DOE-NABIR PI WORKSHOP:
Abstracts

March 15–17, 2004
Warrenton, Virginia

Natural and Accelerated Bioremediation Research Program

This work was supported by the Office of Science, Biological and Environmental Research, U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

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Introduction
DOE–NABIR PI Workshop

March 15–17, 2004

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

The program consists of four interrelated Science Elements (Biotransformation, Community Dynamics/Microbial Ecology, Biomolecular Science and Engineering, and Biogeochemistry). The program also has a cross-cutting Integrative Studies area that supports development of innovative approaches and technologies to support the science elements. An element called Bioremediation and its Societal Implications and Concerns (BASIC) addresses potential societal issues of implementing NABIR scientific findings. The program also funds field projects at the NABIR Field Research Center at Oak Ridge and co-operative NABIR–Office of Environmental Management projects at other DOE sites. The material presented at this year's workshop focuses on approximately 60 research projects funded in FY 2001–2004 by the Environmental Remediation Sciences Division in DOE's Office of Biological and Environmental Research (BER) in the Office of Science. Abstracts of NABIR research projects are provided in this book.

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Agenda
NABIR PI Meeting

Airlie Conference Center
Warrenton, VA
March 15–17, 2004

Monday, March 15

8:30–8:45 AM Welcome—Paul Bayer, NABIR Program Coordinator

8:45–9:00 Ari Patrinos, Associate Director, Biological and Environmental Research

9:00–9:30 Teresa Fryberger, Director, Environmental Remediation Sciences Division, BER

9:30–10:00 Biogeochemistry (Brian Wood, Oregon State)

10:00–10:30 BREAK

10:30–11:00 Biotransformation (Cal Ainsworth, PNNL)

11:00–11:30 Biotransformation (Bill Burgos, Penn State)

11:30–12:00 PM Community Dynamics (Cheryl Kuske, LANL)

LUNCH

Afternoon Session			
2:00–5:00	Posters	Breakout: Bioremediation of Tc (Zachara)	Breakout: Genomics and NABIR (Wall)
5:00–6:00	Meeting of FRC Working Groups (Microbial Communities, Biotransformation, Geochemistry, and Modeling)		
6:00–7:00	Free time		
7:00–9:00	Dinner		

Tuesday, March 16

8:30–9:00 AM Biomolecular Science and Engineering (Tom DiChristina)

9:00–9:30 Biomolecular (Carol Giometti, ANL)

9:30–10:00 Joint Genome Institute

10:00–10:30 BREAK

10:30–11:00 GTL Project (Terry Hazen, LBNL)

11:00–11:30 GTL Project (Derek Lovely, U. Mass.)

11:30–12:00 PM Field Experiment at the Old Rifle UMTRA Site (Phil Long, PNNL/Todd Anderson, U. Mass.)

LUNCH

Afternoon Session			
2:00–5:00	Posters	Breakout: Biodiversity of Bioremediation (Joel Kostka)	Breakout: Transferring lab results to the field (Yuri Gorby and Jack Istok)
5:00–6:00	Free time		
6:00–7:00	Dinner		
7:00–9:00	Poster Session: Biogeochemistry, Biotransformation, FRC projects; NABIR-EM projects. Authors must be at posters.		

Wednesday, March 17 (ALL FRC RESULTS)

8:30–8:45 AM Introduction to the NABIR FRC at Oak Ridge (Paul Bayer, DOE)

8:45–9:00 Update on NABIR FRC activities (David Watson, ORNL)

9:00–9:30 Progress on "Field-scale evaluation of biostimulation for remediation of U-contaminated ground water" (Craig Criddle, Stanford)

9:30–10:00 In-line uranium immunosensor at the FRC (Diane Blake, Tulane)

10:00–10:30 BREAK

10:30–11:00 Progress on "Factors controlling in situ U and Tc bioreduction and reoxidation" (Jack Istok, Oregon State)

11:00–11:30 Progress on "In situ immobilization of U in structured porous media" (Tim Scheibe, PNNL)

11:00–12:00 PM FRC Working Groups Report Out (15 min. each; Kostka, Burgos, Jardine, Parker)

12:00–12:15 Wrap-up; Meeting adjourns

ABSTRACTS

PROGRAM ELEMENT 1
Biotransformation

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

Calvin C. Ainsworth[1] (PI), Zheming Wang[1], Kevin M. Rosso[1], and James K. Fredrickson[2].

[1]Chemical Sciences Division, [2]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA

We have been applying density functional theory calculations to estimate the reorganization energy and electronic coupling matrix element Marcus parameters for reduction of the UO_2^{2+} ion. For the reduction of UO_2^{2+} by AH_2DS, the first electron transfer reaction is considered the rate-limiting step, i.e.,

$$AH_2DS^{2-} + UO_2^{2+} \rightarrow AH_2DS^{-\bullet} + UO_2^{+} .$$

Subsequent deprotonation and transfer of an additional electron between the $AH_2DS^{-\bullet}$ radical and U(V) in UO_2^{+} are assumed to proceed extremely rapidly, within the lifetime of the initial encounter complex for the first electron transfer reaction. The reorganization energy is the energy required to manifest a conformation change in the electron donor/acceptor pair and surrounding material that is consistent with the quantum mechanical requirements of electron transfer. Larger reorganization energies usually lead to slower electron transfer rates because more energy must be supplied in order to excite the formation of the transition state. Calculations of the reorganization energy for one-electron transfer reactions involving UO_2-salicylate complexes indicate that the reorganization energy is sensitive to the presence and number of strong chelating ligands in the equatorial plane. For weaker binding ligands, such as H_2O, the effect is smaller but systematic. The intrinsic reorganization energy calculated for reduction of $UO_2^{2+}(OH_2)_x$ where x = 0 – 6 progressively increases from 0.23 eV for x = 0 to 0.61 eV for x = 6. This suggests that increasing ligand numbers should systematically slow down the reduction rate. We are currently evaluating these effects and related quantities for UO_2^{2+} complexed by other ligands with distinctive chelate properties such as carbonate and the siderophore ligands desferrioxamine E and rhototorulic acid.

Experimentally, reduction rates of U(VI) complexes by AH_2DS typically exhibit reaction half-lives ($t_{1/2}$) of more than 20 seconds, and redox behavior of uranyl complexes are clearly dependent on the stoichiometry, the structure of the complex as well as the nature of the ligand. These results are in reasonable accord with Marcus theory calculations. Fluorescence spectroscopic measurements of U(VI), however, are hindered by the severe quenching effect of either AQDS or AH_2DS. Cryogenic time-resolved laser fluorescence spectrometry (CTLFS) at liquid helium temperatures tends to alleviate the quenching problem. Preliminary CTLFS results indicate that spectral measurements are possible but the much broadened U(VI) spectral bands as well as energy transfer effects from AH_2DS to uranyl prevents accurate identification of fluorescent U(VI) species. On the other hand, the observed energy transfer suggests a complex between the uranyl ion and AH_2DS forms, likely as an encounter complex, over the course of the reaction time frame. An understanding of the nature of the formed complex will assist in delineating the reaction mechanism.

New Insights into the Functional Behavior of Antibodies as Revealed by Binding Studies on an Anti-Uranium Monoclonal Antibody

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As part of an ongoing effort to develop immunoassays for chelated uranium(VI) on a hand-held flow fluorimeter, an anti-uranium monoclonal antibody designated as 8A11 was fluorescently labeled using two different strategies. When 8A11 was coupled via reactive lysines to either ALEXA^{TM} 488 or Cy5^{TM}, the resulting fluorescent antibody conjugate exhibited positive cooperativity in the presence of its antigen, U(VI) chelated with 2,9-dicarboxy-1,10-phenanthroline [U(VI)-DCP]. That is, when one of the two binding sites on the covalently modified 8A11 was occupied with bound antigen, the affinity of the remaining site on the antibody for U(VI)-DCP appeared to increase. Unmodified 8A11 bound U(VI)-DCP with the expected hyperbolic dependence on the concentration of antigen, consistent with independent and equal binding of ligand at both sites. Proteolytic cleavage of the fluorescently conjugated 8A11 to produce the fluorescent monovalent Fab fragment yielded an active preparation that now bound U(VI)-DCP with no evidence of positive cooperativity. Although, in principle, any divalent antibody has the potential to exhibit positive cooperativity in binding interactions with its antigen, very little literature about this type of behavior exists.

Native 8A11 was also noncovalently labeled with highly fluorescent XENON^{TM} reagents. These reagents are fluorescently labeled Fab fragments of goat antimouse antibodies that bind to the Fc portion of 8A11. These high-affinity, monovalent fluorescent reagents permitted the intact 8A11 mouse antibody to be labeled in situ with no covalent modifications. Incubation of the 8A11 with XENON 647 produced a fluorescent protein complex that showed an eightfold higher affinity for U(VI)-DCP than did the free 8A11 alone. Again, very little literature exists about this phenomenon, where agents that bind to the Fc portion of an intact antibody change the affinity of the antibody for the antigen at the structurally distant Fab portion of the molecule.

The addition of protein G, a bacterial protein that also binds to the Fc portion of mouse IgG, to the covalently modified 8A11 produced an antibody preparation that showed a lower affinity for U(VI)-DCP than that observed in the absence of protein G. This protein G-dependent decrease in the affinity of 8A11for U(VI)-DCP was dose-dependent. Similarly, U(VI)-DCP was observed to decrease the affinity between 8A11 and protein G, also in a dose-dependent manner. These reciprocal binding effects between protein G and U(VI)-DCP were taken as further evidence that binding to the Fc portion on the intact 8A11 antibody could influence the strength of the interaction at the antigen binding sites on the Fab portions of the protein, and vice versa.

These practical, development-driven binding experiments have revealed a fundamental facet of antibody functional behavior that appears to have been largely unnoticed. The binding phenomena described for the first time in this report may have physiological relevance and can be purposefully exploited to improve the sensitivity and utility of selected immunoassays.

Biodegradation of PuEDTA and Impacts on Pu Mobility

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

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The contamination of many DOE sites by Pu presents a long-term problem because of its long half-life (240,000 yrs) and the low drinking water standard ($<10^{-12}$ M). EDTA was codisposed with radionuclides (e.g., Pu, ^{60}Co), 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 biodegradation of EDTA in the presence of PuEDTA complexes. The aqueous system investigated at pH 7 (10^{-4} M EDTA and 10^{-6} M Pu) contained predominantly $Pu(OH)_2EDTA^{2-}$. The EDTA was degraded at a faster rate in the presence of Pu. As the total concentration of both EDTA and PuEDTA decreased (i.e., 10^{-5} M EDTA and 10^{-7} M PuEDTA), the presence of Pu decreased the biodegradation rate of the EDTA. Research is currently investigating if this is because of a chemical or radiological effect. The soluble Pu concentration decreased, in agreement with thermodynamic predictions, as the EDTA was biodegraded, indicating that biodegradation of EDTA decreases Pu mobility when the Pu is initially present as soluble Pu(IV)EDTA. A second objective was to investigate how the presence of a second metal will influence the speciation and biodegradation of Pu(IV)EDTA. Results indicate that the presence of Fe(III) will out-compete the Pu(IV) for the EDTA complex. This indicates that Pu(IV) will not form stable complexes with EDTA for enhanced transport of Pu in Fe(III)-dominated oxidative subsurface systems. A third objective is 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. RT-PCR analysis showed that the transporter genes and EDTA monooxygenase gene (*emoA*) are cotranscribed. EppA is one of the transporter genes, and it 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 MgEDTA, CaEDTA, Fe(III)EDTA, MgNTA, CaNTA, and Fe(III)NTA. Our data also suggest that BNC1 uses the same ABC-type transporter for both EDTA and NTA uptake. When the gene cluster was cloned in *Sinorhizobium meliloti*, the recombinant acquired the ability to use NTA as the sole nitrogen source. Results from these studies can provide mechanistic understanding and approaches to assist in the bioremediation of PuEDTA and other radionuclide-EDTA complexes at DOE sites.

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

William D. Burgos[1] (PI), Brian A. Dempsey[1], Je-Hun Jang,[1] Gour-Tsyh (George) Yeh,[2] and Eric E. Roden[3]

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The objectives of our research are to evaluate 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. To achieve these objectives we have been conducting research in a variety of areas including (1) kinetic measurements of biological Fe(III) reduction in natural sediment columns for reaction-based reactive transport modeling, (2) construction of reaction-based reactive transport models, (3) kinetic measurements of concomitant Fe(III) and U(VI) reduction in natural sediments, (4) abiotic reduction of U(VI) by sediment-associated Fe(II), (5) complexation of U(VI) by humic substances using dialysis and ultrafiltration techniques, (6) impact of humic substances of biological U(VI) reduction, and (7) sorption of U(VI) on specimen ferric oxides. Our poster presentation will focus on this last research element.

Studies were conducted to investigate the precipitation of U(VI) oxides and the sorption of U(VI) onto hydrous ferric oxide (HFO) or hematite at pH 5.9 to 7.8, ambient PCO2, 0.1 mM Fe(III), U(VI) to Fe(III) molar ratios from 0.001 to 70, and for reaction times from 15 min to 48 days. "Dissolved" U(VI) was measured using Kinetic Phosphorescence Analysis (KPA) after filtration using 0.2 μm pore-size filters. "Total" U(VI) was measured using KPA after dissolution of solids in 0.5N HNO_3 or $NaHCO_3$. Complete recovery of U(VI) was demonstrated. Ferric oxide precipitates were characterized using Fe-57 Mössbauer Spectroscopy.

Sorption onto HFO decreased with time for conditions that were undersaturated with respect to U(VI) oxides. The decreased sorption coincided with conversion of some HFO to hematite, and the conversion to hematite was catalyzed by U(VI). Based on data from 48 days, the slope of the sorption isotherm at pH 7.8 first decreased and then increased with increasing dissolved U(VI). This indicated that sorption of $(UO_2)^3(OH)_5^+$ became a significant process at the higher concentration, which is consistent with the EXAFS observation of polymeric sorbed U(VI) species by other investigators. Accordingly, sorption was modeled assuming both monomeric and polymeric surface species. Sorption resulted in between 50 and 99% sorption of U(VI), corresponding to significant retardation of U(VI) in groundwater. Due to the change from monomeric to polymeric sorption mechanisms, mobility of U(VI) in oxic groundwater is predicted to be greatest for intermediate U(VI) concentrations at alkaline pH.

Two different U(VI) oxide phases were formed. Fast precipitation at high U(VI) concentrations resulted in a solid with solubility consistent with schoepite, using solubility and complexation constants reported by Langmuir. Slower precipitation of U(VI) oxide with solubility about four times lower than "schoepite" was observed for lower U(VI) concentrations. The development of precipitates over time and the lower solubility for the highest total U(VI) conditions were consistent with slow Ostwald ripening. These results are significant in terms of retardation or precipitation of U(VI). The results also indicate that hydrolysis constants reported by Langmuir are more consistent with experimental data than hydrolysis constants reported by Grenthe.

Anaerobic Microbial Metal Redox Cycling by Members of the Beta Proteobacteria

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As a continuation of our NABIR studies investigating the immobilization of uranium onto biogenically produced iron minerals resulting from anaerobic nitrate-dependant Fe(II) oxidation, we recently isolated a novel *Dechloromonas* sp. strain LT-1 and a closely related organism, *Propionivibrio limicola* strain CR. Both of these organisms were isolated as dissimilatory perchlorate-reducing bacteria and were obtained from enrichments initiated with groundwater and subsurface sediments, respectively. Analysis of the complete 16S rDNA sequence indicated that strain LT-1 was 96% similar to *Dechloromonas* sp. CL24 in the beta subclass of the Proteobacteria. In contrast to all other tested *Dechloromonas* species, strain LT-1 did not oxidize Fe(II) with nitrate as the sole electron acceptor. However, strain LT-1 was capable of coupling growth to the reduction of Mn(IV) with acetate as the sole carbon and energy source. No growth occurred in the absence of manganese. [^{14}C]-acetate was oxidized to $^{14}CO_2$ during this metabolism. Further screening of other *Dechloromonas* species, including *Dechloromonas* strain RCB, the only described organism capable of anaerobic benzene oxidation, indicated that manganese reduction was a common trait in this genus. In addition to acetate, strain RCB could even oxidize [^{14}C]-benzene to $^{14}CO_2$, with Mn(IV) as the sole electron acceptor. Strain RCB is currently being sequenced by the Joint Genome Institute at Walnut Creek, CA. Three, eight, and forty kB libraries have been generated in puc18, p21, and Fosmid vectors, producing sequence reads representing greater than 10X coverage of this approximately 4.2 Mb genome. Gap closure and resolution of misassemblies are currently being conducted via resequencing and primer walking of the clonal libraries. An oligonucleotide library representing the entire genome has been constructed and is currently being used in microarray analyses.

In contrast to strain LT-1, 16S rRNA sequence analysis indicated that strain CR was 97.2% similar to *Propionivibrio limicola.* Phenotypic characterization revealed that in contrast to all previously described perchlorate-reducing bacteria, strain CR was capable of reducing Fe(III)-NTA coupled to the oxidation of acetate. Although another Fe(III)-reducing member of the betaproteobacteria, *Ferribacterium limneticum*, was previously described, this organism has since been lost. In addition to Fe(III) reduction, preliminary phenotypic characterization studies indicated that strain CR was also capable of oxidizing Fe(II) coupled to nitrate reduction. A recently published pure culture study of *Geobacter metallireducens* demonstrated that, similarly to strain CR, this organism was alternatively capable of coupling the oxidation of Fe(II) as well as U(IV) to the reduction of nitrate. Together these results indicate that metal-reducing bacteria may also be capable of oxidizing Fe(II) or other metals coupled to nitrate reduction.

The implications of such metabolic versatility on the long-term immobilization and stabilization of radionuclides such as uranium has yet to be determined.

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

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This research addresses the need to understand the mechanisms of microbial alteration of radionuclide-organic complexes and the resultant impacts on radionuclide solubility and stability under anaerobic conditions. We investigated the mechanisms of biotransformation and fate of uranium complexed with naturally occurring organics such as ketogluconic acid, malic acid, citric acid, protocatechuic acid, catechol, salicylic acid, and fulvic acid by anaerobic bacteria.

Characterization of uranium-organic complexes. Uranium-organic complexes (equimolar and with excess ligand) were prepared at pH 3.5 and 6.0. Potentiometric titrations and UV-vis spectra confirmed the presence and type of complexes formed. Synchrotron-based Fourier transform infrared spectroscopy (FTIR) and extended x-ray absorption fine structure (EXAFS) analyses at the NSLS established the nature of bonding between U and the ligand. Ketogluconic acid formed a mononuclear complex with uranium involving the carboxylate group while malic acid, citric acid, and catechol formed binuclear complexes with U. The catechol was bonded to uranium through the two hydroxyl groups, whereas the hydroxycarboxylic acids were bonded in tridentate fashion to uranium through two carboxylate and the hydroxyl groups.

Biotransformation of U complexed with organic ligands by anaerobic bacteria. The mechanisms of biotransformation and fate of U complexed with malate and citrate under anaerobic conditions by growing cultures and resting cells of *Shewanella putrefaciens* CN32, *Clostridium sphenoides* (ATCC 19403), and *Clostridium* sp. (ATCC 53464) were investigated. *S. putrefaciens* reduced U(VI) bound to citric acid to U(IV), which remained in solution as a U(IV)-citrate complex. In the presence of citric acid or glucose, *C. sphenoides* reduced U(VI) to U(IV). No reduction was observed in the absence of metabolizable carbon source. *Clostridium* sp. did not metabolize the equimolar U-citrate complex, but the addition of glucose resulted in reduction of U(VI)-citrate to U(IV)-citrate. The U(VI)-malate complex was reduced to U(IV)-malate at a rate and extent similar to that of U-citrate complex. EXAFS analysis shows the reduced complex to have the formula U(IV)-cit_2, with uranium exhibiting eightfold coordination with oxygen. These results suggest that the complexed uranium is readily accessible for microorganisms as an electron acceptor despite their inability to metabolize the organic ligand. Characterization and biodegradation studies of U-catechol and U-fulvate complexes are in progress.

Reduction of uranium in FRC groundwater by *Clostridium* sp. We examined the reductive precipitation of uranium in groundwater at acidic pH by *Clostridium* sp. Adding varying concentrations of FRC groundwater (FW024) to a growing culture (18 hr, pH 3.5) resulted in the reduction of uranium, and the Al in the groundwater had no effect on uranium reduction. Adding uranyl nitrate to the growing culture confirmed the reduction of uranium at pH 3.2.

