

**DOE-NABIR WORKSHOP:  
Abstracts**

January 31 – February 2, 2000  
Reston, Virginia

**Natural and Accelerated Bioremediation Research Program**

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**Introduction**  
**DOE–NABIR PI Workshop**  
**January 31 - February 2, 2000**

The mission of the NABIR program is to provide the scientific understanding needed to use natural processes and to develop new methods to accelerate those processes for the bioremediation of contaminated soils, sediments and groundwater at U.S. Department of Energy (DOE) facilities. The program is implemented through seven interrelated scientific research elements (Assessment, Bacterial Transport, Biogeochemical Dynamics, Biomolecular Science and Engineering, Biotransformation and Biodegradation, Community Dynamics/Microbial Ecology and System Engineering, Integration, Prediction and Optimization); and through an element called Bioremediation and its Societal Implications and Concerns (BASIC), which addresses societal issues and concerns of stakeholders through communication and collaboration among all relevant groups, including community leaders and representatives, engineers, scientists, lawyers, etc.

The initial emphasis of NABIR program research is on the bioremediation of metals and radionuclides in the subsurface below the root zone, including both thick vadose and saturated zones. The material presented at this year's workshop focuses on research funded in FY 1998-2000 by DOE's Office of Science through its Office of Biological and Environmental Research. Sixty-eight projects have been funded in the scientific program elements, and two have been funded in the BASIC program. Abstracts of these programs are summarized in this booklet, along with abstracts of other DOE programs related to research in the NABIR program.

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**Agenda**  
**DOE–NABIR PI Workshop**  
**January 31 – February 2, 2000**

**Monday, Jan. 31**

8:30-9 a.m. Welcome, Opening Comments  
 9-9:30 a.m. Biogeochemistry (Fendorf, Stanford)  
 9:30-10 a.m. Biotransformation (Bolton, PNNL)  
 10-10:30 a.m. Break  
 10:30-11 a.m. Biotransformation (Kemner, ANL)  
 11-11:30 a.m. Proposed Field Research Center (Watson, ORNL)  
 11:30-noon Bioremediation and its Societal Implications and Concerns  
 Noon-1:30 p.m. Lunch

Afternoon Session			
1:30 p.m.	Posters: Community Dynamics, BASIC Biomolecular, Bacterial Transport	Breakout: Metal-Microbe Interactions (Gorby-organizer)	Breakout: Scaling from Lab to Field (Hazen-organizer)
3 p.m.		Proposed Field Research Center: Q&A Session (Watson)	
4:30-6:30 p.m.	Poster/Happy Hour: Authors should be at posters		

**Tuesday, Feb. 1**

8:30-9 a.m. Assessment (Blake, Tulane)  
 9-9:30 a.m. Assessment (Chandler, PNNL)  
 9:30-10 a.m. Community Dynamics ( MacNaughton, U. Tennessee)  
 10-10:30 a.m. Break  
 10:30-11 a.m. Community Dynamics ( Konopka, Purdue)  
 11-11:30 a.m. Biomolecular (Clark, UC Berkeley)  
 11:30-noon Biomolecular (Giometti, ANL)  
 Noon-1:30 p.m. Lunch

## Tuesday, Feb. 1, continued

Afternoon Session			
1:30 p.m.	Posters: Biogeochemistry, Biotransformation, Assessment, Systems Integration, Data Management	Breakout: Molecular Methods for Community Fingerprinting (White- organizer)	Breakout: Metal Reducing Microbes (Frederickson- organizer)
3 p.m.		Roundtable: Communicating Bioremediation Research to Non-Scientists. (Bilyard, Bjornstad and Wolfe-organizers)	
4:30-6:30 p.m.	Poster/Happy Hour: Authors should be at posters		

## Wednesday, Feb. 2

- 8:30-9 a.m.            Field Research at UMTRA Sites (Long, PNNL)
- 9-10 a.m.             Bacterial Transport/ Field Research at Oyster, VA (Onstott, Princeton)
- 10-10:15 a.m.        Break

Breakout Sessions		
10:15-2 p.m.	Breakout: Field Research at Oyster, VA (ends at noon) Onstott, DeFlaun	Breakout: Field Research at UMTRA sites (ends at 2 p.m.) Long

- 2 p.m.                 Meeting adjourns

# **ABSTRACTS**

**PROGRAM ELEMENT 1**  
**Biotransformation and Biodegradation**



## Biodegradation of PuEDTA and Impacts on Pu Mobility

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The contamination of many DOE sites by Pu is a long-term problem because of its long half-life (240,000 years) and the low drinking water standard ( $<10^{-12}$  M). EDTA was co-disposed with radionuclides (e.g., Pu, <sup>60</sup>Co), formed strong complexes and enhanced radionuclide transport at several DOE sites. Biodegradation of EDTA should decrease Pu mobility. Our objective is to investigate PuEDTA biodegradation by the bacterium BNC1 and determine the PuEDTA aqueous species, the biodegradation of Pu- and metal-EDTA, the cellular uptake of EDTA, the location of the Pu during and after EDTA biodegradation, and the enzymology and genetics of EDTA biodegradation.

Research has focused on PuEDTA aqueous speciation to allow us to predict Pu(IV)EDTA behavior in the environment and design PuEDTA biodegradation experiments. The solubility of PuO<sub>2</sub>(am) was determined with varying EDTA concentrations and at various pHs with EDTA. The Pu(IV)EDTA species in solution and their equilibrium constants were then determined using the Pitzer ion-interaction model. EDTA greatly enhanced the solubility of Pu, with previously determined stability constants greatly underestimating the Pu(IV)EDTA in solution. The Pu(IV) forms strong Pu(OH)<sub>x</sub>EDTA<sup>x-</sup> complexes (x = 1, 2 or 3), which enhance Pu solubility. Pu(IV) solubility increased with decreasing pH and increasing EDTA. Metal competition for the EDTA or its biodegradation will lower the Pu(OH)<sub>x</sub>EDTA<sup>x-</sup> concentration. Studies are in progress to investigate the biodegradation of EDTA in the presence of Pu and its influence on Pu solubility.

A gene cluster from BNC1 involved in EDTA degradation has been cloned and sequenced. The first operon contained seven genes, a regulatory gene, two genes involved in the oxidation of EDTA to ethylenediaminetriacetate (ED3A) or nitrilotriacetate (NTA) to iminodiacetate (IDA) and four genes possibly involved in cellular transport of EDTA and NTA. Another gene further downstream from the EDTA genes is a gene encoding IDA oxidase, which we have recently identified, purified and characterized. The IDA oxidase oxidizes IDA to glycine and glyoxylate. This gene cluster contains all the genes required to convert NTA to metabolic intermediates. For EDTA degradation, the enzymes required to channel ED3A to IDA or normal metabolic intermediates have not yet been identified. Biochemical characterization of the gene products is in progress.

Current research directions include PuEDTA biodegradation, the cellular uptake of metal-EDTA complexes, EDTA biodegradative pathway and the genetics of EDTA biodegradation. This information will provide mechanistic understanding and approaches to assist in the bioremediate PuEDTA and other radionuclide-EDTA complexes at DOE sites.

## Impact of Iron-Reducing Bacteria on Metals and Radionuclides Adsorbed to Humic-Coated Iron(III) Oxides

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Studies were conducted to investigate the role of natural organic matter (NOM) on bacterial dissimilatory reduction of ferric iron oxides. Natural organic matter has been proposed to enhance dissimilatory iron reduction by two mechanisms: (1) shuttling of electrons from the bacterium to the ferric iron surface, and (2) complexation of ferrous iron. Electron shuttling by soluble quinone components of NOM may enhance both the rate and extent of ferric oxide bioreduction by relieving the requirement for direct bacterial attachment to the oxide surface. The adsorption of biogenic ferrous iron to oxide surfaces may limit further reduction due to oxide surface passivation, while ferrous iron adsorption to cell surfaces may decrease cell viability or activity. The complexation of ferrous iron by NOM may reduce these effects and enhance bioreduction.

NOM collected from a wetland pond (Georgetown NOM, GNOM), a known electron shuttling compound (anthraquinone-2,6-disulfonate, AQDS), and a strong ferrous complexing agent (Ferrozine) were employed to quantify the effects of NOM on iron reduction and to comparatively evaluate the two proposed mechanisms. Test systems consisted of serum bottles sealed with Teflon-faced butyl rubber stoppers and aluminum crimp seals containing 10 mL of medium inoculated to produce a known density of the dissimilatory iron reducing bacterium *Shewanella putrefaciens*, strain CN32. Tests were conducted under non-growth conditions with H<sub>2</sub> as the sole electron donor. Test medium contained 10 mM PIPES, pH = 6.8, 30 μM PO<sub>4</sub><sup>3-</sup>, and 2.0 g<sub>L</sub><sup>-1</sup> of commercial hematite. All test vessels were incubated at 20°C on gyrarotory shakers outside of an anaerobic chamber, while all test preparation was performed in an anaerobic chamber under an N<sub>2</sub>:H<sub>2</sub> (ca. 97.5:2.5) atmosphere. Specific amendments to the test medium were (final concentrations): 100 μM AQDS, 1.0 g<sub>L</sub><sup>-1</sup> Ferrozine and 250 mg<sub>L</sub><sup>-1</sup> GNOM.

Test conditions were replicated in parallel with all treatments and included uninoculated controls. Ferrous iron production and pH were measured in sacrificed samples at various incubation times. Soluble ferrous iron was determined by filtering (0.1 μm) the medium and analyzing the filtrate with Ferrozine. Acid-extractable ferrous iron was determined by allowing a sample of the medium to react with HCl (final normality 0.5 N) overnight, filtering (0.1 μm) the extracted sample and analyzing the filtrate by Ferrozine. The pH of the unacidified filtrate was measured under anaerobic conditions using a combination electrode. Student t-tests were used to determine if significant differences existed in ferrous iron production between treatments. Preliminary results revealed statistically significant differences between both AQDS and Ferrozine treatments compared to unamended inoculated controls and AQDS compared to Ferrozine.

# Immobilization of Heavy Metals and Radionuclides Through Bio-oxidation of Reducing Environments

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Radionuclides and other contaminating heavy metals are readily adsorbed to ferric oxides present in the environment. However, if the environment becomes reducing due to co-contamination with organic compounds, microbial activity will rapidly cause the reduction of the Fe(III)-oxides with a resulting release of the bound metals. Recent studies in our lab have identified several (per)chlorate-reducing bacteria (CIRB) that can couple the anaerobic oxidation of Fe(II) to the reduction of chlorate or nitrate. Oxidized minus reduced difference spectra of whole-cell suspensions of these organisms indicated the presence of *c*-type cytochrome(s). Difference spectra studies on anaerobic H<sub>2</sub>-reduced whole-cells demonstrated that the *c*-type cytochrome(s) are involved in the transfer of electrons onto chlorate or perchlorate by these organisms. Furthermore, difference spectra studies of anaerobic whole-cells in the presence of Fe(II) demonstrated that Fe(II) oxidation was an enzymatic process and was not the result of abiotic reactions with highly oxidized intermediates such as chlorite, which are formed as transient intermediates during the reductive metabolism of (per)chlorate.

When anaerobic sediments from contaminated aquifers or aquatic environments were inoculated with an active culture of one of the CIRB isolates, *Dechlorisoma suillus*, and amended with nitrate, the Fe(II) content of the sediment was rapidly oxidized. Interestingly, if acetate was also added, the rate of Fe(II) oxidation decreased. This was probably due to the stimulation of an indigenous acetate-oxidizing, Fe(III)-reducing community which was reducing the bio-oxidized iron as it was formed. In washed whole cell suspensions of *D. suillus* the Fe(III)-oxides formed precipitate out of solution as a green/orange precipitant. XRD analysis of the precipitant yielded a broad absorbance maximum, which is characteristic of an amorphous structure. Similar XRD diffraction patterns were obtained with fresh, abiotic Fe(III)-oxide precipitants. Characteristic peaks indicative of a crystalline structure began to appear in the XRD spectra as the precipitants aged. Interestingly, the biogenically formed Fe(III)-oxides began to crystallize much sooner than the abiotically formed Fe(III)-oxides and significant absorbance peaks were apparent in the biogenic XRD spectra within two weeks.

Although *D. suillus* could not grow with Fe(II) serving as the sole electron donor, it could oxidize Fe(II) while growing in basal media with acetate as a carbon and additional energy source. Growth was rapid and was directly linked to acetate concentration. However, Fe(II) oxidation continued after growth had ceased as long as there was enough electron accepting capacity in the media. Interestingly, concentrations of Fe(II) greater than 1 mM were inhibitory to growth when *D. suillus* was grown with chlorate as the electron acceptor. With nitrate as the sole electron acceptor, *D. suillus* could grow rapidly and oxidize Fe(II) concentrations as high as 25 mM. In addition, the presence of radionuclides/heavymetals such as uranium or cobalt had no significant effect on the growth of *D. suillus* at concentrations of 100  $\mu$ M. ICP analyses of the soluble uranium and cobalt concentrations indicated that the soluble metal content rapidly decreased as the Fe(III)-oxides were formed and after complete oxidation of the 10 mM FeCl<sub>2</sub> added, 80% of the cobalt was removed from solution.

Our results demonstrate that bio-oxidation of the Fe(II) content of reduced environments by CIRB may offer a novel alternative for the immobilization of heavy metals and radionuclides in impacted environments. Previous studies by our group have demonstrated that these organisms are ubiquitous and we have potentially identified the predominant CIRB in the environment. We have now developed specific molecular probes to the predominant CIRB that can be used to monitor their activity during a remediative strategy.

## The Effects of Cadmium Toxicity on Bacterial Consortia

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The effects of cadmium toxicity on bacterial consortia originating from anaerobic sewage sludge and cultivated under a variety of physiological conditions were studied. The cultures were enriched in minimal media containing all cadmium in soluble form. To enrich for cadmium resistance, cultures were subcultured multiple times, always when an absorbance of 0.2 OD<sub>600nm</sub> was reached. Physiological conditions were varied by providing differing electron donors and acceptors anaerobically, including (electron donor, electron acceptor): acetate, CO<sub>2</sub>; lactate, sulfate; glucose, no electron acceptor; and glucose, sulfate. Aerobic cultures used glucose and O<sub>2</sub>.

We found that for all cultural conditions studied, the level of cadmium resistance of the consortia increased over time. Generally, cadmium tolerance was greatest under aerobic conditions, followed by anaerobic with glucose and sulfate, and then anaerobic with lactate and sulfate. Under sulfate reducing (lactate, sulfate) and methanogenic (acetate, CO<sub>2</sub>) conditions, the consortia exhibited the lowest level of cadmium tolerance. A significant precipitation of cadmium occurred under aerobic and sulfate reducing conditions, presumably increasing the cadmium tolerance of these cultures by lowering the bioavailability of the heavy metal. Under other enrichment conditions cadmium was transformed into a yet unidentified soluble form. In this form the cadmium was detoxified as shown by assays using *E. coli* as a toxicity indicator organism. The addition of iron citrate increased the amount of cadmium precipitated in some enrichments, though precipitation under sulfate reducing and aerobic conditions was decreased.

We hypothesize that under conditions where the soluble cadmium was detoxified, the soluble cadmium was altered via a fortuitous siderophore interaction. Evidence to support this hypothesis includes the presence of free sulfide in solution, decreased resistance in the presence of bioavailable iron, and the presence of cadmium resistance in control cultures after the four cycles of subculturing without cadmium. The 16S rRNA profiles of the anaerobic consortia grown with glucose and sulfate and with lactate and sulfate were followed over time. The 16S rRNA fragments were analyzed by denaturing gradient gel electrophoresis (DGGE). Initial results indicate that the consortia underwent a succession when compared with the profile of the inoculum. This succession stabilized by the fourth cycle of subculturing. Under sulfate reducing conditions (lactate, sulfate), the presence of cadmium seemed to lead to four predominant bands in the DGGE analysis, independent of the iron concentration, whereas in the control (no cadmium) only one predominant band emerged after four subculturing cycles. With glucose and sulfate grown cultures, in the presence of cadmium we found evidence for the enrichment of an organism with DNA of relatively low GC.

## **Biotic and Abiotic Interactions Between Chlorinated Solvents, Microbial Metabolites and Metals: The Example of *Pseudomonas stutzeri* Strain KC**

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*Pseudomonas stutzeri* strain KC, while growing under iron limitation, produces and excretes a novel metal chelator we have identified as pyridine-2,6-bis(thiocarboxylate), or PDTC. PDTC is unusual in that besides strongly complexing metals, it also promotes the dehalogenation and mineralization of chlorinated solvents such as carbon tetrachloride (CT). We have cloned and characterized the genes required for the synthesis of PDTC, and are able to transfer these genes into other species of *Pseudomonas* such that they become able to synthesize and excrete PDTC. This leads to the possible use of *Pseudomonas stutzeri* strain KC (or other organisms expressing the PDTC genes) in mixed waste-contaminated environments to simultaneously address both organic and inorganic contaminant problems, and is an excellent example of a combined biotic and abiotic treatment strategy. However, the chemistry involved is quite complex.

In our recent work we have begun looking at this complexity. PDTC and its metal complexes were synthesized using modifications of known methods. PDTC and its Fe, Ni, Zn, Co, Cu, Au and Mn complexes were prepared and purified by crystallization. All compounds were > 95% pure. Divalent metals such as Cu and Zn formed 1:1 complexes with PDTC, while metals such as Fe, Co, and Mn formed 1:2 complexes. The structures of PDTC and its metal complexes were elucidated using electrospray negative ionization mass-spectrometry (MS) of samples prepared in a water/methanol solution. For further confirmation of structures, daughter fragments were generated using collision-induced ionization with an argon gas-filled collision cell in an MS/MS spectrometer. The PDTC metal complexes then were examined for (a) their ability to dehalogenate CT, and (b) their binding affinities for different metals. These types of data will allow us to predict the usefulness of in-situ biologically produced PDTC in mixed-waste environments for simultaneous degradation of chlorinated solvents and mobilization or immobilization of metals or radionuclides.

# Microbial Reduction and Immobilization of Uranium in Fe(III)- and Mn(IV)-Containing Sediments

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Solid and liquid wastes discharged to the ground over a 40-year period constitute a major environmental problem at Department of Energy (DOE) sites nationwide. Uranium is the most common radionuclide in soils, sediments and groundwater at these sites and therefore is of particular environmental concern.

Dissimilatory iron-reducing bacteria (DIRB) can utilize ferric iron associated with aqueous or solid phases as a terminal electron acceptor coupled to the oxidation of H<sub>2</sub> or organic substrates. DIRB are also capable of reducing other metal ions, including contaminants such as U(VI), Tc(VII) and Cr(VI), significantly altering their solubility and mobility.

The focus of this research project is on laboratory investigations of coupled microbiological-geochemical transformations of U(VI) species in the presence of reactive solid phases (synthetic and naturally-occurring) containing Fe- and Mn-oxides and humic acid-facilitated microbial metabolism and reduction of metals. This research is evaluating three hypotheses pertaining to redox disequilibria, microbial U reduction and nucleation for precipitation of U(IV) solids, and humic acid acceleration of microbial reduction of Fe oxides. These processes are of particular concern for the effective in-situ reduction and long-term stability of contaminants.

