field Research Center (FRC) in Oak Ridge, Tennessee. We are testing the hypothesis that indigenous microorganisms with the capability to reduce U(VI) are present at the site and that their activity can be stimulated by providing exogenous electron donors. An experimental well field has been constructed, and sediment core samples have been collected for use in laboratory column and microcosm experiments. The groundwater geochemistry within the well field is highly spatially variable, with widely ranging values of pH (3.5–7.0), U(VI) (0.008–7.0 mg/L), nitrate (100–9000 mg/L), and Tc-99 (100–30000 pCi/L). Hydraulic conductivities are generally low, with well yields ranging from 0.1 to 1.0 L/min. Twelve tracer tests have been completed. Retardation factors for U(VI) and Tc-99 are being computed for use in dilution corrections during field manipulation experiments. Laboratory microcosm experiments were conducted to identify electron donor additions for use in field manipulation experiments. Results indicated that the subsurface environment at the site is electron-donor limited. In pH 6.8 sediment incubations, the addition of acetate, ethanol, or glucose stimulated complete reduction of nitrate within 10 days, with transient nitrite accumulation. No ammonia was detected, suggesting that nitrate reduction resulted from denitrification. In unamended incubations, nitrate was not completely consumed, and nitrite accumulated to 1 mM. When identical incubations were performed with pH 3.0 sediments and pH 3.0 site groundwater, no denitrification activity was observed in electron-donor-amended or unamended incubations after 98 days. When *Pseudomonas stutzeri* was added to these incubations, denitrification activity was observed in pH 6.8 sediments incubations, but not in pH 3.0 incubations.

A series of field push-pull tests were performed in six wells to determine the effect of electron donor additions on nitrate and uranium reduction, with and without pH adjustment. Injected test solutions consisted of site groundwater containing 2 mg/L nitrate, 1.4 mg/L U(VI), and 31,000 pCi/L Tc-99 amended with 100 mg/L Br⁻ tracer, sodium bicarbonate, and selected electron donors (acetate, ethanol, and glucose). Injected test solutions in control wells consisted of site groundwater, containing tracer and sodium bicarbonate but no exogenous electron donors. In all cases, the addition of electron donor stimulated denitrification, resulting in transient NO₂⁻ concentrations of up to 300 mg/L; however, there was no evidence of U(VI) or Tc-99 immobilization.

A series of additional electron-donor additions are planned in an attempt to further stimulate microbial activity. These electron-donor additions will be followed by an additional series of push-pull tests to measure the effect of repeated electron-donor additions on rates of denitrification and U(VI) and Tc-99 immobilization.

Biogeochemistry and Microbially Mediated U(VI) Reduction at Uranium Mill Tailings Sites, Colorado Plateau, USA

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The NABIR Program research at selected Uranium Mill Tailings Remedial Action (UMTRA) sites is continuing, with considerable success in meeting the goals of identifying (1) the dominant terminal-electron-accepting processes and (2) the biotransformations of metals in a field-scale system. A range of data at the Shiprock, New Mexico, and Gunnison, Colorado, UMTRA sites suggests that microbially mediated reduction of U(VI) to U(IV) occurs locally in the contaminant plumes of both sites. At the Shiprock site, geochemical data permit identification of two specific areas of the Shiprock Floodplain where U(VI) bioreduction is occurring. Specifically, ³H and chloride concentrations were used to constrain a three-component groundwater-mixing model. Areas where measured U(VI) concentrations are lower than predicted by the model are identified as the zones where U(VI) reduction is occurring. These zones are potential future sampling sites for obtaining direct evidence of “natural” bioreduction of U(VI). Detailed vertical profiles from four wells, and microbial data from sediments and groundwater, support this conclusion and provide evidence that U(VI) reduction is occurring independently of sulfate reduction.

Earlier research using enrichments and microcosm studies on sediment samples demonstrated that microbial communities in the subsurface are capable of reducing U(VI) to insoluble U(IV). Where nitrate is present, its removal is a prerequisite to sulfate and U(VI) reduction, but this is rapidly accomplished under laboratory conditions by adding dilute solutions of electron donor (e.g., acetate). Field-scale experimental acceleration of U(VI) bioreduction is the next logical step. We are collaborating with DOE's UMTRA Project to conduct an experiment in which an electron donor is metered into the subsurface under natural gradient conditions. Results to date include a single-well push-pull test at the Gunnison UMTRA Site, in which addition of acetate caused a 60% reduction in U(VI) in 4.5 days. A matching increase in Fe(II) also occurred, reflecting reduction of Fe(III). As expected, a decrease in U(VI) was not observed in a control well. However, in a second acetate-amended well, a significant decrease was not observed, probably explained by the relatively high permeability and associated rapid dilution of acetate at this location. These results demonstrate the importance of pre-test modeling and push-pull tests prior to conducting a field-scale, longer-term injection of acetate.