Response of *Shewanella oneidensis* Strain MR-1 to Fe(II)-Induced Inhibition of Anaerobic Respiration

Yuri A. Gorby (PI), Grigoriy Pinchuk, and Thomas Gihring

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Dissimilatory iron metal-reducing bacteria control the biogeochemistry of anaerobic, nonsulfiogenic environments principally by reducing Fe(III) to Fe(II). The products of this respiratory metabolism are generally recognized as significant contributors to redox reactions that can mediate the degradation or immobilization of certain classes of contaminants, including halogenated organic compounds, nitrate, heavy metals, and radionuclides. However, the products of iron respiration can also impact cellular metabolic activity through mechanisms that are analogous to suffocation in higher organisms.

We previously reported that Fe(II) accumulates on the surfaces of iron-reducing bacteria and that this accumulation inhibited both anaerobic respiration and cell growth. Electron microscopic images revealed that Fe(II)-rich mineral precipitates were removed from cell surfaces by an unknown mechanism. Removal of the precipitates coincided with restoration of cellular respiration and growth. We have extended our investigation to include controlled cultivation methods (chemostats) that allow continuous monitoring and independent control of culture parameters. *Shewanella* was continuously cultured using a defined medium with lactate as the electron donor and fumarate as the electron acceptor. Ammonium was provided as the sole nitrogen source at a growth-limiting concentration. To investigate the response of cells to Fe(II)-induced inhibition, an anaerobic solution of $FeCl_2$ was added to the steady-state cultures to a final concentration of 1 mM, which was sufficient to saturate cell surfaces. Samples for transmission electron microscopy (TEM), mRNA microarray analysis, and Mossbauer spectroscopy were taken at regular time intervals after the addition of Fe(II). TEM revealed the accumulation of iron precipitates evenly distributed over cell surfaces. In the presence of excess soluble electron acceptors (fumarate), the precipitates began to clear within 1 hr after their formation. Surface blebs were also formed over the entirety of the cell surface, as previously documented. However, removal of surface precipitates was not mediated by these blebs, as suggested by our previous work. Instead, the iron precipitates appeared to be internalized by the cells and partially transformed to a less electron-dense phase. Within 2 hr after Fe(II) addition, cells were completely free of cell surface precipitates. The extracellular medium was dominated by protein-rich structures. The protein complement of these structures is being examined by MS-based proteome analysis. Cellular response to Fe(II)-induced respiratory stress at the transcriptome level is underway using mRNA microarray technologies.

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X-ray and Electron Micro(spectro)scopy Investigations of Spatial Distributions of Elemental Concentrations and Valence States in Bacteria

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

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Bacteria and the extracellular material associated with them are thought to play a key role in determining a contaminant's speciation and thus its mobility in the environment. In addition, the metabolism and surface properties of bacteria can be quite different when the bacteria exhibit a planktonic (free-floating) versus a biofilm (surface-adhered) habit. The microenvironment at and adjacent to actively metabolizing cells also can be significantly different from the bulk environment. Thus, to understand the microscopic physical, geological, chemical, and biological interfaces that determine a contaminant's macroscopic fate, we must characterize, at micron and submicron length scales, the spatial distribution and chemical speciation of contaminants and elements that are key to biological processes. Hard x-ray microimaging is a powerful technique for highly sensitive element-specific investigations of complex environmental samples at the needed micron and submicron resolutions. Moreover, the greater spatial resolution of electron microscopy enables investigations of geomicrobial systems beyond those afforded by x-ray microprobes. Combining electron and x-ray microscopies provides even more advantages for investigating geomicrobial systems than using either technique alone. The objectives of the studies presented here are to (1) determine the spatial distribution, concentration, and chemical speciation of metals at, in, and near bacteria and bacteria-geosurface interfaces, (2) use this information to identify the metabolic and/or chemical processes occurring within the microbes, and (3) identify the interactions occurring near the interfaces among the metals, mineral, and bacterial surfaces, and bacterially produced extracellular materials under a variety of conditions. We have used x-ray fluorescence microscopy to investigate the spatial distribution of 3*d* transition metal elements in *Pseudomonas fluorescens* in free-floating (i.e., planktonic) and surface-adhered states. We have also investigated the valence state of Cr associated with these cells after exposure to toxic levels of Cr(VI) (1,000 ppm for ~6 hr). We have used electron and x-ray microscopies to investigate single *Shewanella oneidensis* MR-1 cells that have been shown to produce internal precipitates rich in biomineralized Fe [1]. The x-ray zone plate used in these microscopy experiments produced a focused beam with a cross section (and hence spatial resolution) of 0.15–0.30 μm.

Results from x-ray fluorescence imaging experiments with *P. fluorescens* indicate that the distribution of P, S, Cl, Ca, Fe, Ni, Cu, and Zn can define the location of the microbe. Quantitative analysis of the elemental concentrations within cells can indicate the viability and metabolic state of the cells. X-ray absorption near edge spectroscopy (XANES) investigations of the Cr valence state indicated two different modes of abiotic chemical reduction of the element (depending on the microbe's physiological state). Fe K-edge XANES measurements of internal precipitates in the *S. oneidensis* MR-1 cells identified the valence state of the precipitates. Additional Fe XANES measurements of precipitates external to the *S. oneidensis* MR-1 cells determined an average Fe valence state that is different from the internal average Fe valence state. These results will be presented, with a discussion of the use of combined x-ray and electron micro(spectro)scopies for identifying metabolic states of individual microbes within communities.

1. S. Glasauer, S. Langley, T.J. Beveridge, "Intracellular iron minerals in a dissimilatory iron-reducing bacterium," Science 295 117-119, 2002.

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

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

Our studies will focus on the contaminated subsurface sediments from Hanford and in single mineral and mineral mixtures that represent U(VI)-bearing fractions. We propose to experimentally investigate three strategies for accessing intraparticle U(VI) by DMRB: (1) enzyme U(VI) reduction after U(VI) diffusion out of particle grains; (2) U(VI) reduction by extracellular and/or exogenous electron shuttling compounds (ESC) after bioreduced ESC diffusion into intraparticle U(VI) locations; and (3) abiotic U(VI) reduction by biogenic Fe(II) after Fe(II) diffusion and sorption into U(VI)-bearing intraparticle microfractures. The influence of these three strategies on the bioavailability and overall microbial reduction rates of intraparticle U(VI) will be experimentally determined and numerically analyzed. The speciation and physical location of U(VI)/U(IV) and their effects on the reoxidation rate and long-term stability of precipitated U(IV) will be investigated. Spectroscopic and microscopic techniques will be used to characterize intraparticle mass transport properties including porosity, diffusivity, and tortuosity. Collectively, these experiments and analyses will allow formulation of a kinetic bioreduction/biotransformation model that explicitly addresses mass transfer constraints.

Dissimilatory Metal Reduction by *Anaeromyxobacter* Species

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Although substantial knowledge accrued over the past decade on metal-reducing microbial populations, our understanding of the key players involved in metal reduction at contaminated sites is incomplete. Recent findings suggest that *Anaeromyxobacter* populations play relevant roles in toxic metal reduction and immobilization at contaminated DOE sites. This research effort will characterize *Anaeromyxobacter dehalogenans* strain 2CP-C as well as other *Anaeromyxobacter* isolates in hand, and assess their contribution towards metal detoxification and plume stabilization under environmentally relevant conditions. The distribution and diversity of *Anaeromyxobacter* species at uranium-impacted DOE sites will be explored using 16S rRNA gene-based approaches. Primers targeting regions of the 16S rRNA gene specific to the *Anaeromyxobacter* group are being designed, tested, and refined. Additionally, a Real-Time (RTm) PCR approach is designed to provide information on the abundance of *Anaeromyxobacter* species in the environment (i.e., quantitative assessment). RTm PCR will verify the growth of *Anaeromyxobacter* species with uranium(VI) as a metabolic electron acceptor, and will explore metal reduction in the presence of alternate electron acceptors. The quantitative approach will be employed to investigate the relative contributions of *Anaeromyxobacter* species towards metal reduction in microcosms that contain other metal-reducing populations (e.g., *Geobacter* species and *Shewanella* species). The genome of *Anaeromyxobacter dehalogenans* strain 2CP-C has been selected for sequencing by the DOE Joint Genome Institute. With the complete sequence available, we will use genomic annotation tools to identify genes involved in metal reduction in an effort to elucidate the organism's metabolism as it relates to metal reduction.

The proposed research effort will enhance our understanding of the microbially catalyzed metal reduction process, and will provide information for the rational design and operation of both intrinsic and engineered approaches for enhanced in situ bioremediation of toxic metals at contaminated DOE sites.

This research is conducted as a collaborative project at the Georgia Institute of Technology (Dr. F. Löffler) and the University of Illinois (Dr. R. Sanford).

Biotransformations Involved in Sustained Reductive Removal of Uranium in Contaminated Aquifers

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Microbial reduction of soluble U(VI) to insoluble U(IV) shows promise as strategy for *in situ* immobilization of uranium in uranium-contaminated subsurface environments. However, little is known about how this process works outside the laboratory and how to sustain this process for the long periods of time that may be needed to treat extensively contaminated sites.

In a previous study, acetate addition (1–3 mM) to a uranium-contaminated aquifer at a site in Rifle, Colorado, temporarily promoted removal of uranium from the groundwater via microbial reduction of soluble U(VI) to insoluble U(IV). Uranium removal continued while *Geobacteraceae* remained the predominant organisms, but as Fe(III) was depleted near the injection gallery, Fe(III) reducers could no longer out-compete sulfate reducers. Acetate was consumed via sulfate reduction and U(VI) concentrations increased in the groundwater.

In the 2004 field experiment, conditions were changed in an effort to prolong the activity of *Geobacteraceae* and removal of U(VI) from the groundwater. Acetate concentrations *in situ* were increased to levels comparable to or greater than the ambient sulfate concentration (ca. 9 mM) to prevent complete consumption of acetate by sulfate reducers and ensure acetate transport to downgradient areas containing available Fe(III). Bromide tracer data from monitoring well samples indicated delivery of acetate solution to the subsurface across a broad front but at varying concentrations. Loss of U(VI) after 109 days of acetate injection averaged 34%, 40 %, and 13% (n=5) in wells positioned at increasing distances from the injection gallery relative to control wells. However, the amount of U(VI) removed from the groundwater varied significantly from well to well with U(VI) losses as high as 91% observed in some wells. Loss of U(VI) was greatest (58–91%) in wells where both acetate and Fe(III) were still available, promoting the activity of *Geobacteraceae*. There was little or no U(VI) reduction at sites where Fe(III) was depleted and sulfate reduction predominated.

Analysis of the sediments from the field experiment, as well as studies with laboratory sediment incubations, demonstrated for the first time that the loss of U(VI) from the groundwater could be accounted for as an accumulation of U(IV) in the sediments. Surprisingly, a substantial amount (ca. 40%) of the U(VI) in sediments prior to treatment was adsorbed to the sediments. Unlike the soluble U(VI), the adsorbed U(VI) was not reduced over time, but did remain immobilized.

These results demonstrate that it is possible to promote microbial reductive precipitation of uranium in situ, but further research is required to devise strategies so that U(VI) will be reduced under sulfate-reducing conditions as well as under Fe(III)-reducing conditions.

New Catalytic DNA Biosensors for Radionuclides and Metal Ions

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We are developing new DNA biosensors for simultaneous detection and quantification of bioavailable radionuclides, such as strontium, uranium, technetium, and plutonium, and metal contaminants, such as lead, chromium, and mercury [1,2] They will be applied to the on-site, real-time assessment of concentration, speciation, and stability of the radionuclides and metal contaminants during and after bioremediation. To achieve this goal, we have employed a combinatorial method called "in vitro selection" to search for catalytic DNA molecules that are highly specific for radionuclides or other metal ions [3]. The DNA has been 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 one specifically for a particular metal ion at a certain 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+}, with a quantifiable detection range from 10 nM to 4 μM [4]. Even in the presence of other metal ions, this biosensor displays a remarkable sensitivity and selectivity. Recently we have carried out several studies to further improve the sensor's sensitivity and selectivity. For example, by combining both inter- and intramolecular quenchers, we have improved the signal to noise ratio significantly [5]. To further improve the metal ion selectivity, we have developed a "negative" selection strategy that can significantly improve the metal-binding selectivity in the selection process [6]. To improve the metal-binding affinity, we have developed a new synthetic strategy for making phosphoroselenote DNA/RNA that are capable of binding different metal ions [7]. To provide insight into the metal-binding sites in DNA and to allow the design of metal ion sensors and chelators from the first principle, we have also carried out a detailed biochemical [8] and biophysical [9] study of the DNA lead sensors obtained in the lab. This year we have made a breakthrough by converting the catalytic DNA into colorimetric metal sensors, making on-site, real-time detection even more affordable and achievable because no equipment is needed in the operation [10]. We accomplished this by taking advantage of recent advance in both biotechnology and nanotechnology. Finally, we are making progress in applying the strategy developed in the lab toward making sensors for radionuclides such as uranium and chromium(VI) ions. Now we are poised to make sensors for NABIR applications. The latest results from the lab will be presented.

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Biotransformations of Plutonium and Uranium by Naturally Occurring Bacteria

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This project will characterize and quantify specific biotransformation mechanisms that control the environmental distribution of actinides (Pu and U) and may be used for in situ biostabilization. Radionuclide contaminants may generally follow the pathways of the redox-active major elements, Fe and Mn. For example, Fe^{III} forms discrete complexes with polydentate chelators, such as co-contaminant EDTA and bacterial siderophores, that have 1:1 ligand to metal stoichiometry and low molecular charge. Pu^{IV} forms similar complexes; however, as a consequence of its larger coordination sphere and higher charge, it also forms additional species, including $Pu^{IV}(EDTA)_2$ and $Pu^{IV}EDTA(CO_3)_2$. These similarities and differences have direct consequences on biogeochemical behavior, as demonstrated by the dissimilar biodegradation of Fe- and Pu-EDTA and bioaccumulation of Fe- and Pu-siderophore complexes.

Microbacterium flavescens (JG-9), which utilizes a FhuD-type siderophore receptor, takes up Fe^{III}-desferrioxamine more rapidly and approximately five times more effectively than Pu^{IV}-desferrioxamine. When desferrioxamine complexes of both metals are simultaneously added to the bacteria, the rate of uptake of each is reduced by a factor of two, indicating comparable recognition by the same receptor sites. Towards determining if Pu-siderophore uptake is general, we isolated and purified the pyoverdin siderophore of *Pseudomonas putida*. Under conditions where Fe^{III}-pyoverdin is accumulated by metabolically active cells of *P. putida*, the corresponding Pu^{IV} complex is not. Interestingly, NTA complexes of both metal ions are taken up at the same rate ($k = 6.3 \times 10^{-2}$ min^{-1}) and in the same amount, indicating that pyoverdin is preorganized for metal binding in the receptor and metal uptake is via a ligand exchange mechanism (NTA to pyoverdin-receptor). Further voltametric and spectrophotometric studies showed that, unlike Fe^{III}, Pu^{IV} can form bis-pyoverdin complexes. When Pu^{IV}-pyoverdin/*P. putida* studies were repeated under conditions of lower siderophore-to-metal ratios that would favor the mono-pyoverdin complex (the direct Fe^{III}-pyoverdin analog), then the same amounts of Fe and Pu were taken up at the same rate. These studies indicate that under environmental conditions Pu^{IV} will be accumulated by bacteria via siderophore receptors and may be stabilized with respect to long-range transport.

In addition to forming different species, Fe^{III} and Pu^{IV} also differ in their redox properties, with Pu^{IV} having a much broader potential electrochemical range and more readily accessible oxidation states. These properties suggest that bacteria-mediated redox processes could transform plutonium to either less mobile or more mobile forms. Unlike uranyl, which is reduced to relatively stable $U^{IV}O_2$, plutonyl ($Pu^{VI}O_2^{2+}$ and $Pu^{V}O_2^{+}$) could be reduced beyond Pu^{IV} to Pu^{III}, which is not preferred. We have shown that plutonyl species are reduced by *Geobacter metallireducens* GS15 and *Shewanella putrefaciens* CN32. We are now assessing the ability of these bacteria to respire Pu and comparing their growth rates during Pu and U reduction.

Mesoscale Coupled Transport and Biogeochemical Effects on Reduction of U(VI) and NO_3^- as Co-contaminants in Natural Sediments and Soils

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Since fluids in subsurface environments are not well mixed, even at relatively small scales, the subsurface can be categorized into advective and diffusion-limited domains. Information is lacking on local, diffusion-limited processes that arise from small-scale variations in permeability, preferential flow paths, and soil/sediment structure. The primary objective of this research is to study coupled transport and biogeochemistry of U(VI) and NO_3^-.The redox reactions of these co-occurring contaminants are investigated in realistically heterogeneous model systems. We emphasize direct measurements within diffusion-limited domains at the mesoscale (about 10^{-4} to 10^{-1} m). It is within these diffusion-limited domains that large gradients in microbial activity, chemical potentials, reaction rates, and transport rates can coexist. Thus, the dynamics at this mesoscale can control redox-dependent biotransformations in nature.

Diffusion-limited U biogeochemical transformations. Measurements include determinations of U oxidation state (micro-XANES spectroscopy), soluble U, redox potential profiles, microbial activity, and microbial communities. Strong retardation of the U(VI) diffusion front in initially reducing sediments has been found to persist even after nearly two years without resupply of organic carbon. In oxidizing soils, U(VI) diffusion was relatively rapid under both acidic and slightly alkaline conditions because of relatively weak sorption. The strength of U(VI) sorption can be orders of magnitude less at high concentrations typical of initial waste sources.

U(VI) sorption in soils containing calcium carbonate. Calcite is a common component in slightly alkaline soils and sediments. However, the impact of calcite on U(VI) sorption in whole soil systems (as opposed to model systems comprised of single mineral phases) has not previously been evaluated. We found substantial suppression of U(VI) sorption at neutral to slightly alkaline pH, and showed that the depressed level of U(VI) sorption is fairly well accounted for by the stability of aqueous $Ca_2UO_2(CO_3)_3$, identified by Bernhard et al. [1].

Long-term stability of in situ microbially reduced U(IV). Area 2 sediments containing 200 ppm U, initially primarily as U(VI), were packed into columns and infused with lactate solutions to accelerate reduction. Supporting measurements included analyses of U concentrations in effluents, in situ determination of sediment U(VI):U(IV), redox potential profiles, pore water chemical analyses, microbial activity, and microbial community structure. Nearly complete U reduction was achieved by about 90 days, with effluent U concentrations decreased from initial values in excess of 1,000 ppb down to less than 5 ppb. However, continued organic carbon infusion and monitoring (over 500 days) revealed substantial U remobilization, with later effluent U concentrations exceeding 300 to 500 ppb. Net U reoxidation within the soil columns was supported by micro-XANES analyses. The remobilization of U was attributed to in-situ production of biogenic carbonate which shifts equilibrium towards stable U(VI) carbonate species. A shift in bacterial community structure was observed in the re-oxidation stage (relative to the earlier reducing stage). Thus, in some cases, reduction-based U immobilization can be transient and unreliable. This result also demonstrates the need for experiments that address long-term stability of in situ strategies for remediating actinide and metal contaminants.

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PROGRAM ELEMENT 2

Community Dynamics/ Microbial Ecology

Using Artificial Neural Network Tools to Analyze Microbial Biomarker Data

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New data analysis tools are needed to help understand biomolecular community characterization data. In our project, we have developed 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. Supervised learning is used for prediction and unsupervised learning provides dimension reduction. We have successfully applied these techniques to the analysis of membrane lipids and nucleic acid biomarker data from both laboratory and field studies. Although ANNs typically outperform linear data analysis techniques, the user must be aware of several issues to ensure that the analysis results are not misleading. This poster will provide guidance in the proper use of ANNs.

Overfitting is a potential problem when using ANNs to analyze 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 several techniques to help reduce the problem of overfitting. The new techniques include: (1) a sensitivity-based pruning algorithm to help identify efficient ANN models for nonlinear prediction, (2) cross validation, (3) weight decay, and (4) a global optimization (e.g., simulated annealing) technique to select parsimonious ANNs that generalize well to new data sets. We have also implemented an unsupervised learning technique called the input training technique that can be used to reduce the dimensionality of the input variables for visualization. This method is more efficient than a popular autoassociative ANN technique used for the same purpose.

