DOE-ERSP PI MEETING:
Abstracts

April 3–5, 2006
Warrenton, Virginia

Environmental Remediation Sciences Program (ERSP)

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U. S. Department of Energy
Environmental Remediation Sciences Division
Principal Investigators Meeting

Welcome to the annual 2006 Environmental Remediation Sciences Division (ERSD) Spring Principal Investigators (PI) meeting! The objective of this 2006 ERSD Spring meeting is to provide an annual update of research results, discuss significant research issues, and identify opportunities to interact with other research efforts and make use of new capabilities. The meeting is scheduled for 2 1/2 days, April 3–5, 2006.

As many of you know, on October 1, 2005, ERSD's Natural and Accelerated Bioremediation Research (NABIR) program and Environmental Management Science Program (EMSP) were merged to create the Environmental Remediation Sciences Program (ERSP), in accordance with Congressional direction. The new ERSP will continue to support and build on the substantial research progress developed under the former NABIR and EMSP programs to address some of the nation's most difficult environmental cleanup problems, and it will continue the former NABIR and EMSP program objectives to understand and influence contaminant mobility in the subsurface. The merging of the two former programs does not alter previously existing awards.

ERSD plans to continue holding a Spring PI meeting at the Airlie Center in Warrenton, Virginia (which has been a NABIR tradition), and to add an annual Fall PI meeting. For 2006, the Fall PI meeting will be held in late October at ERSD's Field Research Center (FRC) in Oak Ridge, Tennessee. As with the NABIR and EMSP programs, research findings reported in the presentations and posters at these meetings will continue to provide ERSD program managers with information to assess individual project progress as well as to provide synergistic opportunities among the program's scientists.

As part of ERSD's efforts to integrate the former NABIR and EMSP programs, some of the PI's funded by the former EMSP program have been invited to this Spring PI meeting. For 2006, ERSD has decided to invite PI's conducting non-field-oriented research to the Spring PI meeting and to invite PI's conducting field-oriented research to the Fall PI meeting in Oak Ridge. Future PI meetings will be organized along other "themes" to expose as many of our investigators as possible to research by others, while maintaining the "family atmosphere" that has made these meetings so valuable.

The agenda for the 2006 Spring PI meeting includes plenary sessions in the morning and two concurrent breakout sessions in the afternoon, followed by poster sessions in the evening on both April 3rd and 4th. PI's selected to present during the plenary sessions have been chosen because their research findings are likely to provide information that will be useful during the breakout session discussions. PI's also have been asked to plan, lead, and facilitate breakout sessions. Breakout session reports, plenary session presentations, and posters will be posted on the ERSD web site.

This document contains abstracts of research funded by ERSD during Fiscal Years 2003–2006. Abstracts for this meeting are organized into four categories: Biomolecular Sciences, Microbial Ecology, Biogeochemistry/Biotransformation and Integrative Studies. Abstracts within the Biomolecular Sciences and Microbial Ecology categories are primarily those from PIs funded by the former NABIR program. Abstracts within the Biogeochemistry/Biotransformation and Integrative Studies categories include those from PIs funded by the former NABIR and EMSP programs, as well as those from other efforts funded by ERSD. These additional abstracts include DOE laboratory PIs who are part of the joint ERSD/National Science Foundation Environmental Molecular Science Institutes (EMSI), as well as other DOE laboratory PI's who provide support to environmental scientists at the Advanced Light Source (ALS), Advanced Photon Source (APS), National Synchrotron Light Source (NSLS), and Stanford Synchrotron Radiation Laboratory (SSRL). Approximately 75 of these abstracts will be presented either in the plenary session or in the poster session of this meeting by scientists funded by ERSD. In addition, six abstracts will be presented during the poster session by students funded by ERSD.

On behalf of all of the ERSD program managers, we look forward to discussing your latest research results, and to identifying opportunities to interact with other research efforts and make use of new capabilities.

Paul E. Bayer
ERSD Program Manager and Spring PI Meeting Organizer
February 2006

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Agenda
Environmental Remediation Sciences Division (ERSD) PI Meeting
Warrenton, VA
April 3–6, 2006

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

Sunday, April 2

All day — Arrival of ERSP PIs, Co-PIs, ERSD program staff and guest speakers

Monday, April 3

7:00 AM — **Breakfast** (all meals served at the Airlie Center)
8:00 AM — Welcome and Opening Remarks (Paul Bayer, ERSP Program Manager)
8:10 AM — BER Programs (David Thomassen, Acting Director, BER)
8:20 AM — ERSD Update (Mike Kuperberg, Acting Director, ERSD/BER)

Biomolecular Studies of Metal/Radionuclide Reduction

8:45 AM — Enzyme Design for Cr(VI) and U(VI) Reduction (A.C. Matin, Stanford University)
9:10 AM — Membrane Proteome of *Shewanella oneidensis* MR-1 (Carol Giometti, ANL)
9:35 AM — Biomolecular Mechanisms of Metal/Radionuclide Transformations in *Anaeromyxobacter dehalogenans* (Alex Beliaev, PNNL)
10:00 AM — Genes Involved in Microbial Survival in Aquifer Sediments (Lee Krumholz, University of Oklahoma)
10:25 AM — **Break**

Latest Findings from Microbial Community Dynamics Studies

10:40 AM — Natural Gene Transfer to Develop Resistance to Metal Toxicity in Bacterial Strains and Communities (Jeffrey Fitts, BNL)
11:05 AM — Adaptation of Subsurface Microbial Communities to Mercury (Soren Sorenson, University of Copenhagen)
11:30 AM — Community Structure in Contaminated Habitats: The Dynamic Tension between Selective Forces and Environmental Heterogeneity (Alan Konopka, Purdue University)
11:55 AM — Uranium Immobilization through Microbial Phosphatases (Patricia Sobecky, Georgia Tech)
12:20 PM — **Lunch**

2:00 PM — Introduction of the Genomics: GTL Roadmap (Roland Hirsch, BER)
2:10 PM — Overview of NRC Review of the Genomics: GTL Roadmap (Jennie Hunter-Cevera, University of Maryland Biotechnology Institute)
2:40 PM — **Breakout Sessions**

1) Genomics: GTL Roadmap: Overview and Opportunities (Roland Hirsch, BER, and Jim Fredrickson, PNNL)
2) Coupling Physical, Chemical and Biological Processes (Scott Fendorf, Stanford, George Redden, INL, and Carl Steefel, LBNL)

5:00 PM — **Dinner**
6:30 PM — **Poster Session**
Microbial Ecology, Integrative Studies, Students
9:00 PM — **Adjourn**

Tuesday, April 4

7:00 AM	**Breakfast**
8:00 AM	Announcements and Other Logistics (Paul Bayer, ERSD)
	Reduction of Metals/Radionuclides
8:10 AM	Influence of Geochemistry and Microbial Community Structure on Metal Reduction Rates (Anthony Palumbo, ORNL)
8:35 AM	Influence of Mass Transfer on U(VI) Reduction (Chongxuan Liu, PNNL)
9:00 AM	Stimulating the Microbial Reduction of Chromium (Terry Hazen, LBNL)
9:25 AM	Aqueous Complexation Reactions and Biogeochemical U(VI) Reduction (Scott Brooks, ORNL)
9:50 AM	**Break**
10:05 AM	Transformation of U(VI) under Iron-Reducing Conditions (Edward O'Loughlin, ANL)
10:30 AM	Chromate Bioremediation: Formation and Fate of Organo-Cr(III) Complexes (Luying Xun, Washington State University)
	Grand Challenge in Biogeochemistry
10:55 AM	Overview of the Biogeochemistry Grand Challenge at the Environmental Molecular Sciences Laboratory (Jim Fredrickson, PNNL)
11:20 AM	Mechanisms of Bacterial Metal Reduction (Tom DiChristina, Georgia Tech)
11:45 AM	Electron Transfer at Mineral Surfaces (Kevin Rosso, PNNL)
12:10 PM	**Lunch**
2:15 PM	**Breakout Sessions** 1) Relating Omic **[Ohmic?]** Approaches to Other Field Data (Jizhong Zhou, University of Oklahoma and Matthew Fields, Miami of Ohio) 2) Identifying New Science Opportunities in Biogeochemistry for DOE Sites (John Zachara, PNNL and Eric Roden, University of Wisconsin)
5:00 PM	**Dinner**
6:30 PM	**Poster Session** Biogeochemistry/Biotransformation, Biomolecular Sciences
9:00 PM	**Adjourn**

Wednesday, April 5

7:00 AM	**Breakfast**
8:00 AM	Announcements and Other Logistics (Paul Bayer, ERSD)
	Reduction and Other (Bio)Geochemical Processes
8:10 AM	Uranium Reduction by *Clostridia* (A.J. Francis, BNL)
8:35 AM	Behavior of Sorbed ^{90}Sr in Contaminated Subsurface Sediments (John Zachara, PNNL)
9:00 AM	Heterogeneity Impacts on Contaminant and Microbial Dynamics (Scott Fendorf, Stanford University)
9:25 AM	Reductive Immobilization of Metals by H_2S Treatment (Baolin Deng, University of Missouri)
9:50 AM	Use of Isotopic Tracers at the Hanford Site (Don DePaolo, LBNL)
10:15 AM	**Break**
	Coupled Physical, Chemical, and Biological Processes
10:30 AM	The Biogeochemistry of Pu Mobilization and Retention (Bruce Honeyman, CSM)
10:55 AM	Upscaling Coupled Pore-Scale Reactive Transport Processes to the Continuum Scale (Peter Lichtner, LANL)
11:20 AM	Coupled Flow and Reactivity in Variably Saturated Porous Media (Carl Palmer, INL)
11:45 PM	Breakout Session Summary Presentations (Breakout group leads)
12:30 PM	**Adjourn & Lunch**
1:30 PM	UMTRA Group Meeting
5:00 PM	**All Meetings Adjourn**

ABSTRACTS

Biogeochemistry/Biotransformation

The Interaction of Water with Environmentally Relevant Surfaces

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The goal of this project is to create fundamental molecular-level understanding of environmental interfaces and the important chemical and biological processes that occur at them. Using synchrotron-based spectroscopies under ambient temperatures and relative humidities, we are probing the coverage and chemical speciation of molecules, in particular water, at surfaces under realistic thermodynamic conditions.

We have used ambient pressure photoemission spectroscopy to study the interaction of water with metals and metal oxide surfaces under ambient conditions. Here, we present our *in situ* studies of water adsorption on Cu(111) and Cu(110) at pressures up to 1 Torr, in the temperature range from 0 to 200°C, and compare our results to those obtained under ultra-high-vacuum (UHV) conditions. At a relative humidity (RH) as low as 1%, the Cu(110) surface is covered to saturation by one layer of a mixed H_2O:OH (2:1) phase while no water adsorption is observed on Cu(111) even at a RH of 20 %. The drastic difference in chemistry on the two surfaces is related to the activation barrier for water dissociation. The remarkably high coverage of water and hydroxyl on Cu(110) is explained by the low dissociation barrier for water on Cu(110), leading to a high concentration of strongly bound OH to which adsorbed H_2O attaches via hydrogen bonds. Increasing the temperature of the Cu(110) surface in a 1 Torr H_2O environment leads to a transformation of the H_2O:OH surface phase into a pure OH phase that subsequently reverts into atomic O. This behavior compares well with results of UHV studies. The results of our molecular scale investigations of the difference of water adsorption on Cu(111) and Cu(110) might also help to explain macroscopic phenomena, such as the differences in the wetting of Cu(110) and (111) by water.

We have so far concentrated our investigations on the properties of the first layer of water that is adsorbed at the surface. We will in the future extend these investigations to multilayer water films that grow at surfaces at higher relative humidities and that are of importance to (for example) the solvation of ions and their transport at the surface.

Anaerobic Biotransformation and Mobility of Pu and of Pu-EDTA

Harvey Bolton Jr.[1] (PI), Vanessa L. Bailey[1], Andrew E. Plymale[1],
Dhanpat Rai[1] (Co-PI), and Luying Xun[2] (Co-PI)

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The complexation of radionuclides (e.g., plutonium [Pu]) and ^{60}Co) by co-disposed ethylenediamine tetraacetate (EDTA) has enhanced their transport in sediments at DOE sites. Pu(IV)-EDTA is not stable in the presence of relatively soluble Fe(III) compounds. Since most DOE sites have Fe(III) containing sediments, Pu(IV) is likely not the mobile form of Pu-EDTA. The only other Pu-EDTA complex stable in groundwater relevant to DOE sites would be Pu(III)-EDTA, which only forms under anaerobic conditions. Research is therefore needed to investigate the biotransformation of Pu and Pu-EDTA under anaerobic conditions and the anaerobic biodegradation of Pu-EDTA. The biotransformation of Pu and Pu-EDTA under various anaerobic regimes is poorly understood, including the reduction kinetics of Pu(IV) to Pu(III) from soluble (Pu(IV)-EDTA) and insoluble Pu(IV), the redox conditions required for this reduction, the strength of the Pu(III)-EDTA, how the Pu(III)-EDTA competes with other dominant anoxic soluble metals (e.g., Fe(II)), and the oxidation kinetics of Pu(III)-EDTA. Finally, soluble Pu(III)-EDTA under anaerobic conditions would require anaerobic degradation of the EDTA to limit Pu(III) transport. Anaerobic EDTA-degrading microorganisms have never been isolated. Recent results have shown that *Shewanella oneidensis* MR-1, a dissimilatory metal-reducing bacterium, can reduce Pu(IV) to Pu(III). The Pu(IV) was provided as insoluble PuO_2. The highest rate of Pu(IV) reduction was with the addition of AQDS, an electron shuttle. Of the total amount of Pu solubilized (i.e., soluble through a 0.36 nm filter), approximately 70% was Pu(III). The amount of soluble Pu was between 4.8 and 3.2 micromolar at Day 1 and 6, respectively, indicating rapid reduction. The micromolar Pu is significant since the drinking water limit for Pu is 10^{-12} M. Ongoing experiments are investigating the influence of EDTA on the rate of Pu reduction and the stability of the formed Pu(III). We have also begun to enrich and isolate bacteria capable of aerobic and anaerobic degradation of EDTA. Environmental samples (e.g., sludges, river sediments) were incubated aerobically and anaerobically with EDTA or NTA as the sole carbon and energy source. Aerobic enrichment with EDTA has not resulted in any cultures, but NTA has provided several isolates. Partial 16S rRNA gene sequence and sequence comparison identified four separate strains closely related to *Microbacterium oxydans*, *Aminobacter* sp., *Achromobacter* sp., *Aminobacter* sp., respectively. Anaerobic enrichments with either EDTA or NTA are still in progress since metabolism and growth is relatively slow. In addition to the biotransformation experiments, studies are under way to determine/validate complexation constants of Pu(III) with EDTA and the influence of competing ions on Pu(III)-EDTA complexes. These data are being obtained through solubility studies of $PuPO_4$(s) and $Pu(OH)_3$(s) as a function of time, pH, and EDTA and competing ion concentrations. These results have begun to fill in knowledge gaps of how anaerobic conditions will influence Pu and Pu-EDTA fate and transport to assess, model, and design approaches to stop Pu transport in groundwater at DOE sites.

Anaerobic, Nitrate-Dependent Fe(II) Bio-Oxidation: A Column Study

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Laurie A. Achenbach[2], and John D. Coates[1] (PI)

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Previous studies have demonstrated that nitrate-dependent bio-oxidation of Fe(II) by *Azospira suillium* strain PS results in the formation of crystalline mixed Fe(II)/Fe(III) mineral phases, which results in the subsequent immobilization of heavy metals and radionuclides. Greater than 80% of the U(VI) was sequestered by the most dense, crystalline Fe(II)/Fe(III) mineral phases, which are not readily reduced by Fe(III)-reducing bacteria. Most probable number enumeration revealed nitrate-dependent Fe(II) oxidizing microbial communities in groundwater and subsurface sediments in the order of 0–2.04×10^3 cells mL^{-1} and 2.39×10^2–1.17×10^3 cells (g wet sediment)$^{-1}$, respectively. The efficacy of nitrate-dependent Fe(II) oxidation under advective flow was evaluated in a mesoscale column reactor packed with sterile low iron sand amended with subsurface sediments collected from the ERSD Field Research Center (FRC) background field site (10% mass/mass). Continuous flow of minimal medium mimicked the natural groundwater. Periodic $FeCl_2$ and nitrate injections over a period of 49 days resulted in the retention of 95% of the iron (~20.3 mmol). Extraction of solid-phase Fe revealed a net increase in Fe(III) of 13.2 mmol above background Fe(III) content, indicating that 65% of the injected Fe(II) was oxidized. Differential solubility analysis of 0.5 M HCl-extractable Fe and 3 M HCl-extractable Fe indicated that the oxidation product was crystalline in nature, because only 20% was soluble in 0.5 M HCl. This formation of crystalline biogenic Fe(III) oxides is consistent with our previous studies. Periodic injections of nitrate and acetate did not result in significant changes in Fe(II) or Fe(III) throughout a control column.

Enumeration of the nitrate-dependent Fe(II) oxidizing microbial community in the columns indicated that the Fe(II) and nitrate injection stimulated an anaerobic, nitrate-dependent Fe(II) oxidizing community (7.41×10^5 cells mL^{-1}) just above the injection point (12.5–15 cm depth). This microbial community is ~40% of the heterotrophic nitrate-reducing community and ~350% of the heterotrophic Fe(III)-reducing community. The abundance of the nitrate-dependent Fe(II) oxidizing microbial community enumerated in the column injected with nitrate and acetate was less than 0.0001% of the abundance of the heterotrophic nitrate-reducing microorganisms, suggesting that heterotrophic nitrate-reducing microorganisms were not responsible for Fe(II) oxidation. This result was confirmed by small-subunit 16S rDNA clone libraries. At the point of injection, ~47% of the microbial community was represented by the Acidobacteria and Actinobacteria in the column injected with Fe(II) and nitrate. Whereas the injection of acetate and nitrate stimulated the Betaproteobacteria (86%) and was dominated by *Azoarcus* sp. (66%). The frequency of clones identified as Actinobacteria in the column injected with Fe(II) and nitrate represented the background abundance. However Acidobacteria clones were only observed at the point of injection and represented ~21% of the identified clones. These results suggest that Acidobacteria play a role in anaerobic, nitrate-dependent Fe(II) oxidation in these subsurface sediments. Together these results demonstrate that native subsurface sediments harbor microbial communities capable of nitrate-dependent Fe(II) oxidation under advective flow. The biogenic formation of reactive Fe(III) oxide minerals capable of immobilizing heavy metals and radionuclides presents a plausible bioremediative strategy for contaminated subsurface environments.

Anaerobic U(IV) Bio-Oxidation

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A proposed strategy for the remediation of uranium (U) contaminated sites is based on immobilizing U by reducing the oxidized soluble U(VI) to form a reduced insoluble end product, U(IV). Owing to the use of nitric acid in the processing of nuclear fuels, nitrate is often a co-contaminant found in many of the environments contaminated with uranium. Recent studies indicate that direct biological oxidation of U(IV) coupled to nitrate reduction may exist *in situ*. In an effort to evaluate the potential for nitrate-dependent bio-oxidation of U(IV) in anaerobic sedimentary environments, we have initiated the enumeration of microorganisms capable of catalyzing U(IV) oxidation. Sediments, soils, and groundwater from U-contaminated sites, including subsurface sediments from the ERSD Field Research Center (FRC), as well as uncontaminated sites, including subsurface sediments from the ERSD FRC and Longhorn, Texas, lake sediments and agricultural field soil sites, served as the inoculum source. Most probable number enumeration in these sedimentary environments revealed sedimentary microbial communities exhibiting anaerobic, nitrate-dependent U(IV) oxidizing metabolisms ranging from 9.3×10^1–2.398×10^3 cells g^{-1} sediment in both contaminated and uncontaminated sites. Interestingly, uncontaminated subsurface sediments harbored the most numerous community (2.398×10^3 cells g^{-1} sediment) capable of this metabolism. Given that only 5–225 μM U(IV) was oxidized relative to negative controls, it is unlikely that significant growth was coupled to U(IV) bio-oxidation in the enumeration series. The role of nitrate reduction intermediates in the oxidation of U(IV) cannot be established in the enumeration series and could have indirectly accounted for U(IV) oxidation. Small-subunit rRNA clone libraries constructed from the lowest dilution MPN series revealed a diverse phylogeny of organisms, including gram positive bacteria and members associated with the Alpha, Beta, and Gamma subclass of the Proteobacteria. However, because of limited growth and the low dilutions at which U(IV) oxidation was observed in these experiments, it is impossible to discern the microorganisms catalyzing U(IV) oxidation from the previously established microbial community. Physiological screening of a mixotrophic nitrate-dependent Fe(II) oxidizing bacterium, *Diaphorobacter* sp. strain TPSY, isolated from Area 2 of the DOE ERSD FRC, resulted in the oxidation of 8 μM U(IV) over 24 hours, with nitrate serving as the electron acceptor in washed cell suspensions. Pasteurized control cultures exhibited the abiotic oxidation of 2 μM U(IV). Similarly, the catalysis of U(IV) oxidation (4 μM) was also observed in washed cell suspensions of a previously described freshwater, autotrophic nitrate-dependent Fe(II) oxidizing bacterium, *Cosmobacter millennium* strain 2002. Together with previously published results, these data suggest that anaerobic, microbial catalysis of U(IV) oxidation may be a common metabolism in soil, sedimentary, and groundwater environments that could result in the remobilization of reduced U in anoxic environments.

Renewal: Interfacial Reduction-Oxidation Mechanisms Governing Fate and Transport of Contaminants in the Vadose Zone

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Many soil contamination sites at DOE installations contain radionuclides and toxic metals such as technetium (Tc), uranium (U) and chromium (Cr). *In Situ* Gaseous Reduction (ISGR) using dilute hydrogen sulfide (H_2S) as reductant is a technology uniquely suitable for the vadose zone soil remediation of these contaminants through reduction. It is conceivable that the ISGR approach can be applied either to immobilize pre-existing contaminants or to create a reductive permeable reactive barrier (PRB) for contaminant interception. This project aims to improve our understanding of the complex interactions among the contaminants (U, Tc, and Cr), H_2S, and various soil constituents. Specific research tasks include: (a) examining the reduction kinetics of Tc(VII) and U(VI) by H_2S; (b) measuring the reduction kinetics of Tc(VII) and U(VI) by iron sulfides; (c) characterizing the speciation of immobilized Tc and U and investigate the immobilization mechanisms; (d) assessing the long-term stability of the contaminants immobilized by the ISGR treatment; and (e) validating the pure phase experimental results under natural soil conditions.

Significant progress has been made for all tasks.

1. *Kinetics of Uranium(VI) Reduction by Hydrogen Sulfide in Anaerobic Aqueous Systems***:** Aqueous U(VI) reduction by hydrogen sulfide was investigated by batch experiments and speciation modeling, as well as product analyses by transmission electron microscopy (TEM) and x-ray absorption spectroscopy (XAS). The results show that U(VI) reduction is largely controlled by pH and $[CO_3^{2-}]_T$. Uranium-hydroxyl species are reduced by sulfide, but not the U-carbonate species.
2. *U(VI) Reduction at FeS-Water Interfaces*: U(VI) reduction by FeS particles proceeded via a two-step process: rapid cation exchange between UO_2^{2+} and Fe^{2+}, followed by sorbed U(VI) reduction by sulfide. The reaction was first order with respect to U(VI) concentration, with uraninite as the reduction product.
3. *Uranium Immobilization by Gas-Treated Soil:* Column and batch tests were conducted to evaluate the potential for immobilizing dissolved U(UI) by Hanford formation soil treated with a 200 ppm H_2S/N_2 gas mixture. ISGR-treated Hanford soil is capable of effectively immobilizing U(VI) from simulated ground water. The immobilization is enhanced by soil treatment undertaken with a moisturized H_2S gas mixture.
4. *Pertechnetate Reduction by Sulfide*: Reactions of Tc-99 pertechnetate with sulfide under a variety of conditions were examined to understand the chemistry of these interactions and the reaction kinetics/mechanism. Variables include pH (1–14), sulfide concentration, pertechnetate concentration, buffer and buffer concentration, aerobic conditions, anaerobic conditions, the presence of other anions, ionic strength, and the presence of chelating ligands. Under aerobic conditions, the reaction between pertechnetate and sulfide under acidic conditions might proceed to yield Tc_2S_7, while under basic conditions, the product might be $Tc(S)O_3^-/TcS_4^-$ or Tc_2S_7. Under acidic conditions, a black precipitate formed, with a higher precipitate yield at the lower pH (pH 1 ~83%; pH 2 ~55%; pH 6 ~10%). The reactions were first order in pertechnetate concentration, first order in sulfide concentration, and first order in acid concentration. Under basic conditions, no precipitate formed, and solution analyses showed only the presence of the starting materials.