To probe these complex processes, the reduction of U(VI) by the subsurface bacterium *Shewanella putrefaciens* CN32 was investigated in the presence of goethite under conditions where the aqueous composition was controlled to vary U speciation and solubility. Uranium(VI) as the carbonate complexes UO<sub>2</sub>(CO<sub>3</sub>)<sub>3</sub><sup>4-</sup><sub>(aq)</sub> and UO<sub>2</sub>(CO<sub>3</sub>)<sub>2</sub><sup>2-</sup><sub>(aq)</sub>, in the absence or presence of goethite [FeOOH<sub>(s)</sub>], was reduced by the bacteria to U(IV). Uranium(VI) in PIPES buffer that was estimated to be initially present predominantly as the U(VI) mineral metaschoepite [UO<sub>3</sub>•2H<sub>2</sub>O<sub>(s)</sub>] was also reduced by the bacteria in the absence of presence of goethite. Anthraquinone-2,6-disulfonate (AQDS), a humic acid analog that can be reduced to dihydroanthraquinone (AH<sub>2</sub>DS) by CN32, had a slight moderating effect on U reduction in either buffer. In contrast, only ~30% of the U(VI) associated with a synthetic metaschoepite was reduced by the organism in the presence of goethite with 1 mM lactate as the electron donor, possibly due to the formation of a layer of UO<sub>2(s)</sub> or Fe(OH)<sub>3(s)</sub> on the surface of the metaschoepite that physically obstructed further bioreduction. However, increasing the lactate to a non-limiting concentration (10 mM) increased the nominal reduction of U(VI) from metaschoepite to greater than 80%, indicating that the hypothesized surface-veneering effect was electron donor dependent. Uranium(VI) was also reduced by bacterially-reduced AQDS in the absence of cells, and by Fe(II) sorbed to goethite in abiotic control experiments. In the absence of goethite, uraninite was a major product of direct microbial reduction and reduction by AH<sub>2</sub>DS. These results indicate that DMRB, via a combination of direct enzymatic or indirect mechanisms, can reduce U(VI) to insoluble U(IV) in the presence of solid Fe oxides. Current research is probing the microbial reduction of U(VI) in the presence of Mn oxides.

Preliminary results have demonstrated that biogenic uraninite (UO<sub>2</sub>) is oxidized by pyrolusite (β-MnO<sub>2</sub>) and that the presence of the oxide significantly decreases the rate of microbial reduction of U(VI). Additional experiments are underway to identify and quantify the coupled microbial and geochemical processes in these systems.

## Formation and Reactivity of Biogenic Iron Microminerals

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Radionuclides and heavy metals (e.g., U, Cr and Ni) pose significant environmental toxicity and health hazards in the subsurface at many of the DOE sites involved in the processing of nuclear materials. The fate and transport of these contaminants are controlled to a large extent by redox chemistry of saturated subsurface sediments and by the nature of the mineral phases that are present. Dissimilatory iron-reducing bacteria catalyze many of the reduction reactions in anoxic, non-sulfidogenic environments and are recognized as important agents impacting the migration of metal contaminants in groundwater. We have examined the effect of a variety of environmental parameters and the influence of bacterial metabolism and surfaces on the biogenic transformation of hydrous ferric oxide (HFO) by iron-reducing bacteria.

To evaluate the effect of pH on the products of HFO transformation, 20 mM Fe(III) as HFO was suspended in buffered, anaerobic media with pH values ranging from 5.5 to 8.5. In the absence of CO<sub>2</sub> and with H<sub>2</sub> supplied as the electron donor, *Shewanella putrefaciens* strain CN32 reduced iron in HFO at all pH values tested except 8.5. The transformation products were influenced directly by initial pH. More than 8 mmol<sup>-1</sup> of the 20 mmol L<sup>-1</sup> Fe(III) provided as HFO were reduced at pH values below 6. The crystalline iron oxide goethite was the dominant mineral phase produced during reduction. Biogenic Fe(II) was distributed between the aqueous and mineral phases. Magnetite co-precipitated with goethite at pH 6.5, while magnetite was the dominant phase at pH values >7.0.

Biominerals produced during HFO reduction may be influenced by microenvironmental conditions at the cell-mineral interface. To test this hypothesis, CN32 cells were enrobed in porous sodium alginate beads to physically separate them from the HFO. Anthroquinone-2,6disulfonate (AQDS), an analogue for humic acids, was provided as a soluble electron shuttle between the cells and the HFO. Enrobed cells reduced more than 5 mmol L<sup>-1</sup> of the initial 20 mmol L<sup>-1</sup> Fe(III) at pH 5.5 and 6.0 and goethite was the dominant mineral produced; these results were similar to the cell-mineral interface experiments. At pH 6.5, hematite co-precipitated with goethite without forming magnetite. These results contrast with those from experiments where cells were in direct contact with HFO. We hypothesize that iron reduction increases pH at the cell-mineral interface and that this microenvironment favors the formation of magnetite over other crystalline oxide phases. This hypothesis is receiving further investigation.

The biogenic transformation of HFO into goethite and hematite under iron-reducing conditions has until now gone unreported. This transformation has important implications for the bioavailability of Fe(III) and overall biogeochemistry of anoxic sediments and subsurfaces. Substitution of select heavy metal and radionuclides into the crystal structure of these minerals may provide a mechanism for immobilizing the contaminants. We continue to focus upon the role of the cell-mineral interface in directing the formation of biogenic minerals and how these controls may be manipulated for the in-situ stabilization of contaminants.

# The Role of Natural Organic Matter in Microbial Reduction of Chromate, Pertechnetate and Uranyl: Linking Chemical Structure to Bioavailability and Redox Reactivity

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Our first-year research has focused on the fractionation and structural characterization of natural organic matter (NOM) that may function as electron mediators for microbial reduction of contaminant metals such as chromate ( $\text{CrO}_4^-$ ), pertechnetate ( $\text{TcO}_4^-$ ) and uranyl ( $\text{UO}_2^{2+}$ ). The overall goal of the project is to provide a molecular-level understanding of the roles and mechanisms of heterogeneous NOM in facilitating the reductive immobilization of metal and radionuclide contaminants by anaerobic metal-reducing bacteria. Our specific objectives are to (1) determine the bioavailability of several major organic fractions of NOM by microbes in relation to their unique structural and functional characteristics; (2) quantify the redox-active functional groups and their reaction kinetics of the NOM fractions for accepting electrons from metal-reducing bacteria (such as *Shewanella putrefaciens* and *Geobacter metallireducens*); and (3) determine the microbial reduction and immobilization of  $\text{CrO}_4^-$ ,  $\text{TcO}_4^-$  and  $\text{UO}_2^{2+}$  in both batch and soil column flow-through systems as facilitated by each NOM fraction.

To date, a soil humic acid (Soil-HA) and three aquatic NOM fractions, namely polyaromatic fraction (PP), carbohydrate fraction (CH), and aquatic humic acid (A-HA), have been isolated and spectroscopically characterized for their structural properties and functionality. Both nuclear magnetic resonance (NMR) and infrared (FTIR) spectra revealed that the soil-HA and PP fractions were enriched with polyaromatic organic compounds, particularly the soil-HA. Both Soil-HA and PP gave intense fluorescence and UV absorbance (at <220 nm) in comparison with the NOM-CH fraction. Cyclic voltammetry is being used in an effort to obtain a more direct characterization of the redox properties of the NOM fractions. The results to date indicate weak and irreversible but highly reproducible electrochemical activity at a glass carbon electrode. Reactions between the NOM fractions and a model electron acceptor, chloranil, have been studied and preliminary results indicate that chloranil is reduced to varying degrees with different NOM fractions.

Preliminary experiments were also performed to study the effects of each NOM fraction on the reduction and dissolution of iron oxide, and results indicate that both the PP and soil-HA are more effective in reducing and dissolving iron oxide than the NOM-CH fraction. Additionally, enhanced Fe(III)-reduction rates were observed in the presence of *Shewanella putrefaciens* and anthraquinone-2,6-disulfonate.



# Investigation of the Spatial Distributions and Transformations of Cr, Pb and U Co-contaminant Species at the Bacteria-Geosurface Interface

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The microenvironment at and adjacent to actively metabolizing cell surfaces can be significantly different from the bulk environment. Cell surface polymers (lipopolysaccharides, extracellular polysaccharides), metabolic products, etc., can set up 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 of these environments has been difficult or impossible to define. The behavior of contaminants in such microenvironments can ultimately affect their macroscopic fates. Information about biogeochemical interactions at the microbe-geosurface microenvironment is paramount to predicting the fate of contaminants and effectively designing bioremediation approaches. State-of-the-science x-ray microimaging and spectromicroscopy are powerful techniques for resolving the distribution and speciation of contaminants at the microscopic scale. The objectives of this research are (1) to use x-ray absorption spectroscopy, microimaging and spectromicroscopy to determine the spatial distribution and chemical speciation of Cr, Pb and U near the interfaces of *Pseudomonas aeruginosa* and *Shewanella putrefaciens* with iron (hydr)oxide, and (2) to use this information to identify the interactions among the contaminants, mineral surfaces and microbial extracellular materials that occur near these interfaces.

We have begun a series of experiments at Sectors 2 and 10 of the Advanced Photon Source to apply high-energy synchrotron x-ray microbeams to map the spatial distribution of elements associated with bacteria. Specifically, we have performed x-ray fluorescence (XRF) imaging of a hydrated *P. fluorescens* bacterium, adhered to kapton film at ambient temperature and pressure, with 0.15- $\mu$ m resolution.

XRF imaging studies of a similar hydrated *P. fluorescens* bacterium, exposed to 1000 ppm Cr(+6) solution for 6 hours, has also been accomplished. In all cases, for the measurements made, the highest elemental concentrations occur at the point of adhesion of the bacterium to the kapton film. These results indicate that the combination of the high brilliance of the APS and the use of high-resolution zone plates for focusing enable the identification of the location of a hydrated bacterium on a film and the determination of the relative concentrations of the other elements at the same location. Measurements of quantified standards with known elemental concentrations have been undertaken to enable calculation of the elemental concentrations within and near the bacterium. These results, their implications to the biochemical interactions occurring between the extracellular polysaccharides and contaminant metals, and a discussion of the use of the x-ray spectromicroscopy to investigate the chemical interactions at, near and on a hydrated bacterium will be presented. A discussion of preliminary bulk x-ray absorption spectroscopy studies also will be presented.

## Modeling and Parameter Estimation of Soil Fe(III) Bioavailability

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A two-compartment model developed for chemical transport and distribution in soil systems was modified to incorporate physical and biological characteristics unique to microbiological Fe(III)-reducing regimes. One compartment of the model is an inter-aggregate regime through which advection can occur; the other compartment is an intra-aggregate regime consisting of dead end pores through which there is no advection. Some of the intra-aggregate pores are small enough to exclude bacteria, thus any Fe(III) within these pores is bioavailable only if there exists in that regime a chemical agent (chelating agent or electron shuttle) which can access the Fe(III). This model will be used as a tool in understanding the bioavailability of solid Fe(III) in floodplain soils which undergo cycles of oxidation and reduction. The model describes fluid transport through and around pores, chemical and biological reduction of Fe(III), and the contributions of electron shuttles and chelating agents found in soil organic matter. Biological reduction rate and extent parameters from controlled batch experiments will be presented.

## Charge State Mapping of Mixed Valent Iron and Manganese Mineral Particles Using Scanning Transmission X-Ray Microscopy (STXM)

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The objectives of our NABIR research program involve understanding the relationship between the redox state of metal oxides, and the mobility and redox state of adsorbed counter pollutants such as actinides and transition metals (in particular uranium and chromium for this project). This knowledge will set the stage for planning strategies for bioremediation. The major goals are thus to understand the fine-scale ion of oxidation states of iron and manganese in mixed valent minerals for the host (adsorbing) materials. Once this is possible, the relationship between these redox states and the amounts and kinds (redox states) of U and Cr will be determined, and the distribution of these pollutants will be mapped using similar approaches. Finally, the ability of metal-reducing bacteria in the group *Shewanella* to reduce both the host metal and the bound pollutants will be assessed.

The interfaces between solid mineral particles and water play a crucial role in partitioning and chemical transformation of many inorganic as well as organic pollutants in environmental systems. Among environmentally significant minerals, mixed-valent oxides and hydroxides of iron (e.g., magnetite, green rusts) and manganese (hausmanite, birnessite) have been recognized as particularly strong sorbents for metal ions. In addition, minerals containing Fe(II) have recently been proven to be powerful reductants for a wide range of pollutants. Chemical properties of these minerals strongly depend on the distribution and availability of reactive sites and little is known qualitatively about the nature of these sites. We have investigated the bulk distribution of charge states of manganese (II, III, IV) and iron (II, III) in single particles of natural manganese nodules and synthetic green rusts using Scanning Transmission X-ray SpectroMicroscopy (STXM). Pixel resolved spectra (XANES) were fitted to total electron yield (TEY) spectra of single valent reference compounds. Two-dimensional maps of bulk charge state distributions clearly reveal domains of different oxidation states within single particles of Mn-nodules and green rust precipitates. Changes of oxidation states of iron were followed as a result of reductive transformation of an environmental contaminant ( $\text{CCl}_4$ ) using green rust as the only reductant. In addition, similar approaches were used to follow the fine scale redox distribution of manganese oxides during biological oxidation of manganese II to Mn IV. These experiments revealed unexpected fine-scale heterogeneity in the redox states of Mn during biological oxidation of the metal oxides; oxidation differences that must be understood before pollution remediation strategies are adopted and put in place.

# Environmental Actinide Mobility: Plutonium and Uranium Interactions with Exopolysaccharides and Siderophores of Aerobic Soil Microbes

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Microorganisms are likely to effect the overall environmental behavior of radionuclides through solubility and speciation changes, biosorption, bioaccumulation or other biotransformations. Our goal is to understand how key interactions with aerobic soil microbes affect the speciation and migration of actinides and how they may be exploited to develop remediation technologies. Initially, we have focused on fundamental interactions of environmentally important plutonium and uranium species with common aerobes and the siderophores and extracellular polymers they produce. Specifically, we are studying: (1) siderophore complexation and uptake using *Streptomyces pilosus*, which produces desferrioxamine B (DFB), *Pseudomonas stutzeri*, which produces desferrioxamine E (DFE), and *Rhodococcus rhodochrous* strain OFS, which produces an uncharacterized catecholate siderophore; and (2) the exopolymer binding using the glutamic acid polymer of *Bacillus licheniformis* and the previously uncharacterized polymer of *Rhodococcus erythropolis*.

By studying siderophores in the presence of actinide solids, we have shown that stability constants are not sufficient for predicting solubilization by complex biomolecules. Surprisingly, the siderophores DFE and DFB do not readily solubilize Pu(IV) hydroxide or oxide. They are 100 times slower than EDTA, despite having significantly higher solution formation constants with Pu(IV). Despite being unable to rapidly solubilize Pu(IV), desferrioxamine-Pu(IV) complexes are thermodynamically favored. No matter what oxidation state of Pu (III, IV, V, VI) is present initially, desferrioxamines rapidly and irreversibly form the Pu(IV)DFO complex at environmentally relevant solution pH. In fact, up to 12 equivalents of Pu(VI) can be reduced per DFE/DFB. We have structurally characterized the first Pu(IV) complex of a biomolecule. We have crystallized a Pu(IV)-DFE complex and found the Pu(IV) is nine coordinate and that DFE spans only one hemisphere of the Pu. The structure has very interesting similarities and differences with the structure of the corresponding Fe(III) complex.

Production of the siderophore of *Rhodococcus rhodochrous* is regulated by iron content of the media. We have optimized production of this siderophore, allowing us to characterize it. Amino acid analysis indicates the presence of arginine in the siderophore. <sup>1</sup>H-NMR spectroscopy shows that there are two distinct catechols in the siderophore in a 1:1 ratio. Matrix assisted laser desorption ionization mass spectral (MALDI-MS) results confirm the catecholate functionality and are consistent with a siderophore composed of 2,3 dihydroxybenzoyl arginine. Metal and radionuclide uptake studies of this siderophore and bacteria are underway.

We have purified large quantities of the polyglutamic acid capsule from *Bacillus licheniformis* and determined its stability as a function of temperature and HCl concentration. It is approximately 800 KDa, with approximately 6200 subunits. The surface charge varies with ionic strength and ion type with behavior very different from the surface charge variations typically measured for mineral surfaces. The polymer forms a water soluble U(VI) complex at 1: 10 U:glutamate ratios, but forms insoluble complexes at lower ratios. The conformation of the polymer changes (helical to beta) with varying metal binding, pH and ionic strength.

We have optimized the production and purification of the exopolymer produced by *Rhodococcus erythropolis* that has been identified to be a polysaccharide. The most narrow molecular weight distribution was achieved after 14 hours of growth and is centered at 50 KDa.

# Biotransformation of Mixed Inorganic Ions: Biochemistry, and Contaminant and Species Interactions in Chromate-Reducing Consortia

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Mixtures of metallic and radioactive contaminants, including chromate, constitute a major environmental problem at DOE facilities. Direct microbial reduction of hexavalent chromium to trivalent chromium is one potential treatment technology for such sites. While previous research has shown the potential for this treatment, important questions still remain. Such questions include: (1) which members of an environmental consortium are responsible for chromate reduction; (2) for these active chromate reducers, what biochemical pathways are responsible for chromate reduction; (3) what are the effects of carbon source (electron donor) and of inorganic contaminant ions (e.g., SO<sub>4</sub><sup>2-</sup>, TeO<sub>4</sub><sup>-</sup> and UO<sub>2</sub><sup>2+</sup>) on the system's chromate reduction capacity; and (4) can detailed knowledge of active chromate reducers and their biochemistry be used to facilitate preferential growth of subpopulations with the greatest specific contaminant reduction rates in aquifer systems?

During this year, bacterial enrichment cultures previously isolated from the Hanford site were examined in various ways. These cultures and an *E. coli* wild-type laboratory strain were assayed for their ability to reduce chromate under nitrate reducing and fermentative conditions.

Other studies have focused on the effect of co-contaminants and nutrients on the specific chromate reduction rate. The effect of various co-contaminant concentrations on the microbial growth rate and the chromate reduction rate is being assessed. In these studies, we are concentrating on the effect of sulfate, pertechnetate and uranyl on the growth rate and the specific chromate reduction rate. This community of chromium-tolerant microorganisms, enriched from the Hanford site, was also examined to assess its ability to anaerobically reduce Cr(VI) to Cr(III) using various electron donors. Growth occurred on a range of low-molecular-weight fatty acids and sugars that include acetate, pyruvate, lactate, succinate, citrate, glucose, sucrose and fructose. Chromate was reduced by cultures grown on glucose, sucrose, fructose, acetate and pyruvate — lactate, succinate and citrate are currently under examination. Other electron donors will be evaluated in the near future. Data suggests that this microbial community is made up of at least three cultivable strains. All isolated strains are comprised of oxidase negative, catalase positive, facultative Gram negative rods.

Laboratory-scale soil column studies are also being performed to test our understanding of chromium reduction in the presence of co-contaminants with various nutrients. The column (stainless steel) contains coarse sand inoculated with Hanford site, subsurface bacterial consortia. A clear lexan soil column has been developed that contains micro-oxidation/reduction (ORP) probes and sample collection ports at four locations. This column will allow data to be collected along the column length to enable a more complete understanding of biological activity.