Student Presentations

Mechanisms of Fe Biomineralization Induced by Dissimilatory Iron Reduction

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Iron (Fe) minerals are ubiquitous in nature and play a critical role in the geochemical cycling of trace elements. The Fe(III)-Fe(II) redox couple, for example, is an important electron-transfer mediator for many biological and chemical species. The physicochemical properties of iron oxides, oxyhydroxides, and hydroxides (hereinafter referred to collectively as oxides) result in their significant contribution to redox equilibria and regulation of chemical constituents in soils. In particular, Fe oxides serve as potent sorbents and repositories for nutrients and contaminants; they are also terminal electron acceptors in microbial respiration. Ferrous iron solid phases observed in laboratory batch (closed) systems following microbial respiration of ferrihydrite under varying environmental conditions include siderite (FeCO_3), magnetite ($\text{Fe}^{\text{II}}\text{Fe}^{\text{III}}_2\text{O}_4$), vivianite [$\text{Fe}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$], and green rust [$\text{Fe}^{\text{II}}_{(6-x)}\text{Fe}^{\text{III}}_x(\text{OH})_{12}]^{x+}[(\text{A}^{-2})_{x/2} \cdot y\text{H}_2\text{O}]^{x-}$. The supply rate and resulting aqueous concentrations of ferrous iron are hypothesized to be the controlling factors in resultant biomineralized solids. Here, we test this hypothesis and investigate mechanisms of Fe biomineralization within closed (batch) and open (column) systems containing ferrihydrite. Both abiotic (addition of aqueous Fe(II) to ferrihydrite) and biotic (dissimilatory iron reduction of ferrihydrite) experiments were conducted to determine the role of bacteria in biomineralization pathways. Solids are characterized using x-ray absorption spectroscopy. Spatial relationships between minerals and microbes were resolved using transmission electron and scanning electron microscopies.

Within both abiotic and biotic systems, ferrihydrite converts to goethite and magnetite, with the resulting phase governed, in large part, by Fe(II) concentration. Within biotic systems, goethite forms homogeneously throughout the column within two days of ferrihydrite reduction. The concentration of magnetite, however, increases upgradient corresponding to Fe(II) concentrations. While Fe(II) concentrations decrease over time, magnetite continues to precipitate within the column suggesting that crystal growth rather than nucleation is the dominant mechanism. Similarly, in abiotic batch experiments, magnetite forms only at higher Fe(II) concentrations, while goethite concentrations are equivalent throughout a range of aqueous Fe(II) levels. Although goethite precipitation terminates upon formation of magnetite, goethite concentrations continue to increase in regions lacking magnetite nucleation. Thus, within both biotic and abiotic systems, the conversion of ferrihydrite to goethite appears to be impeded by the nucleation and precipitation of magnetite. The abiotic conversion of ferrihydrite to goethite and magnetite suggests that production of biomineralized solids following microbial reduction may proceed through similar abiotic mechanisms (dissolution/precipitation or solid-state conversion). Accordingly, biomineralization pathways may proceed through a coupled abiotic-biotic pathway, where bacteria act solely as the source and control of Fe(II) concentrations while secondary mineralization occurs through purely abiotic means.

Manipulation of Uranium Speciation for Effective Field-Scale Remediation

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Several geochemical and biological factors greatly complicate microbial reduction of uranyl near the contaminant source zone at the NABIR Field Research Center (FRC) at Oak Ridge, Tennessee. Low pH (approximately 3.4) and high levels of anions and metals evidently limit bacterial diversity (Fields and Zhou, 2001), uranyl complexes of variable bioavailability interact with natural aquifer sediment to differing degrees, and even small increases in groundwater pH result in the formation of large amounts of aluminum hydroxide precipitate.