Isotopic Tracers for Vadose Zone Processes and Contaminant Sourcing: Hanford, Washington

Donald J. DePaolo (PI), John N. Christensen, and Mark E. Conrad

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The objective of this research is to evaluate geochemical approaches to characterizing fluid flow and chemical transport through the vadose zone, using isotopic measurements of natural soils, minerals, pore fluids and groundwater. We have developed and implemented a suite of isotopic techniques, using the elements H, O, N, Sr, and U, to study the interconnection between vadose zone and groundwater contamination at the Hanford Site in south-central Washington. We have been able to use isotopic measurements to establish sources of contamination and place constraints on the rates of transfer through the vadose zone to groundwater. The Hanford Site is situated along an unimpounded portion of the Columbia River, the highest discharge volume river west of the continental divide. Decades of nuclear-related activities have left significant local contamination (e.g., nitrate, U, Cr^{6+}, ^{99}Tc) in the vadose zone and groundwater within the site. Some of this contamination has reached the Columbia River, and there remains the potential for further contaminant migration to the river. Understanding the fate and transport of contaminants has been complicated by the presence of multiple potential sources within relatively small areas. Our multiple-isotopic system approach has proved to be a powerful means to identify sources of contaminants and, once the sources are identified, to understand the subsurface transport mechanisms.

The isotopic composition of nitrate can be used to distinguish high-level tank waste (high $\delta^{15}N$) and low-level process wastes (high $\delta^{18}O$) from the relatively high background concentrations of nitrate in the groundwater at the site. Through mapping of the Sr and O isotopic composition of groundwater, we have been able to provide a picture of groundwater source and movement across the Hanford Site that is independent of the contaminant distributions. The $^{87}Sr/^{86}Sr$ of strontium is typically elevated above background in areas where large volumes of water have been flushed through the vadose zone. Conversely, interaction between high-level caustic waste and feldspars in the vadose zone sediments releases strontium with low $^{87}Sr/^{86}Sr$. High-precision measurements of uranium isotopic ratios ($^{234}U/^{238}U$, $^{235}U/^{238}U$, $^{236}U/^{238}U$) have been particularly useful for distinguishing different generations of nuclear fuel processing, allowing attribution of U-bearing waste in the vadose zone and groundwater to specific known or suspected leaks or spills, and to identify the vadose zone sources of groundwater U plumes.

As illustrations of our research, we will highlight (1) the use of natural U and Sr isotopic systematics in the vadose zone to simultaneously constrain rates of infiltration and weathering, (2) isotopic data bearing on the sources of ^{99}Tc and nitrate contamination in groundwater in the vicinity of the T-WMA tank farm, and (3) the source and flux of contaminant U from the Hanford Site to the Columbia River and its fate.

Composition, Reactivity, and Regulation of Extracellular Metal-Reducing Structures (Bacterial Nanowires) Produced by Dissimilatory Metal-Reducing (and Other) Bacteria

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Redox transformation of heavy metals and radionuclides influences the migration of contaminants in subsurface sedimentary environments. Dissimilatory metal reducing bacteria catalyze the reduction of many valence transformations by poorly understood mechanisms. These organisms produce electrically conductive appendages, which we call bacterial nanowires, in direct response to electron acceptor limitation. Nanowires produced by *S. oneidensis* strain MR-1, which served as our primary model organism, are functionalized by decaheme cytochromes MtrC and OmcA that are distributed along the length of the nanowires. Mutants deficient in MtrC and OmcA produce nanowires that were poorly conductive, as determined by Scanning Tunneling Microscopy (STM). These mutants also differed from the wild type in their inability to reduce solid-phase iron oxides, poor power production in a mediator-less microbial fuel cell, and failure to form complex biofilms at air-liquid interfaces. Nanowires were also produced by other bacteria, including the oxygenic, phototrophic cyanobacterium *Synechocystis* PCC6803. These results demonstrate that electrically conductive nanowires are not restricted to metal-reducing bacteria and may be common throughout the bacterial world, where they serve as structures for efficient electron transfer and energy distribution.

Reduction and Reoxidation of Soils during and after Uranium Bioremediation: Implications for Long-Term Uraninite Stability and Bioremediation Scheme Implementation

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This research focuses on the conditions and rates under which uranium (U) will be remobilized after it has been precipitated biologically, and what alterations can be implemented to increase its long-term stability in groundwater after the injection of an electron donor has been discontinued. Furthermore, this research addresses short-term iron reoxidation as a mechanism to enhance/extend U bioremediation under iron (Fe) reduction, without its remobilization.

The research to date has focused on long-term column experiments involving the biological removal of U from groundwater under Fe- and sulfate-reducing conditions. Aquifer sediment was collected from the background area of the Old Rifle, CO, Uranium Mill Tailings Remedial Action (UMTRA) site and dried and sieved (<2 mm) before being packed into four 15 cm long × 5 cm diameter glass columns. After the natural U was flushed out of the system, a feed media containing 30 mM bicarbonate, 20 μM U(VI) (as uranyl acetate), 3 mM acetate, and 9 μM sulfate was purged with CO_2/N_2 gas (20:80) and supplied to each column at a rate of 0.2 mL/min. Biostimulation was facilitated by the addition of the Fe(III)- and U(VI)-reducing microorganism, *Geobacter metallireducens*. Fe(III) and U(VI) reduction was detected in all four columns after three days of column operation, with effluent Fe(II) and U(VI) concentrations reaching pseudo-steady-state concentrations after 10 and 23 days, respectively. The effluent Fe(II) concentrations for each column ranged from 130 μM to 170 μM, and U(VI) removal from each column was between 58% to 77%. Sulfate reduction was measured in each column with 95% of the initial 9 μM sulfate removed. Phosphate, present in the influent media at a concentration of 13 μM, was removed to non-detect concentrations.

One of the four columns was taken offline after 104 days and destructively sampled. All of the surface-bound Fe (extractable after 24 hours in 0.5M HCl) was reduced, with the average Fe(II) concentration measured to be 20.4 (±7.9) μmol/g dry sediment. The majority (70%) of the surface-bound uranium (extractable after 24 hours in 0.2M bicarbonate) was present as U(IV), with most of the U precipitation occurring at the beginning and end of the column. The speciation and distribution of U and Fe throughout the column is also currently being analyzed using x-ray absorption spectroscopy and Mossbauer spectroscopy. Oxygen will be added to the remaining columns with effluent U(VI), Fe(II), and dissolved oxygen concentrations monitored with time. These results, along with a comparison of the oxidized sediment to the pristine and biologically reduced sediment, will be discussed.

Upscaling Reactive Transport Processes from Pore to Continuum Scales in Porous and Fracture Media

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Modeling reactive flows in porous media is an important tool for understanding and predicting subsurface contaminant migration and evaluating different remediation strategies for contaminated sites. For example, at the Hanford DOE facility, modeling, closely integrated with laboratory and field results, plays an important role in understanding the migration of radionuclides released during leaks of underground storage tanks and the behavior of U(VI) plumes at the 300 Area. Current modeling approaches commonly employ a single continuum description or simplistic dual continuum approach that only allows for a single matrix node. Thus, these approaches do not capture local gradients caused by fast reaction rates and, most importantly, pathways involving secondary porosity and dead-end pores. Further, these approaches rely on heuristic volume averages taken over scales much larger than typical grain sizes, and thus are unable to resolve spatial heterogeneities at smaller scales, potentially leading to inaccurate upscaling of pore-scale processes. In this study, we apply Lattice-Boltzmann and pore-network models to investigate multicomponent reactive transport at the pore scale. By comparing the pore-scale results averaged over a representative elementary volume to continuum scale models, the validity of volume averaging can be ascertained for complex pore geometries. Through upscaling pore-scale processes to the continuum scale, it is possible to identify key parameters and physicochemical processes that control macroscopic phenomena, simultaneously providing constitutive relations needed in continuum models. We hypothesize that pore-scale simulations will enable the most appropriate continuum model—single or dual continuum—to be determined, or will demonstrate that upscaling is in fact not possible—for example, as is expected in the presence of reaction instabilities resulting in wormhole formation. In cases where upscaling is shown to be valid, pore-scale simulations can provide appropriate values for macro-scale properties of the porous medium, such as primary and secondary flow domains and interfacial areas, permeability, tortuosity, dispersivity, and reactive surface area.

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

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Our objectives in this work are (1) evaluate the bioavailability and mechanisms of microbial reduction of sorbed U(VI); (2) investigate fundamental mechanisms of the solute mass transfer process, and (3) develop coupled process models to describe microbial reduction of sorbed U(VI).

The bioavailability and mechanisms of microbial reduction of sorbed U(VI) was investigated using: (1) contaminated sediments from Hanford BX-tank farm that contained U(VI) as uranyl silicate precipitates in micropores and fractures within granitic lithic grains, and (2) alginate beads containing intra-bead synthetic Na-boltwoodite. The experiments were performed with variable cell (*Shewanella oneidensis* MR-1) and U(VI) concentrations. Uranium speciation and distribution was monitored by LIFS and XAS. Biogenic U(IV) precipitates and their bacterial association were examined by transmission electron microscopy (TEM). Our results indicated that U(VI) had to dissolve and diffuse out of intragrain regions before it was microbially reduced. Experimental and modeling results showed strong and sequential coupling of dissolution reactions, diffusive mass transfer, U(VI) aqueous speciation reactions, and microbial reduction of aqueous U(VI). The rates of microbial reduction of aqueous U(VI) that was dissolved/diffused out of intragrain regions in the Hanford sediment were about 2 orders of magnitude slower than that in the control solution without the sediment. The slower bioreduction rate resulted from the dissolution of calcite in the sediment that changed aqueous U(VI) speciation.

Experiments were conducted to evaluate the influence of calcium dissolved from calcite on the coupling of U(VI) dissolution/diffusion, and microbial reduction. Calcium increased the rates of dissolution/diffusion of intragrain U(VI) by increasing local U(VI) solubility, but decreased the rates of microbial reduction of aqueous U(VI). The relative strength of these two effects determined the overall effect of calcium on the rate of microbial reduction of sorbed U(VI). Experimental and modeling studies were also performed to investigate whether bacteria can preferentially use kinetically favorable U(VI) species. Results showed that bacteria (MR-1) randomly used both kinetically favorable and unfavorable U(VI) species as terminal electron acceptors. This presents a challenge to model the kinetics of microbial reduction of U(VI) in systems with time-variable U(VI) speciation.

Experimental and theoretical modeling studies were performed to evaluate the fundamental mechanisms of diffusive mass transfer process in the Hanford granitic lithic fragments and in the Oak Ridge FRC background sediment. We have developed a microscopic two-region multicomponent reactive-ion-diffusion model for the Hanford sediment based on microscopic insights from nuclear magnetic resonance and scanning electron microscopy (SEM) characterization. Model simulations showed that diffusion limitation in the intragrain fractures will allow the long-term persistence of precipitated uranium in the Hanford sediment that could otherwise dissolve relatively rapidly. Reactive diffusion of U(VI) in fine-grained FRC sediment was a strong function of pH. The half life of U(VI) diffusion from the U(VI)-adsorbed sediment was about 4 months at pH 9.5 or 4. The apparent diffusion rate decreased over 100 times from pH 4.5 or 9.5 to 7 because of strong U(VI) adsorption to the sediment at circumneutral pH and possible anion repulsion effects. A model to include anion repulsion was developed to describe ion diffusion in clay materials. The model-derived diffusivity is a complex function of soil electro-chemical properties and aqueous composition, presenting a significant challenge for characterization of the diffusive mass transfer process.

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 (Tc)-99 is a priority pollutant at numerous DOE sites, due to its long half life (2.1×10^5 years), 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. In previous studies we have focused on the fundamental mechanisms of Tc(VII) bioreduction and precipitation, identifying direct enzymatic (hydrogenase-mediated) mechanisms and a range of potentially important indirect transformations catalyzed by biogenic Fe(II), U(IV) or sulfide. These baseline studies have generally used pure cultures of metal-reducing bacteria 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 is needed if *in situ* remediation of Tc(VII) contamination is to be successful at DOE sites.

The aim of this project is to use a multidisciplinary approach to identify the biogeochemical factors that control the mobility of environmentally relevant concentrations of Tc(VII) in ERSD Field Research Center (FRC) sediments, and to assess the effectiveness of strategies proposed to stimulate Tc(VII) reduction and precipitation in the subsurface. Initial experiments focused on obtaining baseline data from FRC "background" sediments. Progressive microcosms incubated with/without added electron donor (20 mM acetate) showed that Tc(VII) reduction occurs concomitant with Fe(III)-reduction. The addition of 10 mM nitrate and 20 mM acetate had little impact on metal reduction, but 100 mM nitrate (with acetate) completely inhibited the reduction of both Tc(VII) and Fe(III). Molecular analyses confirmed the presence of Fe(III)-reducing bacteria known to reduce both Fe(III) and Tc(VII) in axenic culture (*Geobacter* and *Geothrix* species), while nitrate-reducing bacteria were also detected (including *Azoarcus* species) and were present at higher concentrations than Fe(III)-reducing bacteria in MPN dilution series. X-ray absorption spectroscopy identified TcO_2 as the dominant form of Tc in postreduction sediments. Reoxidation of TcO_2 was also studied using nitrate and air as oxidants. Remobilization of Tc was minimal with 100 mM nitrate, but significant (~80%) under air reoxidation conditions, while Fe(II) oxidation was noted in both treatments. Extended x-ray absorption fine-structure analyses of sediments reoxidized with nitrate showed the presence of both Tc(IV) and Tc(VII) immobile phases, suggesting that under anaerobic conditions, Tc(IV) will not remobilize rapidly, even in the presence of high concentrations of nitrate.

Experiments were also conducted using columns containing reduced FRC background sediments with stratified microbial communities. These were challenged with γ-emitting ^{99m}Tc, and the radionuclide was shown to accumulate in zones of Fe(III) reduction (confirmed by microbiological and geochemical analysis) using a γ-camera. Current experiments focus on refining the γ-camera imaging techniques for real-time monitoring of Tc mobility in sediments and also on assessing the biogeochemical controls on Tc solubility in low pH/nigh nitrate sediments from Area 3 of the FRC.

Uranium (VI) Reduction by Anaeromyxobacter dehalogenans

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The project goals are to characterize U(VI) reduction in *Anaeromyxobacter* species and evaluate their contribution to U(VI) immobilization. Previous studies demonstrated growth of *Anaeromyxobacter dehalogenans* strain 2CP-C with acetate or hydrogen as electron donors and Fe(III), nitrate, nitrite, fumarate, oxygen, or ortho-substituted halophenols as electron acceptors. Strain 2CP-C readily reduced U(VI) with hydrogen, but not acetate, provided as electron donor. Quantitative real-time PCR (qPCR) demonstrated that strain 2CP-C grew at the expense of U(VI)-to-U(IV) reduction. Nitrate, Fe(III)citrate, or citrate inhibited U(VI) reduction, whereas 2-chlorophenol and ferric iron (provided as Fe(III) pyrophosphate) had no effect and was concomitantly reduced. In the presence of amorphous Fe(III) oxides, U(VI) reduction proceeded to completion, but at three-fold lower rates compared with control cultures. The genome analysis of strain 2CP-C revealed the presence of 4,313 candidate protein-encoding genes. Among them, 61 putative c-type cytochrome genes with at least one heme binding motif and 17 genes with more than 10 such CXX(XX)CH motifs were identified. A separate ERSP project (PI A. Beliaev) uses microarray technology to explore the *Anaeromyxobacter* transcriptome and elucidate the role c-type cytochromes play in U(VI) reduction.

A sensitive and specific 16S rRNA gene-based qPCR approach was designed to detect, monitor, and quantify *Anaeromyxobacter* species in environmental samples. Using these tools, *Anaeromyxobacter* 16S rRNA gene sequences were retrieved from the Oak Ridge Field Research Center (FRC) site samples. The sequence analysis suggested the presence of multiple *Anaeromyxobacter* strains at the FRC. Microcosms were established with FRC site (Area 1) materials to enrich and isolate *Anaeromyxobacter* species (and other metal reducers) responsible for radionuclide reduction at the FRC site. Numerous sediment-free cultures were obtained, and the enrichment of *Anaeromyxobacter* spp. was monitored with qPCR.

Investigation of the Transformation of Uranium under Iron-Reducing Conditions: Reduction of UVI by Biogenic FeII/FeIII Hydroxide (Green Rust)

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This project addresses fundamental aspects of the effects of coupled biotic and abiotic processes on uranium (U) speciation in subsurface environments where iron (Fe) redox cycling is a significant process. The long-term objective of this research is to evaluate whether reduction of U^{VI} by biogenic green rusts (GRs) is a significant mechanism for immobilization of U in subsurface environments. The ability of synthetic GR to reduce U^{VI} species to insoluble UO_2 suggests that biogenic GRs may play an important role in the speciation (and thus mobility) of U in Fe^{III}-reducing environments. However, little is known about how biogeochemical conditions (such as pH, U concentration, carbonate concentration, and the presence of co-contaminants) and GR composition affect the rate and products of U^{VI} reduction by GRs. It is also unclear which biogeochemical conditions favor formation of GR over other nonreactive Fe^{II}-bearing biomineralization products from the reduction of Fe^{III} by dissimilatory iron-reducing bacteria (DIRB). To address these issues, the following objectives are proposed: (1) identify the geochemical conditions that favor the formation of biogenic GRs from the reduction of Fe^{III} oxides and oxyhydroxides by DIRB (e.g., *Shewanella* and *Geobacter* species); (2) characterize the chemical composition of biogenic GRs (e.g., Fe^{II}:Fe^{III} ratios and interlayer anions) and the effects of compositional variability on the rate and extent of U^{VI} reduction; (3) evaluate the effects of variations in geochemical conditions—particularly pH, U concentration, carbonate concentration, the presence of organic ligands, and the presence of reducible co-contaminants—on both the kinetics of U^{VI} reduction by biogenic GR and on the composition of the resulting U-bearing mineral phases; and (4) determine the potential for coupling the reduction of Fe^{III} by DIRB to the reduction of U^{VI} via biogenic Fe^{II} species (including biogenic GRs).

Our results to date show that a diverse range of *Shewanella* spp. are able to reduce Fe^{III} in lepidocrocite to Fe^{II} when provided with formate as an electron donor. Analysis of the resulting biomineralization product(s) by scanning electron microscopy, x-ray diffraction, and Mössbauer spectroscopy provided results consistent with the formation of GR as the only major solid-phase product. GR was also the only product observed when lactate was provided as the electron donor for lepidocrocite reduction; however, siderite was the main product when either pyruvate or serine was provided. While there are differences in the rate of Fe^{II} production as well as differences in the morphologies of the GR crystals among the *Shewanella* spp. examined, U LIII absorption edge x-ray absorption fine structure spectroscopy indicates that the GRs produced by different *Shewanella* spp. are all able to reduce U^{VI} to U^{IV}, resulting in the formation of nanoscale particles of UO_2. Under our experimental conditions, the reduction of U^{VI} by GR is rapid, with complete reduction typically observed in less than 2 hours. The ability of GRs to reduce U^{VI} appears to be constrained by the nature of the interlayer anion. U^{VI} is rapidly removed from solution in the presence of chloride, sulfate, and carbonate GR. However, while U^{VI} was reduced to U^{IV} by chloride and sulfate GR, U^{VI} was not reduced in systems containing synthetic carbonate GR.

Bioremediation Approaches for Sustained Uranium Immobilization Independent of Nitrate Reduction

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The daunting prospect of complete nitrate removal at DOE sites such as the ERSD Field Research Center (FRC) at Oak Ridge provides strong incentive to explore bioremediation strategies that will allow for uranium (U) bioreduction and stabilization in the presence of nitrate. Typical *in situ* strategies involving the stimulation of metal-reducing bacteria are hindered by the low pH environment and require that the persistent nitrate must be first and continuously removed or transformed. This project investigates the possibility of stimulating nitrate-indifferent pH-tolerant organisms to achieve nonspecific bioreduction of U(VI) despite nitrate persistence.

Enrichments from FRC Area 2 sediments were prepared using a variety of electron donors (ethanol, glycerol, hydrogen, and glycerol) and MOPS/TRIS buffers at pHs ranging from 4.9 to 7. Successful enrichments containing 10–20 mM methanol have demonstrated the nearly complete reduction of uranium (90% reduction at ~10 ppm) with very little loss of nitrate (less than 10% loss at ~850 ppm) from pH 4.9–5.5. Many higher pH enrichments also demonstrated similar U reduction capacity with 5–30% nitrate loss. Bacterial 16S rRNA genes from successful enrichments at pH 5.7–6.7 were amplified and sequenced for phylogenetic analysis. A majority of clone sequences retrieved from enrichment cultures were comprised of *Clostridia*, *Clostridia*-like organisms, and Bacteroidetes.

Further experiments tested the stability of ~2 ppm U(IV) in nitrate or nitrite solutions. When added to water with varying degrees of oxygen removal, U(IV) was stable and oxidized only when exposed to air. The presence of nitrite (100 ppm) or nitrate (1000 ppm) did not induce measurable oxidation over the several-week time scale of measurements.

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

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A concurrent experimental and modeling study centers on the interactions of *Shewanella alga BrY* with plutonium (Pu), the key contaminant of concern at several DOE sites that are being addressed by the overall ERSP program. The goal is to understand the long-term stability of bioprecipitated "immobilized" Pu phases under changing redox conditions in biologically active systems. Our hypothesis is that stable Pu phases will prevail where bioreduction occurs. Understanding the relationships among aqueous speciation, biological effects and interactions, and the fate and immobilization of Pu is the long-term goal of this research.

Experimentally, we have focused on batch experiments to establish the key interactions between actinides and *S. alga* under anaerobic conditions. Our initial emphasis was on the bioreduction of uranium (U) as UO_2^{2+} organic complexes, in the presence of aqueous iron, by *S. alga*. These U studies are being done to develop an experimental approach for the Pu systems and provide a benchmark to evaluate the modeling of anaerobic biological activity with CCBATCH. In the uranyl system, we have established the conditions of growth and growth kinetics, that there are no toxicity effects up to mM U concentrations, and approaches to distinguish the iron from the U chemistry. Additionally, we are showing a strong abiotic component (primarily Fe^{2+} interactions) for Pu, when iron reduction is prevalent, that we predict will lead to complex abiotic-biotic interactions for the Pu system. Future directions are to complete the U batch experiments, model them using CCBATCH, and extend the same batch approach to PuO_2^{+} and PuO_2^{2+} inorganic and organic complexes.

Modeling activities have centered on upgrading the CCBATCH biogeochemical model to include anaerobic growth of *S. alga* and relevant Pu speciation data. New components, complexes, and biological and kinetic parameters were updated in the model as they relate to the species found in the growth media of *S. alga*. One of the challenges was to convert the model to allow bacterial growth anaerobically, so that it depends on Fe^{3+}, not oxygen, as electron acceptor. The problem is that Fe^{3+} complexes with many anionic species in the media, and these complexes may or may not contain bioavailable Fe^{3+}. Although ignoring all Fe^{3+} complexes allows bacterial growth in the model, this is not a realistic representation of the media. Future work will involve determining which Fe^{3+} complexes are bioavailable, expanding the Pu speciation database, and incorporating extracellular polymeric substances (EPS) and SMP into CCBATCH as it relates to *S. alga* growth.

Formation of Acidic and Basic OH on TiO_2(110)

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The adsorption of water on a rutile(110) single crystal was studied with x-ray photoemission at temperatures above 270 K, using a novel instrument that makes it possible to obtain photoelectron spectra in the presence of gases up to a few Torr pressure, thus ensuring that equilibrium conditions can be reached. Two types of OH species were found to form as a result of water dissociation before growth of molecular water. One is acidic, caused by hydrogen (H) attachment to lattice oxygen (O) in bridge positions; the other is basic and is bound to the titanium (Ti) sites. Both groups originate from H_2O dissociation. Molecular water adsorption starts at the Ti sites only after formation and saturation of these OH species. Equilibrium isobars for 6.5 m Torr H_2O have been obtained up to at least eight molecular layers of water in equilibrium with the vapor.

In the future, we plan to continue the studies of water adsorption on oxide surfaces that vary in acidity, from MgO, to Fe_2O_3, V_2O_5, and SiO_2. Emphasis is on obtaining equilibrium phase diagrams (amount of adsorbed water versus vapor p, T), and equally important information on the structure of the water films—for example, the degree of dissociation near the surface, and the bonding structure and orientation of the water. This information will be obtained not only from x-ray photoemission spectroscopy (XPS) measurements but also from near-edge x-ray absorption fine structure (NEXAFS) measurements.