Another challenge in using ANNs is the interpretation of the analysis results. ANNs are often treated as a black box, which is unsatisfactory for understanding relationships between sets of variables. For example, we often want to estimate the relative sensitivity importance of a set of explanatory variables for a given set of predicted variables. However, importance or sensitivity is often an ill-defined concept outside a specific problem context. Many measures are only locally defined or are biased toward large or small values. An unbiased local model-error-based importance index we are currently using is based on the amount of degradation in the accuracy of ANN predictions when a particular input is effectively removed from the model. Inputs that are "important" predictors should severely degrade ANN performance when their effects are removed from the model. We discuss several measures of importance/sensitivity and offer guidance on their use.

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 biostimulation. This objective is being met by coalescing recent developments in 16S rRNA microarrays, microfluidic systems, microparticle chemistries, renewable surface techniques, and suspension array technology. In fiscal year 2003, we simplified the fluidic requirements for integrated biodetection systems by utilizing tunable surface, color-coded microparticles as both an rRNA affinity purification resin in a renewable microcolumn sample preparation system, and as the sensor surface in a flow cytometer detector. The tunable surface detection limit was 1 ng total RNA (~10^4 cell equivalents) in 15 min test tube hybridizations and 10 ng total RNA (~10^5 cell equivalents) in the automated system (30 sec contact time) in both low- and high-salt buffers. RNA fragmentation was essential for achieving tunable surface suspension array specificity, as assessed on a 10-bead prototype metal- and sulfate-reducer array. Chaperone probes reduced but did not completely eliminate cross-hybridization, even with probes sharing <50% identity to target sequences. Unpurified environmental extracts did not irreparably affect our ability to classify color-coded microparticles, but residual environmental constituents from the surface soil significantly quenched the Alexa-546 reporter fluor. Interestingly, sediment extracts from an FRC subsurface sediment could be captured and detected directly on the bead array without influencing the Alexa reporter or measurement. Modulating bead surface charge during the posthybridization wash steps did not influence the interaction of soluble environmental contaminants with conjugated beads. However, the automated hybridization system greatly reduced the effects of fluorescence quenching, especially in the soil background. The automated system was as efficacious as manual methods for simultaneous sample purification, hybridization, and washing prior to flow cytometry detection, as measured by detection sensitivity and hybridization specificity to the 10-bead model array.

From these studies, we determined that a universally applicable, automated bead array system would probably require some level of rRNA pre-purification prior to bead array hybridization in order to remove any residual fluorescence quenchers from sediments (or groundwater) rich in humic acids or organic C. We therefore developed a single-tube rRNA purification, labeling, and fragmentation chemistry for environmental samples that can be automated within the sample preparation fluidics architecture. All chemical operations are performed on a single bed of microparticles. Data show rRNA recovery after combined rRNA purification, labeling, and fragmentation chemistry is approximately 50%, but that sample cleanup removed all residual fluorescence interferents from the surface soil extract. A preliminary fluidics method for automating the universal sample preparation chemistry has been developed and will be further refined during fiscal year 2004, with the principle goal of establishing a fluidics routine to reduce or eliminate backpressure during resin perfusion and wash steps in the automated device.

Ecological Interactions between Metals and Microbes that Impact Bioremediation

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The objectives of this research are: (1) determine the distribution of phylotypes and metal-resistant genes at the scale of spatial heterogeneity observed in microbial community activity; (2) determine the effects of environmental factors on community responses to Cr(VI) contamination (these effects may be mediated by physiological responses, species selection, or gene transfer); (3) determine the role of mobile elements that confer Cr resistance (resistant microbes might function as "bioprotectants" through physiological activity, or reservoirs of transferable resistance genes); (4) identify the novel physiological and genetic bases for bacterial resistance to Cr(VI).

Distinct microbial communities were found in contaminated soils that varied in their concentrations of Pb, Cr, and aromatic compounds. It is difficult to distinguish between their effects as their presence is highly correlated. Microcosms were constructed in which either Pb^{+2}or CrO_4^{-2} was added at levels that produced modestly acute or severely acute effects (50 or 90% reduction). We previously reported on changes in microbial activity and broad patterns of bacterial community composition. These results showed that the addition of an organic energy source selected for a relatively small number of phylotypes and the addition of Pb or Cr(VI) modulated community response. We sequenced dominant phylotypes from microcosms amended with xylene and Cr(VI) and from those with the addition of glucose only. In both cases, the dominant selected phylotypes were diverse. We found a number of distinct *Arthrobacter* strains, as well as several *Pseudomonas* spp. In addition, the high GC-content bands belonged to members of the genera *Nocardioides* and *Rhodococcus*. The focus of amended microcosm work has now shifted to anaerobic processes. The reduction of Cr(VI) to Cr(III) as a detoxification mechanism is of greater interest, as is the specific role of particular physiological groups of anaerobes in mediating Cr(VI) detoxification. The correlation between microbial activity, community structure, and metal level was analyzed on 150 mg of soil collected at spatial scales <1, 5, 15, and 50 cm. There was no correlation between metal content and activity level. Soils <1 cm apart could differ in activity tenfold, and extractable Pb and Cr sevenfold. Therefore, we turned to geostatistical analysis. There was spatial periodicity, which is likely to reflect the heterogeneous distribution of active microbes and metal contaminants. Variograms indicated range of spatial dependence was up to 20 cm. To visualize the spatial relationships between the primary variate (activity) and its covariates (Pb and Cr content), block kriging was used. The kriging maps suggest areas exist where increased metal concentrations have zones of decreased metabolic microbial activity. Cr(VI)-resistant bacteria were isolated from two contaminated sites. Most isolates are *Arthrobacter*, *Rhodococcus*, or *Pseudomonas* spp. A *chrA* gene was cloned from *Arthrobacter* strain CR15 isolated from Cannelton, MI. PCR primers were produced against conserved motifs analyzed from 8 *chrA* sequences. Of the 96 Cr-resistant isolates from Cannelton, 85% gave a positive reaction to these primers. In contrast, none of the 38 isolates from Seymour, IN, were positive. Therefore, at least for the culturable community, a particular resistance determinant appears to be widespread at a geographical site but rare (absent) at another site. The phylogenetic relatedness of the *Arthrobacter* strains is being evaluated via distribution of repetitive elements as well as genome-wide restriction fragment analysis. Work to date on the latter suggests *Arthrobacter* genomes are small (<2.5 Mbp). Gene capture experiments demonstrated that chromate-sensitive Gram-negative bacterial strains could obtain resistance from Cr-contaminated soil. Yet, frequency of transfer is low (10^{-7} to 10^{-8}). Genetic diversity of the acquired chromate resistance mechanism is being assessed.

Subsurface Bacterial Community Dynamics in the Presence of Plutonium and Uranium

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This project has two goals. The first is to determine the effects of Pu(VI) on natural bacterial communities in anaerobic subsurface sediments, and to compare bacterial community dynamics in the presence of Pu(V/VI) or U(VI). The second is to identify groups of indigenous bacteria from subsurface sediments that are active in the presence of Pu(V/VI) or U(VI) and that may be responsible for reduction of Pu(VI) and/or U(VI) in anaerobic subsurface environments. Using subsurface sediments from DOE sites, we will conduct laboratory time-course experiments where sediment is exposed to Pu(V/VI) or U(VI). We will use a combination of methods to specifically analyze Fe(III)- and sulfate-reducing bacterial groups known to reduce U(VI), and to conduct a broader survey of potentially important soil species that are active in the presence of Pu or U. We will measure bacterial abundance of target bacterial groups using real-time quantitative PCR assays, followed by analysis of species composition in those groups found to be active and/or correlated with actinide reduction. In parallel, we will use terminal restriction fragment (T-RFLP) profiling to determine broad changes in the total bacterial community as affected by actinide presence. To insure that potentially important species that are not members of our quantitative PCR groups are not missed, we will also assess total bacterial diversity and composition by 16S clone/sequence libraries, to be conducted at certain points in our time-course experiments. This study will contribute to the NABIR program by providing information on the dynamics of natural sediment communities in the presence of Pu(V/VI) and U(VI) that complement ongoing pure culture and field studies.

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

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Our previous research under the NABIR program has demonstrated that adding acetate to subsurface sediments enhances the growth of *Geobacter* species, which grow primarily via Fe(III) reduction, but simultaneously reduce U(VI), effectively removing it from the groundwater. *Geobacter* species may account for over 80% of the microorganisms in the groundwater during active uranium bioremediation. In order to optimize this uranium bioremediation strategy and broaden its application to other sites it is important to have information on the rates of metabolism and metabolic state of the *Geobacter* species responsible for the bioremediation.

When *Geobacter sulfurreducens* was grown at a range of growth rates in chemostats with acetate as the electron donor and fumarate as the electron acceptor there was a direct correlation between rates of fumarate reduction and levels of mRNA for *frdA*, a gene for the fumarate reductase. Furthermore, when Fe(III) was the electron acceptor, there was a direct correlation between mRNA levels for *omcB*, a gene for an outer-membrane cytochrome required for Fe(III) reduction, and rates of Fe(III) reduction. This result is somewhat surprising because studies with *E. coli* have demonstrated no change or a decrease in mRNA for respiratory genes as growth rates increase with fumarate, dimethylsulfoxide, or nitrate as the electron acceptor. However, the results suggest that it will be possible to estimate in situ respiration rates of *Geobacter* via mRNA analysis.

Studies with sediments from the UMTRA uranium bioremediation field experiment in Rifle, Colorado, demonstrated that mRNA from *Geobacter* species could be recovered with high efficiency, suggesting the potential to evaluate, via mRNA analysis, not only rates of respiration, but also the metabolic state of *Geobacter* species. For example, one concern has been that the growth and activity of *Geobacter*s during uranium bioremediation might be nutrient-limited because acetate, but no nutrients, were added to the groundwater. Analysis of levels of *nifD* mRNA in the sediments demonstrated that *Geobacteraceae* were expressing nitrogen fixation genes, suggesting that growth was limited by the availability of fixed nitrogen. When ammonia was added to the sediments, levels of mRNA for *nifD* decreased three to five orders of magnitude, whereas levels of mRNA for *recA* and levels of *Geobacter* 16S rRNA remained constant. This demonstrated that the *Geobacter*s were fixing nitrogen due to nitrogen limitation when no ammonia was added.

These results demonstrate that monitoring the in situ metabolic state of the microbial community as well as estimating rates of metabolism is feasible via mRNA analysis. This will help move bioremediation from a primarily empirical practice to more of a science.

Introduction of Mercury-Resistant Bacterial Strains to Hg(II) Amended Soil Microcosms Increase the Resilience of the Natural Microbial Community to Mercury Stress

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Heavy metals are among the most important groups of pollutant compounds, and they are highly persistent in the soil environment. Techniques that can be used for the remediation of heavy metal contaminated environments thus need to be evolved. In the present study we introduced two mercury resistant bacterial strains to Hg(II) amended soil microcosms in order to evaluate their effect on the concentration of mercury as well as on the natural microbial community. The two bacterial strains were *E. coli* SØ3539/pJORD70 and *E. coli* SØ3539/pPB117, respectively. pJORD70 is a mercury-resistant conjugal plasmid, whereas pPB117 is a non-mobilizable mercury-resistant plasmid. Nonamended microcosms were similarly set up with the two mercury-resistant bacterial strains, as well as Hg(II) amended and nonamended control microcosms without the introduction of *E. coli* strains. Triplicate soil samples were taken from the microcosms at days 0, 6, 13, and 34, and analyzed for (1) the total concentration of mercury, (2) numbers of transconjugant bacteria harboring pJORD70, (3) total numbers of CFUs appearing on R2A agar +/– 5 ppm Hg(II), (4) day of colony appearance on R2A agar +/– 5 ppm Hg(II), (5) numbers of carbon sources transformed in Biolog EcoPlates +/– 1 ppm Hg(II), and (6) numbers of DNA bands in denaturing gradient gel electrophoresis. We found that pJORD70 was transferred to the indigenous bacterial population; however, only at day 6 were transconjugants detected. It cannot be excluded, though, that transfer to the nonculturable fraction of the microbial community also occurred. Transfer of plasmid pPB117 was not detected. Addition of mercury-resistant bacterial strains had a pronounced effect on the survival of the indigenous microbial population. In the absence of *E. coli* strains, the indigenous microbial population died out and no culturable bacteria were detected after 34 days of mercury exposure. However, in the presence of pJORD70, the bacterial population survived, although at a lower level compared to non-Hg(II)-amended control microcosms. In Hg(II)-amended microcosms, the presence of SØ3539/pPB117 also had a positive effect on the survival of the indigenous microbial population, although this was not as significant as with pJORD70. Similar effects were observed when measuring the number of carbon sources respired in EcoPlates. In addition we found that the microbial community composition had changed and were more dominated by slow-growing bacteria compared to non-Hg(II)-amended microcosms. Thus, the amendment of mercury-resistant bacterial strains to Hg(II)-exposed DOE sediment had a significant effect on the resilience of the natural microbial community, and the presence of conjugative plasmids seemed to increase this effect. In contrast, no effect was measured on the concentration of total mercury in the soil.

Probing for Active Metal-Reducers at the FRC Using Stable Isotope Probing, New Enrichment Strategies, and Metagenomics

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Stable isotope probing (SIP) methods are being developed to identify metabolically active bacteria that may play a role in metal and radionuclide reduction in FRC sediments. SIP methodology involves the addition of ^{13}C-labeled substrates to a mixed microbial community, extraction and density-gradient centrifugation to isolate ^{13}C-labeled nucleic acids of microorganisms that utilized the substrate, and then sequencing of 16S rRNA and rDNA to obtain phylogenetic information. Microcosms of FRC sediments and groundwater are being probed with the same substrates used for field biostimulation of metal reduction (ethanol, acetate). Optimization of SIP methodology, including RNA and DNA extraction methods, density-gradient centrifugation, and isotope-ratio mass spectrometry of nucleic acids, is underway. Highest recovery and quality of RNA and DNA were obtained by bead-beating/grinding sediments in guanidine thiocyanate denaturing solution, extraction at 65C with CTAB and SDS buffer and Sephadex gel filtration, followed by separation of RNA and DNA with Qiagen Tip 100 columns and further purification. RNA and DNA were banded isopycnically, in separate density gradients constructed with cesium trifluoroacetate, and were successfully recovered from fractions.

The community composition of three bacterial enrichment cultures from contaminated subsurface sediment (FW109) was determined with SSU rDNA clonal libraries. Microorganisms closely related to *Anaeromyxobacter dehalogenans* and an uncultured *Anaeromyxobacter* sp. predominated the iron-reducing enrichment, while microorganisms closely related to *Desulfotomaculum* sp. and *Clostridium* sp. predominated the sulfate-reducing enrichment. The nitrate-reducing enrichment consisted of species closely related to *Pseudomonas* sp. C05E and *Citrobacter amalonaticus*. Fosmid libraries have been constructed for the nitrate- and sulfate-reducing enrichments. Over 7,000 clones were obtained from nitrate-reducing enrichment and over 760 clones have been obtained for the sulfate-reducing enrichment. Additionally, fosmid libraries have been constructed from the DNA extracted from the U and acidic well FW005. We have also initiated an analysis of the colonization of surfaces by *Desulfitobacterium hafniense* under iron-reducing conditions. Using a chemostat capable of long-term operation under anaerobic conditions, we have identified several surfaces that support biofilm formation. *D. hafniense* is capable of reaching relatively high cell densities in the chemostat. Based on the genome sequence of *D. hafniense,* we have constructed a full ORF chip and have used this chip to evaluate gene expression under different electron-accepting conditions and under N_2-fixing conditions, and we will use it to study expression under biofilm conditions. Besides metal reduction, *Desulfitobacteria* dechlorinate some chlorinated co-contaminants. We found seven putative dehalogenase genes in the *D. hafniense* genome, and we are evaluating their expression and have cloned and expressed several in *E. coli. Desulfitobacteria* have several properties important to successful remediation at DOE sites.

Defining Conditions for Maximizing Bioreduction of Uranium

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Correlations between modifying electron donor and acceptor accessibility, the in situ microbial community, and bioreduction of uranium at the FRC and UMTRA research sites indicated that significant modifications in the rate, amount, and, by inference, the potential stability of immobilized uranium are feasible in these environments. The in situ microbial community at these sites was assessed with a combination of lipid and real-time molecular techniques providing quantitative insights of effects of electron donor and acceptor manipulations.

Previous microcosm tests conducted with sediment from FRC Area 1 showed little activity at low (<4.0) pH. It was hypothesized that repeated electron donor amendments would stimulate in situ microbial activity and moderate the low pH. The push-pull experiments were performed utilizing coupons containing powder activated carbon (PAC) beads deployed in wells. These coupons were recovered at one-, two-, and three-month intervals, and results are currently being compared to analyses of sediments recovered from these same sites. Microbial biomass on the coupons increased throughout the time of deployment. Non-ester-linked phospholipid fatty acid (NPLFA) profiles in the low pH wells were dominated by monounsaturates in the first month. However, by the third month more diverse NPLFA profiles were evident. Genetic community profiling by 16S rDNA showed distinct differences between control and test wells. Sequences related to *Rhizobium, Caulobacter, Chromobacterium*, and *Bosea* dominated the control well. Sequences recovered from low pH test wells included *Burkholderia*, *Alcaligenes*, *Desulfosporsinus, Azoarcus, and Rhodanobacter*. Community structure as monitored by 16S rDNA changed over time with the appearance of *Cytophaga, Clostridia*, and *Desulfotomaculum* in the test wells by the third month.

A second field-scale electron donor plus acceptor amendment experiment was conducted in 2003 at the Old Rifle Uranium Mill Tailings Remedial Action (UMTRA) site in Rifle, Colorado. Coupons containing PAC beads and beads amended with ferrihydrite were suspended in background and donor impacted monitoring wells. Microbial biomass was consistently higher in the ferrihydrite-amended coupons before donor addition, and RT-PCR showed IRB and SRB only on ferrihydrite PAC. Rates of biomass growth increased at each sampling period with continued donor injection. Ferrihydrite amended beads, which showed higher biomass (a relatively similar community composition), contained more menaquinones and had consistently lower ubiquinone to menaquinone ratios, indicating a lower redox state than the communities that colonized the unamended PAC. Community composition as measured by PLFA was more diverse before the addition of acetate; after the acetate addition, it became less diverse with time, but with an increase of terminally branched saturated PLFA. Real-time PCR specific for *Geobacter* showed none present before donor addition. After two weeks of donor addition there was detectable *Geobacter* colonizing both ferrihydrite and unamended beads. Contrasts between the communities induced on beads and sediments are currently under investigation.

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

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To effectively monitor microbial populations involved in various important processes, a 50-mer-based oligonucleotide microarray was developed based on known genes and pathways involved in biodegradation, metal resistance and reduction, denitrification, nitrification, nitrogen fixation, methane oxidation, methanogenesis, carbon polymer decomposition, and sulfate reduction. This array contains approximately 2,000 unique and group-specific probes with <85% similarity to their nontarget sequences. Based on artificial probes, our results showed that at hybridization conditions of 50°C and 50% formamide, the 50-mer microarray hybridization can differentiate sequences having <88% similarity. Specificity tests with representative pure cultures indicated that the designed probes on the arrays appeared to be specific to their corresponding target genes. Detection limits were about 5–10 ng genomic DNA in the absence of background DNA, and 50–100 ng (~1.3×10^7 cells) in the presence of background DNA. Strong linear relationships between signal intensity and target DNA and RNA concentration were observed ($r^2 = 0.95$–0.99). Application of this microarray to naphthalene-amended enrichments and soil microcosms demonstrated that composition of the microflora varied depending on incubation conditions. While the naphthalene-degrading genes from *Rhodococcus*-type microorganisms were dominant in enrichments, the genes involved in naphthalene degradation from Gram-negative microorganisms such as *Ralstonia*, *Comamonas,* and *Burkholderia* were most abundant in the soil microcosms (as well as those for polyaromatic hydrocarbon and nitrotoluene degradation). Although naphthalene degradation is widely known and studied in *Pseudomonas*, *Pseudomonas* genes were not detected in either system. Real-time PCR analysis of four representative genes was consistent with microarray-based quantification ($r^2 = 0.95$). Currently, we are also applying this microarray to the study of several different microbial communities and processes at the NABIR-FRC in Oak Ridge, Tennessee. One project involves the monitoring of the development and dynamics of the microbial community of a fluidized bed reactor (FBR) used for reducing nitrate and the other project monitors' microbial community responses to stimulation of uranium-reducing populations via ethanol donor additions in situ and in a model system. Additionally, we are in the process of designing a phylogenetic array based on over 5,000 different 16S rRNA genes from microorganisms similar to those in FRC groundwater, and developing novel strategies for increasing microarray hybridization sensitivity. Finally, great improvements to our methods of probe design were made by the development of a new computer program, CommOligo. CommOligo designs unique and group-specific oligo probes for whole genomes, metagenomes, and groups of environmental sequences, and uses a new global alignment algorithm to design single or multiple probes for each gene or group. We are now using this program to design a more comprehensive functional gene array for environmental studies. Overall, our results indicate that the 50-mer-based microarray technology has potential as a specific and quantitative tool to reveal the composition of microbial communities and their dynamics important to processes within contaminated environments.