To elucidate the actual enzyme(s) responsible for chromate reduction, two metal reducing cultures, *Shewanella putrefaciens* MR-1 and *Pseudomonas aeruginosa* PAO1, were selected for further experiments. Both of these bacteria have been fully sequenced, which allows for easier manipulation of the genome. We have also monitored and quantified the rate of chromate reduction during the transition from aerobic to anaerobic conditions. Initial results indicate an increase in the specific reduction rate as the culture grows under fumarate-reducing conditions. This result may indicate that the enzymes responsible for chromate reduction may be induced by such conditions.

# Acceptable Endpoints for Metals and Radionuclides: Quantifying the Stability of Uranium and Lead Immobilized Under Sulfate Reducing Conditions

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The creation of sulfate-reducing conditions to immobilize metals has potential use at many contaminated sites because of the large number of metals (e.g., Hg, Pb, Cd, Cu, Ni, Zn) that form stable sulfide compounds. In addition, effective reduction and subsequent precipitation of U and Cr under these conditions has been shown. However, as with other possible treatments, the long-term stability of the immobilized metals and radionuclides and the factors that affect the immobilization/remobilization process must be quantified to determine whether the treatment can produce an acceptable endpoint.

Methods have been developed to test the hypothesis that the rate of metal remobilization will largely depend on the following factors: (1) aquifer reductive capacity (generated in the form of iron sulfides); (2) distribution and morphology of contaminant precipitates on mineral surfaces; and (3) accessibility of contaminant precipitates to dissolved oxygen.

Experiments using a new fluidized-bed “V-bottom” reactor that examine SRB growth and attachment to grains of quartz and feldspar are in progress. In preliminary anaerobic batch studies using lactate-C medium and quartz, significantly more (2x) growth occurred when quartz was present.

A green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, was introduced into *Desulfovibrio desulfuricans* for direct observation of SRB on hematite using fluorescence microscopy. Unlike fluorescent stains, the GFP allows non-destructive, real-time observation of active SRB colonization and biofilm growth on mineral surfaces (e.g., hematite) in both pure and mixed cultures. The GFP allows us to register the locations of SRB activity without disturbing the progress of experiments.

As another measure of SRB activity in mixed culture biofilms, microelectrode measurements of colony-scale H<sub>2</sub>S profiles on surfaces will also be presented. Biofilms composed of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* were grown on inert glass surfaces. We have measured profiles of H<sub>2</sub>S, pH and local effective diffusivity at two locations in a cell cluster: at the center and near the edge, and at three locations in interstitial voids: in a large void (about 100 microns wide), open to the flow and parallel to the flow direction, in a mid size void, open and perpendicular to the flow direction, and in a small, closed void. The pH in the biofilm was nearly constant, around 7.2, (results not shown). H<sub>2</sub>S concentration varied significantly among locations, indicating a heterogeneity that may be significant in the presence of redox-sensitive minerals.

Specially designed flat-plate flow cells that contain pure and mixed redox-sensitive and insensitive minerals allow us to determine the spatial relationships between SRB colony location and activity, and the location and type of contaminant and sulfide mineral deposits on a hematite, or other aquifer-relevant surface. A hematite coupon surface was exposed to SRB colonization for 17 days, then it was rinsed and examined using X-ray photoelectron spectroscopy (XPS) for surface characterization. XPS data indicate the formation of reduced iron sulfide on the hematite surface and also the presence of numerous sulfur species, including S<sup>2-</sup>, S<sub>2</sub><sup>2-</sup>, S<sub>n</sub><sup>2-</sup>, SO<sub>3</sub><sup>2-</sup> and SO<sub>4</sub><sup>2-</sup>; however, no elemental S or thiosulfate were detected. Our initial results indicate the possible formation of pyrrhotite (Fe<sub>1-x</sub>S, where 0 < x < 0.125) on hematite surfaces when SRBs are grown. We are currently replicating this test and also plan to confirm the result using grazing angle XRD. If confirmed, the presence of pyrrhotite contrasts with the results of most other work in homogeneous SRB solutions where the metastable sulfide phase is mackinawite (FeS<sub>1-x</sub>, where 0 < x < 0.1). Mackinawite is a precursor to both of the thermodynamically stable high-temperature iron-sulfide phases [pyrite (FeS<sub>2</sub>) and pyrrhotite].

## Determination of Long-Term Stability of Metals Immobilized by In-Situ Microbial Remediation Processes

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Most microbial remediation strategies that have been developed for addressing sites contaminated with metals and inorganic radionuclides incorporate anaerobic organisms to achieve in-situ reduction and precipitation of the contaminants. The pollutants are expected to remain immobilized provided that reducing conditions are maintained in the subsurface formation; however, little information has been developed on the stability of these precipitates over very long time periods. This project has investigated the long-term stability of metals and radionuclides immobilized by microbial processes through use of laboratory research and numerical modeling.

The research has focused on arsenic (As), chromium (Cr), selenium (Se) and uranium (U) as being representative of contaminants found at many DOE sites, including uranium mill tailings sites. The laboratory work has involved operation of columns packed with coarse sand, inoculated with anaerobic bacteria and fed a solution containing the metal or radioactive contaminants and a soluble organic substrate. Two different organisms have been used, *Desulfovibrio desulfuricans* and *Shewanella putrefaciens*. The purpose of these columns is to generate sand media containing high concentrations of the contaminants for use in subsequent leaching experiments. They have been operated for more than one year.

To date, samples of the sand media with immobilized pollutants have been subjected to leaching by deionized water solutions and by the weak acetic acid solution specified in the toxicity characteristic leaching procedure (TCLP). The contaminants immobilized by the *D. desulfuricans* culture have been found to be quite stable in a deionized water leach test over a period of time extending up to one week. These contaminants are less stable in the weak acid of the TCLP test. Vigorous mixing of the media as called for in the TCLP test further diminishes the stability of the immobilized pollutants due to the fragile nature of the microbial floc. Testing is continuing with contaminants immobilized by the *S. putrefaciens* culture. Long-term leaching studies are in progress using simulated ground water to generate information on the rate of contaminant release. A one-dimensional coupled contaminant transport and geochemical kinetic code has been selected to use this information to predict contaminant concentrations down-gradient from a site at which in-situ microbial immobilization has been implemented.

# Transformation of Heavy Metal Contaminants in Sulfate-Reducing Sub-Surface Environments: The Role of Thiolated Compounds and Hydrogen Sulfide

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The overall project goal is to seek a better understanding of the speciation and transformation of the toxic heavy-metal ions, particularly cadmium, in anaerobic systems undergoing bacterial dissimilatory sulfate reduction. A major focus will be placed on the chemistry and biochemistry of the interactions between various thiolated compounds and the toxic metal ions. Thiols are of particular importance in bioremediation because they constitute an important class of intracellular biochemicals that organisms produce in defense against metal toxicity. The project research is divided into two major areas: (1) mechanistic and kinetic studies of the interaction between metal ions and natural organic and sulfur compounds in anaerobic systems; and (2) studies on microbial processes on metal resistance under anaerobic conditions.

We investigated the complexation of Cd(II) with a series of low-molecular-weight thiol compounds to better understand the effects of important environmental variables (such as chemical structure, thiol concentration and pH) on the mechanisms of formation and stability of the resulting complexes. A potentiometric method was used to study the reactions between Cd(II) and thiol compounds at two initial ratios of 1:1 and 1:2 over a pH range of 3 to 8. The formation of complexes was found to be highly dependent on pH in which Cd(II) binding decreased with decreasing pH due to competition from protons. The stoichiometry of the binding reactions was affected by both pH and the ratio of Cd(II) to the thiol compound, while the structure and the precipitation/dissolution behavior of the resulting complexes mainly depended on the molecular composition and the structure of the thiol compound. The stability constants for the reactions of Cd(II) with thiol compounds were determined by a model that accounts for both proton competition and Cd(II) speciation. The predicted quantity and speciation of the bound Cd(II) calculated based on the determined stability constants agree well with experimental values.

One of the main objectives of the project is to find a bacterium that can grow in high concentrations of Cd(II) under anaerobic conditions, and transform the metal into stable products, such as cadmium sulfide (CdS). We isolated such a bacterium (strain Cd-1) from coastal sediments that grows in up to 15 mM CdCl<sub>2</sub> under minimal fermentative conditions. The isolate can grow in Cd(II) under a variety of geochemical conditions, i.e., with or without marine level NaCl, at acidic or neutral pH. It also can grow aerobically in Cd(II). Furthermore, it can grow in high levels of many other priority pollutants and co-contaminants including Cr(VI), As(V), Se(IV), Co(II), Pb(II) or Zn(II).

The results from physiological and biochemical methods, plasmid-DNA sequencing and synchrotron x-ray absorption spectroscopy indicate that Cd-1 resists Cd(II) mainly via efflux pumps, although significant immobilization of the metal as CdS also occurs during the stationary phase of the growth. Metabolic data and nearly complete 16S rRNA sequence analyses show that Cd-1 belongs to ubiquitous genus *Klebsiella*, and probably to the species *planticola*. The closely related culture-collection strains, *K. planticola* ATCC33531 and *K. ornithinolytica* ATCC31898, also resisted 5 mM Cd(II), but not as effectively. The implied potential of Cd-1 for rapid bioremediation was further ascertained by comparing its growth with that of eleven new or known strains belonging to the genera *Shewanella*, *Ralstonia*, *Pseudomonas*, *Commamonas*, *Enterobacter* and *Bacillus*. None of them, except *R. eutropha* CH34, grew in high levels of cadmium; however, CH34 did not produce CdS. This is the first report of the ability of a marine eubacterium or a *Klebsiella* sp. to grow anaerobically in high levels of Cd(II) and other stated toxic metals. The bacterium readily transformed aqueous thiosulfate complex of Cd(II) to insoluble CdS under anaerobic conditions. This transformation lends a potential approach for transforming aqueous Cd(II) to CdS in in-situ or above-ground bioremediation applications.



# Mesoscale Biotransformation Dynamics Controlling Reactive Transport of Chromium

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The interdependent influences that sediment structure and microbial communities have on transport and reduction of chromate are being investigated in various batch and microcosm systems of clay (Altamont, Calif.) and fine sand (Savannah River, S.C.) sediments. Diffusive transport of Cr(VI) has been quantified in these systems by macroscopic and synchrotron x-ray microspectroscopic methods. Indigenous microorganisms were grown in these sediments, saturated with neutral salt solutions or dilute nutrient broth (1% or 10% tryptic soy broth), prior to Cr(VI) exposure. Redox potential measurements indicated that all systems developed towards conditions favoring reduction of Cr(VI) to Cr(III) prior to Cr(VI) exposure. Cr(VI) solutions (260 to 5200 ppm) were placed in hydrostatic contact with one boundary of each sediment sample in order to simulate diffusive transport into sediment blocks from contaminant-transporting macropores. The Cr(VI) boundary reservoir was removed following 2 to 3 days of contact time to impose local conditions analogous to those expected during a short contaminant spill event. Spatially-resolved redox measurements in the sediment microcosms showed local oxidation by Cr(VI) within several mm of the exposure boundary. Spatially-resolved micro x-ray absorption near edge structure (micro-XANES) spectroscopy typically showed short Cr penetration distances, with abrupt rather than diffuse termination. Micro-XANES analysis provided direct evidence of Cr(VI) reduction to less toxic Cr(III) forms. The extent of Cr transport into sediment blocks was far less than expected by diffusion without reduction, and proportional to the boundary Cr(VI) concentration.

The microbial communities and populations in sediment microcosms were characterized with DNA fingerprints, direct counting and enrichment culturing. Intergenic Transcribed Spacer (ITS) analyses of the Altamont soil microcosm exposed to 260 ppm Cr(VI) and 1% tryptic soy broth showed that the microbial community composition in the exposure region (2 mm of the microcosm) is different from those in sediments taken from greater depth. Several populations appear only in soil that was exposed to Cr, suggesting that they are chromium resistant and that they may play an active role in Cr reduction. These results were confirmed with Denaturant Gradient Gel Electrophoresis (DGGE), where again, certain bands appeared only in fingerprints taken from soil communities that were exposed to Cr(VI). Several microbial cultures have been enriched from the sediments used in the microcosms on 10% tryptic soy broth in the presence of 100 ppm Cr(IV). These cultures will be further characterized by sequencing their 16S ribosomal genes. Direct counting of microbial populations in the sediment microcosms showed higher population densities in the outer layers for the sample exposed to 260 ppm Cr. More growth occurred in the surface layer sediment due to the availability of oxygen. Analyses of samples from other microcosms are ongoing.

These results show the important microbial and chemical heterogeneity developed from transport-limited reactions within common sediment structure. The need for measurements and models with at least mm-scale spatial resolution was demonstrated for highly nonequilibrium reactive transport in structured sediments.

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

# Vadose Zone Microbial Community Structure and Activity in Metal/Radionuclide-Contaminated Sediments

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The objective of the project is to determine the effect of unsaturated flow rate, exogenous nutrients and metal concentrations on microbial community structure, activity, diversity and dynamics in vadose zones with low natural recharge which have been impacted by anthropogenic recharge. Uncontaminated vadose zone sediments from near the DOE Hanford Site in Washington, and exposed to irrigation water for three years, were used in unsaturated batch and column experiments.

An unsaturated flow column experiment (8 columns) was conducted to evaluate the impact of different levels of unsaturated flow on vadose zone microbiological properties. No nutrients were added to the columns. Both unsaturated (0.15 volumetric water content) and saturated flow conditions increased microbial growth as measured by cell membrane synthesis by a factor of ~5 compared to no-flow columns with 0.05 volumetric water content. Select ribosomal RNA and phospholipid fatty acid (PLFA) analyses are being performed to determine if, and which, specific microbial groups were stimulated by the different flow conditions.

Chromate toxicity to microbial communities was determined under aerobic, saturated and unsaturated conditions, comparing short and long-term exposure effects. The toxicity of chromate to microbial communities was quantified using cell membrane synthesis as the response variable. Chromate concentrations resulting in 50% inhibition ( $IC_{50}$ ) were lower under unsaturated conditions than saturated conditions and lower after long-term exposure (4 weeks) than short-term exposure (3 days).

An unsaturated aerobic batch experiment composed of 27 treatments with varying chromate, nitrate, and carbon levels was conducted to select a subset of treatment combinations to be evaluated in subsequent unsaturated flow column experiments. Chromate concentrations of  $IC_{50}$  and  $IC_{90}$  after long-term exposure were used. Nitrate was included because it is a common co-contaminant at Hanford's chromium-contaminated sites, and organic carbon was added to stimulate bioremediation. Studies by others have shown chromate can be reduced under both aerobic and denitrifying conditions. Samples have been analyzed for aerobic heterotrophic plate counts; cell membrane synthesis; chromium VI(reduced, soluble), VI(adsorbed) and III(oxidized, precipitate); concentration of nitrite reductase genes; and terminal restriction fragment length polymorphism (T-RLFP) analysis of rRNA to identify changes in the composition of the metabolically active microbial community. Chromate, nitrate, and carbon levels — individually and in combination — altered microbial populations, diversity and activity. Select samples will also be further analyzed by both PLFA and nucleic acid probe analysis of flotation films.

In the third year of the project, we will evaluate several chromate bioremediation approaches in unsaturated flow column experiments using the same analytical techniques.

# Ecological Interactions Between Metals and Microbes That Impact Bioremediation

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The project's objectives are to:

- develop an ecological understanding of the interactions among heavy metals (lead and chromium), the physico-chemical environment and microbes capable of remediating organic pollutants to supplant the current empirical approach;
- use microbial community diversity and heavy metal tolerance to determine the load of “bioavailable” metal in contaminated sites;
- use experimental microcosms to test ecological conclusions derived from analysis of waste sites. The purpose of these experiments is to optimize organic bioremediation and provide feedback to field site remediators.

Potential limiting factors (heavy metal concentration vs. available organic C) for microbial biomass were tested in soils from three contaminated sites. Soil contamination consisted of either (a) Cr alone (UP site), (b) Pb alone (Avanti site) or (c) Pb, Cr and petroleum (Seymour site). In each case tested, the addition of degradable organic matter caused increases in microbial biomass (phospholipid-P) and microbial respiration rates. We interpret these results to suggest that carbon availability rather than heavy metal toxicity controls microbial activity in these habitats.

The tolerance of microbial communities to heavy metals has been tested in two ways. With the first method, culturable bacteria were recovered and the minimum inhibitory concentrations of metal for growth of individual isolates were determined. A majority of the culturable bacteria in all of the habitats appeared to be sensitive to the toxic metal. However, significant populations of resistant bacteria were also found. In the case of Pb, resistant strains could grow up to 25–70  $\mu\text{M Pb}^{++}$ . In the case of Cr, bacteria resistant up to 50 mM  $\text{CrO}_4^{2-}$  were isolated. The second method involved measuring  $^3\text{H}$ -leucine incorporation into bacteria extracted directly from soil particles. The functional response of the microbial population was modeled as  $Y = 100 \times I / (\text{IC}_{50} + I)$ , where Y is the percentage of microbial activity (relative to the sample with no added metal), and I is the concentration of added metal. The  $\text{IC}_{50}$  values for lead were in the low (<10)  $\mu\text{M}$  range for all soils tested, whereas  $\text{Cr}_2\text{O}_4^{2-}$  concentrations in the low millimolar (< 5) range were necessary to reduce microbial activity by 50%.

We investigated the correlation between the microbial communities and levels of lead, chromium and various organic contaminants present along a 21.3 m transect at a mixed waste contaminated site (Seymour, Ind). Soil chemical analysis showed that total concentrations of xylenes, methylene chloride, toluene, lead and chromium ranged from high to low along the transect. For community analysis soil microbial DNA was extracted in triplicate from a total of 24 locations along the transect. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA was used to determine bacterial community structure for each sample location. The DGGE patterns indicated that the number of populations at this contaminated site was reduced compared to those typically observed in bulk agricultural soils. Comparison of DGGE community fingerprints using similarity coefficients showed they fell into three groups. The three groups correlated with the concentrations of organic contaminants and not metal concentrations (not totally confirmed, need more organic data). A greater number of high G+C bacteria (indicated by band migration by DGGE) were found in locations with high organic concentrations (e.g., 8,200 ppm methylene chloride, 12,820 ppm toluene, 2,030 ppm xylene). This may indicate populations specifically involved in degradation of high concentrations of these compounds.

# Determination of the Structure of Metal- and Humics-Reducing Microbial Communities in Subsurface Environments Contaminated with Uranium and Other Metals

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The purpose of these studies is to determine the composition and activity of the metal- and humics-reducing community in subsurface environments and elucidate the physiological characteristics of subsurface metal and humics reducers. This will provide information that will aid in bioremediation of metal-contaminated subsurface environments. A combination of culturing, biochemical and molecular approaches is being employed in these studies.

In both laboratory and field studies, stimulation of Fe(III) reduction in diverse aquifers invariably resulted in a dramatic increase in the number of 16S rDNA sequences closely related to known *Geobacter* species. In laboratory studies, the addition of either various organic electron donors or electron shuttle compounds stimulated Fe(III) reduction and resulted in *Geobacter* sequences becoming important constituents of the *Bacteria* 16S rDNA sequences that could be detected with PCR amplification and denaturing gradient gel electrophoresis. Quantification of *Geobacteraceae* sequences with a most-probable-number technique indicated that the extent to which numbers of *Geobacter* increased was related to the degree of stimulation of Fe(III) reduction. *Geothrix* species were also enriched in some instances, but were orders of magnitude less numerous than *Geobacter* species. *Shewanella* species were not detected, even when organic compounds known to be electron donors for *Shewanella* species were used to stimulate Fe(III) reduction in the sediments. *Geobacter* species were also enriched in two field experiments in which Fe(III) reduction was stimulated with the addition of benzoate or aromatic hydrocarbons. The apparent growth of *Geobacter* species concurrent with increased Fe(III) reduction suggests that *Geobacter* species were responsible for much of the Fe(III) reduction in all of the stimulation approaches evaluated in three geographically distinct aquifers. Studies at the Shiprock UMTRA site also suggested that *Geobacter* species are likely to be the dominant metal-reducing microorganisms under Fe(III)-reducing conditions, even when the subsurface is contaminated with uranium. Therefore, strategies for subsurface remediation that involve enhancing the activity of indigenous Fe(III)-reducing populations in aquifers should consider the physiological properties of *Geobacter* species in their treatment design.