Promoting Uranium Immobilization by the Activities of Microbial Phosphates

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The overall goal of this project is to examine the role of nonspecific phosphohydrolases present in naturally occurring subsurface microorganisms, for the purpose of promoting the immobilization of radionuclides through the production of uranium [U(VI)] phosphate precipitates. Specifically, we hypothesize that the precipitation of U(VI) phosphate minerals may be promoted through the microbial release and/or accumulation of PO_4^{3-}. During this phase of the project, we have been conducting assays to determine the effects of pH, inorganic anions and organic ligands on U(VI) mineral formation and precipitation when ERSD Field Research Center (FRC) bacterial isolates were grown in a defined minimal medium. Our experimental results indicate that species such as NH_4^+, CO_3^{2-}, glycerol-3-phosphate, and inositol-6-phosphate influence the precipitation and the toxicity level of U(VI). The mineral $(UO_2)_3(PO4)_{2(s)}$ precipitates at pH 5 and is not influenced by carbonate below pH 6. However, in a minimal medium containing NH_4^+, the mineral uramphite, $(NH_4)(UO_2)(PO_4)_{(s)}$,, forms and is stable over a greater pH range. At pH > 6, carbonate, which is present in the FRC, forms soluble complexes with U(VI), thereby increasing the solubility and mobility of U, thus highlighting the importance of acidic conditions for promoting microbial phosphate-driven precipitation.

The molecular characterization of FRC isolates has also been undertaken during this phase of the project. Analysis of a subset of gram-positive FRC isolates cultured from FRC soils (Areas 1, 2, and 3) and background sediments have indicated a higher percentage of isolates exhibiting phosphatase phenotypes (i.e., in particular those surmised to be PO_4^{3-}-irrepressible) relative to isolates from the reference site. A high percentage of strains that exhibited such putatively PO_4^{3-}-irrepressible phosphatase phenotypes were also resistant to the heavy metals lead and cadmium. Previous work on FRC strains, including *Arthrobacter*, *Bacillus*, and *Rhanella* spp., has demonstrated differences in tolerance to U(VI) toxicity (200 μM) in the absence of organophosphate substrates. For example, *Arthrobacter* spp. exhibited the greatest tolerance to U(VI), while the *Rhanella* spp. have been shown to facilitate the precipitation of U(VI) from solution, and the *Bacillus* spp. demonstrated the greatest sensitivity to acidic conditions and high concentrations of U(VI). In the presence of inositol-6-phosphate (IP6), the toxicity of U(VI) to *E. coli* and the FRC *Rahnella* sp.Y9602 appears to be ameliorated, possibly because of the complexation of U(VI) with the phosphate moieties on the IP6 molecule. Polymerase chain reaction (PCR)-based detection and hybridizations of FRC strains are being conducted to determine if nonspecific acid phosphatases of the known molecular classes [i.e., classes A, B, and C] are present in these FRC isolates. Additionally, these amplified phosphatases are being analyzed to determine whether there is evidence for the horizontal transfer of such genes among subsurface microbial populations. Dissolved U and microbially precipitated U(VI) phosphate minerals will be further analyzed via capillary electrophoresis and extended x-ray absorption fine structure spectroscopy, respectively, to elucidate U speciation.

Scale Dependence of Reaction Rates in Porous Media

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The purpose of this project is to increase our understanding of the scale dependence of (bio)geochemical reaction kinetics in natural porous media. The present lack of understanding limits our ability to develop effective bioremediation schemes for contamination cleanup, to develop predictive models for CO_2 sequestration in deep aquifers, and even to determine the fundamental controls on the rates of chemical weathering, an important long-term regulator of atmospheric CO_2 levels.

The research approach is to compare reaction-rate data using conventional well-mixed flowthrough reactors and less conventional microfluidic-reactive-flow devices so as to interpret effective rates in porous media. As model systems, we are considering (1) the dissolution of calcite (a ubiquitous subsurface phase), (2) the abiotic and microbially mediated reductive dissolution of Fe-hydroxides (important phases in bioremediation and natural attenuation of contaminants), and (3) the dissolution of olivine (a model system with relevance to the problem of CO_2 sequestration). Pore-scale experiments are carried out with engineered single pores containing the reactive phase of interest (calcite, Fe-oxyhydroxide, or olivine), with rates determined by the change in fluid concentration between the injection fluid and the effluent.

The first experiments involved the mineral calcite, within which a 20 μm wide and 40 μm high channel was etched using a femto-second laser. Even given the short residence time in the channel (30 seconds), the extent of reaction was enough to raise the pH of the injection fluid from 5 to 7.5 over the 2 cm length of the pore. Calcium concentrations were close to those expected for equilibrium conditions in the case of stoichiometric dissolution, so it appears that the low pH relative to the expected equilibrium pH (about 9) is caused by the diffusion of CO_2 into the effluent. The equilibration of the calcite is also supported by reactive transport modeling based on a radially symmetric cylindrical pore of analogous dimensions. A microfluidic reactive flow experiment involving the abiotic reductive dissolution of Fe-hydroxide is planned and will involve *in situ* imaging of reactive phases using scanning transmission x-ray microscopy (STXM) at LBNL's Advanced Light Source.

Reactive transport modeling of flow, diffusion, and reaction through a single pore has also been used to evaluate the conditions under which gradients in concentration may develop in single pores. Gradients in concentrations at the pore-scale lead to variations in the local reaction rates, and thus a scale dependence when larger domains are considered. To examine where such scaling issues occur in single pores in natural porous medium systems, we ran simulations with a Darcy velocity of 10^{-6} cm/s, and a medium pore length of 100 μm. Because of the lower flow velocities and the small length scales within such a pore, diffusion becomes the dominant transport process and thus homogenizes the concentration field. Similar results are found for plagioclase. These preliminary results suggest that significant gradients within single pores are unlikely, and that the scale dependence of reaction rates is more likely linked to (bio)geochemical and physical heterogeneities at the pore network scale rather than the individual pore scale.

Mesoscale Biotransformation of Uranium

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Bioreduction of uranium (U) in contaminated sediments is becoming an attractive remediation strategy because of its low implementation cost, and because short-term studies support its feasibility. However, any *in situ* approach for immobilizing U will require assurance of either permanent fixation or of very low release rates into the biosphere. Our long-term laboratory experiments have shown that reoxidation of bioreduced UO_2 can occur even under reducing (methanogenic) conditions sustained by continuous infusion of lactate. The biogeochemical processes underlying this finding urgently need to be understood. Our current research is designed to identify mechanisms responsible for anaerobic U oxidation and identify effects of key factors controlling long-term stability of bioreduced U. We are investigating: (1) the effects of organic carbon (OC) concentrations and supply rates on stability of bioreduced U, (2) the influences of pH on U(IV)/U(VI) redox equilibrium, (3) the roles of Fe- and Mn-oxides as potential U oxidants in sediments, and (4) the role of microorganisms in U reoxidation.

Part of our current work examines effects of varying influent OC concentrations on U mobility under reducing conditions. Through a long-term laboratory column experiment using ERSD Field Research Center (FRC) Area 2 soils, under continuous infusion of OC (lactate, at an OC concentration of 32 mM), our earlier study showed that U was reduced during the first 100 days, then reoxidized. These soil columns were subsequently infused with different concentrations of organic carbon (OC). At Day 500, different solutions were supplied to different columns: 0, 6, 32, and 100 mM OC (0, 2, 10, and 33 mM Na-lactate). Rapid changes in effluent U concentrations occurred in response to these changes in OC supply. Both the 0 and 6 mM OC treatments yielded decreased U concentrations (contrary to conventional expectation), and the 100 mM OC treatment caused even higher levels of U in effluents (also contrary to conventional expectation). The system continuously supplied with 32 mM OC sustained a nearly steady outflow U concentration of about 1 μM. These new results strongly support our hypothesis that carbonate enrichment (from microbial oxidation of OC) promotes U(IV) oxidation because of the stability of U(VI) carbonate complexes. These results also show that U-soil systems can be highly sensitive to OC supply.

Although several factors point to a residual reactive Fe(III) fraction in these sediments as the likely terminal electron acceptor for U reoxidation, we are currently conducting other experiments to further test this hypothesis. These include even longer-term column incubations targeted at completely reducing the reactive Fe(III) fraction in sediments, micro-x-ray absorption spectroscopy for determining distributions of Mn, Fe, and U oxidation states in sediments at various stages of OC-stimulated bioreduction, and use of chemical methods for determining concentrations of Fe(II) and Fe(III) in sediments and pore waters.

Kinetics and Topology of Precipitation on Mineral Surfaces

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The research objectives are to determine the mode of precipitation and kinetics of iron (Fe) oxides on quartz and sapphire substrates, and of silicate sorption and precipitation on hematite.

Current work is dedicated along several lines: (1) the development of grazing-incidence small angle scattering (GISAXS) methods for the study of fast precipitation and aggregation reactions on mineral surfaces—in conjunction with Mike Toney, a SAXS expert, at Stanford Synchrotron Radiation Laboratory (SSRL) and Young-Shin Jun, a postdoc provided through DOE-BER in conjunction with the EMSI at Pennsylvania State University (CEKA); (2) sorption and surface reactions and kinetics for silicate growth on hematite surfaces. This is a combined crystal truncation rod (CTR) surface diffraction and grazing-incidence EXAFS experiment focusing on the way in which silicate passivates and grows on Fe oxide surfaces. Initial results show that monomeric silicate sorbs in an ordered manner on surface positions similar to arsenate and other tetrahedral anions. The time evolution of these sorbates will be examined in continuing work.

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

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Chromate is a common contaminant at DOE facilities; its reduction by microorganisms to less toxic chromium (Cr)(III) is a viable remediation option. We have discovered that soluble organo-Cr(III) complexes, instead of insoluble $Cr(OH)_3$ precipitates, can be formed during bioreduction of chromate. This formation has been demonstrated with four bacterial cultures (*Shewanella oneidensis* MR1, *Cellulomonas* sp. ES6, *Rhodococcus* sp. and *Desulfovibrio vulgaris* strain Hildenborough). Purification and analysis indicates that the organo-Cr(III) complexes are inherently heterogeneous. Enzymatic reduction of chromate in the presence of common cellular metabolites demonstrates that many cellular metabolites can form soluble complexes with Cr(III). The complexes are recalcitrant, but they can be slowly transformed to insoluble Cr(III) precipitates by microorganisms. Structural characterization of the organo-Cr(III) complexes have been performed with synthesized model compounds. A variety of techniques have been used to probe these structures, including extended x-ray absorption fine structure (EXAFS), electron paramagnetic resonance (EPR) and mass spectrometry (MS). Soil column experiments have shown that some organo-Cr(III) complexes are relatively mobile. These findings imply that soluble Cr(III) species in groundwater are likely organo-Cr(III) complexes, resulting from microbial reduction of chromate. Thus, a more complete biogeochemical cycle of Cr should include the production and transformation of organo-Cr(III) complexes as an integral link.

Microscopic Controls on the Desorption/Dissolution of Sorbed U(VI) and Their Influence on Reactive Transport

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This project was first initiated in FY2003. Over its course, eight manuscripts were published on the speciation of uranium(VI) in two different Hanford waste sites and the desorption/dissolution behavior of sorbed U(VI) from contaminated vadose zone sediments. The project scope was revised in lieu of the CY 2005 EMSP call, to which a successful renewal proposal was submitted. The new research that began in FY2006 will investigate the kinetics of U(VI) dissolution and desorption and the scaling of reaction rates using a unique suite of U(VI)-contaminated sediments from the Hanford 300A whose speciation was studied in the first project. Shallow sediments from this location contain coprecipitated U(VI) with calcite, intermediate depth sediments contain precipitated U(VI) in the form of metatorbernite, and the deepest sediments contain an adsorbed U(VI) species. The project focus is to understand how the chemical/physical state of "sorbed" U(VI) in long-term contaminated sediments controls future plume migration.

The research will: (1) identify physical (e.g., diffusion) and geochemical controls (e.g., molecular speciation) on U(VI) reaction kinetics at the microscopic scale, (2) parameterize microscopic rate laws of controlling geochemical reactions and mass transfer rates, and (3) evaluate how the complex, derived microscopic rate laws may be scaled to U(VI) reactive transport in meter-length columns with coarse, field-textured sediment. Detailed characterization measurements on the sediments using state-of-science microscopies and spectroscopies, and batch and column experimentation will parameterize a rigorous, reaction-based, subgrid model that will be imbedded in a dual continuum, reactive transport model. Additional experimentation will explore the coupling of kinetic geochemical processes and water advection using columns of increasingly coarse sediment. Iterative comparisons of model simulations with experimental results of large column studies will allow the evaluation of a central project hypothesis on the scaling of mass transfer rates.

At the 2006 ERSP program meeting, we will describe speciation measurements performed on a depth sequence of 300A sediments using bulk extended x-ray adsorption fine structure (EXAFS), micro-EXAFS and x-ray microprobe, and cryogenic laser-induced fluorescence spectroscopy (CLIFS). These speciation measurements are used to interpret wet-chemical results of batch and column dissolution/desorption experiments with the < 2.0 mm fraction of the sediments that reveal complex kinetic behavior controlled by either mass transfer or chemical kinetic limitations. Lastly, issues of reaction network "scale-up" are highlighted by presenting the results of a large column experiment in which the long-term desorption of contaminant U(VI) was investigated in field-textured materials dominated by coarse river cobble.

Mineralogic Residence and Desorption Rates of Sorbed ^{90}Sr in Contaminated Subsurface Sediments: Implications for Future Behavior and In-Ground Stability

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Strontium-90 desorption processes are being investigated in coarse-textured Hanford sediments contaminated by different waste types, as well as by a reaction-based reactive transport model developed to forecast ^{90}Sr concentration dynamics in response to water infiltration and variations in cation concentrations. Our overall goal is to provide fundamental knowledge on the subsurface hydrogeochemistry of ^{90}Sr to predict future in-ground behavior as required for sound remedial decisions. Preliminary observations suggest that 30-year sorbed ^{90}Sr in coarse-textured sediment resides in fractured interiors of basaltic lithic fragments that comprise approximately 40% of the sediment mass. This unexpected intraparticle retention mechanism defines a new conceptual model for ^{90}Sr retardation that is tentatively attributed to internal domains of phyllosilicates formed from the weathering of basaltic glass. Research is characterizing the spatial locations, composition, and reactivity of these intragrain phyllosilicate domains using spectroscopic, microscopic, and wet chemical methods. Intragrain porosity, diffusivity, and tortuosity are being estimated using emersion experiments coupled with particle imaging (using electron, x-ray, and nuclear magnetic resonance techniques). Desorption rates and extent are being measured from contaminated Hanford sediments of different waste impact in electrolytes that promote isotopic exchange, ion exchange, and/or dissolution. Desorption results are interpreted with a geochemical-physical model that incorporates aqueous speciation, ion exchange, calcite dissolution and precipitation, mass transfer, and other important factors. Batch and column experiments have been performed with ^{90}Sr-contaminanted vadose zone sediments from Hanford's tank farms (B-110, T-106), as well as aquifer sediments from the 100-N groundwater plume, to quantify factors controlling long-term release rates and river stage effects. New-found understanding and geochemical parameters have been incorporated into the FLOTRAN reactive transport code for simulation of ^{90}Sr concentrations in vadose zone pore water and groundwater in response to the passage of uncontaminated waters of different composition.

MICROBIAL ECOLOGY

Microbial Pathways for the Reduction of Mercury in Saturated Subsurface Sediments

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The reduction of inorganic mercury (Hg[II]) to elemental mercury (Hg[0]) may increase the mobility of mercury in groundwater and the vadose zone, because the interactions of Hg(0) with ligands and available surfaces are weakened. Two pathways for Hg(II) reduction are investigated: (1) an inducible reduction by the bacterial-mercury-resistance (*mer*) system and (2) a constitutive reduction by mercury-sensitive metal-reducing anaerobic bacteria. The hypothesis that is tested in this project is that *under anoxic conditions, mer-mediated reduction occurs in highly contaminated sediments, whereas in environments impacted by low concentrations of mercury, the second processes dominates.*

To test this hypothesis, we are examining Hg(II) reduction to Hg(0) in incubations of saturated zone sediments incubated under varied respiratory conditions with high and low concentrations of Hg(II). The taxonomic composition and presence and expression of *mer* genes are examined in active microbial communities using state-of-the-art molecular analyses to assess what pathways dominate under the various incubation conditions. A microbial community from highly contaminated sediment (100's μg Hg/g sediment) incubated under fermentative conditions had a high diversity of *merA*, the gene encoding for the central function of the *mer* operon, the mercuric reductase. Many of these genes belonged to novel clusters in the *merA* gene tree, possibly representing reductases that have specialized in reduction of Hg(II) during anoxia.

In less contaminated environments (ng Hg/g), metal-reducing anaerobic bacteria, whose activities immobilize radionuclides and toxic metals, also reduce Hg(II). This activity was constitutive and occurred while pure cultures of metal reducers grew with fumerate or iron as terminal electron acceptors. However, specific reduction rates were at least three times higher under iron-reducing conditions, suggesting that, as has been shown for other oxidized elements, Hg(II) may be reduced indirectly by ferrous iron produced during iron reduction. If so, more than one pathway for the constitutive reduction of Hg(II) may take place in environments with low levels of Hg contamination. This study is the first attempt to examine how microbial activities control the mobility of Hg in saturated subsurface sediments. and should lead to improved management practices that will minimize groundwater contamination.

Ecological Interactions between Metals and Microbes That Impact Bioremediation

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Objectives

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

Results

Arthrobacter sp. FB24 was isolated from soils contaminated with metals (chromium [Cr] and and lead [Pb]) and aromatic hydrocarbons. This bacterium is most notable for its resistance to extreme concentrations of Cr(VI) (200 mM). The genome of strain FB24 has recently been sequenced and analyzed. A cluster of putative Cr resistance genes is located on a 230 kb megaplasmid. This plasmid is known to also contain genetic determinants for Cd, Co, Hg, Pb, and Zn resistance. In contrast, genes for arsenate and arsenite resistance are chromosomely encoded. Genes for the degradation of phthalate, phenol, and hydroxylated benzoates are also present. Physiological testing revealed these genes are functional. FB24 is able to grow using phthalate, phenol, benzoate, 3-, 4-,3,4-, 2,5-, and 2,4-hydroxybenzoates as carbon sources. It does have some resistance to the metals Cd, Co, Cu, Hg, Ni, Pb, and Zn, but most notable is its resistance to another oxyanion, arsenate (250 mM), and arsenite (5 mM).

More detailed research has been conducted on the mechanism underlying FB24's extreme Cr(VI) resistance. Some level of Cr resistance appears to be constitutively expressed, and growth in Cr reduces growth rate and yield. Among predicted open reading frames (ORFs) is ChrB (40% similarity), two chromate ion transporters (51% and 53% similarity to published ChrA), and a probable Cr-resistance signal peptide (34% similarity). A 10.6 kb region spanning the putative Cr resistance genes is being examined genetically. A focused DNA microarray was conducted to evaluate expression of selected genes within this gene cluster as a function of Cr concentration. A dose-response for *chrA* and *chrB* was seen. Two-dimensional protein gel electrophoresis was used to examine the Cr response of strain FB24 on a global scale. The data revealed up-regulation of five proteins and down-regulation of six proteins with the 5 mM Cr samples, whereas nine proteins were up-regulated and ten were down-regulated in 20 mM Cr. Of these, four proteins were differentially expressed in both Cr concentrations. A number of spots were subjected to sequence analysis. Of the proteins that were down-regulated, two are involved in central carbon metabolism. The down-regulation of proteins involved in carbon metabolism is consistent with the growth aberrations seen when *Arthrobacter* FB24 is grown in increasing concentrations of chromate. Several of the up-regulated proteins had sequences that could not be related to those predicted from known ORFs; others had similarities to genes of unknown function. In addition, two of the up-regulated proteins were identified as involved in biosynthesis (lipid and S-metabolism).

The role of mobile elements in heavy metal resistance has been investigated in *Arthrobacter*. The availability of sequence data from two genome sequencing projects (FB24 and TC1), along with published (pAO1) and unpublished (pSI1, pCR15) plasmid sequences, enables us to use a comparative-genomics approach to look at and analyze the different functions located on *Arthrobacter* plasmids. Similarities in putative replication, partition, mobilization, and functional genes have been found among these mobile elements, as well as unique genetic elements.

Soil Bacterial Community Dynamics in the Presence of Plutonium and Uranium

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The goals of this project are to (1) determine the effects of different forms and concentrations of plutonium (Pu) and uranium (U) on abundance and composition of bacterial species in communities, and (2) to identify bacterial species that are active in the presence of actinide that may be responsible for reduction of actinides in anaerobic subsurface environments. We are conducting replicated laboratory time-course experiments where soil is exposed to Pu(VI)/HCl, Pu(IV)EDTA, or U(VI)/HCl, at actinide concentrations spanning 10^{-3}M to 10^{-7}M, and incubated under aerobic or anaerobic conditions. A suite of DNA- and RNA-based methods has been used to monitor changes in the bacterial community in response to actinides at different concentrations. As expected, soil bacterial species richness and composition shifted after two-week incubation in control laboratory treatments under aerobic or anaerobic conditions. Superimposed on this change, the composition differed greatly between the aerobic and anaerobic incubation treatments. In soil samples incubated with up to 10^{-4}M Pu(VI)/HCl, the effect of the chloride accounted for most all of the observed composition changes, and could result from either the Cl- or pH shifts. The nature of the composition changes differed when monitored using the 16S rRNA gene (DNA), or 16S rRNA (RNA). In anaerobic treatments containing Pu(VI)Cl, HCl, or water, the community became dominated by a few Clostridium species in DNA analysis. However, when measured using the 16S rRNA-based analysis, the dominant members detected were species in the alpha- or beta-Proteobacteria or the Actinobacteria. With the RNA-based measurements, the dominant species differed between the Pu-treated and control (HCl, water) treatments. We hypothesize that the DNA-based assessment is dominated by spore-forming Clostridia that sporulated when the samples were flooded, but may not be active under the incubation conditions. The RNA-based assessment may be a more accurate survey of active species in the samples; this is being further tested. The presence of *Desulfotomaculum*, *Geobacteraceae* and *Shewanella* species were determined in Pu- and U-treated soils using group-specific primer sets. This study will contribute to the ERSP program by providing information on the dynamics of natural soil and sediment communities in the presence of Pu(V/VI) and U(VI) that complements ongoing pure culture and field studies.

Cultivation of Hard-to-Culture Subsurface Mercury-Resistant Bacteria from Lower East Fork Poplar Creek Floodplain, Oak Ridge, TN

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The overall goal of the project is to investigate the effect of mobile genetic elements and conjugal gene transfer on subsurface microbial community adaptation and biotransformation of mercury (Hg). Special emphasis is given to the contribution of the "nonculturable" fraction of the microbial communities. We have previously shown that the Hg tolerance and the functional versatility of the bacterial communities were higher for the topsoil compared to the deeper soils. However, following amendment with Hg^{2+}, no differences between the bacterial communities were observed, indicating the high adaptive potential of the subsurface community. Here, we report data on the diversity of the soil bacterial communities (at 18–22 inches and 36–40 inches depth), and on isolation of Hg-resistance plasmids.

To enhance the culturability of the Hg-resistant soil bacteria, natural growth conditions were simulated by allowing the soil bacteria to pre-grow to microcolonies (mCFU) on polycarbonate membranes placed on a soil substrate spiked with Hg. The soil substrate was renewed every 7 days. Compared to direct plating on agar media (1/10 TSA), the pre-incubation on the membrane filters increased the culturability of the soil bacteria by a factor of up to 180 after 28 days incubation. The genetic diversity of the mCFUs, measured by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), increased during incubation, suggesting induction of culturability of "viable but not culturable" bacteria. A total of 800 isolates were characterized by randomly amplified polymorphic DNA (RAPD), and at least one isolate from each RAPD group was selected for 16S RDNA sequencing. The diversity of the hitherto "unculturable" Hg-resistant bacteria was high and included α, β, and γ-Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. A total of 25 different 16S rDNA sequences were obtained. Of these, approximately one third were represented at both depths. The diversity of the isolates obtained by direct plating was low and dominated by the Actinobacteria (six of the eight different sequences). A clone library of 16S rDNA, by PCR amplification of extracted soil DNA, was also prepared. The most dominant phylum was the Acidobacterial phylum. Other phyla with several representatives included Proteobacteria (α-, β-, and γ-), Nitrospira, Bacteroidetes, Actinobacteria and Firmicutes. Less abundant were Verrucomicrobia, Gemmatimonadetes and Planctomycetes.