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PROGRAM ELEMENT 3

Biomolecular Sciences and Engineering

Isolation and Characterization of Mobile Genetic Elements from Microbial Assemblages Obtained from the Field Research Center Site

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Considerable knowledge has been gained from the intensive study of a relatively limited group of bacterial plasmids. Recent efforts have begun to focus on the characterization of plasmid populations at the molecular level and associated mobile genetic elements (e.g., transposons, integrons) occurring in a wider range of aquatic and terrestrial habitats. Surprisingly, however, little information is available regarding the incidence and distribution of mobile genetic elements extant in contaminated subsurface environments. Such studies will provide greater knowledge on the ecology of plasmids and their contributions to the genetic plasticity (and adaptation) of naturally occurring subsurface microbial communities.

We requested soil cores from the DOE NABIR Field Research Center (FRC) located on the Oak Ridge Reservation. The cores, received in February 2003, were sampled from four areas on the Oak Ridge Site: Area 1, Area 2, Area 3 (representing contaminated subsurface locales), and the background reference sites. The average core length (24 in.) was subdivided into three profiles, and soil pH and moisture content were determined. Uranium concentration was also determined in bulk samples. Replicate aliquots were fixed for total cell counts and for bacterial isolation. Four different isolation media were used to culture aerobic and facultative microbes from these four study areas. Colony-forming units ranged from a minimum of 100 per gram soil to a maximum of 10,000, irrespective of media composition used. The vast majority of cultured subsurface isolates were gram-positive isolates, and plasmid characterization was conducted per methods routinely used in the Sobecky laboratory. The percentage of plasmid incidence ranged from 11% to 80% of all isolates tested. This frequency appears to be somewhat higher than the incidence of plasmids we have observed in other habitats, and we are increasing the number of isolates screened to confirm this observation. We are also characterizing the plasmid populations at the molecular level. It is interesting to note that the frequency of plasmid-containing isolates from the background control site was comparable. Aliquots of samples were also used in enrichment assays to isolate metal-resistant subsurface isolates. Samples were subjected to three different metals (chromium, mercury, and zinc) at two different concentrations, and incubated following a conditioning period in which samples were amended with a carbon, nitrogen, and phosphorus source. Isolates were plated on metal selection and purified to single isolates, and plasmid content was determined.

Facilitated Cr(VI) Reduction by *Deinococcus radiodurans* Engineered for Complete Toluene Mineralization, and Isolation of Environmental Bacteria Able to Grow on Fuel-Derived Aromatic Hydrocarbons under Chronic Radiation

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We aim to develop experimental models for understanding the reduction of metals coupled to organic compound oxidation in aerobic radionuclide-contaminated environments. Toluene and other fuel hydrocarbons are commonly found in association with radionuclides at numerous Department of Energy (DOE) sites, frequently occurring together with Cr(VI) and other heavy metals. In this study, the extremely radiation-resistant bacterium *Deinococcus radiodurans* was engineered for complete toluene mineralization by cloned expression of *tod* and *xyl* genes of *Pseudomonas putida*. The recombinant *tod/xyl* strain showed significant incorporation of carbon from the toluene aromatic ring into cellular macromolecules and carbon dioxide, in the absence or presence of chronic radiation (50 Gy/hr). Although the recombinant strain did not grow on toluene, the organism's native Cr(VI) reduction capabilities were facilitated by toluene when present as the sole carbon and energy source in natural sediment analogues of DOE-contaminated environments. The engineered bacteria were able to oxidize toluene under both minimal and complex nutrient conditions, which is important since both conditions have environmental equivalents in the context of bioremediation processes. An alternative to the engineering approach is to isolate natural organisms proficient at degrading aromatic hydrocarbons under chronic radiation. Using *meta*-toluate plates for selection, we have isolated bacteria from desiccated Arizona desert samples that are able to grow on toluene as the sole carbon source in the presence of 50 Gy/hr. Strain characterizations will be presented. How bacteria are able to grow under chronic γ radiation remains unknown and is a central question revolving around the development of strategies for bioremediation of radioactive waste sites. We have shown that bacteria containing very high intracellular manganese concentrations are capable of growth under chronic radiation, and that Mn restriction renders *D. radiodurans* sensitive to irradiation.

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

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Metal-reducing members of the genus *Shewanella* (e.g., *S. oneidensis* strain MR-1 and *S. putrefaciens* strains 200 and CN32) display remarkable respiratory versatility as they are able to respire a wide variety of compounds as terminal electron acceptor, including U(VI) and Tc(VII). Although *S. oneidensis*, *S. putrefaciens*, and other Tc(VII)-reducing bacteria are able to reduce Tc(VII) in anaerobic liquid culture, anaerobic growth on Tc(VII)-amended agar medium has not been observed [possibly due to Tc(VII) toxicity]. The inability to grow colonies on Tc(VII)-amended agar has hindered development of a genetic system for studying the molecular basis of microbial Tc(VII) reduction: Tc(VII) reduction-deficient (Tcr) mutants may not be rapidly identified by simply testing mutagenized colonies for anaerobic growth on Tc(VII)-amended agar. To circumvent this problem, we have developed an alternate, agar-based screening technique for rapid identification of *S. oneidensis* Tcr mutants. The newly developed Tcr screening technique is based on the observation that wild-type *S. oneidensis* and *S. putrefaciens* colonies produce insoluble Tc(IV) particles on their colony surface during microaerobic growth on Tc(VII)-amended agar.

We have recently applied the Tcr mutant screening technique to isolate a set of *S. oneidensis* Tcr mutants: *S. oneidensis* colonies arising from ethyl methane sulfonate-treated cells were grown microaerobically on Tc(VII)-amended agar, and several putative Tcr mutants were identified by the inability to produce insoluble Tc(IV) particles on their colony surface during microaerobic growth. The Tcr mutants were subsequently found to lack the ability to reduce Tc(VII) in anaerobic liquid culture. The Tcr mutant screening technique is analogous to a method that we recently developed to identify U(VI) reduction-deficient (Urr) mutants of *S. oneidensis*: wild-type *S. oneidensis* colonies produce insoluble U(IV) particles on their colony surface during microaerobic growth on U(VI)-amended agar medium. Urr mutants identified by the inability to produce U(IV) on their colony surface were subsequently found to lack the ability to grow anaerobically in liquid culture with U(VI) as electron acceptor. All Urr mutants also lacked the ability to respire NO_2^-, suggesting that the U(VI) and NO_2^- respiratory pathways share common components. The Urr mutants, however, retained the ability to reduce Tc(VII), indicating that U(VI) and Tc(VII) are reduced via different mechanisms in *S. oneidensis*.

We have also followed a genetic complementation approach to identify the genes and gene products unique to the Fe(III) respiration system of *S. putrefaciens* and *S. oneidensis*. Results from these studies have provided genetic evidence linking Fe(III) respiration and type II protein secretion. Subsequent biochemical analyses indicated that the type II protein secretion system targets Fe(III)-reducing proteins to the outside face of the *S. putrefaciens* and *S. oneidensis* outer membranes where they may act as respiration-linked, Fe(III) terminal reductases. The *S. oneidensis* and *S. putrefaciens* type II protein secretion mutants [i.e., Fe(III) respiration-deficient mutants lacking the Fe(III) reductase on the outside face of their outer membrane] displayed wild-type Tc(VII) and U(VI) reduction activities. These findings indicate that *S. putrefaciens* and *S. oneidensis* do not require functional type II protein secretion or Fe(III) reduction systems to reduce Tc(VII) or U(VI), and support the previous observation that Tc(IV) and U(IV) are precipitated in the periplasmic space (and not on the cell periphery) of Tc(VII)- and U(VI)-reducing *S. putrefaciens* CN32.

Construction and Evaluation of *Desulfovibrio vulgaris* Whole-Genome Oligonucleotide Microarrays

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Desulfovibrio vulgaris Hildenborough has been the focus of biochemical and physiological studies in the laboratory, and the metabolic versatility of this organism has been largely recognized, particularly the reduction of sulfate, fumarate, iron, uranium and chromium. In addition, a *Desulfovibrio* sp. has been shown to utilize uranium as the sole electron acceptor. *D. vulgaris* is a δ-Proteobacterium with a genome size of 3.6 Mb and 3,584 open reading frames (ORFs). The whole-genome microarrays of *D. vulgaris* have been constructed using 70-mer oligonucleotides. All ORFs in the genome were represented with 3,471 (97.1%) unique probes and 103 (2.9%) nonspecific probes that may have cross-hybridization with other ORFs. In preparation for use of the experimental microarrays, artificial probes and targets were designed to assess specificity and sensitivity and identify optimal hybridization conditions for oligonucleotide microarrays. The results indicated that for 50-mer and 70-mer oligonucleotide arrays, hybridization at 45°C to 50ºC, washing at 37ºC, and a wash time of 2.5 to 5 min obtained specific and strong hybridization signals. In order to evaluate the performance of the experimental microarrays, growth conditions were selected that were expected to give significant hybridization differences for different sets of genes. The initial evaluations were performed using *D. vulgaris* cells grown at logarithmic and stationary phases. Transcriptional analysis of *D. vulgaris* cells sampled during logarithmic phase growth indicated that 25% of annotated ORFs were up-regulated and 3% of annotated ORFs were down-regulated compared to stationary phase cells. The up-regulated genes included ORFs predicted to be involved with acyl chain biosynthesis, amino acid ABC transporter, translational initiation factors, and ribosomal proteins. In the stationary phase growth cells, the two most up-regulated ORFs (70-fold) were annotated as a carboxynorspermidine decarboxylase and a 2C-methyl-D-erythritol-2, 4-cyclodiphosphate (MECDP) synthase. Spermidines are polyamines that are typically abundant in rapidly dividing cells and are essential growth factors in eukaryotic organisms. Polyamines are thought to stabilize DNA by the association of the amino groups with the phosphate residues of DNA and can also enhance tRNA and ribosome stability. The MECDP synthase enzyme is essential in *Escherichia coli* and participates in the nonmevalonate pathway of isoprenoid biosynthesis, a critical pathway present in some bacteria and apicomplexans but distinct from that used by mammals. Several of the highly up-regulated ORFs were annotated as conserved hypothetical proteins. Interestingly, an ORF that was predicted to contain a flocculin repeat domain was almost ninefold up-regulated in stationary phase cells compared to logarithmically growing cells. The flocculin domain is commonly observed in fungi, and is thought to play a role during flocculation (nonsexual aggregation of single-cell microorganisms).

These preliminary results have identified possible responses of *D. vulgaris* cells to stationary phase growth and suggest that polyamine production as well as cell aggregation and/or extracellular polymer production are responses of *D. vulgaris* during the stationary phase. Initial microarray results indicate that the recently produced oligonucleotide microarrays are functional. We are currently optimizing growth conditions in order to culture *D. vulgaris* cells in the presence of uranium(VI) and to monitor whole-genome expression levels.

Protein Expression in Wild-Type and Mutant *Shewanella oneidensis*

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Shewanella oneidensis is a facultative anaerobe capable of utilizing a variety of electron acceptors, but researchers know little about the molecular events responsible for this microbe's metabolic versatility. We have been studying changes in the complement of proteins expressed by this microbe in response to growth with different electron acceptors. By comparing the steady-state protein and mRNA expression in lysates from wild-type cells grown either aerobically or anaerobically with fumarate, ferric citrate, or nitrate, we have found that specific metabolic changes occur in response to the available electron acceptor. Several of the proteins found to change in abundance are involved in energy metabolism and substrate transport. In addition, by analyzing steady-state protein and mRNA expression in *S. oneidensis* cells with specific gene deletions (e.g., *fur*, *oxyR*, and a hypothetical protein) and comparing them with findings in wild-type cells, we discovered alterations in the abundance of proteins unrelated to those encoded by the deleted genes. In general, although a small number of specific protein changes have been observed in experiments with whole cell lysates from either wild-type or mutant cells, a majority of the *S. oneidensis* proteins detected are maintained at consistent steady-state levels under all of the growth conditions studied thus far. In comparisons of mRNA and protein expression, we have found good correlation between the steady-state level of some proteins and the abundance of the corresponding mRNAs, but not for all. The lack of correlation suggests that regulatory mechanisms exist that cause some proteins to be maintained at steady-state levels in spite of mRNA abundance.

Diverse terminal reductases and components of the electron transport chain, many of which are located in the cell membrane, have been observed to be expressed differentially in response to different metals. However, the sequence of reactions in the electron transport chain involved in metal reduction (as well as the exact roles of the individual proteins and the regulation of their expression) is not completely understood. To more specifically address the events involving the electron transport aspect of *S. oneidensis* metabolism, we are focusing our analysis of protein expression on membrane proteins isolated from both wild-type and mutant cells. We are using a commercial membrane protein isolation kit II (BioRad), as well as sucrose density gradient centrifugation methods developed by Myers and Myers, to obtain fractions enriched in inner and outer membrane proteins. Thus far, we have identified conserved hypothetical proteins, heme proteins including cytochromes, chaperonins, TonB-dependent receptor proteins, transport proteins, and chemotaxis proteins.

Metabolic Engineering of Microorganisms for Actinide and Heavy-Metal Precipitation

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Heavy metals and actinides are significant problems at a number of DOE sites and industrial locations in the U.S. Many of these sites contain heavy metals, actinides, and organics. Due to 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 be useful in treating waste tanks at such sites as Hanford that contain mixed organics, metals, and actinides.

We have successfully engineered *Escherichia coli*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* to remove heavy metals and actinides from solution and to 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 desulfhydrase 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* to enable 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 and have potential for metal and actinide removal from contaminated waste streams or immobilizing these elements in situ. We examined U(VI) interactions with phosphate-containing species using Raman spectroscopy and time-resolved laser-induced fluorescence spectroscopy (TRLFS). TRLFS of U(VI) showed that *P. aeruginosa*—genetically engineered to accumulate polyphosphate, subsequently degrade it, and secrete phosphate—precipitated U(VI) quantitatively at pH 4.5. The same bacterial strain, not induced to secrete phosphate, sorbed only a small amount of U(VI).

Signature Tagged Mutagenesis (STM) for Identification of Genes Required for In Situ Sediment Survival of *Desulfovibrio* Strain G20 and *Shewanella oneidensis* MR-1

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Sediment-dwelling anaerobic bacteria are capable of utilizing a wide range of electron donors and acceptors, and they play a key role in the bioremediation of radionuclide contaminants in the subsurface. We believe that these subsurface microorganisms have unique genes that are required only during exposure to the natural environment. *Desulfovibrio* strain G20 and *Shewanella oneidensis* MR-1 were used as model organisms for signature tagged mutagenesis (STM) to identify these genes. This is the first report where STM has been used to identify genes which are essential for survival of subsurface microorganisms in the environment.

Six thousand mutants of *Desulfovibrio* strain G20 and 12,000 mutants of *Shewanella oneidensis* MR-1 were generated by tagged Tn10 transposons and assembled into pools of 60 mutants, representing the 60 unique tags in use. Each pool was incubated in sediments to identify potential nonsurviving mutants by a microarray-adapted method we have previously developed. Strain G20 was incubated in subsurface sediments at room temperature for 7 to 9 days. For *Shewanella*, extensive studies were conducted to find a suitable sediment system to use for STM. Addition of lactate was found to be a minimum requirement in order for these organisms to grow and survive in Fe-reducing sediments [subsurface sediments amended with Fe(III) oxyhydroxide]. *Desulfovibrio* and *Shewanella* pools were screened by reisolating cells from sediment when they grew to maximum concentration, or several days following peak growth. Populations of *Desulfovibrio* increase 10- to 50-fold over their initial inoculum during the first week of incubation, and *Shewanella* increase at least tenfold over 3 days. This growth is necessary for selection of nonsurviving mutants.

Using our microarray hybridization technique, approximately 3% of all screened mutants were found to be attenuated in sediment survival for both organisms. These potential nonsurvivors were then tested again in sediment individually or with the wild-type strain. Sequence information for those mutants that did not grow in the same manner as the wild-type strain during the second screening will be presented. We also tested mutant survival using flow-through columns to emphasize certain features of aquifers that are not emphasized in a static system. In aquifer systems, groundwater moves, and bacteria must attach in order to be maintained in the system. In a similar manner, column systems will emphasize attachment.

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

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

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

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

During the first year of this grant we have focused on identifying the Tc(VII) reductase of *G. sulfurreducens*. Biochemical and genetic evidence have shown that the enzyme responsible for Tc(VII) reduction in *G. sulfurreducens* is a NiFe hydrogenase localized in the periplasm. Hydrogen was the sole electron donor used for Tc(VII) reduction, with CO profiling experiments suggesting the involvement of a NiFe hydrogenase. Treatment of whole cells with Cu(II), which is toxic to hydrogenases and can access the periplasm but cannot penetrate the cytoplasmic membrane, completely inhibited Tc(VII) reductase activity. Protease treatment of whole cells resulted in complete inhibition of insoluble metal reduction [e.g., Fe(III) oxides] on the surface of the cell but had no impact on Tc(VII) reduction. These results confirm that Tc(VII) is reduced in the periplasm (which is also supported by transmission electron microscopy studies) via a mechanism that is distinct from that used to reduce Fe(III) oxides. Finally, a *G. sulfurreducens* knockout mutant that is unable to synthesize the principal periplasmic NiFe hydrogenase (obtained from Derek Lovley's group in Amherst) was also unable to couple the oxidation of hydrogen to Tc(VII) reduction, unequivocally identifying the Tc(VII) reductase of *G. sulfurreducens*.

Additional experiments have focused on the mechanism for the reduction of key actinides, including U(VI), Np(V), and Pu(IV). We have previously shown that U(VI) is reduced via a mechanism that involves a periplasmic cytochrome c_7. Ongoing work aims to obtain baseline information on the reduction of other key actinides and metals by *G. sulfurreducens*, prior to determining the role of cytochrome c_7 and other redox active proteins on such transformations. The focus of our current research is to develop analytical tools for monitoring the reduction of penta- and tetravalent actinides in microbial culture, and we have now conducted preliminary experiments using Np(V) as a potential electron acceptor. We have also conducted experiments using an *E. coli* strain engineered to overproduce cytochrome c_7. Initial experiments suggest that this cytochrome confers the ability to reduce metals, including U(VI), on *E. coli*, and may give a useful model for determining the role of cytochrome c_7 in the reductive precipitation of contaminant metals.

Biomolecular Mechanisms for Microbe–Fe(III) Oxide Interactions in *Geobacter* Species

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The most promising strategy for the in situ bioremediation of radioactive groundwater contaminants that has been identified by the NABIR program to date is to stimulate the activity of dissimilatory metal-reducing microorganisms to reductively precipitate uranium, technetium, and radioactive cobalt. Previous studies with a variety of subsurface sediments, including those at uranium mine tailing (UMTRA) sites, have indicated that *Geobacteraceae* are the primary agents for metal reduction and that, even when uranium levels are high, electron transfer to Fe(III) reduction accounts for ca. 99% of the growth of the *Geobacteraceae*. These results suggest that, in order to understand the factors controlling the growth and activity of the predominant U(VI)-reducing microorganisms during subsurface bioremediation, it is important to understand how *Geobacteraceae* interact with the Fe(III) oxides.

Significant progress was made in understanding the role of Type IV pili in Fe(III) oxide reduction. A knockout mutation in *pilA*, the gene for the structural protein of the pili, eliminated the ability of *Geobacter sulfurreducens* to reduce Fe(III) oxide, but not soluble electron acceptors, including Fe(III) citrate. The mutant could reduce Fe(III) oxide if the Fe(III) chelator, nitrilotriacetic acid, or the electron shuttle, anthraquinone-2,6-disulfonate, was added. Complementation of the mutant *in trans* with a functional *pilA* gene also restored the capacity for Fe(III) oxide reduction.

Physically shearing surface-associated proteins from *G. sulfurreducens* revealed two *c*-type cytochromes that were expressed during growth on insoluble Mn(IV) oxide, but not during growth on soluble electron acceptors. The genes for these cytochromes were identified with MALDI-TOF mass spectrometry, and deletion mutations were made in each of the cytochrome genes. The mutants could not grow with insoluble Fe(III) oxide as an electron acceptor, but growth on soluble, chelated Fe(III) or fumarate was not affected. These results suggest that these two cytochromes, which are located on the outside of the cell, are required for electron transport to Fe(III) oxide in *G. sulfurreducens* and that expression of these genes is highly regulated in response to the electron acceptors available for reduction.