The finding that the *Geobacter* species that predominated in the various subsurface environments were closely related to *Geobacter* species already available in pure culture is surprising because the current dogma in environmental microbiology is that the most environmentally significant microorganisms can not be recovered in culture. Thus, we now have an apparently unique opportunity in which we can study the physiology of microorganisms in pure culture which are closely related to the microorganisms that we know are environmentally significant in the subsurface. This suggests that an in-depth characterization of the physiology and biochemistry of *Geobacter* species will provide important insights into the mechanisms for metal reduction in the subsurface.

As part of the investigation of the physiological characteristics of *Geobacter* species, the genome of *Geobacter sulfurreducens* is being sequenced in collaboration with TIGR. Sequence data available to date has already suggested novel metabolic characteristics of *G. sulfurreducens*, such as the ability to fix nitrogen, and has identified genes for proteins we have recently found to be involved in electron transport to Fe(III). The results of a more complete genomic analysis and preliminary comparisons of the *G. sulfurreducens* genome with other *Geobacter* genomes will be presented, as will a model for the current understanding of electron transport to extracellular Fe(III) oxides in cultures and subsurface environments.

## The Structure of Microbial Communities as a Diagnostic Indicator of Ecosystems

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The structure of more than 100 microbial communities has been determined by terminal restriction fragment length polymorphisms (T-RFLP) of 16S rDNA. These data, in conjunction with intensive geochemical profiling of the substrata, have been used to direct culture-independent 16S rDNA comparative sequence analysis of putatively critical phylogenetic groups in selective communities. We report on the current state of three lines of research that employ this general approach.

A chromium contaminated superfund site has been the subject of intensive investigation during the past two years. More than 90 communities from this site, representing a broad range of Cr, Cu and organic carbon concentrations, have been profiled with T-RFLP. Across this significant range of geochemical attributes, we have identified both ubiquitous and endemic populations of bacteria. Initial results suggested that populations of *Cytophaga* or *Flexibacter* were unique to sites with high chromium concentrations. We report on the diversity, as measured by 16S rDNA sequence, of *Cytophaga*-*Flexibacter* from these sites. In addition, a subset of four sites was profiled with T-RFLP at three depths and with three primer sets specific for bacterial, archaeal and cytophaga phylogenetic assemblages.

Community analyses using comparative 16S rDNA sequence analysis as well as T-RFLP were conducted on both pristine and contaminated aquifers. We report here on the phylogenetic diversity between aquifers of different geology as well as within a single aquifer. As many as 23 terminal fragments (populations) were detected in a single community and communities separated by only a meter can show differences in terminal fragment profiles. From the Narrow Channel aquifer in Oyster, Va., 147 rDNA clones were screened and 88 unique ARDRA patterns identified. Partial sequencing has identified at least 12 phylogenetic groups/genera. In addition, based upon rDNA phylotyping, we propose a novel physiology operative in this coastal aquifer.

T-RFLP profiling has also identified populations unique to sandstone and shale strata from 200 m below the surface at Cerro Negro, N.M. Between 11 to 17 populations were detected in these subsurface bacterial communities with six populations present in both shale and sandstone communities, three populations unique to shale, and one population unique to sandstone. The populations appearing unique to each stratum were phylogenetically characterized by 16S rDNA sequence.

In summary, T-RFLP has proven to be a sensitive technique for assessing community structure. It provides a cost-effective approach to the rapid identification of populations that are unique to specific geochemistries. These populations can, in turn, be used as landmarks to assess the phylogenetic and physiologic state of a community.

# Microbially Induced Phosphorus Bioavailability: Effects on Community Ecology and Uranium Sequestration

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We are investigating the precipitation of metals and radionuclides under aerobic conditions using introduced bacteria and organic phosphorus in a joint collaboration with Oak Ridge National Laboratory, Georgia Institute of Technology (P. Sobecky), and the University of Missouri (C. Zhang).

Traditional approaches to the remediation of metals and radionuclides utilize dissimilatory reduction processes. However, in oxygenated environments, such as in the vadose zone and in oxygenated aquifers, dissimilatory reduction is problematic. Thus, new technologies are needed to broaden the scope of available bioremediation approaches. In this project, we have proposed to immobilize contaminants in aerobic environments by coupling the introduction of bacteria (either as GEMS or natural isolates) containing the enzyme alkaline phosphatase. Plasmid pJH123, containing a *pglA-phoA* hybrid gene encoding a fusion protein, was introduced via electroporation into a number of subsurface pseudomonad isolates selected for their potential in field-scale delivery systems. We have strains in which plasmid pJH123 was stably maintained in the absence of selection and conferred significantly higher levels (100-to 850-fold) of alkaline phosphatase activity to the pseudomonad hosts relative to parental, control strains. The increased enzyme activity does lead to uranium precipitation with the introduction of organic phosphate. Following 36h of incubation in glycerol-3-phosphate (G3P) amended medium, 20  $\mu\text{M}$  of uranyl acetate was added to supernatants collected from GEMs and parental control flasks. As much as 69% (13.8  $\mu\text{M}$ ) of the uranyl acetate was precipitated with supernatants from two of the three genetically modified pseudomonads.

These findings demonstrate that overproduction of alkaline phosphatase by subsurface pseudomonads can result in considerable precipitation of uranium from solution. To optimize/enhance the choices of potential microorganisms to be used in this strategy, we have also isolated a number of natural (indigenous) bacteria, including several *Burkholderia* strains and a gram-positive bacteria, capable of using triethylphosphate (TEP) and G3P. Tn5 mutagenesis and genome library expression cloning are being employed in an effort to locate genetic elements associated with TEP utilization. Exconjugates expressing TN5 derived tetracycline resistance and a loss of enhanced growth rate in the presence of TEP have been obtained. Also, studies are underway to determine (1) the effects of phosphate accumulation and GEM introduction on subsurface microbial community structure and function, and (2) the relative mobility of orthophosphate, TEP and G3P in high- and low-iron subsurface sediments.

# Horizontal Gene Transfer as Adaptive Response to Heavy Metal Stress in Subsurface Microbial Communities

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The hypothesis of our project is that microbial communities respond to sub-toxic heavy metal stress by increased horizontal gene transfer and rearrangement. We postulate that such response can lead to useful genetic rearrangements and recombinations that improve the community's ability to resist or cope with the applied heavy metal stress. Our thrust is to directly examine gene transfer in soil microcosms subject to different levels of heavy metal (in our case  $Zn^{2+}$  and  $Cd^{2+}$ ) stress and examine this for soils that are either pristine or have historic heavy metal contamination.

We have been devoting efforts to answer the following: (1) How can we describe the structure of a particular soil microbial community, and how is that structure related to the soil geochemistry? (2) How stable is the microbial community structure, and how does community structure differ as a function of preexisting or applied stress level?

We have been using community ARDRA and community T-RFLP as primary tools to inspect community dynamics in our soil microcosms. Our microcosm experiments methodology aims to mimic the conditions of a semi-continuous oligotrophic carbon flux that exists in the subsurface. In addition, our approach allows a facile periodic sampling of the microbial community.

A first set of microcosm experiments was completed wherein the fate of *Escherichia coli* (pMOL18 with *czc* cassette) was monitored in sterile and non-sterile soil microcosms. The results clearly revealed the competitive pressure of the indigenous soil microbial community on *E. coli* survival, the efficacy of donor-selective enumeration techniques, and the resolving ability of the ARDRA method to see time-varying community profiles. The presence of the *czc* fragment was semi-quantitatively determined using *czcD* specific primers and soil-DNA dilution series. We are, currently, adhering to an MPN-PCR technique to quantify the concentration of the introduced mobile element in the soil microcosm. Another microcosm experiment has been initiated wherein the fate of *Pseudomonas putida* KT2440 and its plasmid TOL::Tn5(*km*) are monitored. This strain, in addition, contains the *gef*-based IPTG inducible suicide gene cassette and we are monitoring the efficacy of suicide induction in the microcosm systems. A large set of microcosm experiments has been initiated wherein the fate of either a conjugal (Tol::Tn5) and non-conjugal (pMOL187) plasmid are being monitored in the presence of varying Cd concentrations (0,10,100,1000  $\mu$ M).

Due to earlier limitations in the P<sub>a104/038</sub>*gef* suicide systems, we have initiated construction of a second suicide gene cassette based on the *genE* lethal gene. In addition, using dedicated microcosm experiments we are testing the efficacy of suicide induction, and are evaluating the mechanism of the high escape mutation frequency.

We are continuing to probe the original site-derived soil samples taken at various locations at a metal contaminated study site. Several isolates were obtained based on resistance to either Cd, Ni, or Zn. Cross resistance to other metals was very high (typically > 80%), while transfer of the Cd phenotype to *Alcaligenes eutrophus* was common. In addition, Cd-resistant encoding phenotypes were isolated from the soil community using *Alcaligenes eutrophus* as a genetic sink, revealing the incidence of the mobile nature of the resistance phenotype. Characterization is ongoing.



# Noncompetitive Microbial Diversity Patterns in Soils: Their Causes and Implications for Bioremediation

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The determination of optimal strategies to remediate mixed waste contamination in soils requires baseline information about the response of microbial communities under these conditions. This goal is complicated by a general lack of understanding about what forces most impact the structure of soil microbial communities, both in contaminated and pristine soils. The objectives of this study are to examine how mixed waste contamination affects the diversity and composition of soil microbial communities, and to determine the forces that most impact the structure of these communities.

Surface, vadose and saturated zone soils, collected from both contaminated and uncontaminated sites were analyzed using a 16S rDNA approach to examine microbial community structure. Both surface and vadose soils exhibited high levels of diversity with no dominance, which we characterize as a noncompetitive diversity pattern. For example, one surface sample from Dover Air Force Base yielded 886 unique rDNA clones out of 920 total. In contrast, soils from the saturated zone showed much less diversity, and were dominated by one or several community members. No differences in community diversity were observed between contaminated and noncontaminated samples, suggesting that wastes are not the key factor controlling microbial community structure at these sites.

Our examination of existing microbial communities suggests that spatial isolation at the soil surface leads to the maintenance of high levels of microbial diversity, whereas greater levels of connectiveness in the saturated zone allow for dominance by one or several community members. We tested this spatial isolation hypothesis by conducting two-species competition experiments in sand microcosms with varying levels of moisture. Competition in liquid or saturated sands resulted in dominance of the species with the most competitive growth parameters in a predictable manner. However, when the moisture content of the microcosms was lowered to increase the degree of spatial isolation and thus mimic the conditions in a surface soil, each species persisted in the microcosm in nearly equal frequency. These results suggest that spatial isolation may be a key determinant in structuring microbial soil communities.

Of concern in our community analysis is the generation of PCR artifacts during the 16S rDNA amplification and cloning. We addressed this problem by examining the severity of artifact formation in a four-species community. The degree of PCR-generated artifacts varied with the type of polymerase used, and increased with increasing PCR cycles, template concentration and species diversity. Based on these findings, an optimal strategy was devised to minimize PCR-generated artifacts in 16S-gene-based community studies.

To determine if sample size affects the observed diversity pattern, surface samples of 0.1, 0.4, 1 and 5 grams were analyzed using the 16S rDNA approach. Despite the wide range in size, each sample showed a noncompetitive diversity pattern. Surprisingly, there was little overlap among the clone libraries from these samples, suggesting that microbial diversity at the soil surface may be even higher than previously expected.

## The In-Situ Assessment of Microbial Community Ecology Within Samples From Chromium and Uranium Contaminated Sites

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Microbial activity is of primary importance in the bioremediation of metal contaminated soils. Microorganisms can alter metal chemistry and mobility through reduction, accumulation and immobilization. Furthermore, the structure and diversity of soil microbial communities is known to change in the presence of heavy metals as the communities adapt to pollutant loads. Our principal objective is to utilize the combination of signature lipid biomarkers (SLB) and PCR-denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA to study the impact of metals and radionuclides on indigenous microbial communities.

Traditionally, methods employed to monitor microorganisms require ex-situ culture analysis. However, these methods poorly represent in-situ microbial communities. Phospholipid fatty acid (PLFA) analysis can be utilized to determine shifts in microbial biomass, nutritional/physiological status and community diversity in situ. However, PLFA analysis does have limitations for the analysis of community structure. To complement this, we have utilized a PCR-DGGE approach employing primers that recognize the 16S rDNA of almost all known and inferred bacterial species. Sequence analysis of individual bands from DGGE gels was used to provide fine-scale biomarkers and loosely infer the identity of the source organisms using database searches and phylogenetic methods. We are also attempting to relate the complexity, band positions and relative band intensities of DGGE patterns to contaminant load.

Results of analyses at two sites currently under investigation by NABIR are presented: (1) the Cannelton industrial site in Michigan (T.L. Marsh, PI) and (2) the Shiprock Uranium Mill Tailings Remedial Action (UMTRA) site in New Mexico (P.E. Long, PI). Contamination (primarily Cr) at the Cannelton site ranges from background levels (0-50 mg kg<sup>-1</sup>) to 200,000 mg kg<sup>-1</sup>. Linear and non-linear techniques were used to map changes in the microbial communities (determined from PLFA/DGGE analysis) correlating with Cr concentration.

Although total biomass showed no correlation with Cr concentration ( $P > 0.05$ ), the ordination of PLFA profiles together with sample characteristics by principal component analysis revealed associations between Cr and specific PLFA. In collaboration with PIs S. Piffner and C. Brandt (ORNL), the association between PLFA/PCR-DGGE profiles and Cr concentration was further pursued using both hierarchical cluster analyses and artificial neural networks (ANN), an artificial intelligence technique. At Shiprock, contaminants include a wide range of metals dominated by U, with other solutes including NO<sub>3</sub><sup>-</sup>, NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup>, and SO<sub>4</sub><sup>2-</sup>. A PLFA/DGGE analysis of groundwater samples indicated the presence of diverse, active microbial communities, containing a high relative proportion of biomarkers indicating the presence of sulfate/metal reducing bacteria (SRB). There was a high degree of correlation between the PLFA and geochemical data, and DGGE revealed dominance of the bacterial communities of most samples by organisms related to known metal-metabolizing species.

**PROGRAM ELEMENT 3**  
**Biomolecular Sciences**  
**and Engineering**

# Molecular and Microcosm Analyses of the Potential for Gene Transfer in Radionuclei and Metal-Contaminated Subsurface Environments

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The objectives of this project are to (1) determine if metal resistance among subsurface microbes evolved by gene transfer; (2) measure the potential for gene transfer in subsurface microbial communities; (3) use microcosms to measure transfer rate; and (4) determine if gene transfer increases the ability of the community to respond to metal and radionuclei stress.

These objectives are accomplished by testing the following hypotheses (in corresponding order to the listed objectives):

Among subsurface microbes the phylogenetic relationships of metal resistance genes do not correspond with the rRNA-based phylogeny of their host strains, indicating that metal resistance evolved by horizontal rather than vertical transfer.

Conjugal plasmids are more abundant in microbial communities of metal and radionuclei contaminated soils than in pristine soils.

The presence of metals and radionuclei stimulates conjugal transfer in the subsurface environment. The spread of metal resistance via conjugal transfer increases the resilience of the subsurface microbial community to metal stress.

Hypothesis (1) is tested by comparing the phylogenetic relationships among subsurface culture collection strains and among metal resistance genes that are carried by these strains. The hypothesis will be accepted if the phylogenetic trees that emerge are dissimilar; similar trees will indicate a coevolution of microbes and their metal tolerance. Hypothesis (2) is tested by using the exogenous plasmid isolation approach. Genetically marked recipient strains are mixed with subsurface soils, isolated bacteria and DNA obtained from these soils, and metal resistant exconjugants are selected. The hypothesis will be accepted if conjugal metal resistance plasmids are more frequently isolated from contaminated as compared to pristine soils. Hypothesis (4) is tested by measuring conjugal transfer rates in metal-spiked subsurface soil microcosms. The hypothesis will be accepted if the transfer rate is stimulated in the presence of heavy metals. Hypothesis (4) is tested by evaluating genotypic, phenotypic and functional diversities of subsurface communities in the presence of metals. Microcosms containing a bacterium with a metal resistance mobilizable plasmid and a conjugative mobilizing plasmid are compared with microcosms containing the same bacterium with only the mobilizable metal resistance plasmid. If a community where transfer of the metal resistance genes occurs at high frequency shows higher diversity compared to a community where transfer is less frequent, the hypothesis will be accepted.

## Metabolic Engineering of Microorganisms for Heavy Metal Bioremediation

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An attractive method for heavy metal remediation is precipitation of the metal on the bacterial cell wall as an insoluble metal complex. Two potential applications for this technology are immobilization of heavy metals in situ and improved flocculation and removal of metals from waste streams. While bioprecipitation of metals would not remove the metal from its environment, it could be used to slow migration of the metal into groundwater. In a reactor, bioprecipitation can take advantage of the improved settling ability of cells over non-biological metal complexes, and thereby decrease the cost of treating heavy metal-contaminated waste streams.

To this end, we have engineered the metabolism of Gram-negative bacteria (namely, *Escherichia coli* and *Pseudomonas aeruginosa*) to secrete sulfides or phosphates for precipitation of cadmium sulfide or uranyl phosphate, respectively, on the cell wall. Two aerobic pathways for sulfide secretion were developed. The first relies on reduction of thiosulfate to form sulfide. The second relies on deregulation of the cysteine biosynthetic pathway and subsequent production of sulfide from cysteine. For phosphate secretion, we engineered the polyphosphate pathways to improve polyphosphate accumulation under phosphate-rich conditions and its subsequent degradation to and secretion of orthophosphate under phosphate-starvation conditions. When secretion of phosphate or sulfide occurs, complete removal of the metal is observed, even at relatively high concentrations. Electron microscopy and energy dispersive X-ray spectroscopy confirmed the presence of the metal precipitate on the outside of the cell. Future work will involve its application to other metals that readily precipitate as phosphates or sulfides.

## Engineering *Deinococcus Radiodurans* for Remediation of Radioactive Mixed Waste Environments

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Immense volumes of radioactive waste, generated from nuclear weapons production during the Cold War, were disposed directly to the ground. The current expense of remediating these polluted sites is driving the development of alternative remediation strategies using microorganisms. *Deinococcus radiodurans* is the most radiation resistant organism known and can grow in highly irradiating environments. *D. radiodurans* strains expressing cloned mercury(II) resistance functions were constructed and shown to be effective at reducing toxic ionic mercury, a frequent constituent of radioactive wastes, to volatile elemental mercury. The mer operon was chosen as a model-system to guide future engineering efforts, particularly in the area of metal remediation.