Approximately 150 of the isolates, representing different RAPD groups, have been screened for content of plasmids. Presently, 15 of these isolates have been confirmed to contain plasmids of a size (>30 kb) that could be self-transmissible. The ability of these 15 plasmids to transfer Hg resistance is currently being tested.

Genomic and Physiological Perspectives on Bioremediation Processes at the ERSD FRC

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A suite of molecular and physiological studies, including metal reduction assays, metagenomics, functional gene microarrays, stable isotope probing, and sequence analyses were applied to investigate organisms involved in bioremediation processes at the ERSD Field Research Center (FRC) and to understand the effects of stress on the makeup and evolution of microbial communities to inform effective remediation strategies.

A 16S rRNA gene library was sequenced from groundwater (FW106) contaminated with nitrate, uranium (U), and other heavy metals and with pH ~3.7. Sequence analyses revealed 10 operational taxonomic units (OTUs) with >90% of OTUs represented by an unidentified γ-proteobacterial species similar to *Frateuria*. The DOE Joint Genome Institute (JGI) conducted metagenome sequencing of the collected DNA. Three clone libraries with different DNA fragment sizes (3, 8 and 40 kb) were constructed and shotgun sequencing produced 50–60 Mb raw sequences, which were assembled into 2770 contigs totaling ~6 Mb that were further assembled into 224 scaffolds (1.8 kb–2.4 Mb). Preliminary binning of the scaffolds suggests one dominant phylotype of the *Frateuria*-like γ-proteobacteria and at least three secondary phylotypes (1 *Frateuria*-like γ-proteobacteria, 1 *Burkholderia*-like β-proteobacteria and 1 *Herbaspirillum*-like β-proteobacteria). Annotation of the metagenome indicates genes necessary for stress responses and survival under the given geochemical conditions are present, specifically denitrifying pathways, metal and pH resistance and general stress response mechanisms. Analysis with functional gene arrays containing ~23,000 probes targeting genes involved in biogeochemical cycling of C, N, and S, metal resistance, and contaminant degradation suggested that the dominant *Frateuria*-like species identified by metagenomic analysis could be biostimulated during *in situ* U reduction experiments at the FRC, and may play a direct or indirect role in the bioremediation of U. Sediment communities from throughout the Area 3 biostimulation zone are also being investigated using 16S rRNA gene clone libraries, stable isotope probing (SIP) and functional gene analyses to identify bacteria active in bioremediative processes and to spatially map microbial communities.

The metal-reducing physiology of *Desulfitobacterium hafniense* (DCB-2) is being investigated for use in bioremediation. A screen of metabolic capabilities revealed that *D. hafniense* (DCB-2) reduces the metals Fe(III), Cu(II), U(VI), Co(III) and Se(IV). Selenium (Se) is reduced under fermentative conditions, while the remaining metals can be the sole electron acceptor under anaerobic respiratory conditions. Growth in the presence of U(VI) and Se(IV) produces unusual cell morphologies, greatly elongated in the presence of U and producing surface vesicles in the presence of Se. The vesicles appeared lipid bound as seen with lipophilic fluors and osmium staining. Moreover, energy dispersive spectrometry reveals high concentrations of Se in the vesicles. The transcriptome of *D. hafniense* (DCB-2) under reducing conditions for each metal will be determined along with the kinetics and stoichiometry of respiratory growth.

Development and Use of Integrated Microarray-Based Genomic Technologies for Monitoring Microbial Community Dynamics During Biostimulation

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Microarray technology provides the opportunity to identify thousands of microbial genes or populations simultaneously, but adapting such technologies for characterizing microbial communities in natural settings is a great challenge. One of the big issues in developing a functional gene array (FGA) for environmental application is to design probes for achieving appropriate specificity of hybridization with environmental sequences. Over the past year, we have developed, tested, and applied a new software tool (CommOligo) for the design of an FGA that vastly improves upon past methods, and has recently been published in *Nucleic Acid Research* (Li et al. 2005, *NAR*, 33, 6114-6123). With this new program, we have developed a quite comprehensive FGA containing >23,000 probes for the genes important for biogeochemical cycling of C, N, S, P, metal resistance, metal reduction, and organic contaminant degradation. The arrays consist of gene-specific probes and group probes that encompass the variation in closely related sequences for which specific probe design was not possible. In most cases, we were able to design multiple probes for each target sequence, and we selected three probes for each of the target genes for array fabrication. This is the most comprehensive array available so far for environmental studies and will also be very useful for biogeochemistry studies in general.

Another great challenge to successful application of FGAs is that current detection sensitivity (approximately 10^7 cells) is often insufficient for detection of the majority of microbial populations in environmental samples. Thus we have developed a novel rolling-circle-amplification (RCA)-based method to be used in conjunction with microarray detection approaches for analyzing microbial community structure within the context of environmental applications. Our results showed that as little as 10 fg DNA is needed for microarray-based detection. This RCA-assisted microarray-based detection is representative and quantitative for community DNA samples within the range of 1 to 10 ng of starting materials.

We have applied these new technologies to evaluate the impacts of contaminants on microbial community structure and the adaptation of microbial communities to environmental conditions in groundwater samples (2 L) from the ERSD Field Research Center (FRC) that contain only ~10^5 cell/mL. As expected, uncontaminated background samples had the highest diversity and the highly contaminated samples had the lowest diversity. In addition, this array has recently been used to monitor microbial population changes after adding ethanol to groundwater to stimulate biological reduction of soluble uranium (U)(VI) to insoluble U(IV). Microbial species and genes such as *Desulfovibrio*, *Geobacter* and multiheme cytochrome genes that have been shown in the laboratory to facilitate U reduction are well correlated with U reduction in a field study. A Mantel test of the FGA data suggested a correlation between denitrification, sulfate reduction, and C-type cytochrome genes and levels of nitrate, sulfate, and U. Additional microcosm studies using different electron donors and acceptors are now under way to identify the specific populations responsible for U reduction.

Biomolecular Sciences

Biomolecular Mechanisms Controlling Metal and Radionuclide Transformations in *Anaeromyxobacter dehalogenans*

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Microbiological reduction and immobilization of U(VI) and Tc(VII) has been proposed as a strategy for remediating radionuclide-contaminated environments. Numerous studies focusing on the reduction kinetics and speciation of these metals have been carried out using contaminated sediment samples, microbial consortia, and pure bacterial cultures. While previous work with model organisms has increased the general understanding of radionuclide transformation processes, fundamental questions regarding radionuclide reduction mechanisms by indigenous microorganisms are poorly understood, especially under the commonly encountered scenario where multiple electron acceptors are present. Therefore, the overall goal of the proposed research is to elucidate the molecular mechanisms of radionuclide biotransformation by *Anaeromyxobacter dehalogenans*, and to assess the effects of relevant environmental factors on these transformation reactions. Members of the *Anaeromyxobacter* genus have been found in a range of undisturbed and contaminated soils and sediments. Importantly, *Anaeromyxobacter* species were shown to occur in the U(VI)-contaminated, acidic sediments of the ERSD Field Research Center (FRC).

This newly funded project will integrate targeted physiological and genetic analyses with microarray expression and genotype profiling studies to elucidate the mechanisms of metal transformation reactions in an environmentally relevant bacterial group. Further, we will determine the effects of co-contaminants (e.g., nitrate, chlorinated solvents) on radionuclide reduction. Established chemostat cultivation techniques will be used to produce cells under precisely controlled and defined conditions. The distribution and diversity of genes involved in metal and radionuclide reduction among different *A. dehalogenans* strains, including those detected in enrichment cultures derived from FRC site material, will be assessed using a microarray-based comparative genomics approach. This research effort will generate novel understanding of the mechanisms involved in metal reduction, and enhance our predictive capability of the processes that govern radionuclide transformation reactions in subsurface environments. Ultimately, these findings will assist the design and implementation of more efficient bioremediation approaches to enhance the reductive transformation and immobilization of radionuclides at contaminated DOE sites.

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

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Metal-reducing members of the genus *Shewanella* respire anaerobically on a wide range of terminal electron acceptors, including oxidized forms of the radionuclides uranium [U(VI)] and technetium [Tc(VII)]. The molecular mechanism of U(VI) and Tc(VII) reduction, however, remain poorly understood. The respiratory versatility of *Shewanella* is attributed in part to a large number of *c*-type cytochromes displaying widely varying midpoint redox potentials (E_0). A previously generated point mutant of *S. putrefaciens* strain 200 (designated U14), originally identified by its inability to grow anaerobically with U(VI) as electron acceptor, was found to grow on electron acceptors with high E_0 [O_2, NO_3^-, Fe(III)-citrate, Mn(IV) and Mn(III)-pyrophosphate], but unable to grow on electron acceptors with low E_0 [NO_2^-, U(VI), DMSO, TMAO, fumarate, Fe(III)-oxide, SO_3^{2-} and $S_2O_3^{2-}$]. Genetic complement-ation and nucleotide sequence analyses indicated that the U14 respiratory mutant phenotype resulted from the mutation of a conserved histidine residue in *ccmB*, encoding the permease subunit of an ABC transporter required for cytochrome *c* maturation. Although U14 retained the ability to respire on electron acceptors with high E_0, the cytochrome *c* content of U14 was <20% of the wild-type strain. Additions of cystine and oxidized glutathione to the culture medium restored growth rates to near wild-type levels. These results suggest that *ccmB* is required for proper redox homeostasis during growth on electron acceptors with low (but not high) E_0.

Microbial reduction of soluble Tc(VII) results in formation of Tc(IV), which precipitates as the highly insoluble hydrous oxide $TcO_2 \bullet nH_2O$, a Tc immobilization process forming the basis of alternate remediation strategies. Although Tc(VII) reduction has been studied in *Escherichia coli* and *Desulfovibrio*, such studies have not been carried out in metal-reducing members of *Shewanella*. To identify genes required for Tc(VII) reduction by *S. oneidensis*, we developed a rapid mutant screening technique for identification of Tc(VII) reduction-deficient (Tcr) mutants. The Tcr mutant screen was based on the observation that wild-type *S. oneidensis* produced a black Tc(IV) precipitate on its colony surface during Tc(VII) reduction, while putative Tcr mutant colonies remained colorless. Six Tcr mutants were identified via application of chemical mutagenesis procedures and the Tcr mutant screen. Based on their ability to respire an array of 13 alternate electron acceptors with H_2, lactate, or formate as electron donor, the Tcr mutants were divided into three classes: (1) deficiency in reduction of Tc(VII) with H_2 or lactate as electron donor, yet retaining the ability to reduce Tc(VII) with formate; (2) deficiency in reduction of Tc(VII), NO_3^-, Mn(III) or U(VI) with H_2 as electron donor, yet retaining respiratory capability on all electron acceptors with lactate or formate; and (3) deficiency in reduction of all anaerobic electron acceptors regardless of electron donor. These results suggest that the Tc(VII) reduction pathway of *S. oneidensis* contains separate branches linked to the oxidation of H_2, lactate or formate, and shares structural or regulatory components with pathways for reduction of NO_3^-, Mn(III), and U(VI). Genetic complementation analysis of the Tcr mutants is currently under way to identify the genes required for Tc(VII) reduction by *S. oneidensis*.

Construction of Whole Genome Microarrays, and Expression Analysis of *Desulfovibrio vulgaris* Cells in Metal-Reducing Conditions: Temporal Transcriptomic Analysis of *Desulfovibrio vulgaris* Hildenborough Transition into Stationary Phase during Electron Donor Depletion

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Desulfovibrio vulgaris was cultivated in a defined medium and biomass was sampled to characterize the shifts in gene expression as cells transitioned from exponential to stationary phase during electron donor depletion. In addition to temporal transcriptomics, protein, carbohydrate, lactate, acetate, and sulfate levels were measured. The microarray data were used for statistical expression analyses, hierarchical cluster analysis, and promoter element prediction. Microarray data were validated via quantitative polymerase chain reaction (PCR). During growth, triplicate cultures were sampled nine times for RNA extraction and global expression analyses. As the cells transitioned from exponential to stationary phase, a majority of the down-expressed genes were involved in translation and transcription, and this trend continued in the remaining time points. Intracellular trafficking and secretion, ion transport, and coenzyme metabolism showed more up-expression than down-expression as the cells entered stationary phase. As expected, DNA replication machinery was down-expressed, and genes involved in DNA repair were up-expressed during stationary phase. Genes involved in amino acid acquisition, carbohydrate metabolism, energy production, and cell envelope biogenesis showed both up- and down-expression. Interestingly, most phage-related genes were up-expressed at the onset of stationary phase. This result suggested that nutrient depletion may signal lysogenic phage to become lytic, and may impact community dynamics and DNA transfer mechanisms of sulfate-reducing bacteria. The putative feoAB system (in addition to other putative iron-related genes) was significantly up-expressed, and suggested the possible importance of Fe^{2+} acquisition under reducing growth conditions for sulfate-reducing bacteria. A large subset of carbohydrate-related genes had altered gene expression, and the total carbohydrate levels declined during the growth phase transition. Interestingly, the *D. vulgaris* genome does not contain a putative *rpo*S gene, a common attribute of the δ-*Proteobacteria* genomes sequenced to date, and other putative rpo factors did not have significantly altered expression profiles. The elucidation of growth-phase dependent gene expression is essential for a general understanding of growth physiology, which is also crucial for data interpretation of stress-responsive genes. In addition, to effectively immobilize heavy metals and radionuclides via sulfate reduction, it is important to understand the cellular responses to adverse factors observed at contaminated subsurface environments, such as the changing ratios of electron donors and acceptors. Our results indicated that genes related to phage, stress response, internal carbon flow, and iron acquisition played important roles as the cells experienced electron donor depletion.

Identification of Molecular and Cellular Responses of *Desulfovibrio vulgaris* Biofilms under Culture Conditions Relevant to Field Conditions for Bioreduction of Heavy Metals: Possible Roles of Extracellular Protein and the Megaplasmid in the Formation of *Desulfovibrio vulgaris* Biofilms

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Desulfovibrio vulgaris ATCC29579 is a sulfate-reducing bacterium that is commonly used as a model for direct and indirect heavy metal reduction, and can also be a causitative agent of metal corrosion. Our objective here is to characterize *D. vulgaris* biofilms and identify key proteins necessary for biofilm formation and maintenance.

During growth with lactate and sulfate, internal carbohydrate levels increased throughout the exponential phase and peaked as the cells transitioned to stationary phase. The carbohydrate-to-protein ratio (C:P) peaked at 0.05 ug/ug as the cells transitioned to stationary phase and then declined to 0.02 ug/ug during extended stationary phase. In contrast, a strain of *D. vulgaris* that does not contain the megaplasmid (rmp) maintained higher internal carbohydrate levels, and the C:P ratio peaked at 0.1 ug/ug (a 2-fold increase compared to wild type). The C:P ratio in extended stationary phase was 0.06 ug/ug (4-fold increase to wild type). Under the tested growth conditions, we observed biofilm formation in wild-type cells, but the rmp strain formed less biofilm (a 2-fold decrease). In addition, carbohydrate levels in the culture supernatant were approximately a 2-fold increase for wild-type cells compared to rmp cells. We hypothesized that carbohydrate was reallocated to the external cell proper for biofilm formation. However, biofilm contained little carbohydrate (0.6 to 1.0 ug/mL) and had a similar C:P ratio compared to wild-type early stationary phase cells. Staining with calcafluor white also indicated the presence of little external carbohydrate in *D. vulgaris* biofilms.

The formation of biofilm was hindered by the presence of protinease K, trypsin, and chymotrypsin, but the growth of planktonic cells was not. In addition, when *D. vulgaris* biofilm was treated with a protease, the biofilm was degraded. In comparison, the biofilm of *Shewanella oneidensis* contained more carbohydrate, and the *S. oneidensis* biofilm was not significantly affected by protease treatment. Electron micrographs indicated the presence of filaments between the biofilm cells, and filaments were susceptible to protease degradation. Biofilm filtrates contained soluble protein, and SDSPAGE analysis suggested different polypeptide profiles between filtrates, planktonic, and biofilm samples. The results indicated that *D. vulgaris* changes carbohydrate distributions in response to growth phase, the megaplasmid contains genes important for carbohydrate distribution and biofilm formation, and *D. vulgaris* biofilms contain extracellular filaments that may be important for the initial stages of biofilm formation.

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

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

Using natural gene transfer, the nickel resistance operon (*ncc-nre*) was introduced into eight different species of nitrate-reducing bacteria obtained from the fluidized bed reactor being used to condition groundwater at the FRC. The *ncc-nre* operon was introduced either on the broad host range IncQ plasmid pMOL222 through *E. coli* CM2034 as donor, or on a single hopper mini transposon through *E. coli* CM2520 as donor. Improved minimal inhibition concentrations for nickel up to 6 mM were observed for the transconjugants compared to the wild type. The Ni-resistant phenotype was stably expressed after growth of the transconjugants under nonselective conditions over 100 generations. A series of continuous percolation microcosms containing sediments from Area 2 of the FRC and amended with Ni-resistant and Ni-sensitive species are being used to determine if natural transfer of the Ni-resistant marker occurs via conjugation.

Since it is expected that natural gene transfer will affect the overall microbial composition and activity, Green Fluorescent Protein (GFP) labeling of the exogenous strains was attempted using both conjugation and electroporation. *Ralstonia metallidurans* CH34 and its heavy-metal-sensitive counterpart AE104 were successfully labeled. In cases where GFP labeling is unsuccessful, species specific DNA probes for the engineered strains and Ni-resistant markers will be used in conjunction with quantitative PCR to follow gene transfer. Changes in the endogenous community structure will be analyzed using Q-PCR and SARST based on the 16S rRNA gene. The community activity and structure will be correlated with physical-chemical parameters including nitrate, sulfate, iron, and uranium reduction and speciation.

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Characterization of the Membrane Proteome of Shewanella oneidensis MR-1

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Shewanella oneidensis MR-1, a metal-reducing bacterium, can utilize a large number of electron acceptors, including both soluble and insoluble metal oxides. Since a majority of the electron transport-related proteins are believed to be associated with the membrane structures of this microbe, our current project is focused on (1) characterization of proteins located in the inner and outer membrane compartments of MR-1, and (2) the identification of proteins found to be altered in abundance in response to growth with different electron acceptors. This project involves the fractionation of the cell membrane compartments to enrich for proteins specifically associated with one compartment or the other. Identification of proteins expressed at the surface of the outer membrane is achieved by extracting biotinylated proteins from intact cells using avidin affinity chromatography. By using a combination of two-dimensional gel electrophoresis for protein detection and quantitation with liquid chromatography coupled to peptide mass spectrometry for protein identification, we are able to identify proteins located primarily in either the inner or outer membranes and to observe differential expression when cells are grown with different electron acceptors.

Differential protein expression is being revealed in the comparison of MR-1 cells grown with oxygen as the electron acceptor or with soluble or insoluble electron acceptors under anaerobic conditions. The protein complement of MR-1 cells grown with soluble electron acceptors has been observed to be significantly different from that of cells grown with goethite or on electrodes of varying composition. One of the major differences observed when MR-1 was grown with goethite (ferric oxide) as electron donor was the significant increase in the abundance of OmpW, an outer membrane protein of unknown function in *S. oneidensis*. Other outer membrane proteins identified include flagellin, flagellar hook protein FlgE, decaheme cytochrome c, TolA, TolB, TolC, TonB-dependent receptor protein, TonB system transport protein ExbD2, pilin protein MshB, OmcA, OmcB, OmpA, OmpH, MtrB, cytochrome c, cytochrome c552 nitrite reductase, Mol/TolQ/ExbB proton channel family protein, GspD, outer membrane porin, several ABC transporter proteins, agglutination protein aggA, multidrug resistance protein (AcrA/AcrE family), and chaperone DnaK and GroEL. Differential expression of these proteins in response to insoluble electron acceptors is currently under investigation.

In Situ Survival Mechanisms of Uranium- and Technetium-Reducing Bacteria in Contaminated Sediments

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In this work, we have the goals of (a) determining the mechanism of uranium (U)(VI) reduction of sulfate-reducing bacteria and (b) determining cell functions needed for sediment survival. We used the signature-tagged mutagenesis (STM) technique to identify mutants in genes of *Desulfovibrio desulfuricans* G20 and *Shewanella oneidensis* MR-1 involved in growth with U(VI) and in sediment survival. Mutated genes will be identified, and some will be further characterized to determine their roles in growth of these bacteria in radionuclide-contaminated sediments.

The STM library of 5,760 mutants was screened for the ability to grow with 2 mM U(VI). Genes disrupted in these mutants included those involved in DNA repair, rRNA methylation, gene regulation, and RNA polymerase renaturation. U(VI) reduction ability was also tested using washed cells of these mutants. Twenty-two mutants had a similar uranium reduction ability to G20. Two mutants, both in thioredoxin-related genes, had an impaired ability to reduce U(VI), with one mutant completely unable to reduce U(VI). The thioredoxin inhibitor ($CdCl_2$ at 50μM) blocked U(VI) reduction by strain G20 in washed cell tests. These results confirm a role for thioredoxin in the reduction of U(VI) by *Desulfovibrio*. The role of thioredoxin in U(VI) reduction will be further characterized.

We further screened the G20 STM mutant library for loss of ability to survive in sediment microcosms. A total of 108 mutants were confirmed to be true nonsurvivors, and their transposon insertion regions have been sequenced. Nonsurvival mutants had insertions in genes including those involved in the synthesis of the cell wall and the outer membrane, transporter proteins related to amino acid uptake, ion uptake (such as phosphate/ phosphonate, chloride, cations) and antimicrobial peptide secretion, bacteriophage genes, and insertion sequences. We also detected two nonsurvival mutants with transposon insertion in genes encoding methyl-accepting chemotaxis proteins (MCP). We are in the process of further characterizing several of these mutants.

Survival genes of *Shewanella oneidensis* MR-1 were also identified by STM screening in anaerobic subsurface sediments. Mutants in sediment-survival genes putatively involved in multidrug resistance (organic compound efflux) were investigated. Growth of the *mexF* transposon mutant was impaired compared to the parent strain in the presence of chloramphenicol and tetracycline. Growth of an in-frame *mexF*-deletion mutant was also significantly impaired by chloramphenicol. It is unlikely that antibiotics are present at significant concentrations in natural aquifers, and therefore these results suggest that there may be another role for multidrug efflux genes in environmental bacteria. This may involve export of endogenously produced organic compounds (electron shuttles?) or protection from environmental toxins. These findings may help us better understand mechanisms used by sediment microorganisms to survive and reduce radionuclides in contaminated environments.

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

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Heavy metal and radionuclide contamination at DOE sites nationwide constitute a major environmental problem. Of particular interest are uranium (U) and technetium (Tc), as well as iron (Fe) and manganese (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- 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 between these bacterial groups, is essential for understanding these mechanisms and assessing bioremediative potential at DOE sites.

We have used cell fractionation techniques to resolve subcellular protein fractions and quantify the purity of proteins within each enriched fraction. While the identification of metal-reducing proteins in cell cultures has been relatively straightforward, a number of additional issues have been encountered in the proteomic characterization of these proteins from the waste sites. The first issue, unrelated to the waste site, is the characterization of cytochrome-containing peptides from metal reducing proteins. We have addressed this issue by the direct characterization of cytochrome proteins isolated from *Shewanella* to determine the site of modification and build a mass tag database for these peptides for use in the field samples. Additionally, the extraction of the proteins from field samples for proteomic characterization has presented a challenge, since most existing extraction schemes utilize surfactants that are incompatible with the mass spectrometry characterization. To this end, we are modifying and applying novel extraction schemes for the characterization of proteins from the Old Rifle, CO, site. Such advances will allow a much more extensive proteomic characterization of microbial communities in such sites for not only the cytochrome proteins, but all the proteins present. The eventual synthesis of all the technologies developed in this project will create a road map for the comprehensive characterization of microbial communities from field sites and the identification of any particular set of proteins displaying an activity of interest.

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

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

Initial work focused on identifying the Tc(VII) reductase of *G. sulfurreducens*, developing the analytical tools for monitoring the reduction of penta and tetravalent actinides in microbial culture, and characterizing the mechanisms of U(VI) and Np(V) reduction in detail. Extensive biochemical and genetic evidence, including studies with a defined mutant from Derek Lovley's group at University of Massachusetts, showed that the enzyme responsible for Tc(VII) reduction in *G. sulfurreducens* is a NiFe hydrogenase localized in the periplasm. In collaboration with Mireille Bruschi's group in Marseille, France, we also partially purified and characterized a suite of hydrogenases in *G. sulfurreducens* as a first step towards studying hydrogenase-mediated radionuclide reduction *in vitro*. We also purified and characterized a periplasmic 40 kDa 12 heme cyctochrome, comprised of four tri-heme cytochrome c_7 domains.