A mutation in a novel secretion system in *G. sulfurreducens* also selectively inhibited Fe(III) oxide reduction, but not reduction of soluble electron acceptors. The mutant continued to form pili, but other proteins were not exported to the outer membrane. The gene for one of these proteins, which has high homology to a manganese-oxidizing protein in *Leptothrix discophora*, is required for growth on insoluble Fe(III) oxides, but not soluble electron acceptors.

These are the first reports of electron transport proteins specifically involved in the electron transport to Fe(III) oxide in any microorganism. This information is leading to a new model for electron transfer from the cell surface to the surface of Fe(III) oxides.

Comparative Biochemistry and Physiology of Iron-Respiring Bacteria from Acidic and Neutral pH Environments

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In contrast to their neutrophilic counterparts, acidophilic dissimilatory iron-reducing bacteria (DIRB) have not been extensively studied. This project is focused on the physiology and biochemistry of a representative acidophilic iron reducer, *Acidiphilium cryptum* JF-5. Preliminary data gathered thus far show *A. cryptum* to be an excellent system for these types of studies. Culturing methods have been developed that result in higher cell yields, and the organism is now routinely cultured in a continuous culture bioreactor, under both aerobic and anaerobic conditions. Preliminary biochemical analysis shows the presence of c-type cytochromes in cell extracts, and molecular "gene-discovery" tools have detected genes encoding cytochromes c. We have also examined the ability of *A. cryptum* JF-5 to reduce Fe(III) and other metals, including U(VI) and Cr(VI), in cell suspensions at pH 2.5. Cells (10^9) reduced Fe(III) at a rate of 1.5 μmol hr^{-1} when glucose was present as an electron donor. In the absence of exogenous glucose, Fe(III) was still reduced, albeit at a slower rate of 0.75 μmol hr^{-1}, indicating the presence of reserve reducing equivalents, possibly as storage carbohydrate. We also examined the reduction of $KCrO_4$ by JF-5. Chromium(VI) was toxic to the cells at conc. ≥ 100 μM. Below this concentration, Cr(VI) was removed from solution within hours, presumably as a Cr(III) species. U(VI) and the humic acid analog AQDS were not reduced during two weeks of incubation. The results indicate that *A. cryptum* may influence the fate of not only Fe, but also Cr in low-pH environments. It thus appears that acidophilic DIRB share certain features and abilities with their neutrophilic counterparts, but additionally have other traits that allow for this type of metabolism in extreme environments. Continuing investigation will include purification of proteins involved in electron transport to metals, characterization of attachment mechanisms to solid phase electron acceptors, and comparison of overall findings to known neutrophilic iron-reducing organisms.

Biomolecular Strategy to Decrease Chromate Toxicity to Remediating Bacteria

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Cr(VI) (chromate) is a widespread, toxic, and soluble environmental pollutant. Bacteria can reduce chromate to insoluble and less toxic Cr(III); thus, chromate-bioremediation-utilizing bacteria are of interest. Chromate, however, is toxic to the remediating bacteria; application of genetic and protein engineering approaches can ameliorate this problem and also improve chromate bioremediation in other respects [1]. Towards this end, we have conducted an extensive study of bacterial chromate reductases [2–6]. A major reason for chromate toxicity is the generation of reactive oxygen species (ROS) during its reduction. Using enzyme purification and in silico approaches, we cloned several bacterial genes with presumptive capacity to encode chromate reductase activity and have examined several characteristics using pure enzymes. The propensity to generate ROS during chromate reduction was examined using rapid scan kinetics, electron spin resonance measurements, as well as H_2O_2 generation. In this respect, these enzymes, all of which are flavoproteins and coexist in bacterial cells, fall into three categories. The first is exemplified by the protein YieF, which belongs to a protein family known for its obligatory two-electron reduction of electrophiles. The YieF dimer did not generate the flavin semiquinone form of the enzyme, nor (likely) also Cr(V) during chromate reduction, and transferred only 25% of the reductant (NADH) electrons to H_2O_2. Thus, YieF tightly maintained its obligatory two-electron transfer characteristic even when catalyzing a three-electron-requiring Cr(VI) to Cr(III) reduction ("tight chromate reductases"). The second category is exemplified by the proteins ChrR and NfsA, which belong to a similar family of proteins. However, flavin semiquinone forms of NfsA and Cr(V) were generated during chromate reduction, and over 25% of the NADH electrons were consumed in generating ROS, indicating these enzymes maintain their obligatory electron transfer characteristic only semi-tightly during chromate reduction ("semi-tight chromate reductases"). ChrR was shown to protect *P. putida* against chromate toxicity, and YieF is likely to be even more effective in this respect. The third category is exemplified by lipoyl dehydrogenase. It was previously shown that large and persistent amounts of Cr(V) are generated during chromate reduction by this enzyme, and we found that 60% of the NADH electrons were directed to ROS generation during chromate reduction catalyzed by this enzyme ("single electron chromate reducers"), which was more than generated by either of the above two enzyme categories. Thus, our strategy to minimize chromate toxicity is to generate bacteria in which the flow of chromate is enhanced through the reductive pathways represented by the tight and semi-tight chromate reductases, so as to preempt its reduction by the single electron reducers of the cell.

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Studies of Multi-Heme Cytochromes from *Geobacter sulfurreducens*

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

Previously, we determined the three-dimensional structure of three-heme cytochrome c_7 PpcA (coded by ORF01023) characterized by Lloyd et al., and we identified in the *G. sulfurreducens* genome ORFs for several of its homologs. Four of the ORFs are the same size as PpcA; three other ORFs are polymers of c_7-type domains, two of which consist of four domains and one of nine domains.

We have now cloned, expressed, purified, and crystallized all four three-heme homologs of PpcA, coded by ORFs 601, 603, 2938, and 1734 that have 77, 62, 65, and 57% identity respectively with PpcA. We have determined the structure of two of them, ORF00603 and ORF01734. Although these proteins are highly homologous to each other, their structures differ: that is, their surface characteristics and the arrangement of the hemes are different. We found that their thermal stabilities are also different, and it is expected that they will have different reduction properties as well. Lovley's group is working on the elucidation of the physiological functions of the above homologs, which will make it possible to determine the functional correlates of the observed structural differences.

We further developed methods to express cytochromes *c* with up to 12 hemes in *E. coli* and to purify them. We cloned and expressed individual domains, two contiguous domains, and recently the complete four-domain protein coded by ORF03300. We purified and crystallized domains C and D, the two-domain protein CD, and the complete four-domain protein. We have determined the x-ray structures of domain C and the two-domain protein CD. These c_7-type domains that form the polymers represent a new family of cytochromes *c* that has not been previously described. Based on the three-dimensional structures of individual domains, they differ from PpcA. While two of the hemes are bis-histidine coordinated, as found in cytochromes c_7 and c_3, the third one is coordinated by a histidine and a methionine that are expected to make its redox potential more positive than those of the other two. Indeed, the midpoint reduction potential of domain C is –105mV, 50mV more positive than that of PpcA, as determined by our collaborator Prof. Carlos Salgueiro (Universidade Nova de Lisboa). The structure of the two-domain protein shows that the domains form a chain, which is as we have predicted based on the packing of the molecules in the crystals formed by PpcA. The arrangement of all four domains in the complete molecule coded by ORF03300 will be of special interest, as we recently collected diffraction data on the complete protein.

Protein Engineering in the Hg(II) Resistance (*mer*) Operon

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NABIR research objectives. The widely found bacterial mercury resistance (*mer*) operons functions in Hg biogeochemistry and bioremediation by converting reactive inorganic [Hg(II)] and organic [RHg(I)] mercurials to relatively inert monoatomic mercury vapor, Hg(0). We study its metalloregulator, MerR, and the two enzymes which mediate these processes, MerA, the mercuric reductase, and MerB, the organomercurial lyase to dissect their basic mechanisms and accelerate their in vivo and in vitro performance.

Results and plans. Cytosolic expression of an engineered single polypeptide (107aa) antiparallel ("hairpin") coiled-coil metal binding domain (MBD) derived from MerR protects *E. coli* (1,2) and *Deinococcus* (2) from Hg(II). Outer-membrane expression of MBD also protects *E. coli* (2). MerR and MBD bind other thiophilic metals with unique coordination geometries distinct from S_3 coordination they use for Hg(II), and they exhibit partly overlapping metal preferences, suggesting enhanced flexibility in MBD. This work defines a novel metallosequestration domain and establishes that MerR's specificity in transcriptional activation does not reside just in metal binding. Near-term work will complete determinations of kinetic and thermodynamic parameters of metal interactions of both proteins. This will be followed by analysis of point mutants with altered metal specificity to define the metal-driven allosteric responses of the MerR coiled-coil and to discern at what steps in transcriptional activation metals are distinguished. We also continue efforts on 3-D structure determination of MerR.

MerB. With UGA crystallographer Cory Momany, we are currently analyzing a data set for MerB-Hg collected to 2 Å (7% Rsym) at the Advanced Photon Source. It has two clear Hg sites, consistent with 2 molecules per asymmetric unit. We expect this data set to provide an initial MerB 3-D structure but will continue growing crystals for both MerB and MerB-Hg (and other metals) as backups.

MerA. The plasmid- and transposon-borne *mer* operon functions in many different cytosolic environments. With Sue Miller (UCSF) we're dissecting its dependence on the cytosolic redox buffering system, which varies widely among prokaryotes. We've found that in *E. coli* lacking its major cytosolic thiol buffer, glutathione (GSH), the specific activity of Hg(II)-reductase (MerA) in cells expressing the complete *mer* operon (MerA plus transport proteins MerTPC) declines by 25%, compared to the parental strain with a consequent 50% decline in the Hg resistance phenotype compared to the wild-type parental strain. In GSH cells expressing only MerA or its catalytic core domain (which lacks the N-terminal metal chaperone domain) the specific activity of both forms of the enzyme declines by ca. 55%, indicating that the metal chaperone domain of MerA cannot compensate for a lack of cytosolic low molecular weight thiols. We have also characterized intracellular reduction of Ag(I) and Au(I/III) by wild-type *E. coli* expressing the complete *mer* operon, and are extending this work to metals of DOE interest, including Co and Cr.

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Elucidating the Molecular Basis and Regulation of Chromium(VI) Reduction by *Shewanella oneidensis* MR-1 and Resistance to Metal Toxicity Using Integrated Biochemical, Genomics, and Proteomic Approaches

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The mediation of metal reduction by microorganisms has been investigated intensively from physiological and biochemical perspectives; however, little is known about the genetic basis and regulatory mechanisms underlying the ability of certain bacteria to transform or immobilize a wide array of heavy metals contaminating DOE field sites. Chromium(VI), for example, is one of several risk-driving contaminants at DOE sites and has been targeted by DOE for bioremediation research. The bacterium *Shewanella oneidensis* MR-1 can potentially be used to immobilize chromium, a toxic and mutagenic metal, by reducing soluble Cr(VI) to the insoluble and less bioavailable form of Cr(III), thus facilitating its removal from contained-storage and natural sites. The overall goal of this study is to integrate targeted biochemical and proteomic analyses with genome-wide gene expression profiling to examine the molecular basis and regulation of chromium(VI) reduction by *Shewanella oneidensis* MR-1. Towards this goal, we will (1) isolate and identify the terminal chromium(VI) reductase and the gene(s) encoding this activity using whole-genome sequence information for MR-1 and liquid chromatography-tandem mass spectrometry (LC-MS/MS) in conjunction with conventional protein purification and characterization techniques, (2) verify the function of the gene(s) encoding the terminal Cr(VI) reductase and compare whole transcriptome data with whole proteome data in order to understand the regulation of chromium reduction, and (3) investigate the molecular stress response and adaptation of *S. oneidensis* to high levels of soluble Cr(VI). Use of whole-genome microarrays for *S. oneidensis* under conditions deemed toxic and attractive will help to describe the global cellular regulation of Cr(VI) reduction. This research will provide important information on the functional components and regulatory mechanisms of microbial metal reduction, which should prove valuable in developing effective assessment strategies for in situ bioremediation, and in genetically engineering desired bacteria for enhanced bioremediation.

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

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Bacteria in the genus *Shewanella* grow by transferring electrons to soluble and insoluble metals for energy production, thereby decreasing metal toxicity and mobility. Humic compounds are known to accelerate the process by which microorganisms transfer electrons to metals. The pigment melanin is a particularly important humic compound in this process, and is produced by *Shewanella oneidensis* MR-1. In the presence of melanin, *S. oneidensis* MR-1 reduces the insoluble mineral hydrous ferric oxide (HFO) at a greater rate than without melanin. This is accomplished because, under anaerobic conditions, melanin serves as a terminal electron acceptor and soluble electron shuttle to iron minerals.

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

To date we have demonstrated the inhibition of melanin production by *S. oneidensis* MR-1 with the enzyme inhibitor sulcotrione [2-(2-chloro-4-methane sulfonylbenzoyl)-1,3-cyclohexanedione]. Sulcotrione is a competitive inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4HPPD). This enzyme is responsible for homogentisic acid , the precursor to melanin production in *S. oneidensis* MR-1. Using the suicide vector system (pDS3.1) developed in our lab, we generated a 700 bp deletion in the gene *melA*, which encodes for 4PHPPD. The *melA* lacking mutant was unable to produce melanin in the presence of tyrosine. Preliminary studies indicate that HFO reduction was not completely halted in the absence of *melA*; however, the HFO reduction rate was decreased. Studies related to the role of melanin production in iron reduction are ongoing.

Genes for Uranium Bioremediation in the Anaerobic Sulfate-Reducing Bacteria

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The ability of members of the genus *Desulfovibrio* to participate in a variety of redox reactions with heavy metals and radionuclides has drawn the attention of those seeking novel approaches to bioremediation of those materials. To realize this objective, the pathway of electrons from various electron donors to sulfate or these alternative electron acceptors must be elucidated so that rate-limiting steps and control points may be identified. Both a periplasmic hydrogenase and the primary tetraheme cytochrome c_3 have been shown to be necessary and sufficient for in vitro U(VI) reduction, with hydrogen as the electron donor. Earlier we provided evidence supporting the in vivo role of the tetraheme cytochrome c_3 in electron flow from hydrogen to U(VI).

To examine the roles of hydrogenases, we have tested mutants and inhibitors. Results with Fe-hydrogenase mutants of *Desulfovibrio* were interpreted to mean that this enzyme was not essential for U(VI) reduction with hydrogen. Nitrite addition, reported to inhibit the Fe- and NiFeSe-hydrogenase of *Desulfovibrio vulgaris*, had no effect on U(VI) reduction with pyruvate but did decrease the ability of lactate or hydrogen to provide electrons by about 50%.

Additional studies with the uncoupler, carbonyl cyanide m-chloro phenyl hydrazone (CCCP), showed differential pathways of energy generation from pyruvate and lactate. CCCP-inhibited pyruvate supported U(VI) reduction in resting cells, and pyruvate supported growth of *Desulfovibrio,* but neither activity was inhibited when lactate was used as a substrate. Both pyruvate and lactate would be predicted to be able to provide energy by substrate-level phosphorylation; thus the differential inhibition was unexpected. However, growth of the mutant lacking the tetraheme cytochrome c_3 also reveals a dramatic difference between the two substrates with growth on lactate being nearly wild type in rate, and extent and growth on pyruvate being greatly impaired.

Clear evidence has now been obtained for compensation for the loss of the tetraheme cytochrome c_3. A large increase in the expression of the high molecular weight cytochrome occurs in the mutant lacking the tetraheme protein. Studies are under way to determine whether this cytochrome is responsible for the residual electron flow to U(VI) from pyruvate or lactate in the mutant.

PROGRAM ELEMENT 4
Biogeochemistry

Biogeochemical Processes Controlling Microbial Reductive Precipitation of Radionuclides

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(Collaborators: A. Beliaev, D. Elias, and J. Zachara)

For microbial reductive precipitation of radionuclides to be useful for groundwater remediation, bacteria must effectively reduce target contaminants, and reduced products must be stable to reoxidation by naturally occurring oxidants present in the system. This project focuses on identifying and quantifying the coupled biogeochemical reactions that control the reductive precipitation of U(VI) and Tc(VII).

Bacterial U(VI) reduction. U precipitates were observed by transmission electron microscopy (TEM) in association with cell periplasm and outer membrane. Subsequent time course analyses indicated periplasm-associated $UO_{2(s)}$ is eventually localized to cell exterior as aggregates of a fine-grained precipitate and in association with fiberlike features that radiate from the cell surface. This led to a hypothesis that metal-reducing *Shewanella* have an active mechanism for exporting nm-sized $UO_{2(s)}$ from the cell envelope. The type II secretion pathway, the terminal branch of the general secretion pathway (GSP), is responsible for the extracellular secretion of proteins including toxins and hydrolytic enzymes. An *S. oneidensis* MR-1insertional mutation in the *gspD* gene (provided by A. Belieav) was characterized with regards to U(VI) reduction. The MR-1 *gspD* mutant was found to reduce U(VI), but at a reduced rate relative to the wild type. When thin sections of the *gspD* mutant were examined by TEM, nm-sized $UO_{2(s)}$ was localized to cell periplasm and the outside of the outer membrane; extracellular U solids were nearly absent. In contrast, MR-1 cells treated in an identical manner initially contained both extracellular and periplasmic $UO_{2(s)}$, but upon extended incubation, U was localized to the cell exterior. These results suggest U(VI) enters the periplasm through the outer membrane as a uranyl carbonate complex and is subsequently reduced. The poorly crystalline nm-sized $UO_{2(s)}$ particles that form are subsequently exported by the cell via an unknown mechanism, and the $UO_{2(s)}$ is removed from the cell surface via a secreted protein. Although additional work is required to establish the precise mechanisms, the results have significant implications with regards to the fate and transport of microbially reduced radionuclides.

Manganese oxides. Mn(III,IV) oxides are among the most powerful, naturally occurring oxidants and may promote the oxidative dissolution of uraninite ($UO_{2,cr}$) and TcO_2. Thermodynamic calculations predict, and batch laboratory experiments confirm, that manganese oxides oxidize UO_2 and TcO_2. Nevertheless, the rate at which this solid–solid redox reaction occurs is poorly known. The oxidation of biogenic uraninite (precipitated and harvested from cultures of *Shewanella putrefaciens* CN32) by well-characterized manganese oxides has been measured. Experiments were conducted at pH 6.9 in 30 mM $NaHCO_3$, using an initial UO_2 concentration of 250 μM and initial Mn-oxide concentration of 100 m^2/L. The measured ratio of Mn(II):U(VI) produced agreed well with the expected ratio based on direct measurement of the oxidation state of Mn and U in the respective starting solids. Production of U(VI) in the presence of the Mn(IV) oxides $MnO_{2,am}$, nsutite (γ-MnO_2), and the mixed oxide hausmannite (Mn_3O_4) was described well by a single pseudo-first-order product curve, whereas U(VI) production in the presence of the Mn(III) oxides manganite (γ-MnOOH) and bixbyite (Mn_2O_3) were described by two simultaneous pseudo-first-order product curves ($P < 0.001$). Addition of Mn(II) to vials containing Mn_2O_3 or prereduction of Mn_2O_3 with AH_2DS had no effect on the rate of U(VI) production. By contrast, treating γ-MnO_2 with AH_2DS prior to introducing UO_2 resulted in a significantly faster rate of U(VI) production; the enhanced rate of U(VI) production was evident with repeated additions of UO_2 to the system. We continue to examine the kinetics of UO_2 oxidation as a function of solution composition and are conducting similar experiments using continuous flow through methods.