To demonstrate how future engineering efforts of mixed radioactive wastes could be achieved, mercury-reducing and toluene-metabolizing functions were combined into the same host, yielding a strain expressing both functions. We have analyzed *D. radiodurans* growth on a chemically defined minimal medium in the presence and absence of continuous radiation. Whereas cell growth was unaffected in the absence of radiation, cells did not grow, and were killed, under continuous radiation. In nutrient-limiting conditions, DNA repair was found to be limited by this organism's metabolic capabilities and not by any nutritionally induced defect in genetic repair. Our growth studies and analysis of the complete *D. radiodurans* genomic sequence support the existence of several defects in *D. radiodurans*' global metabolic regulation that limit carbon, nitrogen and DNA metabolism. We have identified key nutritional constituents that restore growth of *D. radiodurans* in nutritionally limiting radioactive environments.

## Stabilization of Radionuclides by Anaerobic Bacteria

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The genus *Clostridium* is a diverse group of anaerobic, gram-positive, rod-shaped, endo-spore forming bacteria. The taxon is very heterogeneous, comprising organisms with considerable variation in genome size (2.5 to 6.5 Mb) and G+C content (24 to 55 mol%). *Clostridium* sp. BC1 (ATCC No. 53464; hereafter referred to as BC1, an anaerobic, N<sub>2</sub>-fixing member of this group that was isolated from coal-cleaning residues, is a potential candidate for ameliorating radionuclide contamination at DOE sites as it has biochemical pathways that can convert water soluble uranyl ion U(VI) to less soluble U(IV). The objective of this project is to use a whole genome shotgun sequencing approach to obtain large amounts of BC1 DNA sequence information to discover key functional genes and to understand gene sequence/function relationships in this and related *Clostridia*/*Bacillus* species. We are currently sequencing several BC1 clone libraries constructed in a vector, pZIP, we developed for generating sets of bidirectional nested deletions. Fragments in the 10 kbp size range or larger can be sequenced on both strands from ordered sets of nested deletions using universal vector primers to produce highly accurate sequence contigs. We are in the process of annotating several of these contigs encoding genes involved in intermediary metabolism and reductive reactions using a graphics-based gene finder program we developed to aid in analysis of sequence assemblies.

We are also sequencing DNA from *Alcaligenes eutrophus* (*Ralstonia eutropha*) CH34, a gram-negative, non-spore forming bacillus that flourishes in millimolar concentrations of heavy metals. Resistance is conferred by large megaplasmids carrying gene clusters that encode cation-efflux complexes which span both bacterial membranes. The reference stock CH34 harbors two plasmids, pMOL28 (180 kb) and pMOL30 (240 kb), which together confer resistance to Zn, Cd, Co, Pb, Cu, Hg, Ni and Cr. Both plasmids are low copy number, stably maintained even without selective pressure and are self-transferable at low frequencies. About 20% of each plasmid is used to encode metal resistance. Due to the activity of these efflux systems, a supersaturated zone of metals is formed around the cells, which leads to bioprecipitation or biomineralization of heavy metals on the cell envelopes and removal of heavy metals from solution. Other large plasmids in other *A. eutrophus* strains confer resistance to xenobiotics. *A. eutrophus* can use a broad range of substrates as its carbon source or it can grow chemolitho-trophically using molecular hydrogen as the energy source and carbon dioxide as a carbon source. In the presence of nitrate *A. eutrophus* can grow anaerobically. This combination of properties makes this bacterium another attractive candidate for application in several bioremediation scenarios relevant to DOE waste sites.

We are about halfway through sequencing of pMOL28. A remarkable feature of *A. eutrophus* CH34, whose optimal growth temperature is around 30°C, is a high rate of cell death and mutation that occurs when it is grown at 37°C. As part of this project we sequenced a 21.5 kb segment of the *Alcaligenes* chromosome that complements a mutant phenotype of temperature resistant growth at 37°C back to the wild-type temperature sensitive phenotype. In collaboration with D. van der Lelie from the Flemish Institute of Technological Research in Belgium, we are now carrying out experiments to define which of the 17 ORFs within this region causes temperature sensitive growth and the nature of the mutations that occur at elevated growth temperatures.

# Characterization of Environmental Regulation of the Genes and Proteins Involved in Metal Reduction Pathways in *Shewanella Putrefaciens*

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*Shewanella putrefaciens* is a versatile microbe capable of metal reduction in both aerobic and anaerobic environments and is thus of interest for bioremediation of waste sites containing toxic metal compounds. Assessing the usefulness of *S. putrefaciens* for bioremediation, however, requires characterization of the molecular mechanisms and regulation of the metal reduction activity. The U.S. Department of Energy Microbial Genome Program is funding the sequencing of the *S. putrefaciens* genome and the development of microarrays containing all ORFs from *S. putrefaciens* MR-1. NABIR is now funding a project to correlate the output of these Microbial Genome projects with changes in protein expression as a means to characterize the regulatory mechanisms controlling metal reduction activity when *S. putrefaciens* is grown in different environmental conditions. Shifts in the abundance of specific proteins are indicative of gene regulation, while the relative abundance of chemically modified forms of proteins (i.e., phosphorylated, glycosylated, deamidated, or methylated) reveals mechanisms of metabolic pathway regulation.

In this new NABIR project, *S. putrefaciens* MR-1 cells are grown under experimental conditions designed to replicate metal contamination in a variety of pH and temperature environments. Messenger RNA and proteins are extracted from the cells and analyzed using microarrays and two-dimensional gel electrophoresis, respectively. Proteins altered in abundance in cells grown in the presence of metals will be identified and the correspondence between protein changes and changes in the expression of specific genes detected through the microarray analysis will be examined. Post-translational modifications will be characterized and the mechanism of regulation of protein function deduced. Based on identified changes in gene expression in different environmental conditions, knockout mutants will be generated to determine whether the regulated genes are essential for cell survival. The results of the proposed experiments will identify (1) gene sequences required for *S. putrefaciens*' metal reduction activity in a variety of environments, (2) regulatory pathways controlling the abundance of the gene products, and (3) the effects of those genes on cell viability.



## Complete Genome Sequencing of *Shewanella Putrefaciens*

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*Shewanella putrefaciens* grows both aerobically and anaerobically. In the anaerobic phase, it acts as a metal reducer. The use of metal reducing bacteria in bioremediation has several advantages over more standard respiring bacteria, including: (1) their substrates (iron oxides) are solids and thereby can be delivered to a contaminated site without diffusing away; (2) iron oxides are specific substrates, so competition from other bacteria for the electron acceptor will be minimal; (3) in stratified aqueous environments, reduced iron should diffuse upward, be reoxidized by molecular oxygen in the oxic zone, and return to the anoxic zone by gravity, thus acting as a “pump” for oxidizing equivalents.

The 5 Mb *S. putrefaciens* MR-1 genome is being sequenced by the random whole genome strategy used to complete the sequence of multiple bacterial genomes at The Institute for Genomic Research. The random sequencing phase of the project resulted in 70,000 sequences. These were assembled into 125 linked contigs. Currently, gaps are being closed using a combination of PCR and small and large insert clone walking.

## In-Situ Survival Mechanisms of Sulfate-Reducing Bacteria in Polluted Sediments

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The long-term goal of our work is to identify genes and physiological activities essential for the in-situ survival of sulfate-reducing bacteria. Specifically, we are delineating genes from *Desulfovibrio* strains that are essential to its survival within contaminated environments. Our approach allows direct profiling between genes essential for survival in different settings such as normal and contaminated environments. With this method we can identify essential genes which could be missed in standard laboratory media, and identify genes which may important for survival in the presence of specific contaminants (U, Co, As, Pb).

To carry out these studies we are exploiting approaches developed in microbial pathogenesis to characterize genes essential for survival in experimental mouse models, the pathogen in-situ environment. As a complementary approach, we are using an environmentally important microbe and contaminated sediments for our experimental model. This approach involves using a pool of Tn5 based transposons which have been designed to express unique random tags. Thus, every transposon has its own signature and the technique is termed signature-tagged mutagenesis (STM). With STM, transposons can be transformed into the target organism to generate a library of transposon insertions carrying unique tags. Since each mutant carries a unique tag, it can be followed during the selection process. An input pool of STM clones is generated under normal conditions and then grown under the selective conditions. Those that grow in the normal setting, but are lost in the contaminated setting must have transposon disruption in gene(s) important for dealing with that contaminant. This clone is then selected from the input pool and the insertional mutation is characterized.

We have recently started work on this project and are developing transformation protocols for *Desulfovibrio*. We have recently isolated a new *Desulfovibrio* strain from a uranium-contaminated subsurface location. This strain will be used as the model in these studies. Using a combination of approaches, including electroporation and conjugation, we are optimizing the conditions for generating competent cells and obtaining random transposon insertions.

## Genes and Functions Regulated by *adnA* in *Pseudomonas fluorescens*

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The genetic traits that contribute to the survival and competitive fitness of bacteria in soil are poorly understood. One potentially important factor is the ability to adhere to abiotic surfaces, which are nutritionally rich relative to the interstitial spaces. The common soil bacterium *Pseudomonas fluorescens* adheres to sand columns. Screening of 3500 mutants generated by Tn5-insertion mutagenesis identified three strains that adhered < 50% as well as the wild type strain. The Tn5 insertions in these three strains mapped to two genes, *adnA* and *adnB*; *adnA* is a transcription factor of the NtrC/NifA family based on sequence homology. Intriguingly it is most similar to fleQ of *P. aeruginosa* (82% identity), a transcription factor required for adhesion to epithelial cells and mucins. Strains with insertions in *adnA* lack flagella, suggesting that *adnA* regulates flagellar gene expression and perhaps other cell surface structures required for adhesion. Recent work indicates that AdnA expression alone can complement the defects in adhesion and motility. The strain with an insertion in *adnB* is hyperflagellated and has reduced motility. *adnB* is similar to the flagellar motor protein motA, an integral membrane ion channel.

Our goals are to further examine the role of the AdnA by identifying genes that are activated or repressed by *adnA*, and to identify environmental conditions that regulate adhesion. To address the first goal, we are preparing a *P. fluorescens* strain in which wild type *adnA* will be replaced by a copy whose transcription requires an exogenous inducer. Briefly, a plasmid DNA was constructed in which *adnA* is transcribed from the promoter of the *meta*-cleavage pathway operon (Pm) of the *Pseudomonas putida* TOL plasmid pWWO. Transcription from Pm requires the activator protein XylS, and XylS activates transcription only in the presence of an added inducer, in this case 3-methylbenzoate (3-MB). The chromosomal *adnA* promoter will be replaced by the inducible promoter cassette in an otherwise wild type background. The new strain will be tested to insure that adhesion and motility are 3-MB dependent. The strain will then be mutagenized with Tn5-*lacZ* or Tn5-*phoA* transposons and screened for differences in reporter enzyme activity in the presence or absence of 3-MB. Inducer-dependent changes in activity will indicate that the insertion has occurred in a transcription unit whose expression is affected by AdnA. The disrupted genes identified will be candidates for genes that mediate sand adhesion and other traits needed for survival in soil.

Work is also proceeding on characterizing environmental conditions that regulate adhesion. Using a simplified adhesion assay, we find that wild type cells grown in rich or minimal media adhere to borosilicate glass. Various combinations of carbon sources and metal ions were added to minimal media. Preliminary results suggest that cells grown on glucose, glutamate or acetate media are adherent, while cells grown on citrate medium are nonadherent. Citrate is dominant if two carbon sources are used together. Additionally, calcium in the media promotes adhesion, while iron inhibits adhesion. These results provide initial data relating to the nature of the extracellular signals that regulate adhesion. An understanding of the mechanisms promoting or inhibiting adhesion will allow greater flexibility in designing genetically modified organisms suited to particular purposes.

## **Mechanisms for Uranium and Technetium Reduction in *Geobacter sulfurreducens***

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Project work was initiated on Sept. 1, 1999, and the following objectives were achieved in the first month. Mass culture of *Geobacter sulfurreducens* was optimized, allowing generation of sufficient quantities of biomass for initial characterization of the enzyme systems catalyzing U(VI) and Tc(VII). Assays suitable for monitoring the reduction of Tc(VII) and U(VI) were also developed. Finally, mg quantities of the 9.6 kDa c-type cytochrome were purified from the soluble fraction of *G.sulfurreducens*, and shown to reduce U(VI) in vitro.

# Flow Cytometry Technique for Multiplexed Detection, Quantification and Isolation of Nucleic Acids

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The goal of this work is to develop a new polystyrene bead-based capture method to identify specific DNA sequences from mixtures of heterogeneous DNA samples obtained from environmental samples. The methodology uses beads impregnated with different colored fluorescent dyes. The beads are usually coupled to DNA oligonucleotides that are used as capture probes. Following capture of complementary DNA sequences from the environmental samples, the fluorescent beads can be analyzed and separated by flow cytometry. The bead-based method may provide information comparable to information obtained using DNA microarrays, although with potential advantages such as greater sensitivity, quantitation and sequence determination. The method can also be preparative; primers based on the capture probe sequences and universal anchor primers can potentially be used to PCR amplify the captured DNA fragment for cloning and sequence analysis.

In this application, the investigators propose to develop the technology for the identification of DNA fragments from microorganisms that may be involved in metal bioremediation. The specific aims for one year are as follows: (1) Evaluate the bead-based method with respect to sensitivity, sequence discrimination, accuracy and precision in measuring abundances. (2) Determine if the bead-based method can measure abundances of seven genera of bacteria, previously identified to be important for metal reduction. (3) Develop an assay using beads to capture useful genes from environmental samples and prepare the captured material for PCR amplification, cloning and sequencing. (4) Assemble a set of candidate probes for metal reduction genes, and determine if the bead-based method can expedite the development of effective probes for capturing these genes. The sequence length of the capture probe may need to be considered.

Metal and Radionuclide Bioremediation of Mixed Wastes by Starvation Promoter-Driven Combinatorial Bacteria

# Metal and Radionuclide Bioremediation of Mixed Wastes by Starvation Promoter-Driven Combinatorial Bacteria

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This grant deals with two issues: purification of bacterial chromate reductases and cloning of their genes, and improvement of starvation promoter expression of *Pseudomonas putida*.

Chromate reductases: Cr(VI) (chromate) is a widespread environmental contaminant. Bacterial chromate reductases can convert soluble and toxic chromate to insoluble and less toxic Cr(III). Bioremediation can therefore be effective in removing chromate from the environment, especially if bacterial propensity for such removal is enhanced by genetic and biochemical engineering. To clone the chromate reductase-encoding gene, we purified to homogeneity (>600-fold purification) and characterized a novel soluble chromate reductase from *Pseudomonas putida*, using ammonium sulfate precipitation (55-70%), anion exchange chromatography (DEAE Sepharose CL-6B), chromatofocusing (Polybuffer exchanger 94), and gel filtration (Superose 12 HR 10/30). The enzyme activity was dependent on NADH or NADPH; the temperature and pH optima for chromate reduction were 80°C and 5, respectively; and the  $K_m$  was 374  $\mu$ M, with a  $V_{max}$  of 1.72  $\mu$ mol/min/mg protein. Sulfate inhibited the enzyme activity non-competitively. The reductase activity remained virtually unaltered after 30 min exposure to 50°C; even exposure to higher temperatures did not immediately inactivate the enzyme. X-ray absorption near-edge structure spectra showed quantitative conversion of chromate to Cr(III) during the enzyme reaction. Physiological studies strongly suggest that the chromate reductase we have purified has some other role for the bacterium. This premise is supported by the finding that many bacterial enzymes characterized in other contexts have chromate reductase activity — we have cloned one such gene from *Escherichia coli*.

Starvation promoters: The use of these promoters makes it possible to greatly minimize biomass formation and nutrient demand for bioremediation processes. Recognizing the gene that codes for proteins which sense starvation and other stresses in bacteria can greatly assist in manipulating starvation promoter activities in differently stressed DOE sites. We cloned the *flhF* gene of *P. putida*, which encodes a GTP-binding (G-) protein. Its disruption compromises induction of more than 50 starvation proteins and development of the starvation-induced general stress resistance (GR). It appears that this G-protein senses the onset of stresses by changing into its GTP-bound form, thereby activating the starvation/stress promoters. Interestingly, disruption of this gene renders *P. putida* unable to place its flagella at the cell pole and incapable of directional motility; instead it becomes randomly distributed throughout the cell surface. Moreover, overproduction of this gene product greatly increases the number of polar flagella. Thus, this GTP binding protein controls both the stress-resistance response as well as flagellar assembly, opening the way for manipulating motility and chemotactic function as well.

## Cellular Response of *Shewanella Putrefaciens* to Soluble and Solid-Phase Metal Electron Acceptors

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The fate and transport of many multivalent metals and radionuclides can be strongly influenced by a phylogenetically diverse group of microorganisms, termed dissimilatory metal-reducing bacteria (DMRB). The DMRB, *Shewanella putrefaciens* MR-1, a facultative anaerobe that displays remarkable respiratory capacity, is amenable to genetic manipulation and is the subject of a DOE-sponsored microbial genome sequencing project. The research proposed herein is intended to develop an understanding, at the genetic level, of how *S. putrefaciens* derives energy by coupling oxidation of organic compounds or H<sub>2</sub> to reduction of either soluble- or solid-phase Fe(III) oxides. In particular, we intend to investigate specialized functions that we hypothesize are required for utilizing solid phase Fe(III) (oxides or oxyhydroxides).

Hypotheses will be tested through the study of differential transcriptional responses associated with the growth and/or respiration of *S. putrefaciens* MR-1 in the presence of soluble- or solid-phase (such as Fe oxides) electron acceptors. Transcriptional activity will be measured by the use of cloned *Shewanella* promoters fused to the GFP reporter gene. Time-course measurements will reveal the sequence of transcriptional events that mediate the response of *S. putrefaciens* to anaerobic respiration via dissimilatory iron respiration. This research will provide important insights into the response of *S. putrefaciens*, at the cellular level, to metals, both soluble- and solid-phase, as electron acceptors. Ultimately, we expect to gain insights into the mechanisms by which this metabolically versatile organism accesses Fe(III) for respiration from insoluble metal oxides.

## Probing the Proteome with Capillary Isoelectric Focusing-ESI-FTICR Mass Spectrometry

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

We are currently developing technologies that will allow the visualization of the protein complement by obtaining comparative displays for the expression of many proteins simultaneously, based upon stable-isotope labeling. Two versions of each protein are generated and analyzed simultaneously, to precisely establish changes in expression. Capillary isoelectric focusing on-line with Fourier transform ion cyclotron resonance mass spectrometry provides a powerful tool to study the changes in expression (i.e., repression or induction) for hundreds of proteins simultaneously. Further characterization of the proteome can be accomplished by characterization of the proteolytic fragments of the proteins in the organism. For many proteins, these proteolytic fragments can be used as unique mass markers for the identification of the proteins in question. Additionally, the sequence of the peptides can be determined as another identification technique. These combined technologies will enable ultra-sensitive proteome-wide expression profiling to evaluate changes in the complete proteome of the iron-reducing bacterium *Shewanella putrefaciens* strain MR-1 induced by switching from aerobic to anaerobic respiration with heavy metals and radionuclides.



## Optimizing the Metalloregulator MerR for Metallosequestration and Metallosensing

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This is a new project based on our previous work supported by another agency. Its objective is to re-engineer the metal binding domain (MBD) of MerR to recognize metals besides Hg(II), especially those of interest in DOE bioremediation.