X-ray absorption spectroscopy (XAS) was also used to show that *G. sulfurreducens* reduces U(VI) by a one-electron reduction, forming an unstable U(V) species that subsequently disproportionates to give insoluble U(IV). This was confirmed by challenging *G. sulfurreducens* with the Np(V) analogue, which is stable with respect to disproportionation and was not reduced by whole cells. Similar results were obtained *in vitro* using purified cytochrome c_7 from *G. sulfurreducens*, with hydrogenase as the electron donor for actinide reduction. This surprising degree of selectivity for hexavalent actinides illustrates the need for mechanistic understanding and care in devising *in situ* bioremediation strategies for complex wastes containing other redox-active actinides, including plutonium. One-electron transfer reduction mechanisms were also identified for other priority contaminants, with the identification of Cr(V) and Hg(I) as intermediates in Cr(VI) and Hg(II) reduction. Key enzymes involved in these transformations were also identified using mutants from the Lovley group, and a novel mechanism for Hg(II) bioremediation was also identified. Current work focuses on the impact of *G. sulfurreducens* on plutonium in a range of oxidation states, and the structural basis of electron transfer to Cr(VI) and U(VI) by *c*-type cytochromes using *in vitro* techniques (via collaboration with Marianne Schiffer at Argonne National Laboratory.

Outer-Surface Components Involved in Electron Transfer to Fe(III) Oxidesin *Geobacteraceae*

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The most promising strategy for the *in situ* bioremediation of radioactive groundwater contaminants that was identified by the ERSP is to stimulate the activity of dissimilatory metal-reducing microorganisms to reductively precipitate uranium (U), technetium (Tc), and radioactive cobalt (Co), as well as the toxic metal vanadium (V). Previous molecular studies of a variety of subsurface environments, including several U-contaminated DOE sites, have clearly indicated that *Geobacteraceae* are the primary agents for metal reduction and that, even when U levels are high, electron transfer to insoluble Fe(III) oxides accounts for ~99% of the growth of the *Geobacteraceae*. When Fe(III) oxides are depleted, the growth and activity of *Geobacteraceae* stop, and U(VI) is no longer reduced. These results demonstrate that to design strategies to optimize *in situ* bioremediation of metals, it is imperative to understand how *Geobacteraceae* transfer electrons to insoluble Fe(III) oxides.

As reported at last year's meeting, there is substantial evidence suggesting that the electrically conductive pili of *Geobacter sulfurreducens* are the electrical conduit from the cell onto Fe(III) oxides. This contrasts with the nearly universal concept that outer-membrane cytochromes are the proteins that transfer electrons to Fe(III) oxide in Fe(III) reducers. To further investigate this, we studied the pili of *Pelobacter carbinolicus*, which is representative of the *Pelobacter* species that are phylogenetically intertwined with other genera of the *Geobacteraceae*. *Pelobacter* species do not contain the abundant outer-membrane *c*-type cytochromes found in other *Geobacteraceae*, yet they are capable of using Fe(III) oxide as the sole electron acceptor. *Pelobacter* do contain genes for pili that, like those of *G. sulfurreducens* and other *Geobacter* species, are phylogenetically distinct from the pilin genes of microbes outside the *Geobacteraceae*. Analysis of pili from *P. carbinolicus* with a conducting probe-atomic force microscope indicated that the pili had conductive properties similar to those of *G. sulfurreducens*. These results further suggest the pili are important conduits for extracellular electron transfer in *Geobacteraceae*. Further studies of the properties of the pili of other *Geobacteraceae*, as well as the pili of organisms outside this family, are under way to determine the mechanisms by which the pili are conductive.

The potential role of other abundant outer-membrane proteins in Fe(III) reduction are also under investigation. For example, genetic studies have previously demonstrated that the outer-membrane proteins OmcB, a polyheme *c*- type cytochrome, and OmpB, a multicopper protein, are required for the reduction of insoluble Fe(III) oxides. Localization studies using enzymatic and immunological approaches demonstrated that these proteins were exposed on the outer surface of the cell. However, unlike pili, neither of these proteins was localized to just one side of the cell. These results indicate that neither of these proteins forms specific association with the conductive pili, suggesting that their role is not direct electron transfer to the pili. Further characterization of the protein–protein interactions between OmcB and OmpB and with other outer membrane proteins are expected to provide further understanding of the function of these outer surface-exposed proteins during Fe(III) oxide reduction.

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

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This project has addressed a number of questions regarding the physiology and biochemistry of metal transformation in *A. cryptum* JF-5. Among the most significant questions and findings are as follows:

Do acidophilic DIRB produce electron transport proteins and enzymatic activities that are significantly different from their neutrophilic counterparts? Studies with the cell surface fractions of *A. cryptum* have revealed two distinct c-type monoheme cytochromes, of 42 and 48 kDa molecular mass. Periplasmic fractions contain two major monoheme c-type cytochromes of 10.1 and 11.7 kDa molecular mass. Localization experiments and gene sequence analysis confirm that the large mass cytochromes reside in the outer membrane (OM), while the small mass cytochromes are found in the periplasm. Amino acid sequence data has linked each purified protein to a specific gene in the *A. cryptum* genome. Genome-enabled studies have discovered two *Acidithiobacillus ferrooxidans* Cyc2 gene homologues (Gen numbers 2288, 2671). Each has a predicted N terminal signal sequence (PSORT, SignalP), each has strong OM protein localization prediction (PSORT), and each has strong transmembrane structure prediction (PSORT, TMPred). Several other cytochromes c are predicted to be membrane localized. In general, it appears that *A. cryptum* can use OM cytochromes c as electron transfer mediators to minerals, in a similar fashion to neutrophilic Fe-respiring bacteria. Over 60 mg of each periplasmic cytochrome c has been purified for further study.

What ability does A. cryptum have to metabolize or reduce chromium? We found that Cr(VI) reduction in Chelex 100-treated buffer was indistinguishable from reduction in our standard (untreated) buffer. This observation supports our contention that Cr(VI) reduction in *A. cryptum* is enzyme-catalyzed rather than Fe-mediated. Thus, it would appear that *A. cryptum* reduces Cr(VI) by both a direct enzymatic mechanism and a coupled biotic-abiotic mechanism when Fe(III) is present. A putative chromate reductase has been identified in soluble protein extracts of *A. cryptum* JF-5. Initial characterization of the reductase suggests that it is a multisubunit complex containing at least one c-type cytochrome, expressed under aerobic conditions in the presence or absence of Cr(VI). A method for measuring Cr(III) in wastewater, which utilizes the chemiluminescent reagent luminol, was adapted for use in detecting Cr(III) in polyacrylamide gels for the purpose of identifying novel chromate reductase activity *A. cryptum*, and this method can be used for any Cr-reducing bacterium of interest.

Does A. cyrptum form biofilms and attach to mineral surfaces? Comparative studies conducted thus far suggest that both acidophilic and neutrophilic dissimilatory iron-reducing bacteria form biofilms on mineral surfaces. Batch cultures of *A. crptum* and *G. sulfurreducens* were grown using anaerobic conditions with either schwertmannite, ferrihydrite, or hematite as electron acceptor/mineral surface respectively. In all cases, cell attachment was observed, although it is clear that attachment is at least partially surface-dependent at low pH. Flow cell experiments are in progress to more accurately assess biofilm physiology in *A. cryptum*.

Molecular Mechanisms of Uranium Reduction by *Clostridia* and Its Manipulation

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Subsurface contamination by radionuclides and toxic metals is a major problem across the DOE complex. Removal of contaminated media is financially prohibitive. Consequently, innovative, cost effective, *in situ* stabilization technology by exploiting the natural attenuation processes must be developed. Microbial stabilization of actinides (uranium [U], plutonium [Pu], and neptunium [Np]) and fission products (technetium [Tc]) in the subsurface environments is currently being investigated at DOE sites. A wide variety of bacteria, including the strict anaerobic spore forming *Clostridia*, are involved in the reductive precipitation of U and Tc in the subsurface environments. Although the mechanisms of U reduction by dissimilatory metal reducing bacteria (DMRB) *Geobacter* and *Shewanella*, and sulfate-reducing bacteria (SRB) *Desulfovibrio*, have been extensively investigated, little is known of the mechanisms of U reduction by fermentative bacteria such as *Clostridia*. It is postulated that the excess of electrons generated during fermentation of organic materials are used in the U reduction process.

This research addresses the need for detailed studies of the enzymatic mechanisms for reduction of radionuclides and/or metals by fermentative microorganisms. The overall objective of this research is to elucidate systematically the molecular mechanisms involved in the reduction of uranium by *Clostridia*. We propose to (1) determine the role of hydrogenases in U reduction, (2) purify the enzymes involved in uranium reduction, (3) determine the mechanisms of reduction, e.g., one or two electron transfer reactions, and (4) elucidate the genetic control of the enzymes and cellular factors involved in U reduction. Speciation and intermediate oxidation states of U will be determined electrochemically and by X-ray absorption near edge structure (XANES) at the National Synchrotron Light Source (NSLS) at BNL.

Fundamental knowledge resulting from molecular assessment of radionuclide and metal reduction will allow us to exploit naturally occurring processes, enabling us to attenuate radionuclide and metal contaminants *in situ* within subsurfaces dominated by low and high pH, high nitrate, and/or organic matter where the dissimilatory metal-reducing bacterial activity will be limited. This is a collaborative study between BNL and Stanford University, involving expertise in molecular biology, biochemistry, microbiology, and electrochemistry.

Generation of a Novel High-Activity Enzyme with Combined Cr(VI) and U(VI) Reductase Activities Using Directed Evolution and Rational Design

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Chromate [Cr(VI)] and uranyl [U(VI)] are toxic to plants and animals. Bacterial reduction of these compounds is a promising approach for minimizing their bioavailability and toxicity. The aim of this research is to increase the ability of bacteria to reduce these compounds by generating more efficient enzymes. We report that the bacterial NAD(P)H Cr(VI) reductases that we have extensively studied can also reduce U(VI) to the more stable and "safe" U(IV). Using error-prone polymerase chain reaction (PCR), we generated a modified form of the *E. coli* Cr(VI) (quinone) reductase,Y6, with V120A, Y128N, T160N, and Q175L substitutions. On a pure protein basis, Y6 possessed 30- and 13-fold higher activity for Cr(VI) and U(VI) reduction, respectively, than the wild type enzyme YieF. The H_2O_2 formation as a by-product of Cr(VI) reduction by Y6 was decreased to 12.5% of available electrons, compared to 25% by YieF—indicating that redox cycling also was decreased in the Y6-catalyzed reaction. X-ray absorption near-edge structure (XANES) analysis demonstrated that the end product of Cr(VI) reduction by both YieF and Y6 is Cr(III). Site-directed mutagenesis was conducted to identify the amino acid(s) contributing most to the increased catalytic efficiency of Y6. Re-substitution at positions A120V, N160T, or L175Q made little difference to the chromate-reducing activity of Y6. However, the reversion mutant N128Y showed a significant decrease in this activity, suggesting that it is a key residue in the active site of the enzyme. Substitution of Y128N in the YieF amino acid sequence produced an enzyme, Y128, with 17-fold further increase in chromate reducing ability over Y6. Applying the kinetic data obtained from a collection of evolved improved enzymes to a stochastic "fitness" model resulted in prediction of a further improved candidate with Y128N and G150S substitutions. The resulting enzyme, Y150, showed 840-fold improvement in V_{max} over YieF.

The development of a high-activity enzyme with joint capacity for Cr(VI) and U(VI) reduction is novel, and will greatly facilitate remediation of these pollutants in mixed waste environments such as the DOE and Superfund sites. *Pseudomonas putida*, which is native to polluted sites, was transformed to express YieF, Y6, or Y128. The resulting cells expressing the evolved enzymes showed improved chromate reduction, although to a lesser degree than seen on a pure protein basis. Since lysates of these cells gave much higher activity, we propose that the permeability barrier of the cells to chromate is a factor in decreasing Cr(VI) reduction. Studies aimed at improving *P. putida* permeability to Cr(VI) are currently in progress.

Molecular Mechanism of Bacterial Attachment to Fe(III)-Oxide Surfaces

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As a result of their high surface-area-to-volume ratio, the outer membrane (OM) of bacterial cells plays a significant part in cell function: the OM may control ingress and egress of nutrients and other chemicals as well as having important chemical- and mechanical-reception properties. For cells capable of dissimilatory reduction of solid electron acceptors, the OM is also thought to be important as the site for cytochrome expression, allowing proximity to solid terminal electron acceptors, thus enabling electron transfer (ET). Cell attachment at Fe(III) mineral surfaces should facilitate direct ET from cell to mineral, an important process in subsurface environments with low dissolved organic carbon, since extrinsic electron shuttling capacity is likely to be low. Mechanistically, there is also a clear role for OM surface structures to exert significant influence upon cell-mineral interaction beyond ET.

We propose that gaining greater insight into the biomolecular basis for cell attachment to iron oxide minerals is essential, not only in understanding the predominant mechanism for direct electron transfer in subsurface environments lacking in sufficient extrinsic electron shuttles (i.e., humic and fulvic acids), but also in predicting the transport of bacterial cells through porous vadose and subsurface environments. Our results to date indicate that wild-type *Shewanella oneidensis* MR-1 and *S. putrefaciens* 200R present cell surfaces largely free of extensive exopolymer when grown on fumerate as terminal electron acceptor (TEA), but that different organic TEA have a significant affect upon exopolymer production (inferred from cell electrophoretic softness). Employing targeted deletion mutagenesis, we have also established that expression of putative OM-associated proteins predominantly influence the surface charge density, having a reduced effect upon exopolymer production.

Advances in Understanding Microbial and Abiotic Electron Transfer at Mineral Surfaces

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Several research efforts are under way at PNNL under the new Stanford Environmental Molecular Sciences Institute (EMSI). The overall goal of the Stanford EMSI is to develop a molecular-level understanding of important chemical and biological processes occurring at environmental interfaces. The PNNL research component focuses on fundamental investigations of electron transfer (ET) processes occurring in certain environmentally important microbial and abiotic interfacial ET systems. This work involves three visiting graduate students and two resident post-docs working with Rosso at PNNL.

ET pathways and mechanisms for dissimilatory reduction of iron(III)-bearing oxide phases by microorganisms such as *Shewanella oneidensis* remain poorly understood. At the cell/mineral interface, a role for the outer-membrane proteins OmcA and MtrC as possible terminal electron donors is hypothesized. Anaerobic incubation of *Shewanella* with electrically conducting single crystals of hematite serving as the electron acceptor yields strongly attached cells. Upon lysing attached cells to produce residual attached membrane fragments, current-sensing atomic force microscopy is being utilized to map the electrical conductivity of these fragments with the goal of probing the ET machinery of intact cell walls. This approach allows us to seek connections between the electrical properties of the cell wall and incubation conditions.

In a complementary study, using ambient scanning tunneling microscopy and spectroscopy, we have interrogated the ET properties of individual OmcA and MtrC proteins immobilized by covalent linking to Au (111) substrates. These proteins are decaheme cytochromes with the propensity for heme centers acting as resonant or off-resonant intermediary tunneling sites. Current-voltage (I/V) relations for the two proteins are distinctly different, and the differences can be explained using the theory of incoherent multistep ET. Physical quantities controlling the ET kinetics are being generated from model fits to I/V data.

Abiotic ET mechanisms and kinetics for the reduction of both uranium(VI) and chromium(VI) by Fe(II) in solution or occurring in mixed-valent iron oxides by homogeneous and heterogeneous ET have been modeled and experimentally probed. We are also examining the kinetics of charge transport by small polaron hopping through surface environments of hematite and green rust analogues. Using a combination of molecular dynamics simulations, Hartree-Fock/density functional theory calculations, and Marcus ET theory, we have computed driving force free energies, reorganization energies, and electronic coupling matrix elements for a variety of elementary ET steps that underlie the rates of reductive immobilization of U(VI) and Cr(VI). Small polaron transport in iron oxides may also be intrinsically linked with the migration of electrons injected during bioreduction and the turnover of electron accepting sites for microbial respiration. Hence, the mineral-surface ET calculations are also assisting our work addressing the complexity of the bioreduction process.

Structure and Function in Hg(II) Metalloregulation and Enzymology

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Mercury (Hg) is mobile and toxic in all forms. Its geochemical mobility reflects abiotic and biotic processes; bacteria are key in the latter because they have evolved defenses against its toxicity. The widely found bacterial mercury resistance (*mer*) operon functions in Hg biogeochemistry and bioremediation by converting reactive inorganic [Hg(II)] and organic [RHg(I)] mercurials to relatively inert monoatomic mercury vapor, Hg(0). We study the metalloregulator, MerR, and the key enzymes MerA, the Hg(II) reductase, and MerB, the organo-mercurial lyase to dissect their mechanisms and enhance their performance.

MerR is a very Hg-specific *in vivo* regulator, but *in vitro* and over-expressed *in vivo* it binds other thiophilic metals despite competing thiols, even when bound to its operator, MerO. Thus, MerR must respond distinctly to each metal's preferred coordination. With an NMR probe, 2-fluorotyrosine, we have found that one of MerR's highly conserved tyrosines (Tyr) changes uniquely in response to Hg(II) when MerR is free or complexed with MerO and a second conserved Tyr alters in response to Hg(II) in the MerR-MerO complex. Cd(II) can mimic Hg(II)'s effects on the first Tyr, but Zn(II) cannot. Mutations in either Tyr destroy MerR's DNA binding, though one of them lies >13Å from the DNA binding domain (by homology modeling on CueR/ZntR structures). Both Tyrs are >15Å from the coiled coil metal binding domain (MBD), but even without added Hg(II), two Cd(II)-responsive coiled coil mutants provoke allosteric changes in these Tyrs like those of Hg(II) binding. Thus, changes in these Tyrs reflect the distinct coordination preferences of the Group 12 metals and are propagated from the metal center to the DNA recognition helix. This is the first evidence of an inducer-specific allosteric change in any MerR-type regulator. By isothermal titration calorimetry all three metals bind with similar free energies, but Hg(II)-binding is ΔH-driven and Cd(II) and Zn(II) binding are ΔS-driven, consistent with the latter two provoking oligomerization as confirmed by ultracentrifugation. MerR's affinity for Hg(II) is ~10-fold higher than for Cd(II) and Zn(II), but the latter two have higher stoichiometries. All three metals best fit a two-site model, though this may reflect a slow equilibrium between “bindable” and “nonbindable” states of the dimer; we strive to deconvolute these processes. Metal-binding to the MerR-MerO complex is in progress to complete this first-ever kinetic and thermodynamic analysis of any MerR family member.

MerB: Our high resolution x-ray structure of Hg(II)-bound-MerB identifies Cys96 and Cys159 as ligands, as we predicted (*Biochemistry* 41:10287). This improvement over our recent NMR structure (*Biochemistry* 43:8322) reveals possible proton donors and a pocket for the organic group. We can now prepare crystals of mutant C96S with substrate pHMB, which bodes well for seeing the organic side-chain binding pocket. **MerA**: The N-terminal domain (NmerA) provides both kinetic and survival advantages by removing Hg(II) from cell proteins when glutathione is depleted, as occurs in Hg(II) exposure (*Biochemistry*, 44:11402). Two conserved residues of NmerA are uniquely involved in positioning it for optimum Hg-transfer to core. In cells, *mer* operon expression accelerates Au(III) reduction and pure MerA catalyzes Au(III) reduction in two novel steps, each distinct from the one-step Hg(II) reduction. Extended x-ray absorption fine structure (EXAFS) will be used to identify intermediate and final complexes. As both a substrate and inhibitor, Au(III) is proving valuable in probing MerA's enzymatic plasticity. Lastly, matrix-assisted laser-desorption ionization-mass spectrometry (MALDI-MS) indicates that natural *in vivo* cleavage of NmerA from Core MerA occurs near the end of its homology to MerP. We are screening protease mutants for their effect on this unique cleavage and will randomly mutagenize the cleavage region to assess its contribution to Hg resistance.

Global Molecular Characterization of the Chromate Stress Response in *Shewanella oneidensis* MR-1: Identification of a Putative DNA-Binding Response Regulator and Azoreductase Involved in Cr(VI) Detoxification

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Shewanella oneidensis MR-1 is a model environmental organism that possesses diverse respiratory capacities, including the ability to reduce soluble Cr(VI) to sparingly soluble, less toxic Cr(III). Chromate is a serious anthropogenic pollutant found in subsurface sediment and groundwater environments due to its widespread use in defense and industrial applications. Effective bioremediation of chromate-contaminated sites requires knowledge of the molecular mechanisms and regulation of heavy metal resistance and biotransformation by dissimilatory metal-reducing bacteria. Towards this goal, our ERSP-funded work is focused on the identification and functional analysis of genes/proteins comprising the response pathways for chromate detoxification and/or reduction. Our previous work utilized transcriptomic profiling and whole-cell proteomic analyses to characterize the dynamic molecular response of MR-1 to an acute chromate shock (up to 90 min) as well as to a 24-hour low-dose exposure. In addition, we have examined the transcriptome of MR-1 cells actively engaged in chromate reduction. These studies implicated the involvement of a functionally undefined DNA-binding response regulator (SO2426) and a putative azoreductase (SO3585) in the chromate stress response of MR-1.

Here we describe a detailed functional analysis of SO2426 and SO3585 in order to begin to understand the role of these proteins in the cellular response to chromate. The protein products encoded by genes *so2426* and *so3585* were expressed and detected only in chromate-shocked samples as determined by multidimensional high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Both genes were also highly induced (>46-fold) in MR-1 cells actively reducing chromate, based on whole-genome microarray analysis. We have created in-frame deletions of the *so2426* and *so3585* loci in the MR-1 chromosome using a *cre-lox*-based recombination system and have characterized the phenotype of the resulting mutants in the presence of varying concentrations of chromate, copper, cobalt, strontium, and hydrogen peroxide under aerobic respiratory conditions. Growth studies indicated that the *so2426* deletion mutant was more sensitive to heavy metals compared to the wild-type (WT) reference, and chromate reduction by the *so2426* mutant was impaired significantly. The growth response of the mutant to H_2O_2 was similar to that of WT MR-1. To gain insight into the regulon of this response regulator, MR-1 microarrays were used to explore dynamic changes in the WT and *so2426* mutant transcriptomes during chromate stress and reduction.

The *so3585* deletion mutant resembled the WT in terms of growth; however, this mutant was able to reduce chromate at a substantially faster rate compared to the WT strain. Based on its genomic proximity and co-regulated expression profile, we predict that SO3585 functions in a complex together with the proteins SO3586 (glyoxalase family) and membrane-associated SO3587 (hypothetical protein). Future studies will include purifying SO3585 to determine whether it can reduce Cr(VI) and whether it interacts with SO3586 and SO3587.

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

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Humic compounds (byproducts of the natural degradation of organic matter) are known to accelerate the process by which microorganisms transfer electrons to (i.e., reduce) toxic metals, thereby decreasing metal toxicity and mobility. The pigment pyomelanin is a particularly important humic compound in this process. It is produced by bacteria in the genus *Shewanella*, which occur in subsurface soil. Pyomelanin associated with the bacterial surface increases hydrous ferric oxide reduction rates by as much as tenfold (1). This is accomplished because, under anaerobic conditions, pyomelanin serves as a terminal electron acceptor and soluble electron shuttle to iron minerals (2). The capacity for some species of bacteria to reduce metals offers a strategy for bioremediation of metal and radionuclide contaminated environments. One of the most challenging forms of metal contamination is when the contaminant metal is associated with solid-phase minerals. The overall hypothesis of this investigation is: Pyomelanin production in the genus *Shewanella* is related to external tyrosine concentrations and plays a significant role as a mechanism of electron transfer to solid-phase metals, resulting in immobilization of these inorganic contaminants.

An understanding of the role of pyomelanin in metal reduction may lead to technologies for accelerated remediation rates of solid-phase metal and radionuclide contamination in the environment. We have confirmed the role of tyrosine in pyomelanin production and demonstrated increased rates of metal oxide reduction by a pyomelanin-overproducing mutant compared to a pyomelanin-deficient mutant. Based on electrochemical studies of whole cells, the presence of bacterial-produced pyomelanin increased the rate and amount of electrons transferred from the cell surface to a solid electrode. The primary focus of our continued investigation is on the role of pyomelanin on solid-phase metal and radionuclide reduction. We expect to demonstrate that pyomelanin-based electron shuttling and its associated metal chelation properties contribute significantly to biogeochemical activity at the microbe-mineral interface.