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

We have continued our investigations of the redistribution of actinide species during aerobic dissolution of Fe-bearing minerals. One example is as follows: *Pseudomonas putida* is a common siderophore-producing bacteria in soil and subsurface environments. We began our experiments with *P. putida* by assessing the toxicity of adsorbed U(VI). Fe-deficient medium (FeDM) was prepared by adding the following analytical grade ingredients to 1.0 L of distilled water in acid-washed and rinsed flasks: 0.67 g of glycerol-2-phosphate; 1.0 g of NH_4Cl; 0.2 g of $MgSO_4 \bullet 7\ H_2O$; 0.05 g of $CaCl_2 \bullet 2\ H_2O$; 5.0 g of succinic acid disodium salt anhydrous, and 4.19 g MOPS. The medium was then chelexed with 10 g/L of chelating resin for one hour and filtered through a 0.22 μm membrane. After filtering, 0.125 mL of trace elements (0.005 g of $MnSO_4 \bullet H_2O$; 0.0065 g of $CoSO_4 \bullet 7\ H_2O$; 0.0023 g of $CuSO_4$; 0.0033 g of $ZnSO_4$; and 0.0024 g of MoO_3 per 100 mL of distilled, deionized water) was added to the medium. The pH of the medium was ~7.45. The medium was then transferred into acid-washed/rinsed 250 mL polycarbonate flasks to a final volume of 50 mL. Hematite (29 m^2/L) was added to the necessary flasks. The flasks were sterilized by autoclave, and filter-sterilized U(VI) (0.055 mM) was added, approximately 54 hours prior to inoculation. The initial cell density of *P. putida* was 3×10^{-6} cells/mL. The growth of *P. putida* was not greatly impacted by the addition of 0.055 mM U(VI) to media containing hematite. Growth may have been slightly inhibited in the cultures containing U(VI), but the maximum cell density was similar for both cultures. We are currently determining the redistribution of U in these experiments, and expect U to be mobilized by bacteria similar to previously reported studies.

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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The focus of this project is to elucidate the processes that lead to enhanced stabilization (i.e., immobilization) of dissolved and colloidal Pu species through the action of microbial communities. Significant results of this work include the following.

Production of exopolysaccharides (EPS). The production of EPS by *Pseudomonas fluorescens, Clostridium* sp., and *Shewanella putrefaciens* has been optimized, and the EPS has been characterized by a variety of techniques, including GC-EI-MS. In general, carbohydrates (TCHO) and proteins are the major compounds of the EPS, the TCHO pool is dominated by uronic acids, and, while some acid polysaccharides are found in the EPS fraction, other components remain to be identified. The distribution of EPS compounds varies, depending on the microbial source. For example, the TCHO and protein fractions of *Pseudomonas fluorescens*, *Shewanella putrefaciens*, and *Clostridium* sp. are of the order of 40% and 10–40%, respectively, depending on the batch and preparation method.

Pu complexation with the EPS. Experimental methodologies were developed to evaluate the binding of Pu to the microbially generated EPS. In all cases, Pu appears to be complexed in the +IV valence state. Differences in the composition of the EPS are reflected in the binding of Pu to the ligands. A general sequence of binding affinity for Pu(IV) with the microbially produced EPS is *Clostridium* sp. > *Shewanella putrefaciens* > *Pseudomonas fluorescens*.

Biotransformation of Pu–citrate Complex. Pu(IV)–citrate affected the rate and extent of citric acid metabolism by *Pseudomonas fluorescens.* Reduction in citrate metabolism (>50%) was observed in medium containing 10^{-5} M 1:10 Pu:citric acid. The processes responsible for the inhibition are being investigated. Structural characterization of Pu-citrate complex is being determined by extended x-ray absorption fine structure (EXAFS) at the NSLS and electrospray ionization-mass spectrometry (ESI-MS). Initial studies with ESI-MS indicate Pu is added as Pu(IV)-nitrate forms a bi-ligand Pu-cit_2 complex in the presence of excess citric acid. In addition to citric acid, the presence of oxidized organic fragments of lesser molecular weight suggests the degradation of citric acid by Pu. These fragments also form a complex with the Pu, resulting in the formation of Pu-mixed ligand complexes.

Effect of microbial processes on Pu mobilization and immobilization and Pu speciation in contaminated soils. Plutonium-contaminated soils collected from the Rocky Flats Environmental Technology Site (RFETS) 903 pad (>100 pCi Pu per gram of soil), one in which Pu is predominantly associated with the organic fraction, the other with the oxide fraction, were chosen for this study. Both soils showed association of Pu with phases containing iron. The soils were incubated under anaerobic conditions with water (no added carbon source), or amendments with either glucose or lactate. The pH, Fe species in solution, organic acid production, headspace gas, and Pu in the solution phase were analyzed. Iron reduction and dissolution occurred to the greatest extent in glucose-amended samples. The greatest amount of Pu was detected in the solution phase of glucose-amended soil. These results show that fermentative microbial activity can mobilize stable Pu in contaminated soil possibly due to dissolution of iron phases. Sequential selective extraction will be performed on soils after microbial action to examine the redistribution of Pu, and sequential microfiltration of the solution phase will be used to identify the predominant colloid size fraction with which the Pu resides. Similar experiments with soils incubated under aerobic conditions are underway.

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

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Trace-metal and/or radionuclide bioremediation schemes require that specific redox conditions be achieved at given zones of an aquifer. Tools are therefore needed to identify the terminal electron acceptor processes (TEAPs) that are being achieved during bioremediation in an aquifer. Dissolved hydrogen (H_2) concentrations have been shown to correlate with specific TEAPs during bioremediation in an aquifer. Theoretical analysis has shown that these steady-state H_2 levels are solely dependent upon the physiological parameters of the hydrogen-consuming microorganisms, with H_2 concentrations increasing as each successive TEAP yields less energy for bacterial growth. The objective of this research is to determine if H_2 can still be used as an indicator of TEAPs during a bioremediation scheme when an organic substrate is injected into the subsurface, and organisms may simultaneously consume H_2 and carbon.

Batch experiments were conducted to study the utilization of acetate and H_2 by *Geobacter sulfurreducens* under iron-reducing conditions when either acetate or H_2 is the sole electron donor, and when both are present and utilized simultaneously. The results indicate that though the Monod kinetic coefficients describing the rate of H_2 utilization under iron-reducing conditions correlate energetically with the coefficients found in previous experiments under methanogenic and sulfate-reducing conditions, conventionally measured growth kinetics do not predict the steady-state H_2 levels typical for each TEAP. In addition, with acetate and H_2 as simultaneous electron donors, there is slight inhibition between the two electron donors for *G. sulfurreducens*, and this can be modeled through competitive inhibition terms in the classic Monod formulation, resulting in slightly higher steady-state H_2 concentrations under steady- state conditions in the presence of acetate. This dual-donor model indicates that the steady-state H_2 concentration in the presence of an organic electron donor is not only dependent on the biokinetic coefficients of the TEAP, but also on the concentration of the organic substrate, and that the H_2 concentration does not start to change very dramatically as long as the organic substrate concentration remains below the half-saturation constant.

Column experiments were performed to measure steady-state H_2 concentrations under iron-reducing conditions using NABIR Field Research Center background soil. Steady-state H_2 concentrations in a flow-through column were higher than observed for iron-reducing conditions in the field, even though evidence suggests that iron reduction was the dominant TEAP in the column. Additional column experiments were performed to determine the effect of iron bioavailability on steady-state H_2 concentrations using the humics analogue, AQDS (9,10-anthraquinone-2,6-disulfonic acid). Slightly lower steady-state H_2 levels were observed in the presence of AQDS, indicating that even though iron reduction does occur, a decreased bioavailability of iron may inhibit iron reduction such that H_2 concentrations increase to levels that are more typical for less energetically favorable reactions (sulfate-reduction, methanogenesis).

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

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

The aim of this new project is to use a highly multidisciplinary approach to identify the biogeochemical factors that control the mobility of environmentally relevant concentrations of Tc(VII) in FRC sediments. We will use a combination of geochemical, mineralogical, microbiological, and spectroscopic techniques to determine the solubility and phase associations of Tc in batch sediment experiments ("progressive microcosms") where a sequence of terminal electron accepting processes is separated by time. Additional column experiments utilizing FRC sediments containing discrete biogeochemical zones will be challenged with low concentrations of ^{99m}Tc, and the mobility of the radionuclide imaged using a γ-camera. By comparing the pattern of Tc immobilization, with high-resolution studies of the mineralogy, geochemistry, and microbial ecology of the columns, we will further characterize the biogeochemical controls on Tc mobility in FRC sediments. Column experiments will also utilize similar approaches to determine the stability of immobilized reduced phases of Tc in the presence of oxidizing agents including nitrate. The effectiveness of strategies proposed to stimulate Tc(VII) reduction and precipitation in the subsurface will also be determined in both batch and column experiments. Finally the experimental results will be used to calibrate a modeling approach employing an established coupled speciation and transport code to provide parameters that could potentially be used to make predictions of the mobility of Tc in FRC sediments and other subsurface environments.

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

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

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

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This project builds on our prior research to integrate laboratory and field studies focused on microbial U reduction at hematite surfaces. In the field, sampling for characterization of subsurface microbial communities is restricted by drilling costs, contaminated groundwater disposal, and difficulty with aseptic techniques. As an alternative, Biofilm Coupons (stainless-steel mesh cylinders loaded with mineral particles) were placed in the FRC-contaminated aquifer. A microbial community that attached to hematite surfaces was dominated by putatively metal-tolerant *Alcaligenes* spp. and acidophilic *Frateuria* spp., reflective of the high level of dissolved metals and low pH at the site. These populations were not a significant part of the community in nearby pristine groundwater. Current work is focused on understanding the influence of mineral type on attached community structure, and on the relationship between mineral coupon communities and those in the surrounding groundwater and sediments.

We are evaluating specific physiological activities of subsurface communities that develop on the coupons, and have focused on hydrogenase enzyme activities, as this enzyme is likely involved in electron transfer between metal-respiring bacteria and metal electron acceptors in the subsurface. To date, we have identified the DNA sequence of a 420 bp fragment of the [NiFe] hydrogenase gene from the sulfate-reducing bacterium *Desulfovibrio alaskensis,* and have shown successful in situ PCR amplification of the [NiFe] hydrogenase gene and 16S rDNA.

In the lab, in the presence of hematite and quartz, U(VI) was reduced to nanometer-sized uraninite particles by *Desulfovibrio desulfuricans* G20. Observations made at EMSL using HR-TEM, selected area electron diffraction, and energy-dispersive x-ray spectroscopic analysis confirmed that precipitated U associated with cells was uraninite with particle diameters of 2.6–3.6 nm. However, under electron donor–limited conditions, bioreduced U was partially reoxidized, likely by Fe(III) present in hematite. Using the same techniques, cell-free precipitates were found to be iron sulfides of varying Fe:S ratios. The *d*-spacings of randomly selected Fe-rich precipitates were most closely related to greigite. With G20 in column reactors containing hematite, dolomite, and calcite, over a period of two months of operation, columns with dolomite and calcite showed more than 99% U(VI) removal, while approximately 60% of U(VI) was removed from columns in the presence of hematite and a 10 mM bicarbonate buffer.

Comparison of the abiotic rates of reduction of uranyl ions by sulfide and sorbed Fe(II) (separately and together) is under investigation using an anoxic groundwater that closely simulates conditions found at the NABIR FRC (pH 6.4, high carbonate). Solid sorbents in these studies include hematite and quartz sand. Work has focused on sorption of Fe(II), which is low under these conditions, and on uranyl ions in the presence and absence of aqueous carbonate.

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, but experimental research systems with transport and biologically mediated redox reactions is severely lacking. We have proposed an examination of the reduction of U(VI) in the presence of nitrate and Fe(III)-containing minerals under conditions representative of biostimulation. We will present a summary of research to date for this project, which has focused on the biogeochemical processes that affect the transport of U(VI) in the subsurface. In particular, we will focus on results from three experimental systems that have been examined to date.

Experimental system 1. Uranium(U) sorption was studied using unreduced, bioreduced (with *Shewanella putrefaciens* CN32), and abiotically reduced (with DCB) FRC sediments. Factors including pH, ionic strength, and U(VI) and Fe(II) concentrations were studied on their influence on U sorption. Iron mineral phases in the sediments were determined by Mössbauer spectroscopy. Results showed that the bioreduced and unreduced FRC sediments had similar U sorption isotherms at pH below 7.8, despite the fact that they contained different iron mineral phases from the unreduced sediments. Above pH 8.0, however, U sorption was stronger in the bioreduced than unreduced sediments. The influence of Fe(II) on U sorption was minimal, suggesting that abiotic reduction of U(VI) by Fe(II) was not important in these experiments.

Experimental system 2. The factors of CO_2 concentration, pH, Fe(II) concentration, and aging of the biogenic U(IV) mineral phases were studied to determine their influence on the rate and extent of biogenic U(IV) remobilization in bioreduced sediments when they are reoxidized by atmospheric O_2. Results showed that the immobilized uranium was the most stable at circumneutral pH, and its stability increased with increasing Fe(II) concentration, implying that Fe(II) and biogeochemical conditions in subsurface sediments may be manipulated to maximize the long-term stability of immobilized uranium.

Experimental system 3. Sorption experiments have been conducted under flowing conditions to determine the U(VI) sorption rate to Fe-oxides under two carbonate concentrations (1 and 10 mmol/L) and a range of pH values (4–9). Carbonates can form complexes with U(VI), and these complexes appear to adsorb most strongly at circumneutral pH. Other NABIR researchers have previously reported the equilibrium sorption isotherms for U(VI) in batch systems. We are extending this work to examine the importance of the kinetics of U(VI) sorption under conditions of transport. Our experiments have been conducted in two DOE site sediments (Hanford) at groundwater velocities relevant to field remediation. The systems have been constructed at intermediate scale (50 cm long), and have been using artificial groundwaters that approximate geochemical conditions found at Hanford and the FRC. Our results so far indicate that the sorption of U(VI) in natural sediments cannot be treated as an equilibrium process at the groundwater velocities that are used here. This is an important observation, because it indicates that under conditions that apply to bioremediation, it may not be possible to assume equilibrium partitioning of U(VI) species to the solid phase. We are currently assessing whether the sorption is a diffusion- or rate-limited process.

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

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Chromium contamination exists at 13 of the 18 U.S. Department of Energy (DOE) facilities studied; microbial reduction to form insoluble trivalent Cr, Cr(III), is a potential treatment for such sites. In our prior research on Cr(VI) reduction, we discovered that soluble organo–Cr(III) complexes are likely formed and then, perhaps, further transformed to insoluble Cr(III) precipitates. The production of organo–Cr(III) complexes from chromate reduction is documented in eukaryotic systems because the stable DNA–Cr adducts are mutagenic. However, the formation of soluble organo–Cr(III) complexes from microbial reduction of chromate has only recently been discovered, bringing up the necessity for investigating the significance of the soluble complexes in Cr bioremediation. The proposed research is aimed at (1) characterizing the scope and extent of organo–Cr(III) complex formation by chromate-reducing microorganisms, (2) evaluating cellular components that can potentially form organo–Cr(III) complexes, (3) addressing the stability and biodegradability of these organo–CR(III) complexes, and (4) assessing the fate and transport of these compounds in soils. The results will provide scientific guidance on whether organo–Cr(III) should be considered during application of Cr bioremediation. The information will also help establish a more complete biogeochemical cycle for Cr, which is currently lacking organo–Cr(III) complexes.

High-Resolution Mineralogical Characterization and Biogeochemical Modeling of Uranium Reduction Pathways at the NABIR Field-Research Center

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The effectiveness and feasibility of bioremediation at the field scale cannot be fully assessed until the mechanisms of immobilization and U speciation in the solid matrix are resolved. However, characterization of immobilized U and its valence states is extremely difficult, because microbially mediated mineral precipitates are generally nanometer (nm)-sized, poorly crystalline, or amorphous. To address this difficult, we are (1) developing combined field emission gun-scanning electron microscopy (FEG-SEM, at Indiana University) and FEG transmission electron microscopy (FEG-TEM, at Johns Hopkins) to detect and isolate uranium-containing phases; (2) developing methods for FEG-TEM sample preparation and parallel electron energy loss spectroscopy (EELS) determination of uranium valence; and (3) using the state-of-the-art 300-kV FEG-TEM to determine the speciation, fate, reactivity, and valence states of immobilized uranium.

We have obtained preliminary results on contaminated sediments from Area 3 at the Oak Ridge Field Research Center (FRC). FEG-TEM results show that the sediments contain numerous minerals, including quartz, mica/clay (muscovite and/or illite), rutile, ilmenite, zircon, and an Al–Sr–Ce–Ca phosphate mineral, none of which contain uranium above the EDS detection limit. Substantial U (up to ~2 wt.%) is, however, clearly associated with two materials: (1) the Fe oxyhydroxide, and (2) clots of a chemically complex material that is likely to be a mixture of several nm-scale phases.

The Fe oxyhydroxide was identified as goethite from its polycrystalline SAED pattern and EDS analysis, showing it to be very Fe-rich; the aggregate also displays one of several morphologies that are common for goethite. Uranium is strongly sorbed to goethite in the FRC sediment, and the ubiquitous association with phosphorous suggests that complexes containing both U and P may play an important role in that sorption. Results from bulk analysis and SEM had previously demonstrated the association of U with Fe and thus suggested that U may be sorbed by Fe oxide or oxyhydroxide (Dr. Roh, image presented by David Watson). However, rigorous identification of the host minerals for U requires FEG-TEM results such as imaging, electron diffraction, and spectroscopic analysis.

An even higher concentration of U occurs in the chemically complex material noted above. These "clots" are high in Fe but also contain C, O, Mg, Al, Si, P, S, Cl, K, Ca, Mn, and U. This chemical complexity strongly suggests that these clots consist of aggregates of carbonate, silicate, phosphate, and sulfate phases, and TEM images also suggest that they may be intergrowths of numerous exceedingly small nanoparticles. EELS and Energy-Filtered TEM (EFTEM) studies should be able to resolve these various components and identify precisely where the uranium is in these complex materials.

From the results, it is clear that FEG-SEM and FEG-TEM can readily detect uranium in the FRC samples. The FEG-SEM allows a wide field of view of the samples and can detect U-rich aggregates as small as 20-30 nm. The FEG-TEM can then focus on these aggregates and use SAED, EDS, EFTEM, and PEELS techniques to determine the valence states, structures, and compositional data for these aggregates. This research will provide a crucial component for a complete understanding of the efficacy of uranium bioremediation.

Integrative Studies

Heterogeneity in Iron Biomineralization and the Resulting Impacts on Contaminant and Microbial Dynamics

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Iron (hydr)oxides have a profound effect on contaminant dynamics, being a dominant substrate for metal sorption. They additionally serve as electron acceptors in anaerobic bacterial metabolism and, as a result, undergo dissolution and mineralogical transformation with the onset of reducing conditions. We have observed the rapid and near-complete conversion of 2-line ferrihydrite to goethite and magnetite under advective flow during active metabolism of *Shewanella putrefaciens* strain CN32. The predominant factor controlling (bio)mineralization of ferrihydrite is the aqueous concentration, and resulting surface loading, of Fe(II)—a mechanism supported both by abiotic experiments and those with alternate iron-reducing bacterial species. Reaction of ferrihydrite with ferrous iron results in the rapid transformation to goethite at aqueous concentrations less than 0.3 mM, and to magnetite at higher concentrations.

Despite the importance of ferrihydrite in contaminant attenuation, many environments, particularly those that are classified as mineralogically mature, are dominated by well-ordered iron oxide phases. We have accordingly compared the reducing capacity and Fe(II) retention mechanisms of goethite and hematite to 2-line ferrihydrite under advective flow. Introduction of dissolved organic carbon upon flow initiation results in the onset of dissimilatory iron reduction of all three Fe phases. While the initial surface area normalized rates are similar for all three phases (ca. 10^{-11} moles Fe(II)/m^2 g^{-1}), the total amount of Fe(III) reduced, along with the mechanisms and extent of Fe(II) sequestration, differ among the three iron oxide substrates. After 16 days of reaction, the amount of Fe(III) reduced within the ferrihydrite, goethite, and hematite columns is 25, 5, and 1%, respectively. Following an initial, more rapid reduction period, the three Fe oxides support similar aqueous ferrous iron concentrations, bacterial populations, and microbial Fe(III) reduction rates. Sustained microbial reduction of 2-line ferrihydrite, goethite, and hematite appears to be controlled, in large part, by changes in available reactive surface sites, which is influenced by microbial reduction and equivalent secondary Fe(II) sequestration processes (sorption and magnetite precipitation), regardless of structural order (crystallinity) and surface area.

Using rate parameters derived for the transformation of ferrihydrite to goethite and magnetite, in conjunction with reduction rates and secondary reactions of goethite and hematite, we have constructed simulations of biomineralization processes. Our simulations are projected onto physically complex media representative of natural environments having variations in hydraulic conductivity, such as those noted within the bedded limestone-shale saprolite of Oak Ridge National Laboratory. Gradients in dissolved organic carbon, and resulting metabolic products, will result along diffusive zones emanating from advective flow-paths, and will lead to large variations in biological activity and resulting (bio)mineralization processes. Metabolic rates are greatest along advective flow paths and will diminish with progressive distance into the diffusive domains. As a consequence, bioreduction of both contaminants such as uranium and mineral constituents such as ferric (hydr)oxides are localized along advective flow paths with a progressive decrease entering diffusive pore domains. Thus, appreciable spatial heterogeneity will result in contaminant sequestration, both in terms of degree and mechanism, within soils and sediments.