In preliminary toxicity studies, high levels of uranium (50 mg/L) delayed, but did not prevent, microbial growth in denitrifying enrichments. Co-inhibition by nickel (15 mg/L) and uranium (50 mg/L) delayed growth to a greater extent than either metal independently. To assess indigenous capacity for U(VI) reduction, metal reduction was examined in site groundwater samples. After adjustment of pH from 3.4 to 7.5 by the addition of sodium carbonate, supernatant was separated from precipitate and amended with 2.3 g/L lactate, 0.6 g/L ethanol, and 5 mM iron. Denitrification and iron reduction were observed. Over the course of several weeks, 95% of the initial soluble uranium was reduced, indicating that microbes at the site have the ability to reduce uranium. Similar experiments are underway with FRC sediment.

Studies of uranyl sorption indicate that interactions with aquifer solids and resulting bioavailability depend upon the nature of the solid phase. FRC sediment effectively sorbed uranyl in the neutral pH range, but high carbonate levels decreased the extent of sorption. Formation of the weakly sorbing uranyl carbonate complex would be enhanced by bicarbonate produced during denitrification, which suggests a way to potentially reduce uranyl sorption and enhance its bioavailability.

HYDRAQL modeling was used to evaluate conditions under which microbes reduce uranyl and conditions under which precipitates form. Reduction is expected at pe levels below 3. This supports the need for complete removal of strong oxidants, such as nitrate and its reduction products. Most of the aluminum is expected to precipitate as a hydroxide above pH 4, and calcium precipitates as calcium carbonate above pH 6. Uranyl is expected to sorb to aluminum hydroxide and co-precipitate with calcium carbonate. Addition of base to neutralize acid creates conditions conducive to uranium removal as U(VI). Modeling also predicted precipitation of uranyl phosphate at low levels of inorganic phosphate and uranyl hydroxide at neutral pH in the absence of carbonate. Experiments confirmed these predictions.

Manipulation of conditions to promote uranium reduction within the source zone at the FRC will be based on laboratory studies and modeling. A long-term objective is to develop a model that accurately represents site hydrogeology, geochemistry, and microbiology, and to determine the sensitivity of model predictions to input parameters. Such information will be used to identify terms that are most deserving of future research emphasis.

Assessment of Iron(III)- and Sulfate-Reducing Microbial Communities and Their Impacts on Uranium Reduction

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Among microbial communities in the subsurface, both iron(III)-reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) are likely to be important to the transformation of uranium. This research project addresses the role of these iron(III)- and sulfate-reducing bacteria in the biotransformation of minerals and uranium(VI) in subsurface sediments from the NABIR Field Research Center (FRC) at Oak Ridge, Tennessee.

During the first year of the project, a variety of enrichments was carried out for both FeRB and SRB from contaminated and pristine FRC sediment at pH 7 and at the *in situ* pH (4–5). Using a most probable number (MPN) approach, SRB counts appeared to be negligible at both the contaminated and pristine sites, and low sulfate reduction-rate measurements at both sites confirmed this estimate. Iron(III)-reducing bacteria were found in the pristine enrichments at both pHs. Therefore, focus was shifted to the enrichment and characterization of FeRB.

Various growth conditions were tested during initial enrichments to cultivate the FeRB, including a range in Fe forms, electron donors, and medium composition. In subsequent enrichments, sediment was washed because the excess of nitrate might hinder other respiration pathways such as Fe(III) reduction. Through MPN studies with glycerol, acetate, or lactate as carbon source, FeRB were enumerated in some (34%) of the contaminated and the majority (92%) of pristine sediments. Enumeration of FeRB yielded cell counts up to 46 cells/ml for the contaminated sediments, and up to 230 cells/ml for pristine sediments. Screening of clones from terminal positive MPN enrichments of contaminated sediments revealed that the predominant FeRB were deep-branching members of the delta Proteobacteria, showing <80% sequence identity to all 16S rRNA sequences in current databases. Six pure cultures of FeRB isolated from contaminated sediments showed >99% sequence identity to *Clostridium celerecrescens*. Further study of the FeRB is being conducted through sediment slurries and additional MPNs. These studies are evaluating impacts of different carbon sources (glucose, acetate, and lactate) on potential activity over a range of pH.

As enumeration and characterization studies continue, the ability of FeRB to reduce uranium is being analyzed. Initial experiments using FeRB enrichments of FRC sediments have shown substantial uranium reduction as compared to killed controls. Bacterial abundance, as well as environmental factors such as pH, nitrate concentration, and available carbon source, appears to affect uranium reduction by FeRB.

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