INTEGRATIVE STUDIES

Mobility of Source Zone Heavy Metals and Radionuclides: The Mixed Roles of Fermentative Activity on Fate and Transport of Uranium and Chromium

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Our objective is to determine the effect of carbon and energy flow through simulated waste environments on metal and radionuclide migration from waste pits and trenches across the DOE complex. Metals and radionuclides can be mobilized by infiltration of water into waste storage sites. Cellulolytic and noncellulolytic fermentative microorganisms have been chosen as the focus of this research, because their activity is a critical first step that we hypothesize will control subsequent fate and transport in contaminated natural systems. Microbial communities of lignocellulose degrading and fermenting microorganisms present in the subsurface of contaminated DOE sites can significantly impact migration, by directly reducing and immobilizing metals and radionuclides while degrading complex organic matter to low-molecular-weight organic compounds. These low-molecular-weight organic acids and alcohols can increase metal and radionuclide mobility by chelation (i.e., certain organic acids) or decrease mobility by stimulating respiratory metal reducing microorganisms.

In work done thus far, our team has shown that:

- Removal of UO_2^{2+} from aqueous solutions is possible using fermenting *Cellulomonas* sp. strain ES6 under anaerobic, nongrowth conditions in bicarbonate and PIPES buffer.
- *Cellulomonas* spp. can significantly contribute towards the reduction of not only Cr(VI) and U(VI), but also a variety of iron minerals and dissolved ferric iron sources, including hydrous ferric oxide, goethite, maghemite, hematite, magnetite, and Fe(III)-citrate.
- The presence of quinones increases the electron transport from *Cellulomonas* spp. cells to the iron minerals and also to the dissolved compounds such as Fe(III)-citrate, U(VI), Cr(VI), and TNT.

Our future research will focus on:

- Characterizing the production of fermentable substrates and low-molecular-weight organics from organic debris in low-level waste by the activity of cellulolytic and noncellulolytic fermentative microbial populations and studying their effect on the mobility of U(VI) and Cr(VI)
- Understanding the response of microbial communities to carbon and electron flow through these natural and simulated environments
- Using this information to develop updated conceptual models for carbon and electron flow in waste systems and the associated effect on Cr(VI) and U(VI) transport in the subsurface.

Environmental Remediation Science at the Stanford Synchrotron Radiation Laboratory

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The SSRL Environmental Remediation Sciences Program (ERSP) supports BER-ERSP **[ERSD?]** funded environmental remediation scientists and their collaborators at SSRL. This is accomplished via direct user support from a scientific staff member, Dr. Sam Webb, user education and outreach, the development of innovative techniques for ERSD research, and collaborative scientific research. Major techniques supported by this program include x-ray absorption spectroscopy (XAS), x-ray diffraction (XRD), microbeam x-ray fluorescence chemical imaging (μ-XRF), and microbeam XAS/XRD. This program helped to support 11 BER-ERSD projects that conducted research at SSRL in the past year. These projects used a total of 112.5 eight-hour shifts, which represents 11% of the total time available at the beam stations utilized (stations 2-1, 2-3, 11-2, and 11-3).

A major project at present is the development of an x-ray microprobe for μ-XAS, μ-XRD, and μ-XRF measurements on radionuclides of interest to BER researchers including uranium (U), neptunium (Np), plutonium (Pu), americium (Am), and technetium (Tc). The system will also provide experimental capability for a range of heavy metals, including chromium (Cr), arsenic (As), lead (Pb), and strontium (Sr). Initial hardware design, fixture fabrication, instrument procurement, and assembly were completed in the past year, which was the first year of the project. The microprobe was found to produce a focused beam of 2 μm diameter, which was the design goal for the system. The micro-extended x-ray absorption fine structure (μ-EXAFS) transmission feasibility measurements at 20 KeV on a 10 μm diameter Mo wire show excellent reproducibility and low noise, suggesting that the mechanical stability of the system is adequate for routine EXAFS measurements. Our goals for the current year are to initiate user commissioning of the system and to procure/commission an area x-ray detector for μ-XRD measurements.

A notable collaborative research project concluded in 2005 was the characterization of the mechanism of uranium(VI) sequestration within bacteriogenic Mn oxides. These high-surface-area natural materials are common in sediments and natural waters and have high sorptive capacities for heavy metals, including U(VI). Synchrotron-based EXAFS, *in situ* XRD measurements, and uptake measurements show that U(VI) sequestration by these materials occurs via the formation of a strong bidentate surface complex at low U(VI) concentrations (<1 μM U(VI)) and via structural incorporation in the tunnels of bacteriogenic manganese oxides at low to moderate U(VI) concentrations (>4 μM U(VI)). These observations imply that manganese oxidizing bacteria may be of practical significance to the natural and engineered remediation of subsurface U(VI).

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

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Despite the promise of bioreduction as a remediation strategy, the factors that enhance or inhibit the rate and extent of biogeochemical uranium(VI) reduction under representative environmental conditions are not well defined. Previously, we reported the inhibition of bacterial U(VI) reduction in the presence of environmentally realistic concentrations of soluble calcium (Ca) (Brooks et al., 2003). The effect was attributed to the formation of aqueous $CaUO_2(CO_3)_3^{2-}$ and $Ca_2UO_2(CO_3)_3$ species, although the precise mechanism of inhibition remains undetermined. Subsequent work has now demonstrated that the inhibitory effect of Ca is alleviated with the addition of ethylenediamene tetraacetic acid (EDTA) as a competing ligand for Ca. The effect is proportional to the concentration of EDTA. Measured pseudo-first-order rate constants for U(VI) bioreduction are negatively correlated with the fraction of U(VI) present as Ca-U(VI)-CO_3 species. Although bacteria effectively reduce U(VI) to U(IV) in the presence of EDTA, the biogenic U(IV) does not precipitate but remains in solution as an U(IV)-EDTA complex, as confirmed by wet chemical, XANES, and EXAFS analysis. Reoxidation kinetics of biogenic U(IV) (either precipitated or U(IV)-EDTA) under atmospheric conditions (21% O_2) decreased with pH and in the presence of Ca or EDTA.

We have identified previously undescribed M-UO_2-CO_3 complexes for the remaining alkaline earth elements (M = Mg, Sr, Ba) and quantified their formation constants, enabling more complete speciation predictions for prepared and environmental samples. Early results indicate that U(VI) bioreduction is also inhibited in the presence of the other alkaline earth elements; the effect is proportional to the magnitude of the M-UO_2-CO_3 formation constants and thus to the predicted U(VI) aqueous speciation. By understanding these important key equilibria, more predictable and effective approaches can be established for *in situ* bioremediation of U under realistic field conditions.

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

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Our research is focused on developing mechanistic, phenomenological descriptions of important reactions and mathematical formulations to model reactions for the *in situ* immobilization of uranium (U) promoted via microbial iron(III) reduction. Experimental conditions have been designed to match those in saturated zone sediments at Area 2 of the ERSD Field Research Center (FRC) in Oak Ridge, TN. Our research has pursued three major objectives: (1) elucidate the mechanisms of reduction of solid-associated U(VI) in Area 2 sediment at the ERSD FRC; (2) evaluate the potential for long-term sustained U(IV) reductive immobilization coupled to dissimilatory metal-reducing bacterial (DMRB) activity in Area 2 sediments; and (3) numerically simulate the suite of hydrobiogeochemical processes occurring in experimental systems so as to facilitate modeling of *in situ* U(IV) immobilization at the field-scale. Our research is based on the following hypotheses: (1) the biological and chemical reduction of sediment-associated U(VI) is fundamentally controlled by its mineralogic and coordination environment; (2) the addition of humic substances can stimulate the reduction of solid-associated U(VI); (3) coupled Fe(III)/U(VI) reduction can be sustained in long-term flow-through reactor experiments with hydrologic residence times comparable to those expected in pore domains colonized by DMRB in Area 2 sediments; (4) modest levels of nitrate input will not significantly inhibit coupled Fe(III)/U(VI) reduction in Area 2; and (5) the kinetics and thermodynamics of simultaneous biogeochemical reactions can be described by reaction-based kinetic and equilibrium formulations, where rate formulations/parameter estimates derived from batch experiments will be applicable to flow-through reactor experiments.

Research progress has been made in the following areas: (1) Batch slurry experiments with Area 2 sediments have been completed and used to develop a reaction-based model of Terminal Electron Accepting Processes and other biogeochemical reactions in a hypothetical Representative Elementary Volume of Uranium-contaminated subsurface sediment (referred to as TEAPREVU). The model is capable of simulating time-dependent microbial population dynamics in relation to the abundance of various oxidized and reduced species and mineral phases, which in turn are a function of the input of external electron acceptors/donors and other aqueous species. This model has been up-scaled and applied to the Area 2 field site using the fully coupled, 3-D reactive transport model HYDROGEOCHEM. (2) Column reactors (2.5 cm dia, 125 cm bed length) packed with Area 2 FRC sediments have been constructed and have been running since June 2005. Reactors have been fed an Area 2 artificial groundwater at low flow rate (~0.1 pore volume/day) with varied concentrations of ethanol (0, 1 and 10 mM). Column experiments will be modeled using HYDROGEOCHEM. (3) Semi-continuous culture experiments with Area 2 sediments will begin shortly and will provide further data to scale batch-to-column and column-to-field model predictions. (4) U(VI) sorption to specimen Al oxides and illite have been completed. U(VI)-loaded solid phases have been analyzed by x-ray absorption spectroscopy (XAS) at Argonne and by cryogenic laser-induced fluorescence spectroscopy (CLIFS) at PNNL. Bioreduction of solid-phase U(VI) will be tested with *Geobacter sulfurreducens* in upcoming experiments. (5) A Penn State graduate student spent 7 weeks in Summer 2005 at the Environmental Molecular Science Laboratory (PNNL) assisting collaborative experimental efforts.

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

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Molecular analysis of subsurface microbial communities requires some combination of sample collection, concentration, cell lysis, nucleic acid purification, polymerase chain reaction (PCR) amplification, and specific detection in order to address fundamental questions of microbial community dynamics, activity, and function in the environment. However, changes in microbial community composition and/or abundance are still insufficient to detect or make conclusions regarding specific microbial activity. Thus, fieldable methods for the direct analysis of RNA are still required. Effort in FY2005 focused on microbial community profiling in low-biomass Uranium Mill Tailings Remedial Action (UMTRA) and the ERSD Field Research Center (FRC) sediments before, during, and after biostimulation, utilizing an 85-probe rRNA-targeted bead array for metal- and sulfate-reducing bacteria. Total RNA was extracted from subsurface sediments and interrogated directly, without employing a PCR step. Microbial community structure and dynamics through time and space were consistent with previous clone library data acquired at the sites, where indigenous sulfate- and iron-reducing bacteria and *Desulfotomaculum* near neighbors were the most responsive to a change in injected acetate concentrations. Interpreting bead array data was best accomplished by analyzing the relative change in probe response over spatially and temporally related samples, and by only considering the response of one probe to itself in relation to a background (reference) environmental sample. By limiting the data interpretation in this manner and placing it within the context of supporting geochemical and microbiological analyses, we conclude that ecologically relevant and meaningful information can be derived from direct microarray analysis of rRNA in uncharacterized environmental samples, even amidst current analytical uncertainty surrounding individual probe behavior on tunable bead arrays. Effort through FY2006 will continue applying the microbial community profiling methods to other relevant DOE field studies and developing analytical methods for the direct, automated detection and array-based analysis of mRNA from environmental samples.

Coupled Biogeochemical Process Evaluation for Conceptualizing Trichloroethylene Co-Metabolism

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Chlorinated solvent wastes (e.g., trichloroethene or TCE) often occur as diffuse subsurface plumes in complex geological environments where coupled processes must be understood in order to implement remediation strategies. Monitored natural attenuation (MNA) warrants study as a remediation technology because it minimizes worker and environment exposure to the wastes and because it costs less than other technologies. However, to be accepted, MNA requires "lines of evidence" indicating that the wastes are effectively destroyed. Our proposal will study the coupled biogeochemical processes that dictate the rate of TCE co-metabolism in contaminated aquifers first at the Idaho National Laboratory and then at Paducah or the Savannah River Site, where natural attenuation of TCE is occurring. We will use field-deployed flow-through *in situ* reactors to investigate the rate of methanotrophic co-metabolism of TCE and the coupling of the responsible biological processes with the dissolved methane flux and groundwater flow velocity. We will use new approaches (e.g., stable isotope probing, enzyme activity probes, real-time reverse transcriptase polymerase chain reaction, proteomics) to assay the TCE co-metabolic rates, and interpret these rates in the context of enzyme activity, gene expression, and cellular inactivation related to intermediates of TCE co-metabolism. By determining the rate of TCE co-metabolism at different methane concentrations and groundwater flow velocities, we will derive key modeling parameters for the computational simulations that describe the attenuation, and thereby refine such models while assessing the contribution of microbial relative to other natural attenuation processes. This research will strengthen our ability to forecast the viability of MNA at DOE and other sites that are contaminated with chlorinated hydrocarbons.

Characterizing the Catalytic Potential of Bacteria Isolated from Contaminated Subsurface Environments of the Hanford Site

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For bacteria representative of the Hanford Site, our objectives are to: (1) characterize the relationship between metal reduction, metal assimilation, and radiation resistance; (2) define environmental factors that govern these characteristics; and (3) correlate intracellular metal distributions with function using electron- and synchrotron-based microscopies.

Several groups of environmentally robust bacteria, including *Deinococcus* and *Arthrobacter* spp., have been isolated from radioactive sediments, from a high-level nuclear waste plume in the vadose zone of the DOE's Hanford Site in south-central Washington State. Organisms that belong to these groups are known for their radiation and desiccation resistance, and our recent work has established a strong link between intracellular accumulation of manganese (Mn)(II), metal reduction, and resistance to ionizing radiation (IR). Metal reductase activities in these organisms appear to facilitate the assimilation of Mn(II) from environmental Mn(III,IV) oxides. We have probed the nature of Mn(II)-facilitated IR resistance in *D. radiodurans*, which together with other IR-resistant bacteria have very high intracellular Mn/iron (Fe) concentration ratios compared to IR sensitive bacteria. The results identify a previously undescribed mechanism of IR-driven dioxygen (O_2) and hydrogen peroxide (H_2O_2) generation in the presence of Mn(II), show that *D. radiodurans* releases H_2O_2 during irradiation, and reveal that IR can induce growth of the strictly aerobic *D. radiodurans* under anaerobic conditions. *In vitro* in the presence of IR, Mn(II,III) redox-cycling favors superoxide ($O_2^{\bullet-}$)-scavenging with intermediate release of H_2O_2, whereas Fe(II,III) redox-cycling favors the production of $O_2^{\bullet-}$ and decomposition of H_2O_2 to hydroxyl radicals ($HO^{\bullet}$). *In vivo*, *D. radiodurans* displayed the hallmarks of IR-driven Mn redox-cycling observed *in vitro*. Thus, high intracellular Mn and low Fe concentrations could forestall the generation of reactive oxygen species (ROS) during irradiation. The great efficiency of *D. radiodurans* recovery pathways following IR appears to be based on Mn(II)-dependent protection of its proteome, which is being examined at Pacific Northwest National Laboratory in comparison to the IR-sensitive, Fe-accumulating bacterium *Shewanella oneidensis* (MR-1), using the high-mass-measurement accuracy of Fourier Transform Ion Cyclotron Resonance (FTICR). Most recently, we have combined light-, electron-, and synchrotron-based microscopies at Argonne National Laboratory and have shown a global distribution of Mn and sequestration of Fe in *D. radiodurans* cells. Collectively, these results support that: (1) proteins are the principal targets of IR *in vivo*; (2) metal distributions in bacteria profoundly affect resistance to IR and environmental stress conditions; and (3) modulating Mn and Fe homeostasis in bacteria is an approach that could be used to increase the resistance characteristics of bacteria slated for bioremediation.

Heterogeneity in Bioreduction and Resulting Impacts on Contaminant and Microbial Dynamics

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Soil and sediments are complex mineral assemblages hosting a diverse microbial community, all within a convoluted physically heterogeneous setting. Consequently, biogeochemical processes will exhibit high spatial variability, owing to chemical conditions dictated by local mineralogical and physical proximity. Bioreductive stabilization of contaminants is dependent on the convoluted coupling of biological, chemical, and hydrologic processes that will vary spatially from the micro- to macro-scale. We have therefore been conducting a series of studies that encompass increased chemical and physical heterogeneity on bioreductive processes.

In aerobic environments, uranium (U) is generally found in the hexavalent state and is quite soluble in carbonate-bearing solutions. Reduction of U(VI) to U(IV), principally induced by dissimilatory metal reducing bacteria, diminishes aqueous concentrations of U through precipitation of the sparingly soluble UO_2 phase (uraninite). Uranium reduction, however, is predicated on a viable reduction pathway that may be constrained by competing oxidants and the salient uranyl species. Two dominant factors influencing U reduction are the formation of calcium-uranyl-carbonato complexes that may hinder electron transfer and the presence of iron (hydr)oxides that may serve as competing electron acceptors and, potentially, even as oxidants of UO_2. We compared the impact of calcium concentration on U(VI) reduction between systems containing iron (hydr)oxides (ferrihydrite, goethite and hematite) having a range of surface areas and stabilities. A linear correlation exists between the extent of reduction and calcium concentration at reaction times less than 200 hours, and the correlation exists for all three iron (hydr)oxides. At longer reaction times (528 hours), calcium has a varying effect depending upon the type of iron (hydr)oxide. Imparting an important criterion on U reduction, goethite and hematite surprisingly tend to diminish the effect of calcium by decreasing the dissolved concentration of calcium; ferrihydrite acts as a competitive electron acceptor or oxidant, and thus, along with calcium, decreases U reduction. Uraninite oxidation by Fe(III) (hydr)oxides, however, is thermodynamically favorable only under limited geochemical conditions. Our analysis reveals that goethite and hematite have a limited capacity to oxidize $UO_{2(biogenic)}$, while ferrihydrite can lead to $UO_{2(biogenic)}$ oxidation. The extent of $UO_{2(biogenic)}$ oxidation by ferrihydrite increases with increasing bicarbonate and calcium concentration, but decreases with elevated Fe(II)(aq) and U(VI)(aq) concentrations.

In addition to examining the impact of chemical complexity on bioreductive processes, we have also been investigating metal-reducing bacterial communities and operative geochemical processes within constructively and natively structured soils and sediments. Bioreduction of mineral constituents such as ferric (hydr)oxides are dominant along advective flow paths, with a progressive decrease entering diffusive pore domains. Within four soil horizons from the Melton Branch watershed at ORNL, having variations in pH, organic matter fraction, mineralogy, and water content, a mixture of bacterial communities was noted within enrichment cultures, ranging from affiliated with previously known (e.g., *Geobacter*) and unknown (e.g., *Anaerovibrio*) Fe(III)-reducing bacteria. Increased saturation resulted in a progressive increase in goethite at the expense of ferrihydrite, and chromate reduction was predicated on iron reduction, thus being enhanced in soils/sediments with higher water content. Appreciable spatial heterogeneity will therefore control contaminant mobility or attenuation (both in terms of degree and mechanism) within soils and sediments, depending on the prevailing biogeochemical conditions and operative microbial metabolisms.

Biomolecular Mechanisms of $U(IV)O_2$ and $Tc(IV)O_2$ Nanoparticle Formation by *Shewanella oneidensis* MR-1

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The reduction of uranium(VI) and technetium(VII), which often exist as highly soluble anions in oxic environments, generally results in the formation of poorly soluble and significantly less mobile $U(IV)O_{2(s)}$ or $Tc(IV)O_{2(s)}$ particles. Dissimilatory metal-reducing bacteria such as *Shewanella oneidensis* MR-1, can effectively transform U(VI) and Tc(VII) complexes to UO_2 and TcO_2 nanoparticles; however, the biomolecular mechanisms of reduction and controls on nanoparticle formation are not well understood. *S. oneidensis* MR-1 produces a cadre of multiheme *c*-type cytochromes, including those localized to the outer membrane (OM) that are required for the reduction and electron transfer to both complexed and solid metal oxides such as Fe(III) and Mn(IV). Additionally, *S. oneidensis* MR-1 produces several diversified electron transport networks to facilitate the reduction of a variety of other organic and inorganic substrates. Various hydrogenases and reductases may also constitute integral parts of the terminal reductase complexes for U(VI) and Tc(VII) reduction. We have determined that the *c*-type cytochromes of *S. oneidensis* MR-1 are also essential for the biogenic reduction of U(VI) and that inactivation of a combination of two specific OM cytochromes, MtrC and OmcA, significantly affected the rate of U(VI) reduction.

Interestingly, the reduction of Tc(VII) was not similarly affected by deletions of these or other *c*-type cytochromes. Therefore, we investigated the involvement of hydrogenases and reductases produced by MR-1 in Tc(VII) and U(VI) reduction. A NiFe hydrogenase was identified as the physiological uptake hydrogenase in MR-1, since mutants lacking this protein or the ability to properly secrete it were unable to utilize H_2 for the reduction of U(VI) or Tc(VII). The subcellular localization of UO_2 and TcO_2 nanoparticles were also determined by high-resolution transmission electron microscopy (TEM). TcO_2 nanoparticle localization was predominantly periplasmic, but also seen on outside of the OM surface, whereas UO_2 nanoparticles localization was more complex. There were also distinct differences in subcellular localization of UO_2 between MR-1 and the OMC mutants. Small aggregates of loosely packed UO_2 nanoparticles external to cells were present in MR-1 and an OmcA mutant, but absent in cell suspensions of MtrC or MtrC/OmcA mutants. Similar to the wild type, the mutants accumulated UO_2 nanoparticles extracellularly to high densities in association with an exopolymeric substance that exhibited "fiber-like" features. This UO_2-binding exopolymer exhibited glycocalyx-like properties and contained an integral outer membrane protein in addition to MtrC and OmcA. Moreover, MtrC and OmcA were observed by immune localization to be in close physical association with the UO_2 nanoparticles, providing further evidence of their role in U(VI) reduction and, likely, control on particle size. Additional research is being conducted to determine the composition and properties of the extracellular polymeric material and its effect on reactivity of radionuclide nanoparticles in regards to O_2-promoted oxidation.

Long-Term Chromium Bio-Immobilization at the Hanford 100H Site: Geochemical and Microbiological Response to Slow Release Electron Donor

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The focus of these studies is to understand the coupled hydraulic, geochemical, and microbial conditions necessary to maximize Cr(VI) bioreduction and minimize Cr(III) reoxidation in groundwater. Here we present the application of slow-release electron donor during a field-scale treatability study over an 18-month period. Samples were taken at intervals pre- and post-injection of a ^{13}C-labeled slow-release polylactate compound (HRC) used to stimulate indigenous microbial populations to immobilize hexavalent chromium. Redox potential, pH, dissolved oxygen (DO), nitrate, chromium (VI), and sulfate concentrations in groundwater were monitored. Stable isotope enrichment in dissolved inorganic pools was followed, and a fluorescent antibody was used to visualize the presence of a sulfate reducer. Following HRC injection (27 days) reducing conditions (-130 mV) had established with a corresponding disappearance of DO and nitrate. Cr(VI) concentrations declined steadily over 6 weeks. Analysis of delta ^{13}C ratios in dissolved inorganic carbon confirmed microbial metabolism of the labeled HRC. Hydrogen sulfide production was first observed after about 20 days post-injection; this corresponded with the enrichment of a *Desulfovibrio* species identified using fluorescent antibodies. Bacterial densities have remained high ($>10^7$ cells/mL); Fe(II), and Cr(VI) concentrations in the monitoring and pumping wells have remained below up-gradient concentrations for the first 12 months. Lower parts of the aquifer have maintained sulfate-reducing conditions, and when pumping was applied to downgradient wells, DO declined significantly in the upper parts of the aquifer. A number of sulfate reducers have been isolated from the deeper parts of the aquifer. HRC was still present more than a year after initial injection and was maintaining the overall Cr (VI) levels at nondetectable, suggesting that this may be a long-term bioimmobilization of Cr (VI). Geochemical analysis of groundwater coupled with stable isotope and microbial monitoring allowed for accurate tracking of microbial processes during this field treatability study.

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

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The overall objective of this research is to understand the biogeochemical cycling of plutonium (Pu) in environments of interest to long-term DOE stewardship issues. The hypothesis underlying this project is that microbial activity is the causative agent in initiating the mobilization of Pu in near-surface environments: (1) through the transformation of Pu associated with solid phases, (2) production of extracellular polymeric substances (EPS) carrier phases, and (3) the creation of microenvironments. Brief summaries of some of the work conducted this year follow.