Expression of the Tn21 mercury resistance (*mer*) operon is regulated by a high affinity, high specificity metal-sensing repressor-activator, MerR, which represses transcription of the structural genes, *merTPCAD*, in the absence of Hg(II) and activates their transcription in the presence of Hg(II). MerR contains three domains: An N-terminal DNA binding domain, a C-terminal Hg(II)-metal-binding domain (MBD), and a coupling domain which lies between them. The MBD consists of a helical region from Cys82 to Cys117 followed by a loop from Cys117 to Cys126. Dimerization of MerR is effected by the formation of a coiled-coil employing these C-terminal helical regions of each monomer. Independent, Hg(II)-binding centers lie at each end of the coiled-coil and are comprised of Cys82 from one monomer and Cys117 and Cys126 from the other. This novel inter-subunit, trigonal metal binding center is highly specific for Hg(II) over other Group 12 metals, Cd(II) and Zn(II). We have previously described 11 single point mutants which allow MerR to respond to Cd(II), but not to Zn(II).

As the next step in manipulating the metal-responsiveness of MerR, we are asking how the loop region between Cys117 and Cys126 influences metal specificity by replacing the loop region of MerR with the ZntR loop region. ZntR, is a MerR-like regulator that is more responsive to Zn(II) and Cd(II) than to Hg(II), but unlike MerR, it contains a histidine residue in the middle of its loop region. Histidines are very often found as ligands to Zn(II) in other proteins, so we hypothesize that the ZntR histidine residue may provide a fourth ligand required for Zn(II)-or Cd(II)-binding. To avoid possible artifacts when using metals other than Hg(II), we have switched from using a hexa-Histidine affinity tag to using a streptavidin affinity tag (Sigma-Genosys) for protein purification. We are screening these mutant proteins for their ability to bind Hg(II), Cd(II), Zn(II), and ions of metals involved in radiation bioremediation, including Co and Pb, using a facile DNA gel mobility shift assay to detect metal-induced allosteric changes in MerR. When mutants exhibiting optimum metal affinity characteristics are identified, their MBD domains will be produced alone (lacking the DNA-binding and coupling domains) and subject to precise metal-binding quantification via equilibrium dialysis and isothermal calorimetry. Further optimization of these minimal MBDs will employ phage display technology.

## Genes for Uranium Bioremediation in *Desulfovibrio*

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Members of the species *Desulfovibrio* have been shown to be capable of uranium reduction. We have initiated a genetic approach to identify components of the reduction pathway to this metal in *Desulfovibrio desulfuricans*. Because experiments from Derek Lovley's laboratory point to the role of hydrogenases and cytochrome  $c_3$  in electron flow to uranium in *D. desulfuricans*, two mutants of the strain G20 lacking a [NiFe] hydrogenase and one lacking the [Fe] hydrogenase have been constructed and screened for uranium reduction. Quantitative assays have indicated that these mutants retained the ability to reduce uranium at the same rate as the parental strain. A mutant of G20 with a plasmid inserted in the *cycA* gene encoding cytochrome  $c_3$  has been generated and characterized. While the growth rate of the mutant on lactate is essentially the same as the parental strain, there is a dramatic decrease in growth on pyruvate. The mutant produces copious hydrogen when incubated with pyruvate. Surprisingly, the *cycA* mutant is still capable of uranium reduction, but does so with a rate that is about 50% that of the wild type, regardless of the electron donor, lactate, pyruvate or hydrogen. We interpret these results to suggest that cytochrome  $c_3$  is involved in the reduction of uranium but that other pathways for reduction are possible. Studies to elucidate the regulation of *cycA* are in progress.

## Single-Molecule Studies of Flavin Enzymes

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The high mobility of radionuclides complexed with chelating agents such as ethylenediaminetetraacetate (EDTA), nitrilotriacetate (NTA) and diethylenetriamine pentaacetate (DTPA) represents a major environmental concern at DOE sites. Biodegradation of the chelating agents by microorganisms can help immobilize radionuclides in the environment. Recently, an NTA monooxygenase from an NTA degrading microorganism and an EDTA monooxygenase from an EDTA-degrading microorganism have been purified and characterized. Both NTA monooxygenase and EDTA monooxygenase are FMNH<sub>2</sub>-utilizing monooxygenase and they need a flavin reductase to supply FMNH<sub>2</sub>. FMNH<sub>2</sub> produced by the reductase is rapidly oxidized by free O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>. However, both NTA and EDTA monooxygenases can compete with free oxygen for FMNH<sub>2</sub> oxidation. Since FMNH<sub>2</sub> is unstable, an immediate question is whether there is metabolic channeling between the reductase and NTA monooxygenase or EDTA monooxygenase. We are studying this question by both conventional biochemistry and single-molecule microscopy. For biochemistry studies, we need some mutant NTA monooxygenases that cannot oxidize NTA due to their lack of binding to NTA. The mutant proteins will be used to compete with the wild type NTA monooxygenase. If there is metabolic channeling, the flavin reductase and NTA monooxygenase will be in contact and pass FMNH<sub>2</sub> directly from the reductase to the monooxygenase. The mutant protein is unable to bind NTA but should still form a contact with the reductase. If there is any metabolic channeling, the mutant protein will quench the wild type monooxygenase for NTA oxidation. We have used a chemical reagent to modify arginine residues. After modification, the enzyme is inactivated. When the enzyme has bound NTA, the enzyme is not inactivated by the chemical reagent. Several arginine residues that are protected by NTA have been identified and mutated to other amino acid residues by site-directed mutagenesis. Eight mutants that cannot oxidize NTA have been obtained. We are in the process of purifying all the mutant proteins to check their ability to quench the wild-type NTA monooxygenase for NTA oxidation. This experiment will provide some evidence on whether FMNH<sub>2</sub> is directly channeled from the reductase to the monooxygenase.

We are also using single-molecule microscopy to investigate whether there is metabolic channeling. We have demonstrated that the single-molecule microscopy is a very useful tool to monitor enzymatic reactions associated with the oxidation and reduction of a prosthetic flavin group of enzymes. For NTA oxidation, FMN is not a prosthetic group but a substrate and end-product. We are trying to observe the continuous reduction and oxidation of a single FMN molecule by the reductase and O<sub>2</sub>. The emission of the single FMN molecule is monitored as it hops among many reductase molecules and O<sub>2</sub> molecules. NTA monooxygenase will be added to monitor the transfer of FMNH<sub>2</sub> from the reductase to NTA monooxygenase. Mutant NTA monooxygenases will also be used in this experiment.

These experiments will provide necessary evidence to prove whether there is metabolic channeling during NTA oxidation. If there is metabolic channeling, we have to use the specific flavin reductase to supply FMNH<sub>2</sub> for either NTA monooxygenase or EDTA monooxygenase. If not, any flavin reductase can be used to supply FMNH<sub>2</sub> for the two enzymes. This is important information for constructing genetic engineered organisms for the biodegradation of chelating agents.

**PROGRAM ELEMENT 4**  
**Biogeochemical Dynamics**

# Microbiological Controls on the Fate and Transport of Chelated Radionuclides: Multiscale Investigations in Unsaturated Structured Media

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The overall goal of this project is to provide an improved understanding and predictive capability of the mechanisms that allow chelate-degrading bacteria to be effective in the bioremediation of subsurface environments contaminated with toxic metals and radionuclides. The study is motivated by the likelihood that vadose zone microbial activity can effectively consume chelating organic ligands, thus facilitating the immobilization of released metals and radionuclides via sorption and precipitation reactions. The central hypothesis of this study is: in structured soils and aquifers, the biodegradation of metal-chelating ligands is limited by the diffusive mass flux of contaminants from pores too small to be accessed by bacteria to larger pores where bacteria reside.

Our objectives are to (1) develop an improved understanding and predictive capability of the mechanisms governing the biodegradation of cobalt (Co)-NTA in unsaturated, structured media; (2) quantify the microbial and hydrologic conditions that influence the biodegradation of metal-chelating ligands for the purpose of contaminant containment and remediation in heterogeneous, structured media; and (3) provide integrated experimental and theoretical methodologies for the scale-up of biogeochemical processes from the microscopic scale to the macroscale (pedon) scale. Our approach involves the use of a variably saturated dynamic flow technique to quantify the biodegradation of Co<sup>II</sup>NTA as a function of pore class size in structured media and existing models that couple microbial and hydrogeochemical processes to conduct multiscale process and parameter upscaling studies. The experimental results will provide new insights concerning the relationship between the biodegradation of radionuclide-chelate complexes and the pore structure and hydrologic connectivity in heterogeneous subsurface environments. Further, these results will enhance our ability to upscale laboratory- and pedon-scale biodegradation processes to the field scale.

Our results to date demonstrate some of the complex biogeochemistry that influences the fate and transport of chelated metals and radionuclides. The interaction of Co<sup>II</sup>NTA with common soil minerals results in the rapid formation of a variety of highly stable oxidized products, including Co<sup>III</sup>NTA, (Co<sup>III</sup>NTA)<sub>2</sub>, and two isomers of Co<sup>III</sup>(IDA)<sub>2</sub>. These products have been observed in batch systems and in column displacement studies using undisturbed cores of structured saprolite. The stoichiometry of reaction products is a function of soil properties (e.g., pH). There is no direct evidence for the formation of geochemical dissociation products such as Fe<sup>III</sup>NTA or Al<sup>III</sup>NTA. Furthermore, Co(III) complexes exhibit a low affinity for the solid phase. Accordingly, Co injected into undisturbed cores (as Co<sup>II</sup>NTA) exhibits more rapid transport with increasing pH, indicating that the mobile cobalt remains as an anionic ligand-metal complex during transport through the column. Bacterial strains that can degrade NTA and some metal-NTA complexes (e.g., Co<sup>II</sup>NTA) cannot degrade NTA complexed with Co(III). This effect is presumably the result of the kinetic and thermodynamic stability of Co<sup>III</sup>NTA. These observations illustrate some of the complex biogeochemistry that influence the fate and transport of chelated metals and radionuclides. These observed oxidation reactions have far-reaching implications for the transport of Co-NTA in soils and groundwater. The Co(III) complexes have a lower affinity for the solid phase than the corresponding Co(II) complexes resulting in enhanced transport of cobalt. The Co(III) complexes are much more stable, effectively competing with geochemical dissociation reactions and the concomitant formation of surface reactive Co(II). The Co(III) complexes are resistant to biodegradation.

# Complimentary and Inhibitory Coupling of Biological and Geochemical Processes in Metal and Radionuclide Reduction

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Reductive processes, whether abiotic or biotic, may serve to stabilize toxic heavy metals and radionuclides such as chromium and uranium. Direct enzymatic reduction of chromate and uranyl during bacterial respiration is highly desirable as it should lead to reduced products having limited solubilities. Similarly, reduction of  $\text{Co(III)EDTA}^-$  to  $\text{Co(II)EDTA}^{2-}$  serves to diminish the transport of cobalt. Competing electron acceptors present in natural environments may impede or enhance the reduction of a target phase; metabolic products formed during the reduction of alternate electron acceptors may in some cases lead to an enhanced chemical pathway for the reduction of the target element. Here we discuss and demonstrate the influences of competing electron acceptors on the reduction of uranyl, chromate and cobalt(III)-EDTA<sup>-</sup> by dissimilatory iron (DIRB) or sulfur reducing (SRB) bacteria.

Ferric iron is ubiquitous in nature; in many suboxic soils, sediments and subsurface environments it is likely to be the most abundant terminal electron acceptor for microbial respiration. We investigated the microbially mediated, indirect reduction of chromate by *Shewanella alga* strain BrY by injecting a continuous stream of chromate into a suspension of BrY and hydrous ferric oxide minerals. In such systems, Fe(II) produced as a result of microbial respiration serves as a catalyst, reducing Cr(VI) and thus being reoxidized; the production of Fe(II) also provides a means for bacterial tolerance of chromate—an important factor for DIRB sensitive to chromate such as BrY. The prevalence of DIRB implies that chromate reduction may proceed through bacterially mediated pathways in any site containing iron and having limited molecular oxygen. Specific rates for various ferric (hydr)oxides thus provide a means to estimate the extent to which reduction will take place given an influx rate and residence time of chromate contaminated waters.

Similarly, sulfate reducing bacteria generating dissolved sulfide may serve to catalyze the reduction of  $\text{Co(III)EDTA}^-$ . We observed that dissolved sulfide in fact leads to the rapid reduction of  $\text{Co(III)EDTA}^-$  through the formation of highly reactive polysulfide intermediates. Furthermore, in excess sulfide a solid CoS phase is produced. Thus SRB may directly or indirectly lead to the reduction of Co(III) and produce a cobalt phase having limited solubility.

In contrast, ferric iron may potentially serve to restrict the enzymatic reduction of uranyl. Microbial dissimilatory reduction of the highly soluble uranyl ion ( $\text{U}^{\text{VI}}$ ) to relatively insoluble uraninite ( $\text{UO}_2$ ) offers the potential for in situ stabilization. Uranium stabilization by this process is dependent on uranyl being used as the terminal electron acceptor (TEA) in microbial respiration. We examined the reduction of uranyl by BrY in the presence (and absence) of iron hydrous oxides. When cell suspensions of BrY were added to uranyl acetate, uranyl was rapidly removed from solution. Similarly, uranyl adsorbed on goethite underwent dramatic reduction with active BrY cells. In contrast, however, limited reduction was noted when ferrihydrite or amorphous ferric hydroxides were present in suspension. Our results demonstrate that alternate or competing electron acceptors present in soils may modify the reduction of toxic elements; they may either promote or retard the reduction of the target element. In either case, one must consider the specific site geochemistry when evaluating the potential for in-situ reduction of uranyl or chromate.

# Influence of Microbial Nitrate Reduction on Subsurface Iron Biogeochemistry and Contaminant Metal Mobilization

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Our objectives are to determine (1) the extent to which nitrate will inhibit microbial reduction of iron minerals; (2) the effect of mineral reduction on the mobility of representative toxic metals; and (3) the kinetics of nitrate-dependent, microbial oxidation of Fe(II). In the past year, we have examined (i) the effects of  $\text{NO}_3^-$  on microbial reduction of synthetic iron oxides, and the effect of iron reduction on the mobility of zinc which had previously been sorbed to the oxides; (ii) the effects of goethite on  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction; (iii) competitive interactions between nitrate and Fe(III) oxide reduction in enrichment cultures of natural sediment bacteria; (iv) microbially-catalyzed nitrate-dependent oxidation of solid-phase Fe(II) compounds at circumneutral pH; and (v)  $\text{Zn}^{2+}$  sorption properties of Fe(III) oxides generated via biological nitrate-dependent oxidation of aqueous Fe(II).

In batch experiments in an artificial groundwater medium containing 10 mM lactate, we previously showed that production of Fe(II) via microbial reduction of synthetic goethite and lepidocrocite is inhibited by the presence of  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . When Zn was adsorbed onto the surface of the goethite or lepidocrocite prior to inoculation with *S. putrefaciens*, the weakly bound Zn (soluble in cold, 0.5 N HCl) was sequestered within a strongly bound phase (soluble in 6 N HCl, but insoluble in 0.5 N HCl). Zn immobilization was not observed in cultures containing  $\text{NO}_3^-$  or  $\text{NO}_2^-$  in which  $\text{Fe}^{2+}$  production was inhibited. Current work is in progress to identify the biogenic minerals responsible for Zn incorporation, to determine if metals other than Zn behave similarly, and to ascertain if similar processes take place during reduction of natural sediments.

Recent work with slurries containing both  $\text{NO}_3^-$  and goethite have demonstrated that the presence of goethite also inhibited  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction. Based upon these results and the enhanced production of  $\text{N}_2\text{O}$  in the presence of goethite, we hypothesize that microbially generated  $\text{NO}_2^-$  and Fe(II) abiotically react to form Fe(III) and  $\text{N}_2\text{O}$ . The Fe(III) oxides precipitating on the cell surface can then potentially inhibit transport of  $\text{NO}_2^-$  within the cell. Experiments utilizing differences in  $^{15}\text{N}_2\text{O}$  to discriminate between biogenic  $\text{N}_2\text{O}$  and chemically generated  $\text{N}_2\text{O}$  are underway.

Studies with an autotrophic, circumneutral Fe(II)-oxidizing enrichment culture have demonstrated the potential for rapid, biologically-catalyzed, nitrate-dependent oxidation of solid-phase Fe(II) compounds generated via microbial reduction of amorphous Fe(III) oxide, goethite and two Fe(III) oxide-rich subsoils. Only very low concentrations (~ 15  $\mu\text{M}$ ) of nitrite were detected in most cultures, suggesting that Fe(II) oxidation was the result of direct enzymatic catalysis rather than abiotic oxidation of Fe(II) coupled to reduction of nitrite generated from the partial reduction of nitrate. Biological nitrate-dependent Fe(II) oxidation has the potential to generate large quantities of reactive Fe(III) oxide surfaces under anaerobic conditions, a phenomenon which has broad-ranging implications for the mobility of metals in saturated subsurface sediments.

We have also examined sorption of  $\text{Zn}^{2+}$  onto Fe(III) oxides generated via microbially-catalyzed nitrate-dependent oxidation of soluble Fe(II) in culture medium containing three different levels of inorganic phosphate (0.05, 0.5 or 1.5 mM). The presence of increasing levels of P led to formation of Fe(III) oxides which were more susceptible to dissolution in dilute (0.5M) HCl during short-term (1 hr) extractions. XRD studies and specific surface area measurements showed that the oxides formed under the different P conditions were all essentially amorphous and had similar specific surface areas (ca. 250  $\text{m}^2/\text{g}$ ). In addition, the different oxides had comparable  $\text{Zn}^{2+}$  sorption properties which were also similar to those determined for high-surface-area goethite preparations.

## The Immobilization of Radionuclides and Metals in the Subsurface by Sulfate-Reducing Bacteria

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This research is designed to evaluate biotechnological approaches to remediating subterranean environments contaminated with radionuclides and heavy metals. Our work specifically focuses on the ability of sulfate-reducing bacterial communities to immobilize U and heavy metal co-contaminants that may be present at selected DOE study sites. Experiments have been designed to (1) elucidate the mechanisms that anaerobes employ to immobilize these materials by reaction with biologically produced sulfide, and (2) evaluate the radionuclides or heavy metals as terminal electron acceptors for subsurface microorganisms. In either eventuality, the environmental risk associated with contaminant migration will be mitigated as the materials will be immobilized and/or precipitated and not easily transported in the subsurface.

This project will involve the pursuit of several objectives, including an evaluation of: (1) the factors influencing desirable immobilization bioconversions (i.e., both natural and co-contaminating electron donors and acceptors, and other site specific variables); (2) the spatial heterogeneity associated with the requisite microbial populations in the subsurface; and (3) the potential for the anaerobic reoxidation and consequent remobilization of the reduced products of the bioconversions.

Groundwater, alluvial floodplain sediments and Mancos shale samples were collected from the UMTRA site at Shiprock, N.M. The goal for this phase of the study was to determine which microbial activities were present in subsurface sediments and groundwater, and whether sulfate reduction activity could be stimulated with the ultimate goal of designing a bioremediation strategy based on the activity of sulfate reducing bacteria.