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

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The overall objective of our project is to understand the microbial and geochemical mechanisms controlling the reduction and immobilization of U(VI) during biostimulation in subsurface sediments of the Field Research Center (FRC), which are co-contaminated with uranium and nitrate. The focus will be on activity of microbial populations (metal- and nitrate-reducing bacteria) and iron minerals that are likely to make strong contributions to the fate of uranium during in situ bioremediation. The project will (1) quantify the relationships between active members of the microbial communities, iron mineralogy, and nitrogen transformations in the field and in laboratory incubations under a variety of biostimulation conditions, (2) purify and physiologically characterize new model metal-reducing bacteria isolated from moderately acidophilic FRC subsurface sediments, and (3) elucidate the biotic and abiotic mechanisms by which FRC aluminosilicate clay minerals are reduced and dissolved under environmental conditions resembling those during biostimulation. Active microbial communities will be assessed using quantitative molecular techniques along with geochemical measurements to determine the different terminal-electron-accepting pathways. Iron minerals will be characterized using a suite of physical, spectroscopic, and wet chemical methods. Monitoring the activity and composition of the denitrifier community in parallel with denitrification intermediates during nitrate removal will provide a better understanding of the indirect effects of nitrate reduction on uranium speciation. Through quantification of the activity of specific microbial populations and an in-depth characterization of Fe minerals likely to catalyze U sorption/precipitation, we will provide important inputs for reaction-based biogeochemical models that will provide the basis for development of in situ U bioremediation strategies.

In collaboration with Jack Istok and Lee Krumholz, we have begun to study the change in microbial community composition of FRC sediments during in situ biostimulation in single well push-pull tests. Microbial communities were stimulated in the acidic subsurface via pH neutralization and addition of electron donor to wells. Examination of sediment chemistry in cores sampled immediately adjacent to treated wells revealed that sediment pH increased substantially (by 1–2 pH units), while nitrate was largely depleted. Following the in situ biostimulation, previously cultured metal-reducing delta-proteobacteria 16S rRNA gene sequences substantially increased from 5% to nearly 40% of clone libraries. Quantitative PCR revealed that *Geobacter*-type 16S rRNA gene sequences increased in biostimulated sediments by one to two orders of magnitude at two of the four sites tested, thereby corroborating information obtained from clone libraries, and indicating that members of the delta-proteobacteria (including *Anaeromyxobacter dehalogenans*-related and *Geobacter*-related organisms) are important metal-reducing bacteria in FRC sediments.

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

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Technetium-99 is an important DOE subsurface contaminant. It is long-lived ($t^{1/2} = 2.13 \times 10^5$ yr) and exists in groundwater as the mobile pertechnetate anion [$Tc(VII)O_4^-$]. Pertechnetate can be immobilized by reduction to insoluble $Tc(IV)O_2 \bullet nH_2O$. The half-cell potential for this reaction is "intermediate" in environmental redox space.

Our past NABIR research demonstrated that Fe(II) resulting from the activity of dissimilatory iron-reducing bacteria (DIRB) can reduce and immobilize pertechnetate from high-nitrate waters in Hanford and Oak Ridge sediments. The global biological reduction of Mn(III/IV) oxides that were present in both sediments was a prerequisite for Tc(VII) reduction as these phases oxidize Tc(IV). The reduction kinetics depended on the biogenic Fe(II) concentration and its molecular and mineralogical environment in the sediment. The reduction rate was rapid in batch systems with half-lives ranging from hours to several days. A kinetic model was developed from extensive reduction data sets that described the rate of Tc(VII) reduction as a function of Fe(II) concentration and speciation.

Newly initiated research is investigating the oxidation/remobilization reaction with oxygen, and quantifying mineralogical, biogeochemical, and microbiological factors that may control it during extended in-ground residence times. Biotic, biogenic, and abiotic $Tc(IV)O_2 \bullet H_2O$ phase assemblages are being generated in model systems and Oak Ridge and Hanford sediments using knowledge from our first three years of research; the oxidation kinetics of Fe(II) and Tc(IV) in these are being studied in batch and column systems before and after (1) geochemical aging and (2) biogeochemical transformation by Fe(II) oxidizing, NO_3^- reducing bacteria. Spectroscopic and microscopic techniques are being used to define Fe and Tc speciation, and their physical locations and forms, as a basis for interpretation of oxidation rate. A linked equilibrium/kinetic biogeochemical transport model will be applied to identify the reaction network, and to quantify the interdependent kinetic reactions involved in the oxidation process. Initial results have shown that insoluble $Tc(IV)O_2 \bullet H_2O$ oxidizes relatively rapidly in bioreduced Oak Ridge sediment. However, the oxidation rate and extent depended on biogenic Fe(II) concentration and its biogeochemical speciation. Slow rates of Fe(II) oxidation consistent with what may happen in the field yielded a large residual Tc(IV) concentration that was resistant to further oxidation.

NABIR–Environmental Management

In-line Uranium Immunosensor

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The goal of this project is to develop an in-line uranium immunosensor that can be used to determine the efficacy of specific biostimulation procedures by measuring the amount of hexavalent uranium [U(VI)] present at varying times during the treatment process. An alpha prototype of this sensor has been developed. The instrument consists of a capillary flow/observation cell fitted with a microporous screen. An antibody "capture ligand" is immobilized onto uniform particles, and the particles are subsequently deposited above the screen in a packed bed. A mixture that contains a fluorescently labeled antibody and soluble ligand is rapidly passed through the bead pack. Only those antibody molecules without soluble ligand in their binding sites are available to bind to the "capture ligand," and the fluorescent signal generated is inversely proportional to the amount of soluble ligand in the original mixture of antibody and ligand. A fresh bed of beads is used for each determination. For the measurement of U(VI), the ligand is hexavalent uranium complexed with 2,9-dicarboxyl-1,10-phenanthroline [U(VI)–DCP], and the antibody is one of three monoclonal antibodies (8A11, 12F6, or 10A3) that recognizes this metal-chelate complex.

Stability tests of the reagents used in the assay (capture ligands, chelators, diluent buffers) has shown that these assay components are stable for at least six weeks at 4°C. Frozen antibody stocks are stable for more than two years at –80°C, and diluted stocks for approximately two weeks at 4° C. An alpha prototype of this sensor has the ability to autonomously run a standard curve for hexavalent uranium from stock reagents, and to prepare environmental samples for analysis. When the 8A11 monoclonal antibody is utilized, the assay measures hexavalent uranium at concentrations from 0.5 to 4.8 nM (0.12–4.8 ppb). The coefficients of variation (CV) in the assay range from 3.5 to 5.9%, with an average of 4.6%. The immunoassay results are comparable to those obtained using the Kinetic Phosphorescence Assay (KPA).

The spike-and-recover experiment using natural groundwater from the ORNL FRC background site, and an artificial groundwater designed to stimulate other FRC samples, have indicated that high concentrations of calcium stimulate the binding of the 8A11 antibody to the "capture ligand." Such stimulation may lead to false negatives in the analysis of environmental samples. The binding of the 12F6 monoclonal antibody to the capture ligand is neither stimulated nor inhibited by calcium concentrations up to 31 mM, and future assay development will most likely utilize this antibody.

Field Investigations of HRC®-Stimulated Bioreduction of Cr(VI) at Hanford 100H

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The overall objective of this project is to carry out field investigations to demonstrate the feasibility of a cost-effective, in situ remediation technology, using lactate-stimulated bioreduction of dissolved Cr(VI) to form insoluble Cr(III) precipitates at the Hanford 100H area. Lactate will be provided in situ via injection of Hydrogen Release Compound (HRC®). Specific goals include (1) the design of a field test to develop design criteria for full-scale deployment of in situ Cr(VI) bioreduction via biostimulation for the use at DOE sites, (2) providing field testing and monitoring (including geophysical methods) of the effects of biostimulation on microbial community activity, redox gradients, transport limitations, and other reducing agents, and compare the field results with those of our previous laboratory work, (3) the assessment of kinetic rates and conditions that may cause reoxidation of Cr(III) to Cr(VI) after biostimulation is terminated, and (4) the assessment of the use of bioremediation in conjunction with other alternative remediation technologies, such as a pump-and-treat approach for the Hanford 100H site. Sediment samples from the field site have shown that the microbial densities are quite low ($<10^4$ cells/g), but are easily stimulated with HRC or sodium lactate to densities $>10^8$ cells/g in just a week. As part of that investigation, we collected sediment from the Hanford site and exposed it to 1,000 ppb Cr(VI) and to sodium lactate, HRC, HRC Primer, HRC-Extended Release (HRC-X™), Metals Remediation Compound (MRC™), and no carbon. HRC is a polylactate compound with different degrees of polymerization to control its viscosity and solubility in water. HRC Primer is a low viscosity polylactate, and provides a rapid release of lactate into groundwater. HRC-X is a high viscosity polylactate, and has an extended lactate release profile. MRC is an organosulfur and polylactate combination that reacts directly with the Cr, at least initially. In less than one week, MRC reduced Cr(VI) to undetectable concentrations, though the abiotic MRC control had only been reduced by 65%. By three weeks, all the biotic HRC and lactate combinations had undetectable Cr(VI), while the abiotic controls for each had 40–61% of the Cr(VI) remaining. The sediment with no additional carbon source also showed a 64% reduction in Cr(VI) after three weeks, whereas the abiotic control had only a 12% reduction in Cr(VI). The HRC compounds performed equally well, while the MRC gave a faster response, although much of this response was abiotic. Phospholipid fatty acid analyses (PLFA), terminal restriction fragment length polymorphisms (TRFLP), clone libraries, direct cell counts, and 16s rDNA microarray analysis demonstrated that the initial densities of microbes is very low (less than 10^4 cells/g), but after biostimulation was typically greater than 10^8 cells/g. The low densities made extraction for PLFA and DNA analysis difficult, so a cell concentration technique was developed to increase yields. These studies confirmed that the microbial diversity was low but that sulfate reducers, *Arthrobacter* spp. and *Geobacter* spp., dominated the samples. ^{13}C-labeled lactate was also used to show use of the lactate by specific groups of bacteria. The results demonstrate that even in low biomass and diversity environments, biostimulation of Cr-reducers can occur and that their functional relationship can be evaluated by various molecular techniques. These studies, along with field studies in progress, will be used to develop a conceptual model of chromium bioreduction in groundwater at a field scale, and to provide recommendations for field deployment of engineered bioremediation.

Potential for Immobilization of ^{99}Tc at the Hanford Site by Stimulation of Subsurface Microbiota

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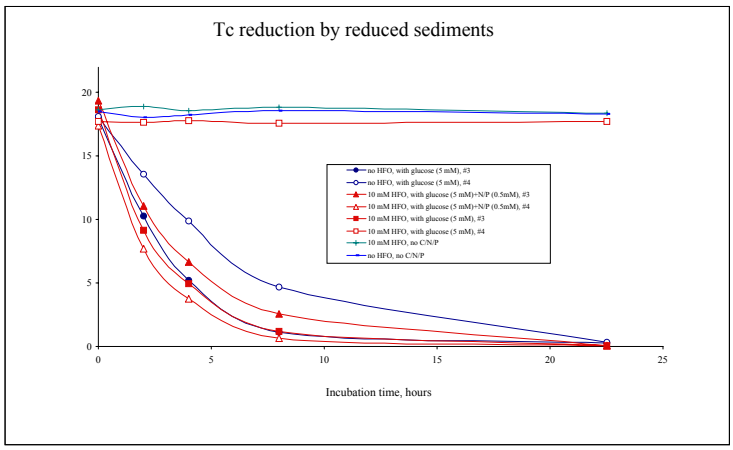
The objective of this project is to evaluate the applicability of direct and/or Fe(II)-facilitated ^{99}Tc reduction by microbes to the problem of ^{99}Tc groundwater contamination at the Hanford site. Our initial research focused on laboratory studies with field samples from the Hanford site to establish the existence and metabolic requirements of microorganisms native to Hanford subsurface sediments that are capable of directly or indirectly mediating $Tc(VII)O_4^-$ reduction. We have successfully biostimulated Hanford formation sediments, and have demonstrated the bioreduction of native and exogenous Fe(III). These bioreduced sediments also effectively remove $^{99}TcO_4^-$ from solution, presumably via biogenic Fe(II)-facilitated reduction. Subsurface samples from below the water table beneath the former North Process Pond in the 300 Area at Hanford were incubated with a wide range of electron donors with or without nutrient amendments. A key result from these incubations is that initial biostimulation in the laboratory required ~120 days to achieve significant levels of Fe(III) reduction. However, once biostimulation was achieved (marked by reduction of oxidized iron in the incubation tubes), $Tc(VII)O_4^-$ was readily reduced. Lactate, glucose, and glucose with N+P promoted the greatest Fe(III) and $Tc(VII)O_4^-$ reduction. Furthermore, $Tc(VII)O_4^-$ added to incubation tubes after bioreduction was reduced in a matter of hours (see figure). A number of anaerobic bacteria have been isolated from the 300 Area sediments that can grow with lactate with fumarate as the electron acceptor or with glucose with Fe(III) as the electron acceptor. These cultures are currently being characterized with regards to their phylogeny and ability to reduce $Tc(VII)O_4^-$.



Because our laboratory-scale experiments show response to biostimulation, and we now know that the kinetics of the initial stimulation process are relatively slow, we are evaluating the use of repeated single-well push-pull tests for determining the viability of in situ reduction of ^{99}Tc by native microorganisms (in collaboration with Jack Istok, Oregon State University). Our long-term goal is a field-scale ^{99}Tc bioreduction experiment to verify, under large-scale field conditions, laboratory results indicating that in situ bioremediation of ^{99}Tc may be feasible and ultimately lead to a relatively low-cost means of decreasing risk from ^{99}Tc at the Hanford site.

BASIC:

Bioremediation and Its Societal Implications and Concerns

Testing a Stakeholder Participation Framework for Fielding Bioremediation Technologies

Robert P. Anex[1] (PI) and Will Focht[2]

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This research is investigating 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 are testing 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 are assessing conflict among stakeholders regarding the acceptability of in situ bioremediation (i.e., immobilization) as a means to reduce risks posed by radionuclides and metals in the environment. Third, we are assessing the role that awareness of risk exposure plays in the willingness of stakeholders to engage in problem-solving and making risk tradeoffs. Fourth, we are assessing 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. This poster presentation describes the results of empirical tests of hypotheses related to the first three objectives. Data used in these tests are the result of more than 75 interviews and several hundred telephone surveys performed in the regions around Oak Ridge, TN; Los Alamos, NM; and Hanford, WA. After processing all data, we will be communicating our results to the interested and affected parties by holding workshops for DOE employees and contractors, and for community members in each of the three study areas.

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

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This project was designed to identify the range of concerns held by the public about bioremediation strategies for in situ cleanup at U.S. Department of Energy sites (DOE). Our interdisciplinary team worked with members of the public and other experts to identify DOE characteristics that contribute to public perceptions of risks associated with bioremediation. Two primary methods were used to collect data. First, a "consensus conference" was conducted with a panel of lay members who worked with panel-identified experts to articulate their concerns about bioremediation in general, and at the specific study site (Idaho National Engineering and Environmental Laboratory). Using information from the consensus conference, a structured survey was created and administered via computer-aided telephone interview (CATI).

From the survey, we found that while only about 32% of respondents from the general population in the four counties around INEEL knew about bioremediation, 85% trusted the DOE "a lot" or "some" to experiment with new cleanup technologies. When asked to identify the "most important" characteristic of DOE that engendered trust in the agency, 26% of the respondents reported that technical competency was the most important, 25% identified attention to human health issues, and 23% said the most important thing was for DOE to be honest about its activities.

When asked about the risks posed by radioactive contamination at INEEL, more respondents said there was a greater risk to future generations than to themselves personally or to workers at INEEL. Demographic variables such as education, gender, and religious affiliation help explain some of the variation in perception of risk, as do variables that measure attitudes and values such as the perceived importance of INEEL to the local economy and values placed on the environment. Our analysis also suggests that perceptions of "recreancy" (i.e., the failure of an organization to act responsibly) may not be a good predictor of risk perception in this context.

The Determinants of Social Acceptability of Bioremediation Technologies

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We report findings from continued analyses of a series of quasi-experimental simulation workshops in which participants were charged with determining the conditions under which proposed bioremediation field research or deployment would be acceptable. This work represents the culmination of our previous NABIR BASIC research in which we also developed a framework for considering social acceptability, analyzed data from a series of DOE site-specific advisory board meetings, and reviewed opportunities for public involvement in decision-making about remediation options at DOE sites. Each simulation workshop consisted of four parts: (1) background information about DOE legacy wastes, remediation challenges, and cleanup options for subsurface chromium, mercury, plutonium, uranium, and technetium, (2) Hypothetical Scenario 1 on proposed field bioremediation field research, (3) Hypothetical Scenario 2 on proposed deployment of genetically engineered microorganisms to immobilize selected subsurface contaminants, and (4) debriefing. Scenarios were fictitious, but realistic. A total of six simulation workshops were conducted, involving three sets of scenarios. We manipulated selected variables among subgroups within and across workshops. Subgroups were instructed to answer short sets of questions associated with each scenario, and to report those answers to the larger group at the end of the workshops.

Groups exhibited different strategies for determining their answers (consensus, averaging members' answers, reporting individuals' answers, etc.). Nevertheless, while there was considerable variation among groups in their discussions and responses, some conclusions can be drawn,

Field Research Activities

Biostimulation of In Situ Uranium Reduction at the NABIR Field Research Center Using a Nested Recirculation Scheme and Aboveground Groundwater Conditioning

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This project is designed to assess the rate and extent of uranium reduction in a complex field environment under controlled conditions. Previously we characterized hydrogeology, geochemistry, and microbiology at a site in Area 3 of the NABIR Field Research Center at Oak Ridge, Tennessee. This site is adjacent to the S-3 pond cap and contains high levels of uranium (~30 mg/L in the water with 300–400 mg/kg on the soil), nitrate (8–10 g/L), aluminum (~0.5 g/L), low pH (~3.6), plus a range of other metals and volatile organics. We have installed two injection wells and two extraction wells along a line parallel to the direction of background groundwater flow, and have screened over a region of high flow. One well pair defines an outer flow cell. The second well pair defines an inner flow cell nested within the outer cell. The outer cell is connected to an aboveground treatment system that includes a vacuum stripper to remove volatile organics and dissolved gases, a two-step precipitation scheme to remove aluminum and calcium, and a fluidized bed reactor to remove nitrate.

Hydraulic conductivity and residence time distributions were determined from breakthrough profiles for a bromide tracer study. Rates of desorption of ions from the soil matrix were determined by modeling the rebound of ion concentrations after the tracer study. The treatment zone was then flushed with acidified tap water to displace aluminum and nitrate, and pH of the inner cell was adjusted to create an environment suitable for U(VI) reduction. Following pH adjustment, x stimulation of U(VI) reduction was initiated by intermittent addition of ethanol into the inner cell. This process is still ongoing. We expect to be able to alter rates of U(VI) reduction by manipulating biomass and carbonate concentrations.

To provide more unambiguous interpretation of our field results, we have performed batch and column microcosm studies, and we have evaluated U(VI) removal under a range of pH conditions. Experiments are ongoing to identify microorganisms responsible for U(VI) reduction, and to quantify rates of reduction and desorption.