Pu-contaminated soil was obtained from the Rocky Flats Environmental Technology Site (RFETS) and characterized using synchrotron techniques including μ-x-ray fluorescence, μ-XANES and x-ray diffraction. These studies showed discrete iron particles and the presence of quartz and iron mineral phases and Pu (100 pCi/g) below detection. In order to study the biotransformation of Pu by indigenous anaerobic bacteria, the RFETS soil was spiked with ^{242}Pu (8 nCi/g soil) and incubated with glucose as a carbon source. Mineralogical association of Pu was determined by selective sequential extraction before and after microbial activity. In the unamended sample, ~80% of the added ^{242}Pu was associated with the iron oxide fraction, while in the glucose-amended sample, it decreased to 50%. Under highly reducing conditions (Eh of 100 mV) and at pH5, the indigenous and tracer-Pu was not soluble (<0.45 μm). However, microbial activity resulted in the formation of colloidal ^{242}Pu as determined by ultrafiltration, and it was correlated with particulate and soluble carbohydrates. These studies demonstrate that anaerobic microbial activity in contaminated soil can redistribute Pu from labile phases to either less-reactive phases or colloidal forms.

The role of the protein content of bacterially produced EPS in Pu binding to the EPS was also a focus this year. It is likely that bacteria can regulate the surface hydrophobicity of their EPS by regulation of the protein content, and thus, the mobility of EPS-bound metals such as plutonium. Protein-poor varieties of EPS from *Pseudomonas fluorescens* Biovar II were produced through pronase treatment subsequent to their separation by repeated alcohol precipitation. The molecular weight distribution was found to be almost identical for EPS with and without proteins. On the other hand, using hydrophobic contact area (HCA) determination by hydrophobic interaction chromatography, it was found that the HCA of protein-containing EPS from *Pseudomonas fluorescens* Biovar II is considerably higher than that of the protein-poor variety, i.e., 24.5 versus 2.6 $Å^2$ molecule^{-1}. This significantly higher HCA of protein-containing EPS demonstrates their higher surface hydrophobicity in water. Pu(IV) binding to EPS from *Pseudomonas fluorescens* Biovar II was found to be dependent on protein content; Pu(V) binding to EPS was significantly less than that of Pu(IV) and less dependent on protein content of EPS than the Pu(IV).

Static columns (5 mL volume, "stop/flow" columns) were used to assess the potential mobility of ^{239,240}Pu from Rocky Flats soil under advective flow conditions. Incubation of the static column systems with glucose as the carbon source showed substantial mobilization of the Pu relative to control cases (~4x the control cases). In contrast, ^{241}Pu, added as Pu(V), showed no statistical difference in the control versus incubated columns unless bacterially produced EPS was introduced. In the latter case, EPS was able to mobilize ^{241}Pu relative to control columns.

Experiments were conducted on the binding of Pu to EPS produced by *Pseudomonas fluorescens, Clostridium sp.* and *Shewanella putrafaciens*. The order of binding, treating bacterial surfaces as ligands using a discrete ligand-affinity-distribution approach, is: *Clostridium sp.* ≈ *Shewanella putrefaciens* > *Pseudomonas fluorescens.*

Multiscale Monitoring and Prediction of Biostimulation Using Geophysical Methods

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To advance solutions for remediation of DOE contaminated sites, approaches are needed that can elucidate and predict reactions associated with coupled biological, chemical and hydrologic processes, over multiple scales and in heterogeneous environments. In this study, we bring together a multi-institutional research team with expertise in geophysics, geochemistry, hydrogeology, and geomicrobiology to develop such tools and approaches. With DOE Environmental Management Science Program (EMSP) support, we have recently investigated the utility of the following different geophysical techniques for detecting various system transformations at the column scale:

- *Radar velocity measurements for detecting onset and evolution of gas during denitrification*. This experiment indicated that the radar measurements are sensitive to the onset and extent of gaseous end products of denitrification, and by extrapolation, methanogenesis.
- *Seismic measurements to detect onset of gas evolution during denitrification.* This study showed that seismic amplitudes are a good indicator of the early onset of denitrification.
- *Self Potential (SP) measurements for characterization of redox conditions.* We have used the SP method to track the onset and location of microbial sulfate-reduction in saturated sediments at the laboratory scale during conditions of organic carbon amendment. The experimental results suggest the ability to measure the changes in the spatiotemporal location of sulfate-reduction during bioremediation.
- *Induced Polarization (IP) methods to track changes in iron mineralogy during remediation.* We have used IP methods to track physiochemical changes in iron-bearing clays resulting from microbial respiration, which correlated with the exhaustion of bioavailable ferric compounds. This study shows that IP methods may be a reasonable approach for noninvasively monitoring the sustainability of prolonged iron-reduction under stimulated conditions.
- *Combined seismic and IP methods to monitor biomass accumulation.* We investigated the use of seismic and IP methods to characterize the accumulation of biomass within saturated sediments. The experimental conditions were similar to those used during our studies of metal sulfide precipitation, with the exception that no metals were added to the influent solution. Although destructive evaluation of the sediments revealed significant biofilm development, only modest changes in IP and seismic signatures were observed.

Our studies to date indicate that minimally invasive, high-resolution geophysical methods hold significant potential for monitoring and elucidating processes that occur during remediation. Our new ERSP project builds upon this research to explore the geophysical responses to stimulation under more realistic conditions (i.e., at multiple scales, in the presence of competing reactions, and using native groundwater and sediments). Laboratory studies will be performed to mimic conditions at the Old Rifle, CO, field site, where we will perform field experiments in conjunction with ongoing projects. An estimation framework will be developed that enables estimation of the dominant processes given various geophysical attribute responses. The extensive estimation and monitoring datasets, collected at both the laboratory and field scales, will be coupled with reactive transport models to improve the use of both geophysical monitoring and geochemical modeling approaches for understanding complex subsurface systems.

An Integrated Approach to the Characterization of Microbial Exudates and Investigation of Their Role in Transformations of Uranium (U)

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The microenvironment at and adjacent to actively metabolizing cell surfaces can be significantly different from the bulk environment. Microbial polymers (polysaccharides, DNA, RNA, and proteins), whether attached to or released from the cell, can contribute to the development of steep chemical gradients over very short distances. It is currently difficult to predict the behavior of contaminant radionuclides and metals in such microenvironments, because the chemistry there has been difficult or impossible to define. The behavior of contaminants in such microenvironments can ultimately affect their macroscopic fates. Information about biotransformations and biogeochemical interactions at the microbe–mineral interface is paramount for predicting the fates of contaminants and designing effective bioremediation approaches.

The overarching goal of our work is the *integration* of a number of microbiological, chemical, and physical techniques, to develop a more holistic approach to understanding the role of microbial exudates in the biotransformations and biogeochemical interactions of contaminant metals and radionuclides at the mineral–microbe interface. We are using U LIII-edge x-ray absorption fine structure spectroscopy, x-ray fluorescence microscopy, and scanning electron microscopy to investigate interactions occurring near the mineral–microbe interface, among a contaminant (U); a mineral surface (thin film lepidocrocite deposited on kapton film); and microbes (*Shewanella oneidensis* MR-1). We have also begun using U LIII-edge x-ray absorption spectroscopy and titration approaches to investigate competitive interactions among U, iron (oxy)hydroxides, and aerobically produced exudates from *S. oneidensis* MR-1. Results of these experiments will be presented.

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

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Our overall objective is to understand the microbial and geochemical mechanisms controlling the reduction and immobilization of uranium(VI) during biostimulation in shallow subsurface sediments co-contaminated with uranium (U) and nitrate. The focus is on the activity and community composition of microbial populations (metal- and nitrate-reducing bacteria) and iron (Fe) minerals that are likely to make strong contributions to the fate of U during *in situ* bioremediation. Our integrated approach was applied to sediment cores and microcosms of site materials from Area 2 of the ERSD Field Research Center (FRC) at Oak Ridge, TN. Substantial differences were observed in the abundance and activity of microbial groups depending upon the electron donor (glucose or ethanol) used for biostimulation. Viable counts revealed that Fe(III)- and nitrate-reducers are abundant (10^4 to 10^5 per g wet) in Area 2 sediments, and counts were shown to be carbon substrate dependent. U(VI) and Fe(III) were reduced concurrently in the glucose but not the ethanol treatments. One to 2 orders of magnitude more Fe(III)-reducers were observed in ethanol- as compared to glucose-amended treatments in parallel with enhanced U(VI) removal in ethanol treatments. Cultivatable Fe(III)-reducing bacteria in the ethanol treatments were numerically dominated by *Geobacter* sp. while those cultured on glucose were dominated by fermentative organisms. Efforts are under way to associate *in situ* activity at the FRC with the bacteria from the microcosms by fingerprinting ribosomes in groundwater samples.

Iron minerals were characterized by Mossbauer spectroscopy over a wide range in temperature (4 to 298 K) to fully determine the form and speciation of Fe. Spectra at room temperature (298 K) exhibited no sextet pattern, thus excluding the presence of hematite, magnetite, and maghemite. At 77 K, the amount of Fe(II) doubled from 15 to 30% in ethanol- and glucose-amended relative to unamended microcosm sediments, in parallel with wet chemical extractions and counts of Fe(III)-reducing bacteria. Poorly ordered or Al-substituted goethite was identified and appeared to be dissolved by microbial activity. However, silicate bound Fe(III) clearly predominated over the Fe minerals reduced.

Novel iron(III)- and sulfate-reducing organisms were isolated from the contaminated FRC subsurface that shared high sequence identity (96 to 99%) to *Geobacter bremensis* and *Desulfotomaculum ruminis*, respectively. The *Desulfotomaculum*-related isolate utilizes Fe(III) as well as sulfate as an electron acceptor. The draft genome sequence of *Geobacter* strain FRC-32 has been completed by the Joint Genome Institute and annotation is currently under way.

Our results have the following implications for U bioremediation in the FRC subsurface: (1) the microbially catalyzed mechanism of U(VI) reduction is electron donor dependent, (2) silicate-bound Fe is an important oxidant that is transformed by indigenous microbial populations in the Area 2 subsurface, and (3) *Geobacter* sp. predominate over other Fe(III)-reducing bacteria during biostimulation with ethanol as an electron donor.

Acceleration of Field-Scale Bioreduction of U(VI) in a Shallow Alluvial Aquifer

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Field-scale biostimulation experiments for bioreduction of uranium(VI) at the Old Rifle, CO, uranium (U) mill tailing site conducted over the last four field seasons have consistently shown loss of U(VI) from groundwater synchronous with growth of *Geobacter* after amendment of the subsurface with acetate at concentrations of either ~3 mM or ~10 mM. Pre-amendment differences in the distribution of U(VI) in groundwater, other groundwater geochemical parameters, and complex resistivity geophysical surveys suggest that naturally occurring differences in redox status and possibly the rate of endemic bioreduction control the initial U(VI) concentrations. Electron donor amendment and associated *Geobacter* growth dominate the system as long as spatial and temporal variability in groundwater flow and injection processes permit delivery of electron donor to the subsurface. Microbially mediated iron (Fe) reduction consumes bio-available Fe(III) solids, eventually limiting *Geobacter* growth. Because there is ~10 mM sulfate in the groundwater, sulfate reduction dominates the system after bio-available oxidized Fe is consumed, resulting in extensive precipitation of $FeS_{0.9}$, particularly near the point of injection and in the deeper parts of the aquifer. Typically, U(VI) concentrations rebound with the onset of extensive sulfate reduction, but, remarkably, after acetate amendment is stopped, U(VI) concentrations fall again and can remain at ~20% of background levels for >1 year. Our current hypothesis is that the extent of post-amendment U(VI) removal is correlated with the amount of $FeS_{0.9}$ precipitated. However, recent laboratory studies (see abstract by Lovley et al.) indicate that $FeS_{0.9}$ cannot reduce U in these sediments without microbial mediation, suggesting that a microbial community different from either the Fe or sulfate reducers is coupling $FeS_{0.9}$ oxidation and U(VI) reduction.

Geophysical measurements (complex resistivity) during the last two field seasons indicate that noninvasive monitoring of both initial heterogeneity of redox status and microbially mediated changes in subsurface mineralogy may be possible. Inversion of complex resistivity data from 2004 shows a marked anomaly below the water table near the injection gallery, interpreted as a response to the changed redox status of Fe-bearing mineral coatings on detrital grains. In contrast, in an adjacent experimental plot, the phase shift in the complex resistivity results suggests that sulfides may have been present prior to electron donor amendment, and that the abundance of sulfide increased toward the end of electron donor amendment. In future experiments, more extensive surface and borehole resistivity measurements will be obtained to provide detailed tracking of Fe redox status and mineralogy in response to *in situ* bioreduction.

Multicomponent biogeochemical reactive transport modeling of biostimulation events, including the formation of $FeS_{0.9}$ and the sorption of Fe(II), successfully captures the concentration changes observed in the field experiments, except for the unexpected long-term U(VI) loss after electron donor amendment. Modeling also identified a propensity for the slightly higher-density acetate solution to sink, resulting in higher concentrations near the aquifer bottom. We anticipate that incorporation of microbial mediation of $FeS_{0.9}$ oxidation will allow quantitative matching of the long-term U(VI) loss. A combination of future field experiments and reactive transport modeling both geared toward quantitatively assessing the long-term removal of U(VI) will enable us to assist the U.S. DOE in decision making for cleanup of the Weapons Production Complex.

Biotransformations Involved in Sustained Reductive Removal of Uranium in Contaminated Aquifers

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Field studies of *in situ* bioremediation of uranium (U)-contaminated groundwater at the field study site in Rifle, CO, have indicated that there are three distinct phases following the addition of acetate to stimulate microbial respiration. In Phase I, *Geobacteraceae* are the predominant organisms, Fe(III) and U(VI) are reduced, and U is removed from the groundwater. In Phase II, Fe(III) is depleted, sulfate is reduced, but U(VI) is not, and sulfate-reducing bacteria predominate. In Phase III, U(VI) removal resumes and continues even after acetate additions are stopped. The removal of U in Phase III was a surprise, and the mechanisms involved in this phenomenon are unknown. To investigate the mechanisms for uranium removal in Phase III, flow-through columns of subsurface sediments from the Rifle site were amended with acetate to simulate the *in situ* U bioremediation that has been carried out in the field. The three phases observed in field studies were successfully replicated in the column studies. Sediments from Phase III were sampled under anaerobic conditions and incubated in serum bottles. Heat-sterilizing these sediments inhibited removal of U from the groundwater, whereas U continued to be removed in sediments that contained a viable microbial community. These results suggest that U removal in highly reduced sediments cannot be attributed to abiotic processes such as reduction of U(VI) by iron sulfides. To further evaluate the apparent microbial mechanisms for U(VI) removal, the composition of the microbial community is currently being evaluated by assessing which 16S rRNA gene sequences predominate, and the speciation of U in the sediments is being determined. These observations suggest that there is a biological mechanism for long-term removal of U from contaminated groundwater that is distinct from the *Geobacteraceae*-catalyzed reductive precipitation of U. This unexpected process enhances the cost effectiveness of *in situ* U bioremediation.

As reported at last year's meeting, our previous studies have demonstrated that electrodes poised at the proper potential can serve as electron donors for microbial reduction of U(VI) to U(IV). This has the potential benefit of the U(IV) producing precipitates on the electrode surface. Thus, when the electrode is removed from the subsurface, the U is also removed from the environment. In studies conducted in a 200-liter drum designed to simulate the Rifle subsurface environment, supplying electrons via an electrode effectively removed U from the groundwater. For reasons not yet understood, precipitates formed on electrodes incubated in the field at the Rifle site and U(VI) was not removed. Additional studies to evaluate U(VI) reduction with electrodes at the Rifle site are warranted. Preliminary laboratory studies suggested that electrodes may be a suitable electron donor for removing U from contaminated groundwater at the ERSD Field Research Center at Oak Ridge.

A new acetate-addition field experiment was run at the Rifle study site in 2005. The results demonstrate that it was possible to effectively reproduce the microbiological and geochemical changes in response to added acetate that were observed in the previous year. This provides the opportunity to carry out field-scale experiments in a reproducible manner, and to reliably compare the effect of changes in the design of bioremediation strategies on a field scale.

Stabilization of Plutonium in Subsurface Environments via Microbial Reductionand Biofilm Formation

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Contaminant plutonium (Pu) is present at DOE sites that have complicated hydrogeology, and redox environments are affected by direct and indirect microbially mediated processes. The influence of these processes is difficult to predict because of the complicated redox behavior and rich chemistry of Pu. Specifically, bacteria can affect Pu speciation and environmental mobility through the following mechanisms: (a) biotransformation and cell internalization by aerobic bacteria, (b) accumulation and immobilization in biofilms, and (c) immobilization via direct enzymatic and indirect biogeochemical reduction of Pu species by metal-reducing bacteria.

Biotransformation of Pu species. Plutonium is accumulated by *Pseudomonas putida* via an active siderophore recognition and uptake process. The pyoverdin (pvd) siderophore produced by *P. putida* strain ATCC 33015 was found by isotopic labeling, mass spectroscopy and multinuclear, multidimensional nuclear magnetic resonance (NMR) experiments to contain 2,3-diamino-6,7-dihydroxyquinoline linked by a catechol to a six amino acid peptide chain. Pvd stabilizes forms highly stable binary and ternary Pu(IV) molecular complexes in solution. Cells of *P. putida* individually presented with a range of metal-pvd complexes accumulated 1:1 Pu(IV), Th(IV) and Fe(III) pvd complexes at similar rates and amounts, but not pvd complexes of several other metal complexes or 1:2 Pu:pvd complexes. These studies indicate that under environmental conditions, Pu^{IV} will be accumulated by bacteria via siderophore receptors—encouraging us to investigate Pu accumulation by bacteria in biofilms.

Stabilization of Pu within biofilms. Bacteria in nature grow mostly in biofilms, surface associated cells enveloped by hydrated extracellular polymeric substances (EPS). Plutonium may be sequestered within biofilms; conversely, bacterial growth and EPS production may be affected by the radionuclide. Prior to studying Pu, the mechanisms of biofilm-radionuclide interactions are being identified and characterized using redox-active metals whose chemistry is better known. Unsaturated biofilms of *P. putida* are being cultivated on membranes overlaying solid media with the individual addition of soluble metal (Cr, U, or Pu), mineral (hematite), and metal + mineral. Biofilms are characterized by imaging techniques, and then separated into cell and EPS fractions for subsequent inorganic speciation and biomolecule analyses. Both Cr and U have specific distribution between cells and EPS. The affect of Cr and U on intra- and extracellular proteins, DNA, and carbohydrate content have also been determined.

Bacterial reduction of Pu(VI/V). Plutonyl (Pu(V) and Pu(VI)) species are the most soluble Pu molecular compounds formed under environmental conditions. Their reduction by metal-reducing bacteria to Pu(IV) solids could stabilize Pu *in situ* with respect to migration. Cell suspensions of *Shewanella oneidensis* MR1 and *Geobacter metallaredceans* GS15 rapidly reduce Pu(V) and Pu(VI) to form a solid that has spectroscopic and physical features consistent with small grained PuO_2 particles, as determined by optical and x-ray methods. Recent experiments indicate that plutonyl and even chelated forms of lower-valent Pu and may also support growth of these bacteria.

Plutonium (Pu) Speciation, Solubility, and Migration under Environmental Conditions

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Actinide contaminants at nuclear material production and reprocessing sites are likely released to the far field slowly and at low concentrations. The presence of redox-active minerals and the interactions occurring at their solid-solution interfaces can affect actinide speciation and overall rates of migration. One of the key goals of this project is to determine the mechanisms of interaction between actinides and manganese/iron (Mn/Fe) minerals and to model this behavior using surface complexation modeling techniques. These results improve our ability to predict the fate and transport of actinides into the environment and to implement effective remediation strategies.

Oxidizing mineral phases such as birnessite, δ-Mn(IV)O_2, have the ability to completely oxidize Pu(V) in solution and stabilize it as a hexavalent inner-sphere complex on the mineral surface. Time-dependent adsorption studies conducted with Pu(VI) and U(VI) at [AnO_2^{2+}] = 10^{-4}–10^{-6} M show rapid adsorption under all conditions for both actinides. The adsorption capacity for Pu(VI) onto δ-MnO_2 is 0.16 mmol Pu(VI) / g MnO_2 at pH 4.0, roughly one tenth the adsorption capacity for Cu(II) under similar conditions. The adsorption of Pu(VI) onto δ-MnO_2 is highly pH dependent, and the adsorption edge occurs between pH 2.0 to 5.0. The adsorption edge for U(VI) is very similar, but occurs at slightly lower pH, reflecting the more complex hydrolysis behavior of U(VI) and the lower pH where the onset of hydrolysis begins. In contrast, when U(VI), Np(V), and Pu(VI) were reacted with hematite (Fe_2O_3) and goethite (FeOOH), reducing mineral phases, the adsorption edges for Pu(VI) and Np(V) are identical, indicating that the adsorption mechanisms may be similar, while the shape and position of the adsorption edge for U(VI) is very different. Following interaction between the Pu and Np with goethite, UV/Vis spectroscopy shows that Pu(VI) is reduced in solution to Pu(V), and as expected Np(V) does not change oxidation state.

The adsorption of Pu(VI) onto δ-MnO_2 is accurately modeled using three hexavalent Pu surface complexes. Initial attempts to prepare fits of the experimental data for Pu(VI) adsorption onto the Fe minerals required the use of two Pu(IV) and two Pu(VI) complexes. Uranium(VI) species adsorbed onto the δ-MnO_2 in differing stoichiometries and structures from the adsorbed Pu(VI) species, reflecting differences in the hydrolysis of the cations. Further, the results for Pu adsorption onto iron oxides reflect the differences in the redox behavior between Pu and U, reinforcing the conclusion that there are no functional or structural analogs for Pu environmental chemistry.

Environmental Remediation Science Program at the Advanced Light Source

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The Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory (LBNL) is one the U.S. DOE laboratories with special experimental capabilities for environmental research. In 2005, the Environmental Science Program at the Advanced Light Source was established to provide support to ERSP PI's who wish to use the Advanced Light Source in their investigations. Based on expressed and documented ERSP PI research needs, this program offers support across a suite of environmental beamlines at the ALS. The capabilities of the facility include infrared spectromicroscopy on beamline 1.4.3, micro-tomography on beamline 8.3.2, micro-x-ray absorption spectroscopy and micro-diffraction on beamline 10.3.2, and scanning transmission x-ray microscopy (STXM), x-ray photoelectron spectroscopy (XPS), and near-edge x-ray absorption fine structure (NEXAFS) spectroscopy on beamline 11.0.2. These beamlines provide an energy range from sub-eV to 30 keV while covering a length scale from tens of nanometers to centimeters. More information about the Environmental Program at the ALS is given at http://esd.lbl.gov/ALS_environmental/index.html

While several of the beam lines in the environmental suite are heavily subscribed, two of them (8.3.2 and 1.4.3) are readily available for environmental users and also have the potential to advance our investigative capabilities. For example, beam line 8.3.2 permits rapid three-dimensional imaging of solid samples through computed x-ray tomography. This represents a new and powerful capability to image the topology and to monitor processes at the pore scale in near-real time, such as how the growth of bacteria and bacterial biofilms alter the three-dimensional structure of the pore spaces within soil samples.

In addition to describing the new opportunities and user assistance provided by the new ALS program, we will illustrate examples of the use of the beamlines for different environmental investigations, as well as the combined synergy offered through the use of multiple beam lines for a single, integrated experiment.

Coupled Flow and Reactivity in Variably Saturated Porous Media

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Improved models of contaminant migration in heterogeneous, variably saturated porous media are required to better define the long-term stewardship requirements for U.S. Department of Energy (DOE) lands, and to design effective vadose-zone remediation strategies that significantly decrease contaminant migration. To better understand contaminant migration processes, we have been using the 2-meter radius geocentrifuge capabilities at the Idaho National Laboratory (INL) to conduct unsaturated transport experiments. The experimental approach using the geocentrifuge provides data in a much shorter time period than conventional methods, allowing us to complete more experiments and explore a wider range of moisture contents. Through our review of the theory of unsaturated flow in the geocentrifuge, we have derived an expression for the fluid potential in a centrifugal field that is different from the expression in constant gravity field. The difference in these expressions suggests that caution should be used in applying simple scaling laws to experimental results. Collaborating with others, we have developed numerical tools that allow us to simulate flow and transport in a centrifugal field. Unlike the smaller centrifuges, the INL geocentrifuge allows more control of the experimental packages. Both the upper and lower boundary of the soil columns is autonomously controllable using the on-board computers. We have designed and constructed an in-flight fraction collector system capable of withstanding the acceleration on the geocentrifuge. The solute breakthrough curve can be determined using either in-line chemical sensors or effluent samples collected using the robust fraction collector system. We have also improved the design of a soil moisture probe, making it more suitable for column experiments. Tracer tests conducted on the geocentrifuge demonstrate that detailed information can be obtained in-flight in relatively short time periods of time. Through the application of our work on flow under enhanced acceleration, modified models, and improved experimental techniques, we continue to improve our ability to conduct and analyze vadose-zone transport experiments on the geocentrifuge.