Populations of microorganisms were enumerated in sediment samples. Significant numbers of methanogens ( $3 \times 10^5$ ), sulfate- ( $3 \times 10^5$ ) and nitrate-reducing ( $2 \times 10^5$ ) bacteria were observed. Smaller numbers of acetogens ( $1 \times 10^4$ ), sulfide-oxidizing ( $2 \times 10^3$ ), Fe(III)-reducing ( $4 \times 10^1$ ), and anaerobic nitrate-dependent Fe(II) oxidizing ( $4 \times 10^2$ ) microorganisms were also present. Time course studies were carried out on sediment samples. Sediments (20 g) were incubated in serum bottles containing 30 ml of filter-sterilized anoxic groundwater under an atmosphere of  $N_2/CO_2$ . Nitrate was rapidly exhausted (within 4 days) in the incubations. After approximately 15 days, sulfate reduction became apparent based on a blackening of the sediments and a decrease in the dissolved sulfate concentration. Sulfate reduction continued over the course of the incubation, indicating that sulfate reduction and consequent metal immobilization was possible in these sediments.

A series of experiments was also performed analyzing for the effect of the presence of clay on sulfate reduction activity in sediments. A general inhibition effect was observed using source clays and commercial clays. Unwashed (but hydrated) clays displayed the most inhibition (4-fold to 65-fold), while washed clays were reduced (1.2-fold to 7.25-fold) in their effectiveness. The most inhibitory included barasym (40-fold) and bentonite (65-fold). Kaolin had a similar effect whether unwashed or washed (10.4 vs. 7.25-fold).



# Biogeochemistry of Technetium: Effects of Enzymatic Metal Reduction on Speciation and Potential for Transport and Remediation in Porous Subsurface Media

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Development of remedial measures for technetium (Tc) and other contaminants that are transported in aerobic groundwaters as oxyanions will require a fundamental understanding of the mechanisms of microbial and abiotic reduction and the factors controlling the effects of these processes on form and mobility. The effect of electron donor on the rate and extent of pertechnetate ion [Tc(VII)O<sub>4</sub>] reduction by the subsurface metal-reducing bacterium *Shewanella putrefaciens* CN32 was determined, and the aqueous and solid phase reduction products formed in the presence and absence of inorganic and organic complexing ligands (bicarbonate, DTPA, EDTA, citrate) were evaluated through a combination of high resolution transmission electron microscopy, X-ray absorption spectroscopy and thermodynamic calculations. The kinetics of these reactions were contrasted to Tc(VII) reduction by Fe(II)<sub>aq</sub> and Fe(II) sorbed on goethite.

When H<sub>2</sub> served as the electron donor, dissolved Tc(VII) was rapidly reduced to amorphous Tc(IV) hydrous oxide, which was largely associated with the cell periplasm and the outer cell surface in unbuffered 0.85% NaCl and with extracellular particulates (0.2 – 0.001 μm) in bicarbonate buffer. The reduction rate was much lower when lactate was the electron donor, with extracellular Tc(IV) hydrous oxide also the dominant solid-phase reduction product. In bicarbonate systems, soluble (< 0.001 μm) electronegative Tc(IV) carbonate complexes were also formed that exceeded Tc(VII) in electrophoretic mobility. Thermodynamic calculations indicated that NaCl solutions without complexing ligands were oversaturated with respect to Tc(IV) hydrous oxide which would be present from pH 4 to 9 and at Eh values <50 mV, and that negatively-charged aqueous Tc(IV) carbonate species would dominate in carbonate solutions from pH 5.5 to 10.5 and at Eh values <200 mV. Thus, carbonate complexes may represent an important pathway for Tc transport in anaerobic subsurface environments where it has generally been assumed that Tc mobility is controlled by low solubility Tc(IV) hydrous oxide and adsorptive, aqueous Tc(IV) hydrolysis products. The presence of organic complexing ligands during microbial reduction of Tc(VII) in NaCl resulted in the formation of aqueous complexes of Tc(IV,V) and investigations are underway to further define their chemical speciation and potential for transport in groundwaters.

In abiotic experiments, Tc(VII) was reduced slowly in the presence of Fe(II) in water, but was rapidly reduced to Tc(IV) by Fe(II) sorbed on goethite. At higher sorbed Fe(II) concentrations (near saturation), the rate of Tc(VII) reduction exceeded direct microbial reduction even with H<sub>2</sub> as the electron donor, suggesting that sorbed Fe(II) resulting from biogenic processes may be an important reducing agent for Tc(VII) in groundwater systems. The effective redox potential and kinetics of electron transfer for biogenic Fe(II) will depend specifically on the chemical/mineralogic environment in which the Fe(II) resides. Investigations are underway to determine the relative importance of direct and indirect reductive processes for Tc(VII) in static and advective experimental systems.

## Solubilization of Radionuclides and Metals by Iron Reducing Bacteria

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Iron oxides are ubiquitous mineralogic components of vadose zone and aquifer sediments, typically existing as particle coatings and intergrain cements. Fe(III) oxides are reactive, act to buffer subsurface redox capacity, and are strong sorbents of metallic and radionuclide contaminants. Iron oxides serve as a major in-ground repository of contaminants on DOE lands. Ascertaining the long term stability of contaminant-Fe(III) oxide associations and devising means to effectively extract sorbed contaminants are important scientific needs for DOE site remediation and closure that are being investigated by this project.

Research is investigating hydrogeochemical controls on the activity of dissimilatory iron-reducing bacteria (DIRB) in subsurface sediments and the potential of DIRB for solubilizing sorbed metals associated with Fe(III) oxides under anoxic conditions. The project focuses on bacterial ferrous iron mineralization and the impact of biomineralization on redox buffering capacity and contaminant solubility. Batch and stirred-flow reactors with synthetic and subsurface materials are inoculated with DIRB to investigate how different C-sources and e-donors, interfacial chemical reactions and water advection meter the activity of DIRB. The reductive solubilization of sorbed metals by DIRB is investigated using synthetic and subsurface materials spiked and aged with important DOE contaminants [Ni(II), Hg(II), Co(II/III) and Eu(III)], and a contaminated subsurface material. A novel biogeochemical modeling approach is used for experiment interpretation whereby surface and aqueous complexation reactions and precipitation are linked with Monod kinetics and water transport. Mossbauer spectroscopy, X-ray adsorption spectroscopy and other techniques are used to characterize the chemical and mineralogic nature of the Fe(III) oxides, biomineralization products and biogenic contaminant host phases as a basis for chemical model development.

In the first project year we have shown that: (1) hematite and goethite in subsurface sediments are more bioavailable (to DIRB) than synthetic goethite and hematite because of crystallite disorder and surface heterogeneities, and (2) Co(III) and Ni(II) can be solubilized from crystalline Fe(III) oxides by DIRB in mole fraction excess to iron leading to their net depletion from the oxide phase. In the second year, we have investigated the bioreduction of fresh, aged and heated hydrous ferric oxide (HFO) spiked with Ni and Co. Aging and heating produces a poly-phase Fe(III) oxide association much like those found in subsurface materials. Using a combination of Mossbauer spectroscopy and high resolution electron microscopies, we have found that sorbed contaminants inhibit the crystallization of HFO, alter bioreduction through a combination of physiologic and surface chemical effects, and direct the formation of different biomineralization suites, some of which are highly effective at contaminant capture through coprecipitation. We have determined biogeochemical conditions favoring both mobilization and immobilization of contaminants and are now linking geochemical and microbiologic modeling to quantitatively describe these processes.

# **PROGRAM ELEMENT 5**

## **Assessment**

# Antibodies and Antibody-Based Sensors for Hexavalent Uranium and Chelators

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The development of sensors that use antibodies as the recognition element appeals to a large number of potential end-users, since these devices may be used to monitor a very broad range of analytes. The variety of molecules that can be quantified by immunosensors is virtually unlimited, and depends primarily upon the binding affinities and specificities of the antibodies incorporated into the devices. The high sensitivity and selectivity of such sensors makes them attractive for situations where both speed and accuracy are required. Previous studies from our laboratories have demonstrated the feasibility of isolating monoclonal antibodies that recognize specific metal ions. The goals during the current grant period are to (1) isolate and characterize antibodies that recognize the most mobile form of uranium,  $\text{UO}_2^{2+}$ ; (2) assemble, test and validate a new field-portable immunosensor based on these antibodies and a hand-held flow fluorimeter; and (3) prepare new monoclonal antibodies to the primary chelators (EDTA and DTPA) found in DOE wastes.

Three hybridoma cell lines have been generated that synthesize and secrete monoclonal antibodies that bind tightly and specifically to  $\text{UO}_2^{2+}$  complexed to 2,9-dicarboxyl-1,10-phenanthroline (DCP). Cloning and sequencing of the cDNAs that code for the light and heavy chain variable regions of these antibodies demonstrated that all three have distinct binding sites. Two antibodies (8A11 and 12F6) were selected for further studies, based upon their affinity for the  $\text{UO}_2^{2+}$ -DCP complex and their resistance to changes in pH and ionic strength.

A prototype competitive immunoassay for  $\text{UO}_2^{2+}$  was developed that accurately monitored  $\text{UO}_2^{2+}$  at concentrations from 10 to 120 nM in buffers amended with 12.5  $\mu\text{M}$  DCP. These assays were conducted on the KinExA<sup>TM</sup>, a semi-automated flow fluorimeter designed for sophisticated studies in the laboratory. As part of an effort to adapt this and related immunoassays for use in the field, an experimental version of a field-portable KinExA flow fluorimeter was assembled for testing purposes. Issues currently under investigation using this *alpha* unit include: (1) the effects of flow rates of liquid reagents on the accuracy and sensitivity of the immunoassay; (2) the necessity for pre-treatment of the sample prior to analysis; (3) practical limits on the volume of sample to be introduced into the field instrument; (4) the effects of lyophilization and reconstitution of disposable reagents on the performance characteristics of the assay. It is anticipated that these studies could generate a useful marketable product, a collection of portable field tests for uranium and related wastes that could be exploited both in government and private sectors.

# Artificial Neural Networks as a tool for the Assessment of Microbial Communities

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Microbial communities in soils can be characterized by the analysis of various biomarkers such as terminal restriction fragment length polymorphisms (T-RFLPs) and signature lipids. Biomarkers may be useful in assessing microbial communities during in-situ bioremediation since they have been shown to change in response to environmental conditions. However, the changes in biomarkers are often complex, nonlinear and not readily amendable to traditional statistical analyses.

One of the objectives of our research is to develop new data analysis techniques that can help in assessing microbial community structure from T-RFLPs and other biomarkers. We are evaluating artificial neural networks (ANNs) as a tool for relating changes in microbial T-RFLP patterns to the concentration of heavy metals. ANNs are nonlinear, nonparametric analysis methods that can learn from experience to improve their performance.

We tested ANNs on T-RFLP data obtained by T.L. Marsh and his colleagues from an abandoned tannery located in the upper peninsula of Michigan. Soil samples were subjected to chromium and T-RFLP analyses as described by Marsh et al. in this and previous proceedings. The abundance of the T-RFLP fragments were converted to a sample proportion, and those fragments with a maximum proportion of one percent or less were removed from the data set. The final data set of 51 samples was used to construct bootstrap training sets (41 samples) and a validation sets (10 samples) for subsequent data analysis.

Several feed-forward architectures were tested by training bootstrap samples with 88 input variables (T-RFLP fragments) and a single output variable (chromium). Generalization performance of these networks was unsatisfactory, and we selected a subset of 25 fragment sizes for subsequent training. The most robust architecture with a limited number of inputs contained seven hidden nodes. One hundred realizations of this ANN architecture were tested. For each realization, we trained the ANN using a cross-validated early stopping procedure that terminated training prior to convergence when the error in the validation set no longer decreased. The best ANN explained more than 98% of the variance in the training data and more than 95% of the variance in the validation data set.

We are currently conducting sensitivity analyses to identify those T-RFLP fragments most strongly related to chromium concentrations. We are also exploring the use of autoassociative ANNs as a means of reducing the number of T-RFLP fragments prior to use in the predictive analysis.

# Coupled Use of DNA Microarrays, Voltammetry and X-Ray Studies for Profiling Changes in Microbial Community Structure and Metal Speciation in Response to Metal Contamination

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The objectives of this project are to develop and apply DNA array technology to profile and monitor microbial communities through time in process-level microcosms and naturally contaminated sediments treated with increasing Cr, Pb and/or Zn concentrations, and correlate microbial community structure to changes in metal speciation and mobility. The central hypotheses relate to: (1) the sensitivity and specificity of tunable surface microarrays relative to standard hybridization chemistry; (2) the biochemical process associated with metal speciation in artificial mesocosms and natural environments; and (3) changes in microbial community composition in response to increasing metal contamination.

Natural sediments (ca.  $10^9$  cells  $g^{-1}$ ) were obtained from three sites in Lake DePue, Ill., which contains substantial zinc, copper, cadmium, lead and arsenic. Three sites were characterized and used to establish microcosms, ranging from 50 ppm to 30% Zn. Methanogenic archaea and sulfate-reducing bacteria were quantified by MPN enrichments, and methanogens, iron-reducers and heterotrophic anaerobes isolated using acetate as an electron donor. Microbial biomass was significantly higher at the more contaminated site. Isolates are currently being evaluated for growth and Zn metabolism at different Zn concentrations, with the intent to compare biologically-governed Zn speciation in monoculture to Zn speciation observed in Lake DePue sediments.

Mesocosms designed to select for metal-reducing bacteria or methanogens were established and monitored for pH, acetate, metal concentrations (Zn and Mn) and headspace gasses (carbon dioxide, methane and hydrogen), with weekly samples taken for DAPI counts and DNA analysis. Differences in acetate consumption and methane generation were observed between sediment microcosms. X-ray absorption spectroscopy of bioreactor solids was used to investigate changes in Zn and Mn speciation due to Zn and Mn amendments. Total nucleic acids are currently being extracted from mesocosm sediments for analysis by T-RLFP to compare changes in microbial community structure with Zn addition. Predominant, novel or unexpected T-RFLP signatures will be cloned and sequenced to identify species-specific probes for a 'metal reducer' microarray.

Microarray research during year one focused on specific technical challenges associated with the *direct* detection (i.e., no PCR amplification) of full-length rRNA targets relative to PCR-amplified, functional gene targets. Surface chemistry and probe attachment methods, target labeling and detection strategies, and novel solution conditions were evaluated to achieve specific and reproducible hybridization of *Geobacter chapelleii* 16S rRNA to universal and species-specific 16S rRNA probes. The specific detection and allelic discrimination of PCR-amplified, functional gene targets from an *E. coli* model system was very successful, regardless of probe attachment chemistry, labeling or detection strategy. However, the specific, reproducible hybridization of full-length 16S rRNA or rDNA targets to a 2-dimensional (versus 3-dimensional membrane or gel-pad) microarray remains a significant technical challenge. Sandwich hybridization and chaperone systems have been developed and compared to direct chemical labeling reporter systems. Short rRNA fragments (ca. 300 bp) hybridized with greater signal intensity than full-length (1500 bp) products, although both size fragments generate anemic signals relative to similar functional gene target assays. We postulate that the high degree of secondary structure, large target fragments, steric constraints at a two-dimensional surface and limited range of ionic strengths amenable to DNA:DNA hybridizations may limit the efficacy of DNA probes in a 16S rRNA microarray. To address these possible limitations of DNA probes for community-level 16S rRNA analysis, we are evaluating peptide nucleic acid probes under low-salt, high temperature buffer conditions to overcome steric constraints associated with large, 16S rRNA targets.

## Rapid Gene Probe for Microorganism Monitoring by Novel MS Approaches

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The goal of this work is to develop new assessment technologies by using laser desorption mass spectrometry (LDMS) for microbial community analysis. The approach involves technology developments and the application of innovative mass spectrometry technologies for microbial DNA analysis. For technology development, we have achieved the following major tasks: (1) initiation, design, installation and test of the idea of laser induced acoustic desorption; (2) demonstration of hybridization probes detected by LDMS; and (3) demonstration of direct DNA sequencing by LDMS with selective fragmentation. For microbial DNA analysis, we have demonstrated: (1) evaluation of mass spectrometry based detection methods for analyzing the RFLP patterns of 16S rRNA genes amplified from microbial communities; (2) development of quantitative PCR methods and coupling with LDMS for measuring copper nitrite genes in environmental samples; and (3) development of methods for simultaneous extraction of RNA and DNA from soil samples.

The principle of laser induced acoustic desorption is based on the shaking force for biomolecule desorption. Since no direct absorption of laser photons to raise the temperature of substrate or matrix molecules occurs, soft desorption can be achieved. We recently obtained mass spectra with better mass resolution by laser induced acoustic desorption compared to matrix-assisted laser desorption/ionization. At present, most hybridization to probe DNA sequence is pursued as one hybridization reaction per site. The hybridization probed needs to be tagged with either radioactive material or fluorescent dye. With mass spectrometry for DNA probe detection, more than one reaction per site can be pursued. We have demonstrated the detection of multi probes on a single hybridization site by mass spectrometry. Since thousands of probes can be used for hybridization on a single chip, it is important to have a quick and reliable method to sequence short DNA probes. For short ss-DNA probes, it is extremely difficult to use Sanger's enzymatic method to prepare DNA ladders for sequencing. We have developed the selective fragmentation for sequencing DNA probes. Future work will be concentrated on the mass resolution improvement for laser-induced acoustic desorption, increase of multiplexing on hybridization detection and direct sequencing of probes on chips.

The size of the intergenic regions between 16S and 23S ribosome genes varies among different bacterial species. We measured the replicated DNA products in this region with the size of ~1600 bp, which is the largest DNA fragments detected by ultraviolet laser desorption. We further measured RFLP to demonstrate that mass spectrometry technology can be used for microbial population determination by the patterns of RFLP. Future effort will be placed on the demonstration of various probes designed to characterize microbial populations in soil and sediment samples.

To examine the potential for mass spectrometric detection in quantitative PCR assays, we developed primers directed to the eubacterial glutamine synthetase (EGS) gene. PCR primers were designed to recognize positions that exhibit the least divergence (highest similarity) among individual EGS DNA sequences in a multiple sequence alignment. This approach allowed us to design PCR primers that generate a 153 or 156 bp product from representatives of a maximum range of evolutionary divergence. Tests have demonstrated that a 174 bp internal standard molecule yields a quantitative result based on assays using known copy numbers of *Escherichia coli* DNA. We are currently in the process of comparing quantification results based on gel electrophoresis with results obtained using mass spectrometry.

# Development and Evaluation of Stable Isotope and Fluorescent Labeling and Detection Methodologies for Tracking Injected Bacteria During In-Situ Bioremediation

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The goal of this research is to develop new methods for tracking bacteria in the subsurface. Methods to label bacteria with a variety of fluorescent dyes will be tested. The effects of each dye on cell culturability and adhesion to sediment, as well as the stability and longevity of the fluorescently-labeled bacteria in microcosms, will be assessed. The level of detection for fluorescently-labeled cells using microplate spectrofluorimetry will be determined. The fluorescent tracking method will be evaluated during both transport experiments using intact sediment cores and in the field, and compared with several other detection/enumeration methods.

Additionally, tracking methods involving the analysis of stable carbon isotopes incorporated into cells will be examined. Bacteria will be grown on <sup>13</sup>C-only substrates to achieve very highly <sup>13</sup>C-enriched cells. Standardization between the number of cells and the amount of <sup>13</sup>C in membrane fatty acids, determined by either gas chromatography/chemical reaction interface mass spectrometry (GC-CRIMS) or high performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS), will be performed. The stable isotope tracking method will be evaluated during transport experiments using intact sediment cores and in the field, and compared with several other detection/enumeration methods.