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

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Extensive in situ (in ground) field testing using the push-pull method has demonstrated that indigenous microorganisms in the shallow (< 8 m) aquifer in FRC Areas 1 and 2 are capable of coupling the oxidation/fermentation of injected ethanol, glucose, or acetate to the reduction of U(VI) and Tc(VII). Despite highly variable initial (prior to testing) contaminant concentrations [pH 3.3–7.2; 0.1–140 mM of nitrate; 1–12 μM of U(VI); 200–15,000 pM of Tc(VII)], sequential donor additions resulted in increased rates of microbial activity (Denitrification: 01.–4.0 mM/hr; sulfate reduction: 0–0.03 mM/hr; U(VI) reduction: 10^{-4} to 10^{-3} μM/hr; Tc(VII) reduction: 4–150 pM/hr) in all wells tested. Tc(VII) reduction and denitrification proceeded concomitantly in all tests. U(VI) reduction was concomitant with Fe(II) production in Area 1, but little Fe(II) was detected under sulfate-reducing conditions in Area 2. Reoxidation of U(IV) (precipitated in the vicinity of the wells during previous tests) but not Tc(IV) was observed when injected test solutions contained initial nitrate concentrations greater than ~ 20 mM. Field data and laboratory studies suggest that U(IV) is likely oxidized by Fe(III) minerals produced by enzymatic Fe(II) oxidation or by Fe(II) oxidation by nitrite. U(IV) reoxidation rates (10^{-3} to 10^{-2} μM/hr) were somewhat larger than U(VI) reduction rates, indicating that sustained nitrate removal will be necessary to maintain the stability of U(IV) in this environment. The production of metal sulfides following addition of sulfate was shown to reduce U(IV) reoxidation rates in field tests conducted in Area 2. Added FRC humics increased the rate of U(VI) reduction in some field tests but not in laboratory microcosms, although added humics increased the survival of FRC isolates in low pH groundwater. Several denitrifying organisms that tolerate relatively low pH (4.5–5.5) have been isolated from Area 1 (see abstract by A. Spain). Increased pH resulting from microbial activity during biostimulation in portions of the site with low (<4) initial pH results in the formation of ~1–2 g/L of metal hydroxide precipitates that gradually reduce hydraulic conductivity and create locally high concentrations of potentially toxic metals (Al, Ni). Interpretations of field tests are supported by the results of laboratory studies with groundwater and sediments, and by the results of geochemical and reaction path modeling performed on selected field tests. In addition, detailed descriptions of pore water chemistry are being obtained through the use of Multilevel Samplers (MLS) installed in sets of wells in Areas 1 and 2. The MLS data provide information on small-scale vertical variations in groundwater chemistry and a quasi-three-dimensional description of the portion of the aquifer interrogated during push-pull tests. The MLS will also be used to investigate geochemical and microbial community changes resulting from donor additions in prepared sediments, and to directly monitor U(IV) reoxidation.

This research collectively suggests that bioimmobilization of Tc(VII) and U(VI) should be possible using a permeable reactive barrier consisting of three defined zones: (1) pH adjustment, (2) denitrification and Tc(VII) reduction, and (3) U(VI) reduction. This hypothesis is being tested in intermediate - scale (~2 m) physical models deployed at Areas 1 and 2. Preliminary results indicate that essentially complete U(VI) and Tc(VII) removal can be achieved with pore water velocities of ~ 8 cm/day, which are comparable to site groundwater velocities.

Acceleration of Microbially Mediated U(VI) Reduction at a Uranium Mill Tailings Site, Colorado Plateau, USA

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A second field-scale electron donor amendment experiment was conducted in 2003 at the Old Rifle Uranium Mill Tailings Remedial Action (UMTRA) site in Rifle, Colorado. The objective of the 2003 experiment (done in collaboration with the U.S. Department of Energy's UMTRA Groundwater Project) was to test the hypothesis that the amendment of an increased concentration of electron donor would result in an increased export of electron-donor downgradient, which in turn would create a larger zone of down-gradient U(VI) bioreduction sustained over a longer time period relative to the 2002 experiment (1). During the experiment, ca. 3 mM of acetate was amended to the subsurface over a period of three months in a 15 m × 18 m × 2.5 m volume comprised of three upgradient monitoring wells, 20 injection wells, and 15 down-gradient monitoring wells. Preliminary results indicate that acetate was exported further downgradient, resulting in a larger zone of microbial U(VI) reduction than for the 2002 experiment, in which one-third as much acetate was injected. However, the pattern of metal reduction followed by sulfate reduction after consumption of the bioavailable Fe(III) occurred again in the 2003 experiment. The onset of sulfate reduction in the subsurface results in precipitation of amorphous FeS, creating a marked darkening of subsurface sediments that wedges out downward from the injection gallery. This Fe(II) reservoir buffers redox conditions after cessation of electron donor amendment, slowing reoxidation, and apparently maintaining a low rate of biotic or abiotic reduction of U(VI) entering the injection zone from upgradient. We are currently monitoring the system post-injection to assess bulk reoxidation rates as a function of dissolved oxygen concentration and other system parameters, particularly during spring runoff, which raises the water table and introduces a layer of oxygenated water at the top of the water table. During the spring of 2003, this layer mixed with underlying water to create a dissolved oxygen gradient that is consistent with the wedge-shaped distribution of FeS developed during acetate injection.

Uranium contamination at Old Rifle occurs in a groundwater flow system consisting of alluvial sands and gravels of the Colorado River overlying the relatively impermeable Wasatch Formation. During electron donor amendment, subsurface sediment samples were collected by drilling. The Advanced Photon Source (APS) PNC-CAT beamline was used in x-ray microprobe mode to assess the distribution and redox state of U in sediment samples from upgradient and downgradient from the injection gallery. Results suggest that U(IV) is more abundant on grain surfaces downgradient than upgradient, consistent with microbial-mediated U(VI) reduction in the zone of electron donor amendment.

1. Robert T. Anderson, Helen A. Vrionis, Irene Ortiz-Bernad, Charles T. Resch, Philip E. Long, Richard Dayvault, Ken Karp, Sam Marutzky, Donald R. Metzler, Aaron Peacock, David C. White, Mary Lowe, and Derek R. Lovley. 2003. Stimulated in situ removal of U(VI) from groundwater of a uranium-contaminated aquifer, Applied And Environmental Microbiology, Vol. 69, No. 10, p. 5884–5891.

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

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We are performing a series of tasks leading to an in situ field-scale biostimulation experiment at Area 2 of the NABIR Field Research Center (FRC). The field experiment will evaluate the feasibility of stimulating microbial U(VI) reduction activity in targeted pore fractions of structured porous media. During the first year, we identified a target study area within Area 2, and performed a series of characterization efforts to determine the suitability of the site for the planned experiment. Surface seismic and resistivity surveys identified an interface between an upper disturbed saprolite interval (depths to ~20 ft) and a lower undisturbed saprolite interval (depth to ~20 to 28 ft). Twelve new wells were installed, mostly in the lower zone. Sediment cores were collected from the intact saprolite, and metal extractions were performed. These showed relatively low levels of uranium, sparsely distributed. Groundwater samples from the lower zone exhibit 1–4 μM concentrations of U(VI). Nitrate levels were much higher than anticipated, in some cases approaching 1,000 ppm. Pumping tests were performed to determine the hydraulic conductivity and connectedness of wells in the intact saprolite. Measured hydraulic conductivity (K) was on the order of 10^{-7} cm/sec along geologic strike, and exhibits strong anisotropy (lower K perpendicular to strike). Based on these observations, we plan to focus our efforts on the upper, disturbed saprolite interval. This zone is more highly conductive, contains higher levels of U(VI) and relatively low levels of nitrate (~20 ppm). Although it does not retain the original structure of the fractured saprolite, we expect that there exists significant secondary and tertiary porosity, and this zone will therefore be an effective target for the field experiments.

Three large intact core specimens were collected from an excavation in the background area (uncontaminated), and have been instrumented for long-term laboratory flow-through and biostimulation experiments. Bulk samples of background area sediments have also been used for a variety of geochemical and microbiological characterization activities.

Sediment samples from the lower zone at the Area 2 field site [low U(VI) levels] have been incubated in slurry experiments (with and without added electron donor) to determine the extent and rate of metal reduction by native microorganisms. Removal of nitrate, reduction of iron, and reduction of added U(VI) has been observed. Slurry experiments are ongoing, and microbial community analyses are being performed on samples collected over the course of the experiments.

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NABIR research objectives and results. To encourage hypothesis-based field research and process-level understanding, the NABIR program has established a Field Research Center (FRC) for NABIR investigators. The FRC provides a site for investigators to conduct field-scale research and to obtain DOE-relevant subsurface samples for laboratory-based studies of bioremediation. Currently, the NABIR program has a single Field Research Center (FRC) located on the U.S. Department of Energy's Oak Ridge Reservation (ORR) in Oak Ridge, Tennessee. Staff from Oak Ridge National Laboratory's Environmental Sciences Division has operated the FRC since April 2000. Both contaminated and background (uncontaminated control) areas are located on the ORR's Y-12 National Security Complex in Bear Creek Valley. The initial focus of research at the FRC has been on in situ biostimulation experiments to promote the immobilization of uranium and technetium.

The FRC is used by NABIR investigators for various purposes including the following:

- A source of subsurface samples.
- Hundreds of groundwater and sediment samples (cores and composites) have been collected and shipped from the background and contaminated sites for use by over eight national laboratories and 15 universities.
- Characterization and source of humic material.
- Evaluation of new characterization and monitoring methods.
- Deployment of coupons (or bug traps) for rapid assessment of in situ microbial activity (University of Tennessee, ORNL, INEEL, and others).
- Microcosm studies, microbial enrichments, and analyses of DNA, RNA, and PLFAs.
- Development of microarray technology for assessment of community dynamics.
- Improvement of mathematical models for prediction of community structure and dynamics.
- Field-portable immunoassay instruments and reagents to measure chelators and mobile forms of uranium (Tulane University).
- Characterization of the subsurface with surface and crosswell geophysics (ORNL and LBNL).
- In situ uranium assay with downhole NaI detector (ORNL).
- Multidisciplinary in situ accelerated bioremediation research projects.
- In situ uranium reduction experiments using push-pull techniques (Oregon State University and Oklahoma University, located in Areas 1 and 2).
- Field-scale bioreduction of uranium (Stanford and ORNL, located in Area 3).
- In situ immobilization of uranium in structured porous media via biomineralization at the fracture/matrix interface (PNNL, ORNL, and University of Alabama located in Area 2).

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

Student Presentations

Growth of *Shewanella oneidensis* MR-1 in Sediment Microcosms for Identification of Sediment Survival Genes

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The ability of *Shewanella oneidensis* MR-1 to reduce radionuclides, including technetium and uranium, has generated great interest in its use for bioremediation. Optimization of in situ bioremediation processes require knowledge of the interaction between MR-1 and its natural environment, a relationship that has not been investigated extensively. We are currently using signature tagged mutagenesis (STM) to examine this interaction at the genetic level by identifying genes that are essential only during exposure to its sedimentary habitat. We assessed survival of MR-1 through growth studies in various sediment systems that simulated the natural environment, as would be used in bioremediation processes. For further studies with STM, we chose the system that allowed some growth of *S. oneidensis* MR-1 in order to select against mutations in genes required for survival.

MR-1 growth and survival was examined in microcosms simulating aerobic conditions, transition of aerobic to Fe-reducing conditions, and Fe(III)-reducing conditions with amendments, including AQDS (anthraquinone-2,6-disulfonate), NTA (nitrilotriacetate), and lactate. For Fe(III)-reducing microcosms, Fe(III) oxyhydroxide was added to subsurface sediments. Survival in the presence of natural microbial communities was tested, along with survival under axenic conditions. Under each of the respiratory conditions, MR-1 grew to higher numbers, and generally survived better in autoclaved sediments than in live sediments, increasing in number: 60-fold in aerobic sediments, tenfold in transition sediments, and less than tenfold in Fe(III)-reducing sediments without amendments. In sterile, Fe(III)-reducing sediments with lactate added, MR-1 population increased 80-fold, and with both lactate and either AQDS or NTA, the increase was approximately 1,500- and 300-fold, respectively. Under all respiratory conditions, when no electron donor was added to live sediments, MR-1 concentrations remained steady or gradually died below the initial inoculum concentration within a week, with the exception of aerobic microcosms. In live, Fe(III)-reducing sediments, yeast extract increased MR-1 populations approximately 2.5-fold. When added together, lactate and either AQDS or NTA allowed MR-1 to grow in live sediments to a concentration nearly as high as achieved in autoclaved sediments. Addition of lactate alone also allowed these organisms to grow at least tenfold and to survive in live, Fe(III)-reducing sediments for approximately one week before the concentration fell below inoculum levels. Additionally, under simulated Fe(III)-reducing conditions, the appearance of Fe(II) was delayed until after peak growth was observed. Experiments are being conducted to determine which *Shewanella* may have been utilized as an electron acceptor prior to using Fe (III) in lactate-only amended microcosms. Based on our growth studies, the live, Fe(III)-reducing sediment system with lactate added demonstrated the best survival of MR-1 with minimal amendments and was therefore chosen for STM experiments. Our results also indicate that successful in situ bioremediation with MR-1 may require the addition of an electron donor.

Reaction-Based Reactive Transport Modeling of Biological Iron(III) Reduction in Natural Sediments

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Previously, we demonstrated that diagonalized reaction-based models could be applied to batch systems in which parallel kinetic reactions were operative if separate experiments were used to independently formulate and parameterize kinetic rate expressions. Currently, our aim is to demonstrate that reaction-based models can accurately simulate complex biogeochemical systems under advective flow conditions using rate formulations/parameters obtained from independent batch experiments. For demonstration purposes we selected biological Fe(III) reduction in natural sediments. All experiments were performed with a hematite-rich sand from Eatontown, NJ (366 μmol DCB-extractable Fe g^{-1} sediment), and the dissimilatory metal-reducing bacterium (DMRB) *Shewanella putrefaciens* CN32 with lactate as the sole electron donor in a buffered (pH = 6.8) background electrolyte (BE) containing 45 mM PIPES, 1 mM $CaCl_2$, 0.1 mM NH_4Cl, 0.01 K_2HPO_4, 0.01 mM $MgSO_4$, and 0.1 g L^{-1} yeast extract (growth conditions). A series of batch kinetic experiments were performed to systematically measure iron reduction as a function of iron oxide surface area, cell density, and electron donor concentration to independently formulate a kinetic rate expression for the biological iron reduction. Batch experiments were conducted with variable sediment concentrations (0.007–2.0 g mL^{-1}), variable initial DMRB concentrations (10^7–10^9 cells mL^{-1}), and variable lactate concentrations (1–50 mM). Reactors were incubated at 20°C on a shaker table for 7 days, and kinetic data were collected on a logarithmic scale. For each time point, soluble Fe(II) (0.2 μm), 0.5 N HCl Fe(II), lactate, acetate, and pH were measured. At the final 7 day point, iron mineralogy (by Mössbauer spectroscopy) was also measured. For all batch experiments, the overall reaction rate of Fe(II) production was zero-order (R^2 values for [0.5 N HCl Fe(II)]-vs-time ranged from 0.905 to 0.993). The overall Fe(II) production rate was directly dependent on sediment concentration (e.g., proportional to reactive iron surface area) and not on lactate concentration (i.e., system was never electron-donor limited). The effect of DMRB concentration on the overall Fe(II) production rate was complex and nonlinear, suggesting a growth term may be required to accurately capture this effect. Future work includes using these data and reaction models to determine the most appropriate rate formulation/parameters for modeling the column bioreduction experiments.

Eatontown sand and the bacteria were carefully wet-packed into 1 cm diameter glass chromatography columns with a packed bed length of ca. 7.5 cm. All column experiments were conducted in triplicate or quadruplicate and run for 20 day. The only experimental variable was flow rate, which ranged from 1 to 12 pore volumes day^{-1}, corresponding to Darcy velocities of 7.5 to 90 cm day^{-1}. Column effluent samples were collected daily and used to measure soluble Fe(II), lactate, and acetate. At the final 20 day point, column sediment samples were used to measure cell concentration, iron mineralogy, and 0.5 N HCl Fe(II) as a function of column length. In most experiments, the effluent biogenic Fe(II) concentrations increased over the first 10 days and then remained relatively constant. From these pseudo-steady-state conditions, the biogenic flux of Fe(II) [μmol Fe(II) hr^{-1}] was calculated as the product of the "plateau" concentration (μmol L^{-1}) times the average flow rate (L hr^{-1}) and clearly demonstrates that hydrologic conditions affect biologic reactions. Based on calculations of the Peclet number for the various flow rates, a step-function increase in biogenic flux occurs when transport processes switch from diffusion-controlled to advective-controlled. These results highlight the intriguing linkages between hydrology and biogeochemistry that we hope to quantitatively simulate using reactive transport reaction-based models.

Diversity of Uranium Reduction Processes in

Oak Ridge Source Zone Sediment

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The stimulation of microbial uranium reduction at the field scale presents several challenges relative to the bench scale. First, bioremediation in the field will take place in the presence of heterogeneous sediment, which contains multiple components that can react geochemically with uranium. Next, a complex and changing community of bacteria will be responsible for uranium reduction in subsurface environments, not single strains or enrichments studied in the laboratory. Finally, multiple electron donors and acceptors are potentially available in a field setting, which complicates predictions regarding microbial metabolism. To investigate these issues, community fingerprinting and x-ray absorption spectroscopy were used to analyze microbial uranium reduction in a heterogeneous environment. Microcosms were established containing sediment samples from Area 3 of the Oak Ridge Field Research Center, ethanol, denitrified synthetic groundwater, and, as an inoculum, the effluent of a denitrifying fluidized bed reactor.

In most microcosms, soluble U(VI) and ethanol concentration decreased as acetate accumulated. Uranium associated with the solid phase was also reduced, as indicated by x-ray absorption near-edge structure (XANES) spectroscopy of sediment from sacrificed microcosms. This transformation was apparently mediated either directly or indirectly by bacterial activity, as uranium was not reduced in sterilized microcosms. Little iron was reduced during this time, but sulfate reduction proceeded concurrently with uranium reduction. Sulfate reduction may have been favored due to greater bioavailability of soluble sulfate over ferric iron, or due to greater initial numbers of sulfate-reducing bacteria than iron-reducing bacteria. As these factors are relevant to the Oak Ridge source zone, sulfate-reducing bacteria may reduce uranium in the field. Results indicated that, under sediment and groundwater conditions representative of the source zone during treatment, the amendment of ethanol can stimulate uranium reduction.

In other microcosms, however, soluble U(VI) concentration rebounded or leveled off after an initial decrease. XANES analysis revealed the reduction of sediment-associated uranium in these microcosms was also incomplete. A rebound in soluble uranium concentration implies the rate of uranium desorption from sediment exceeded the rate of its bioreduction. Biomass growth may have been inhibited in these microcosms, as little DNA was obtained from tubes in which uranium reduction ceased. Differences in patterns of uranium reduction between replicate microcosms highlight the significance of sediment and/or inoculum heterogeneity.

Changes in the microbial community upon biostimulation were monitored with terminal restriction fragment length polymorphism (T-RFLP) analysis. A comparison of T-RFLP profiles indicated a significant shift in community structure occurred as uranium was reduced. In addition, profiles showed that different community members were dominant in microcosms with complete versus incomplete uranium reduction. Further work is needed to elucidate the impact of microbial community composition on the function of uranium reduction.

Uranium and Technetium Bioimmobilization in Intermediate-Scale Permeable Reactive Barriers

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

Microbial Communities Involved in Uranium Reduction under Sulfate-Reducing, Iron-Reducing, and Methanogenic Conditions

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Biological reduction of soluble U(VI) to insoluble U(IV) is an important bioremediation strategy in uranium-contaminated sediments, as push-pull tests at the Field Research Center (FRC) have demonstrated. Although many species have the ability to reduce U(VI), it has not been clearly shown which microorganisms are involved in U(VI) reduction in sediments. Microbial U(VI) reduction was investigated under several terminal electron-accepting conditions to identify groups of bacteria involved in subsurface reductive processes. 100 μM U(VI) was added to anaerobic sediment microcosms in which sulfate-reducing (SR), iron-reducing (FeR), or methanogenic (Meth) communities were previously stimulated. After 26 days, nitric acid and bicarbonate extractions were done to determine amounts of U(IV)/gram sediment. Community phospholipid fatty acids (PLFAs) were extracted and quantified to estimate biomass and to create community composition profiles, as different groups of microorganisms are composed of different types of PLFAs. Uranium reduction occurred to near completion under all conditions, indicating SR, FeR, and Meth conditions are all sufficient for U(VI) reduction. In SR bottles, there was a positive correlation between mid-chain branched saturates (indicative of sulfate reducers) and U(VI) reduction (r=0.466), and also between monounsaturates (indicative of gram-negative bacteria) and U(VI) reduction (r=0.926). In FeR bottles, there was a positive correlation between branched monounsaturates (indicative of sulfate or iron reducers) and U(VI) reduction (r=0.886). Also, in Meth bottles, there was a positive correlation between terminally branched monounsaturates (indicative of Gram positives) and U(VI) reduction (r=0.999). These positive correlations were interpreted, as groups of organisms may be involved in subsurface U(VI) reduction. These data suggest that U(VI) reduction can occur under SR, FeR, and even Meth conditions, and that different populations may be involved in U(VI) reduction under these three conditions. Therefore, in designing an efficient bioremediation strategy at the FRC, it may prove useful to understand the microbial processes and organisms that dominate the natural system.

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