An Integrated Assessment of Geochemical and Community Structure Determinants of Metal Reduction Rates in Subsurface Sediments

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The objective of our research is to examine the effect that microbial community structure and geochemistry has on metal reduction rates in subsurface sediments. Many microorganisms can change the geochemical conditions of their environment such that metal reduction becomes an energetically favored reaction. In these cases, factors such as mass transport, oxygen level, and nitrate concentration will likely dominate the rate of microbial change in the redox potential, and the importance of the microbial community structure may be minimal. Alternatively, some microbes may directly catalyze the necessary reactions. In this case, the composition of the community may be more important. Our results will help determine if the type of electron donor is important in the bioremediation of metal-contaminated environments.

To date, we have done an extensive investigation of the effects of different electron donors on the rates of uranium and nitrate reduction using microcosms containing slurries of sediment and groundwater collected at the ERSD Field Research Center (FRC). We have seen significant and consistent differences in rates. For instance, we found that uranium reduction was significantly influenced by the type of electron donor, but that nitrate and sulfate reduction were influenced to a lesser degree. Ethanol and glucose promoted rapid reduction of uranium and nitrate, whereas methanol promoted nitrate but not uranium reduction. Preliminary characterization of the microbial community indicated that there were substantial differences in the community structure related to the type of electron donor added. In a more limited set of experiments, we have examined the effect of adding humic material on the rates of iron and sulfate reduction. The addition of humics did not increase rates of uranium reduction in treatments where uranium reduction was already rapid (e.g., with addition of ethanol).

We are currently examining the microbial community composition in completed experiments using several molecular methods. Changes in community composition will be related to uranium reduction rates by nonlinear data analysis techniques. In subsequent experiments, we will test resource ratio theory to determine if changes in carbon/phosphate ratio can significantly impact the community and rates of uranium reduction. In addition, we will continue to investigate the interactions between humic additions and changes in community composition.

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Coupling between Flow and Precipitation in Heterogeneous Subsurface Environments and Effects on Contaminant Fate and Transport

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This project is aimed at understanding how solute transport in heterogeneous porous media is impacted by precipitation and dissolution events in two ways: (1) directly via chemical interactions with precipitation processes (broadly defined so as to potentially include colloid filtration and biological growth), and (2) indirectly as a consequence of coupling between the precipitation process and flow. The project will involve both physical experiments and computational model development and testing, at multiple scales.

We hypothesize that precipitation/co-precipitation, encapsulation, isolation from flow, and alteration of reactive surfaces will contribute to altering contaminant mobility during precipitation events, and that predicting the release of contaminants during dissolution requires an understanding of how precipitates are distributed and how contaminants are released from the different compartments over time. Initially using calcium carbonate as a model system, we will use physical experiments and modeling at the pore scale and continuum scale to improve the conceptual approach to predicting the impact of flow-precipitation coupling on solute migration. Column and two-dimensional intermediate-scale experiments with constructed physical and chemical heterogeneities will be used to investigate the movement of fluids and reactive solutes during different types of mixing events that lead to calcium carbonate supersaturation and precipitation. Smoothed particle hydrodynamic modeling will be used to simulate pore-scale mixing and precipitation in heterogeneous porous media and to help estimate continuum-scale parameters. Continuum-scale modeling will be used to test conceptual models and associated effective parameters that simulate the macroscopic behavior of the experimental domains.

Although computational models for the fate and transport of subsurface contaminants can incorporate many of the individual physical and biogeochemical controls on solute mobility and transformations, advanced predictive models must also account for coupling between physical, chemical, and biological processes over a range of spatial and temporal scales. One challenge involves constructing correct conceptual and quantitative relationships between individual processes that are impacted by spatial relationships at different scales. Another challenge is being able to draw connections between molecular, pore, and cellular level information and macroscopic behavior. From a practical standpoint, a hierarchy of models must be established.

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David Watson

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Multidisciplinary teams of researchers from across the United States and overseas working at the ERSD Field Research Center (FRC) in Oak Ridge have shown that microorganisms found in subsurface environments can be used to reduce health risks at DOE waste sites by transforming radionuclides, such as uranium and technetium, and other contaminants into chemical forms that are less mobile or less toxic in groundwater. FRC researchers found that introducing naturally occurring humic substances (organic matter found in soil) can accelerate the chemical reduction and immobilization of these contaminants. At the same time, the researchers demonstrated that co-contaminants in the subsurface, such as nitrate, and elevated concentrations of other chemicals, like calcium, can inhibit the chemical reduction process and can reoxidize uranium, making it more mobile.

Extensive work has been conducted to identify the microorganisms present in the harsh FRC subsurface environment (an environment that is acidic and that contains high concentrations of nitrate and metals that tend to be toxic to most microorganisms). Work conducted to date has begun to determine which specific microorganisms can be used to promote the chemical reduction of radionuclides directly or indirectly. This research relies on genomic sequencing; cutting-edge techniques such as the use of functional gene arrays; and such novel devices as “bug traps,” coupons that trap microbes below the ground’s surface.

In addition to investigating naturally occurring microbial communities in the FRC’s subsurface, researchers have developed novel geophysical, hydraulic, and tracer techniques for characterizing and monitoring subsurface processes and groundwater flow. For example, they have developed inexpensive surface geophysical techniques in which seismic waves and electrical currents are used to create three-dimensional images of the subsurface geology and of contaminated groundwater plumes. Taken together, FRC research findings have contributed to the DOE Office of Biological and Environmental Research goal of understanding the processes that influence the transport and fate of subsurface contaminants, the effectiveness and long-term consequences of extant remediation options, and the development of improved remediation strategies—especially for currently intractable contaminants or conditions. The FRC has also provided valuable opportunities to researchers engaged in genomics research. As examples, researchers associated with the Joint Genome Institute and the DOE Genomics:GTL program use FRC samples in their investigations.

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

Lipid Analysis of Microbial Processes and Communities at a Uranium Bio-Immobilization Site Using ^{13}C-Labeled Acetate Amendment

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At the Old Rifle uranium mill-tailing site in eastern Colorado, a test of acetate injection to stimulate the reductive immobilization of uranium was monitored by using ^{13}C-labeled acetate. Sediment, groundwater and Bead samples were collected and analyzed for polar lipid fatty acids, ^{13}C-incorporation into polar lipid fatty acids, and respiratory quinones. Upon acetate addition, the average viable microbial biomass of sediment samples within the treatment area increased from 193 to 240 pmole phospholipid fatty acid (PLFA) /g, a 24% increase. Increases were also seen in the proportions of fatty acids (16:1ω7c) and quinones (MK-8) of *Geobacter*, indicating that *Geobacter* was responsible for much of the increase in biomass upon acetate amendment, as suggested by earlier work at this site. Similarly, the sample-to-sample variation in microbial community structure in sediment samples was much greater than the shift towards *Geobacter* with treatment, as seen in PLFA and quinone profiles. The average percent of cellular carbon originating from the acetate addition in treated sediment samples was estimated as 20%, ranging from 4% to 56%. The viable microbial biomass of groundwater samples was more responsive to acetate amendment than the sediment samples. Viable microbial biomass of groundwater samples approximately 1 m downgradient from acetate injection was 293 pmole PLFA/L before injection of acetate, and increased to 23,958 pmole/L by 10 days later. By 28 days postinjection, it had decreased to 1,765 pmole/L. At the peak of groundwater biomass, the estimated percent of cellular carbon from the labeled acetate was 87%, while by 28 days postinjection it had fallen to approximately zero.

The sample-to-sample variation in microbial community composition by PLFA or quinone profiles was much greater in the groundwater samples than in the sediment samples, making generalizations about the increase in *Geobacter* populations more difficult to quantify in the groundwater subsurface compartment. The proportions of the PLFA i15:0, 16:1ω7c, and 16:0 (the three most abundant PLFA in *Geobacter*) generally paralleled the increase and subsequent decrease in biomass after acetate injection. Bead samplers were exposed to the subsurface environment before and after acetate injection. The viable microbial biomass in Bead samplers after acetate amendment were approximately twice as great as those from before amendment. The within-treatment variation in microbial community structure was less for the Bead samplers than for sediment or filter samples, and the increase in the proportion of *Geobacter* with acetate addition was clearly seen in the increase in the respiratory quinone MK-8, characteristic of *Geobacter*. The three types of samples analyzed from this system—sediments, groundwater, and Beads—gave different views of the *in situ* microbial community, which has implications for future subsurface monitoring programs.

Biogeochemical Coupling of Iron and Technetium Speciation in Subsurface Sediments: Implications to Long-Term Technetium Immobilization

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Technetium (Tc)-99 is an important DOE subsurface contaminant. It is long-lived ($t^{1/2} = 2.13 \times 10^5$ years), and exists in groundwater as the mobile pertechnetate anion [$Tc(VII)O_4^-$]. Pertechnetate can be immobilized by biotic and abiotic reduction to insoluble $Tc(IV)O_2 \bullet nH_2O$ that exhibits a solubility of 10^{-8} mol/L or less. The half-cell potential for this reaction is "intermediate" in environmental redox space.

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

At the 2006 ERSP meeting, we will present new experimental results on the reaction of aqueous and mineral-associated Fe(II) with Tc(VII) in the presence and absence of actively metabolizing DIRB. We have found that Tc(IV) strongly associates with precipitated Fe(III)/Fe(II) forms, even when conditions appear conducive for enzymatic Tc(VII) reduction. The extremely rapid and complete heterogeneous reduction of Tc(VII) by sorbed Fe(II) outcompetes the biotic reaction under the conditions of our study. Extended x-ray absorption fine structure (EXAFS), Mössbauer spectroscopy, and transmission electron microscopy have been used to characterize the morphology of the Fe/Tc precipitates and the valence and structural environment of both Fe and Tc within them. Tc(IV) exhibits a unique spectral signature in the co-precipitates intermediate between an adsorption complex and a structural substituent. These results are complemented with oxidation rate studies of precipitated Tc(IV) from both cell and Fe/Tc mineral phases of different type and crystallinity. It is shown that the oxidation rate of precipitated Tc(IV) can vary over many orders of magnitude, and that some co-precipitated Fe/Tc forms exhibit strong recalcitrance to oxidation.

Student Presentations

Structure and Function of Metal- and Nitrate-Reducing Microbial Communities in the ERSD Field Research Center Subsurface

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The overall goal of this study is to evaluate structure-function relationships of sedimentary microbial communities likely to regulate U(VI) reduction and immobilization in the subsurface of Area 2 at the ERSD Field Research Center (FRC), Oak Ridge, TN. Microcosm experiments were conducted under near *in situ* conditions with FRC subsurface materials co-contaminated with high levels of U(VI) and nitrate. The activity, abundance, and community composition of microorganisms was determined in microcosm samples, stimulated with ethanol or glucose, and compared to those from sediment cores and unamended controls. Activity was assessed by monitoring terminal electron accepting processes (TEAPs; nitrate, sulfate, uranium, and iron reduction) as well as electron donor utilization. Microbial functional groups, nitrate- and iron(III)-reducing bacteria, were enumerated during the nitrate- and metal-reduction phases of the incubation and in sediment core samples using a most probable number (MPN) serial dilution assay. U(VI) and Fe(III) were reduced concurrently in the glucose but not the ethanol treatments. In ethanol-amended microcosms, U(VI) was reduced during a 4-day lag phase between nitrate- and Fe(III)-reduction phases. Biostimulation resulted in 3 to 5 orders of magnitude higher counts of Fe(III)-reducing bacteria, whereas populations of nitrate-reducers were enhanced by 1 to 3 orders of magnitude. One to 2 orders of magnitude more Fe(III)-reducers were observed in ethanol- as compared to glucose-amended treatments in parallel with enhanced U(VI) removal in ethanol treatments. Cultivatable Fe(III)-reducing bacteria in the ethanol treatments were dominated by *Geobacter* sp. while those cultured on glucose were dominated by fermentative organisms, i.e., *Tolumonas* sp. Currently, carbon substrate utilization is being examined through HPLC analysis of microcosm porewaters. In addition, changes in the overall microbial community composition are being assessed using cultivation-independent techniques, including fluorescence *in situ* hybridization (FISH), terminal restriction fragment length polymorphism analysis (T-RFLP), and cloning/sequencing of structural and functional genes. Our results indicate that the microbially catalyzed mechanism of U(VI) reduction is electron-donor-dependent and that more effective U(VI) removal is achieved in parallel with an enrichment of *Geobacter* sp. upon treatment with ethanol.

Exposure of *Desulfovibrio vulgaris* Cells to Chromium(VI) Temporarily Decouples Lactate Oxidation from Sulfate Reduction

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Desulfovibrio vulgaris is an anaerobic sulfate-reducing bacterium (SRB) able to reduce toxic heavy metals such as chromium and uranium, and *D. vulgaris* represents a useful SRB model for the bioremediation of heavy-metal contamination in anaerobic sediments. Although much work has focused on Cr and U reduction via individual enzymes (e.g., hydrogenase, cytochromes), less is known about the cellular response to heavy metals in *Desulfovibrio* species. When exponential-phase cells were washed to remove hydrogen sulfide carry-over and inoculated into fresh medium with different levels of Cr(VI), lag time increased as the levels of Cr increased. Cells lagged approximately 5, 40, and 55 hours in the presence of 20, 50, and 100 μM Cr, respectively. When cells were transferred to 50 μM Cr, Cr(VI) levels declined within 2 hours and lactate was consumed, but sulfate did not decline until growth was initiated approximately 40 hours later. Lactate continued to be consumed at a slow rate during the lag, but sulfate levels remained unchanged. When cell growth was initiated, lactate utilization rate increased, sulfate was consumed, and acetate levels increased. Similar trends were observed for cultures treated with 20 or 100 μM Cr(VI) that corresponded to the different lag times. During the lag phase when lactate was consumed, the production of hydrogen was detected. However, the amount of hydrogen produced with or without Cr(VI) was not significantly different. These results indicated that hydrogen production alone could not account for the utilization of lactate in the absence of sulfate reduction. The results indicated that lactate oxidation was decoupled from sulfate reduction in the presence of Cr(VI). In addition, the Cr(VI) exposure caused a recovery time in the cells even after the Cr(VI) was reduced, and cells had prolonged recovery times when the initial Cr(VI) levels were increased.

Changes in Bacterial Community Structure Correlate with *in situ* Uranium Immobilization

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Former radionuclide waste ponds at the ERSD Field Research Center in Oak Ridge, TN, pose challenges for U(VI) bioremediation. The site is marked by acidic conditions, high concentrations of NO_3, chlorinated solvents, and heavy metals. A series of recirculating wells establish a subsurface bioreactor to stimulate microbial growth for *in situ* U(VI) immobilization. Well FW-104 is the injection well for the electron donor (ethanol); Well FW-026 is the extraction well for the recirculation loop; Well FW-101 is the center of biostimulation; and FW-024 and FW-103 are upstream and downstream wells, respectively. Bacterial community composition and structure of the groundwater from the wells were analyzed via clonal libraries of partial SSU rRNA gene sequences over time. LIBSHUFF analyses for the clonal libraries from FW-104 and FW-101 showed that bacterial communities of the two wells were initially similar but developed changes through time in parallel. The two wells had reduced diversity at high levels of NO_3 and U(VI) with comparable population composition. FW-101 had increased diversity at intermediate levels of NO_3 and U(VI), but diversity was reduced upon NO_3 and U(VI) reduction. This data supported an intermediate-disturbance theory for perturbation effects on the community. Diversity continued to increase in FW-104. LIBSHUFF analysis for the clonal libraries of the five wells on Day 535 showed that the bacterial communities of the two wells (FW-101 and FW-026) immediately downstream from the injection point were more similar to the injection well than the outer-loop wells. Diversity indices on Day 535 showed that the upstream and injection wells had reduced diversity, whereas the treatment zone and the immediate downstream well both had increased diversity. The furthest downstream well had the lowest diversity compared to other wells. The results indicated that the bacterial community composition and structure changed upon stimulating for metal-reducing conditions, and that sequences representative of the metal-reducers *Ferribacterium*, *Desulfovibrio* spp. and *Anaeromyxobacter* were detected in wells that displayed a decline in both NO_3 and U(VI).

Lead Resistance in Arthrobacter sp. SI-1 Is Mediated by a Plasmid Borne P-Type ATPase

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Despite being numerically dominant in many soils, relatively little is known about *Arthrobacter* in the environment. Two strains of *Arthrobacter* sp. (SI-1, SI-2) were isolated from a metal (Pb, Cr) impacted site. Both strains were found to be resistant to lead, cadmium, and zinc; however, the two strains exhibit distinct resistance profiles to these metals. At pH 6.0 in a minimal media, the growth yield of SI-1 is reduced at lead concentrations above 25 μM and is inhibited at 200 μM. In contrast, the growth yield of SI-2 was largely unaffected by lead at similar concentrations. Additionally SI-2 exhibited increased resistance to cadmium and zinc. In both strains, the metal-resistance phenotype could be mobilized from the wild type to a lead sensitive strain. A plasmid, pSI-1, was isolated from SI-1 and sequenced to determine if metal resistance genes were located on the plasmid. Attempts to isolate a plasmid from the SI-2 strain have been unsuccessful. A cluster of five genes sharing homology to an *arsR* type regulator, a P-type ATPase (*cadC),* a signal peptidase, *ccdA*, and a *cadD* like gene were identified on pSI-1. A similar cluster of genes have been found on another plasmid from *Arthrobacter* sp. FB24, suggesting that the five genes may form a lead resistance operon. A cloned fragment with all five genes was able to rescue lead resistance in a sensitive *E. coli* strain. Deletions of the genes downstream of *cadC* do not affect the resistance, however deletion of either the regulator or the P-type ATPase results in a lead sensitive phenotype. This indicates that only the regulatory gene and the P-type ATPase are required for resistance.

Horizontal gene transfer plays a significant role in metal resistance; it is therefore important to better describe plasmids that harbor resistance genes. The recent availability of plasmid and genome sequences from different *Arthrobacter* strains have allowed us to utilize a comparative-genomics approach to study the genes on pSI-1; of interest are genes needed for plasmid survival such as replication, stability, and conjugation. Genes whose putative functions are related to plasmid transfer and replication are interspersed with many previously undescribed genes, suggesting they may also be involved in these functions. The pSI1 sequence contains a group of approximately 11 genes that includes homologs of *traG*, *virB*, and *trbL*; the same group of genes is found in FB24 and *Arthrobacter* plasmids pAO1, and to a lesser extent in pAA1. In addition, these three plasmids share similar genes for partitioning of the plasmid; currently, we are working to establish the origin of replication. The similarity of these three plasmids with regard to their maintenance genes suggest they may share a common backbone, and may represent an *Arthrobacter* family of plasmids.

Characterization of *Anaeromyxobacter dehalogenans* and Related Metal-Reducing Bacteria by MALDI-TOF MS

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Analysis of whole cells by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is a promising technique for rapid characterization and classification of microorganisms. The goal of this study is to explore the utility of MALDI-TOF MS in the development of new alternatives in environmental monitoring.

Whole cells were harvested from anaerobically grown cultures of metal-reducing bacteria, including *Anaeromyxobacter dehalogenans* strains 2CP-C, 2CP-1, 2CP-3, and R, *Desulfuromonas michiganans* strain BB1, and *Geobacter* sp. strain SZ. All cultures were grown in defined mineral salts medium-amended with acetate and fumarate; *A. dehalogenans* strain 2CP-C was also grown with nitrate and 2-chlorophenol as electron acceptors. Washed cell pellets were suspended in 0.1% trifluoroacetic acid, mixed with sinapinic acid matrix, and deposited onto wells of a stainless-steel MALDI plate. Spectra were acquired with an ABI 4700 TOF-TOF Proteomics Analyzer in linear positive ion mode. Instrument parameters were set to preferentially detect proteins and peptides in the 2 to 14 kDa range. Using customized search algorithms, ten spectra obtained from each well were baseline-subtracted, summed, smoothed, and denoised. Several datasets were obtained for each organism and analyzed with the Random Forest classification algorithm.

Comparison of spectra indicated that *A. dehalogenans* can be readily distinguished from members of the *Desulfuromonas* and *Geobacter* genera. Upon denoising, mass profiles could also be used to discriminate between four *Anaeromyxobacter* strains with at least 98% 16S rRNA gene identity. In addition, strain 2CP-C yielded spectra that were distinguishable according to the electron acceptor used for growth. These results led to the correct classification of a group of blinded cultures; this group included cultures of different *A. dehalogenans* strains and cultures of 2CP-C grown with either nitrate or fumarate. Classification was successful both by strain and by substrate used for growth. Preliminary results suggest that substrate-specific peaks appear in spectra of 2CP-C cultures upon amendment and utilization of that substrate, regardless of the substrate previously utilized. Only slight differences were detectable within a single culture at progressive stages of growth.

Whole-cell MALDI-TOF MS fingerprinting offers high-throughput capabilities, provides the specificity required for monitoring closely related organisms, and may provide information on the metabolic status of these organisms in environmental systems. Future work will focus on identification of the proteins giving rise to peaks in the spectra, analysis of consortia, and adaptation of the method for environmental samples.

Dosage-Dependent Proteome Response of *Shewanella oneidensis* MR-1 to Chromate Insult

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Shewanella oneidensis MR-1 is a gram-negative, facultatively anaerobic bacterium that can utilize metal ions, such as manganese, uranium, and chromate, as alternative terminal electron acceptors during anaerobic respiratory processes. Our overall goal is to identify the gene/protein components comprising the response pathways for chromate resistance and biotransformation, in order to assess the potential of *S. oneidensis* MR-1 for effective bioremediation. We previously have demonstrated that cells exposed acutely and chronically to chromate reveal striking changes in differential protein expression, with those proteins putatively involved in chromate detoxification and stress responses present at a much higher level than proteins found in control cells. The goal of this set of experiments was to compare the initial proteome response of *S. oneidensis* cells to different sublethal doses of chromate (0, 0.3, 0.5, and 1.0 mM). Chromate concentrations in excess of 2 mM were shown to be lethal to *S. oneidensis* under aerobic conditions in complex media (Brown et al., manuscript submitted).

S. oneidensis cells were grown in LB to an outer diameter (OD) of 0.5 and then shocked with three different chromate concentrations: 0.3, 0.5, and 1.0 mM. Cells were harvested 30 minutes postshock. In each case, cells were lysed by sonication, and two protein fractions (a soluble and a membrane protein fraction) were extracted for characterization by mass spectrometry (MS). The protein fractions were digested with trypsin and analyzed with a multidimensional high-pressure liquid chromatography (HPLC)-NanoESI-MS/MS protocol utilizing a LCQ Deca XP Plus 3-D ion trap mass spectrometer operated in the data-dependent mode. Peptide identifications were accomplished using the SEQUEST search engine. Differentially expressed proteins were identified as up- or down-regulated, using a method of semiquantitation taking into account protein sequence coverage, peptide count (number of peptides detected for each protein), and spectral count (number of tandem mass spectra identified for each protein). The criterion for differential expression was as follows: greater than 30% sequence coverage, 4 or more unique peptides, and/or 2X more tandem mass spectra identified.

Examples of up-regulated proteins due to chromate exposure from our previous acute exposure studies included a putative azoreductase (SO3585), glyoxylase (SO3586), and a membrane-associated hypothetical protein (SO3587) whose corresponding genes are organized in a predicted operon; evidence of a DNA-binding response regulator (SO2426) that is induced after exposure to chromate; and proteins involved in metal ion uptake (TonB dependent receptors, AlcA, TonB, and ExbB). In this study, we identified a total of 1,150 proteins from the control (no chromate exposure) and three dosage conditions, where each condition averaged between ~630 and 860 proteins. AlcA and a putative TonB-dependent receptor were found up-regulated in all of the experimental dosage conditions, which were also identified in the acute exposure study. Interestingly, under the dosage conditions presented here, preliminary results suggest other proteins differentially expressed that are unique to a dosage response and were not induced under the microarray analysis.

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