Once developed, these methods will allow the movement of bacteria injected during in situ bioremediation to be accurately and easily assessed, thus improving the overall effectiveness of bioaugmentation efforts.

During the past year, the fluorescent tracking method has undergone extensive evaluation. A green fluorescent compound, 5-(and-6-)carboxyfluorescein diacetate, succinimidyl ester (CFDA/SE), was shown to stain bacteria without significant effects on cell viability or adhesion to sediment. The stained cells remained fluorescent for at least 21 days in both groundwater and sediment microcosms. CFDA-stained cells were quantifiable by epifluorescent microscopy, flow cytometry and microplate spectrofluorimetry. Optimization of the microplate enumeration method resulted in a lower detection limit of approximately 10<sup>5</sup> CFDA-stained cells per well. Cell concentrations in the effluent of intact cores during bacterial transport experiments, as determined by viable plate counts, scintillation counting, direct microscopic counts of CFDA-stained cells, and microplate enumeration, were all similar. Evaluation of the fluorescent tracking method during a field-scale bacterial transport experiment was also performed, with near real-time measurement of the cell concentrations in individual samples using the microplate reader.

Work on the CRIMS stable isotope tracking method has been limited by difficulties with the prototype instrument. However, an alternative stable isotope method using HPLC/ESI/MS is being pursued in collaboration with D.C. White (Univ. of Tennessee). Intact core and field-scale bacterial transport experiments using <sup>13</sup>C-labeled cells have been performed, and samples are currently being analyzed.



# **An In-Situ Tracer Method for Establishing the Presence and Predicting the Activity of Heavy Metal Reducing Microbes in the Subsurface**

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The objective of this study is to establish nondestructive in-situ tracer methods for detecting the presence, distribution and activity of subsurface heavy-metal-reducing microorganisms.

Research efforts have focused on the critical areas of: (1) the identification and characterization of potential biotracers; (2) the development of mathematical models; and (3) the development of methods to extend biotracer technologies to the field.

With regard to the first effort, of identifying and characterizing potential biotracers, a series of bacterial enrichment cultures, batch experiments and flow-through column studies were initiated to identify electron donor-acceptor systems that would likely lead to efficient reduction of chromium and iron under anaerobic conditions in a test soil. The ultimate goal of these studies was to identify electron donors and/or electron accepting systems that would serve as indicators of Cr(VI)-reducing activity in this soil. It was thought that electron donors leading to Fe(III) and Cr(VI) reduction might be candidates for reactive biotracers indicative of Cr(VI)-reducing activity, and that electron accepting systems might prove useful as conservative tracers of these processes. Recent research efforts have examined anthraquinone disulfonate (AQDS) as a conservative electron acceptor and possibly an electron shuttle. Preliminary experiments have shown the reduced form of AQDS, anthrohydroquinone disulfonate (AHQDS), reduces Cr(VI) and Fe(III). Thus, AQDS/AHQDS shuttle has been identified as a potential system of biotracers for iron- and chromium-reducing activity.

Data from the first effort are immediately used in the second to development models that will serve as tools to characterize subsurface microbial distribution and activity through the fate and transport of biotracers. Modeling efforts have focused on the developing analytical and numerical multi-component batch reactor models and a 3-dimensional multi-component finite element transport model. Numerical methods originally developed for large-scale atmospheric transport models have been applied here to solve systems of nonlinear reactive transport equations. Data generated from batch and column experiments have been used to formulate these models with appropriate stoichiometric and kinetic relationships.

The third and final area of focus is a recently initiated effort to address issues pertinent to extending biotracer technologies to potential field applications. Research is under way to develop in-situ methods for measuring water, tracer and chromium fluxes in the subsurface. At a minimum, the technology will quantify changes in tracer mass in 3-dimensional transient flow field. Preliminary laboratory tests show evidence of success; however, a field test has not been performed.

## SIMS for Direct Interrogation of Microbe/Mineral Interfaces

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The goal of this work is to evaluate static secondary ion mass spectrometry (SIMS) as a tool for direct assessment of microbial populations at mineral surfaces. Because SIMS is a sensitive, surface analysis technique, it has the potential to directly interrogate interfacial interactions between microorganism and the mineral substrate. A number of controlled microbial samples have been characterized; this benchmark research is leading toward the microbial surface characterization of sediments found at the Uranium Mill Tailings Remedial Action (UMTRA) site near Shiprock, N.M.

The basis for our approach is to use static SIMS to probe phospholipid fatty acids and other biomolecules associated with the cell membrane of intact microorganisms. We hypothesize that since static SIMS probes only the top layers of the sample surface, it could be used to collect unique mass spectral signatures of the cell membrane chemistry of a microorganism by analyzing intact cells (no sample preparation). In order to test this hypothesis, SIMS spectra of >50 microorganisms were collected and the results were compared to a standard method for probing the cell membrane chemistry (Microbial Identification System, MIS). The mass spectral results from the SIMS analyses showed marked differences in spectral features.

Comparing the SIMS results to the fatty acid profiles generated by MIS, many of the fatty acids were identified on the basis of specific anions observed in the SIMS data. By applying principal component analysis to the SIMS data, microbes having similar phospholipid compositions could be statistically grouped.

A second approach for microbe identification is to utilize mass spectrometry/mass spectrometry (MS/MS) to detect specific biomarker molecules which are contained in the cell membrane. Results from the early stages of this research will be reported as part of this presentation.

Currently, we are investigating detection limits and how specific microorganisms can be typed (groups, species, subspecies) by SIMS. We are also investigating isolates collected from the Shiprock UMTRA site, and plan to discuss those results as part of this presentation.

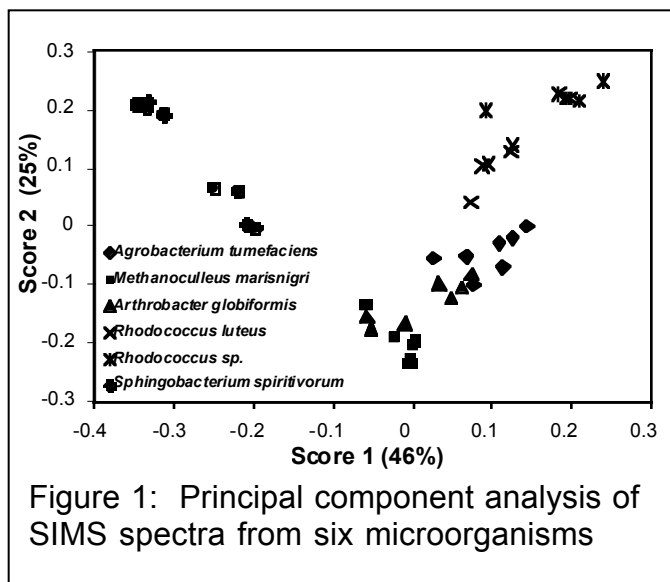


Figure 1: Principal component analysis of SIMS spectra from six microorganisms

## In-Situ Determination of Microbial Metabolic Activity

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The goal of this project is to develop and apply the single-well, “push-pull” test method for measuring in-situ rates of microbial metabolic activity in the subsurface. Activities are measured as the rate of transformation of an injected substrate to a specific product. Push-pull test assays are being developed and field tested to: (1) estimate the size of the metabolically active microbial biomass, and (2) quantify rates of  $\text{SO}_4^{2-}$  and Fe(III)- reduction, at metals-contaminated groundwater aquifers within the Department of Energy complex.

To estimate microbial biomass, assays were developed to measure activity expressed by a broad spectrum of subsurface microorganisms, including those that express aerobic respiration, glucosidase and phosphatase activity, and hydrogen utilization. Laboratory and field experiments were conducted to examine the correlation between activity measured with the push-pull test and independent measures of biomass (e.g., lipid-bound phosphate) obtained for groundwater and sediment. As part of this work, push-pull tests were designed to obtain in-situ estimates for Michaelis-Menton kinetic parameters that describe the transformation of substrate to product. Experiments were conducted at non-DOE sites as well as UMTRA sites, including Shiprock, N.M., and Gunnison, Colo. Field push-pull assays will be used to monitor changes in biomass that occur following growth-substrate addition. In collaboration with other NABIR investigators, an extensive series of field tests was conducted to quantify rates of sulfate reduction in the presence and absence of exogenous electron donors.

## Microbiological and Biogeochemical Characteristics of Subsurface Sediments at Uranium Mill Tailings Sites at Gunnison, Col., and Shiprock, N.M.

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Uranium Mill Tailings Remedial Action (UMTRA) sites provide an opportunity to study the microbiology and biogeochemistry of field sites contaminated with metals and radionuclides. Subsurface sediments at UMTRA sites have been in contact with contaminants for 30 to 50 years, a time period comparable to that for contaminants at DOE weapons complex sites. Contaminants include a wide range of metals dominated by uranium. Other anthropogenic solutes include nitrate, ammonium and sulfate. Given these characteristics, DOE's NABIR Program is collaborating with the UMTRA Program to study selected UMTRA sites.

The objectives of this research are to: (1) determine the dominant electron accepting processes at sites with long-term metal contamination, and (2) define the biogeochemical transformations that may be important to either natural or accelerated bioremediation. Sampling of sediments and groundwater has been completed at Shiprock, N.M., and Gunnison, Colo. Preliminary results for Gunnison indicate: (1) low to moderate microbial activity (based on p-nitrophenol production rates), and (2) enrichment of viable sulfate-reducing, nitrate-reducing and Fe(III)-reducing bacteria. A peak in sulfide, acetate and p-nitrophenol activity in a single sample suggests that microbial activity is relatively heterogeneous at this site.

Preliminary results for the Shiprock site include: (1) phospholipid fatty acids ranging from 50 to 200 picog/g sediment, indicating the presence of a diverse, active microbial community, including SRBs and actinomycetes; (2) viable anaerobic bacteria, including NRB, SRB and methanogens in flood plain sediments; (3) low levels of Fe-oxidizing bacteria that use nitrate as an electron acceptor under anaerobic conditions; (4) flood plain sediments also exhibit U-reducing bacteria but none in shale samples; (5) DGGE results from groundwater filtrate demonstrate a diverse microbial community with representatives from most functional groups occurring in samples from a single well. These results suggest the importance of biogeochemical processes in the natural attenuation of uranium in alluvial sediments, particularly at the Shiprock UMTRA site. They also suggest that indigenous microorganisms present at both sites could contribute to in-situ stabilization of U via reduction to insoluble U(IV) species.

# Core-Scale Interrogation of Permeability and Geochemical Heterogeneity for Assessment of Bioremediation Effectiveness

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Quantitative, field-scale understanding of reactions between microbes and natural porous media is critical to solving many contemporary subsurface environmental problems. Because these reactions occur at water-mineral-cell interfaces and are strongly controlled by local biogeochemical conditions, knowledge of small-scale variations (heterogeneity) in natural porous media properties and their net effect on field-scale transport is needed. However, small-scale heterogeneity of physical properties such as permeability and porosity combines with that of biogeochemical properties to give rise to complex behaviors that are difficult to quantify at relevant field scales. Detailed descriptions of small-scale heterogeneity and observations of their relationship to bacterial attachment are needed to form a defensible foundation for quantitative modeling and theoretical developments.

Progress has been made on integration of a number of innovative, core-scale imaging technologies which will significantly enhance detailed assessment of physical and biogeochemical heterogeneity at sub-core scales. The technologies used have been applied, in varying degrees, to geological characterization problems, but have not been integrated and applied to quantify joint physical and biogeochemical core- and outcrop-scale heterogeneity. Basic issues to be addressed by this research include: (1) the interpretability of mineral abundance in natural porous media from spectral response of sediments; (2) relationships among observations of physical properties (especially permeability) at several scales; (3) the significance of preferential flow paths in microbial transport and attachment; and (4) determination of optimal moisture contents for estimation of permeability using air mini-permeameters and infrared imaging methods.

Significant progress has been made in developing a detailed dataset describing millimeter- to centimeter-scale joint physical and biogeochemical heterogeneity. Specifically, we have collected ultrasensitive IR images (256x256 pixels), high-resolution color scanner images and air permeability data of both halves of a core from Oyster, Va., used for a bacterial transport experiment. The microorganism used in the transport experiment was *Comamonas* sp. DA001 (Fuller et al., 1999; DeFlaun et al., 1990). We are currently in the process of obtaining 20-um resolution x-ray microtomography (XMT) data on core segments and 3-um resolution synchrotron light source XMT on subcores. When the physical parameter measurements are integrated with microbial distribution and transport data for the same core, we expect to identify specific controls on microbial attachment in heterogeneous porous media. These results will fill a key gap in the knowledge required for assessment of in-situ field-scale bioremediation.

# Spatial Heterogeneity of Microbial Iron Reduction Potential at the South Oyster Focus Area

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<sup>3</sup>Lawrence Berkeley National Laboratory, Berkeley, Calif.

We are addressing the spatial distribution of subsurface microbial iron reducers at the field-scale, which will provide significant information required to understand and predict the effect of heterogeneity on the reduction and immobilization of radionuclides and metals by iron-reducing bacteria. In our initial research we have intensively sampled three boreholes at the South Oyster Focus Area, a DOE/NABIR analog site near Oyster, Va. The samples are being analyzed for their microbial iron reduction potential (MIRP) using a low-cost batch measurement method. We have also measured the extractable iron oxyhydroxide content, the hydraulic conductivity, the bulk density, the concentration of organic matter, and the grain size of the samples. We will also determine the detailed mineralogy of the solid mineral phases present for a subset of the samples which are representative of the different biogeochemical sediment types detected in the boreholes. High-resolution crosshole seismic data was recorded between each pair of boreholes.

Preliminary results indicate that the heterogeneity of the site is more pronounced than that of other DOE-sampled locations in the Oyster area, all of which were dominated by sandy sediments. The sediments at the site include fine-grained lagoonal and back-bay sediments as well as sand-rich sediment layers. The fine-grained sediments include both black organic-rich peat layers and light to medium gray clay beds that contain relatively high concentrations of extractable Fe(II) and organic matter. Three distinct types of sand layers appear to be present at the field site, based on differences in the presence of MIRP, the hydraulic conductivity and the concentration of extractable Fe(III).

The variations in physical properties of the sediment layers appear to be traceable on geophysical data recorded at the site, which should make it possible to predict the sediment types between the boreholes. Geostatistical analysis will be used to predict the levels of MIRP between the existing boreholes using stochastic simulation techniques that integrate the MIRP data with geological, geochemical and geophysical data. Further drilling and sampling will then test the predictions. The research will provide a model for the distribution of microbial iron reduction potential on the Atlantic Coastal Plain, and a methodology that can be applied to develop similar models for other locations.

# **In Situ Assessment of Effective Reactive Surface Area of Chemically Heterogeneous Porous Media**

*Robert W. Smith*

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Idaho Falls, Idaho*

The relationship between effective reactive surface area (i.e., the surface area that reacts with locally advected solutes) of heterogeneous porous media and advective groundwater will be evaluated using reactive tracer experiments on cores collected at Oyster and Abbott's Pit. Inverse reactive transport modeling techniques relating tracer breakthrough to effective reactive surface area will be developed. Research results will provide a validated physicochemical scaling approach to assess the role of variable reactive surface area for field-scale contaminant and bacterial transport.

The characteristics and amount of reactive surface areas for subsurface materials have long been recognized as key to controlling the adsorption of contaminants (e.g., metals, radionuclides, organic ligands) and dissolved constituents (e.g., electron donors/acceptors, nutrients) required by subsurface microorganisms. In addition, the activity of (e.g., FIRB) and the retention of (bacterial transport) bacteria in subsurface are also influenced by the reactive surface area of subsurface materials. In water-saturated homogeneous systems devoid of advective fluxes (e.g., batch experiments), the surface area available for reaction is similar to the surface area (as measured by conventional means) of the subsurface media. However, in physically and geochemically heterogeneous systems with advective fluxes, the effective reactive surface area is smaller than the laboratory measured surface area and is a complex function of advective velocity, which is in turn a complex function of the correlation structures of the physical and chemical heterogeneities.

We propose to investigate the coupled relationships of small-scale (e.g., microfractures or sedimentary laminae) geochemical heterogeneity (spatial distribution of reactive minerals) in the presence of nonuniform groundwater flow and the effective reactive surface area of intact weakly consolidated sedimentary media. The experimental focus of the proposed research will be the use of cationic and anionic reactive tracers to estimate effective reactive surface areas in repacked columns with controlled heterogeneity of hydrous metal oxide coated sands, and intact core of from the South Oyster Site (Oyster, Va.) and Abbott's Pit, (Mappsville, Va). The selection of tracers and approaches will be done so that intermediate-scale (IS) reactive tracer experiments can be conducted using Oyster site materials in the latter part of the second year of the project (FY 2000). This research builds upon our extensive investigations of geochemically heterogeneity at the Oyster sites and will expand our previous studies of correlation structure to assess the effects of this structure on reactive transport of contaminants and bacteria.

# **PROGRAM ELEMENT 6**

## **Bacterial Transport**



# Top-Down Controls on Growth and Transport of Groundwater Bacteria From a Coastal Plain Aquifer

Fred C. Dobbs

Department of Ocean, Earth and Atmospheric Sciences, Old Dominion University, Norfolk, Va.

The overall research goal is to examine the importance of protozoan predation and viral lysis, processes effecting so-called “top-down control,” on the abundance and growth of groundwater bacteria at the DOE study site in South Oyster, Va. These findings will contribute to our increased understanding of bacterial transport within aquifers. The specific objectives are: (1) to quantify and characterize the indigenous microbial communities in both South Oyster groundwater and sediment samples; (2) in so doing, to provide a baseline for assessing the impact of transport experiments on the indigenous microbial community; (3) to quantify protozoan bacterivory of injected bacteria in South Oyster intact cores (with M. DeFlaun and M. Fuller, Envirogen, Inc.); (4) to assess the community response of protozoans to injections of aerobic bacteria into the South Oyster flowfield; (5) to quantify protozoan predation and viral lysis of groundwater bacteria in the aerobic portion of the South Oyster aquifer; (6) to compare bacterial community loss rates caused by viral lysis with those visited upon bacteria by protozoans; (7) to assess the ecological significance of protozoan predation and viral lysis through comparison with bacterial production. The methods used to study these processes will include a series of experimental manipulations on groundwater and sediment samples collected from the study site. Whole-core experiments will be performed as well. An initial emphasis will be placed on assessing top-down control of free-living bacteria, with the intent of switching to a focus on particle-attached bacteria in the latter half of the study (2001-2002).

Study results are as follows:

- The study area (South Oyster, Va.) is characterized by low microbial biomass. Protozoans (principally flagellates) ranged in abundance from 10 to 100 cells/g of sediment and from 1 to  $8 \times 10^3$  cells/liter of groundwater. Bacterial densities varied between  $<1$  to  $10 \times 10^6$  cells/g of sediment and from  $<0.5$  to  $1.5 \times 10^8$  cells/liter of groundwater. Finally, virus-like particles (VLPs) were present in densities between 0.2 to  $1.2 \times 10^6$  per g of sediment and about  $4 \times 10^8$  per liter of groundwater. For each microbial taxon, there were differences in abundance, and for protozoans—in composition, between and within the two study sites.
- The aquifer exhibits high concentrations of organic carbon and nitrate; laboratory experiments indicate the bacterial community is phosphorus-limited.
- In laboratory experiments, protozoans' uptake and clearance of bacteria were  $2.69 \text{ nl protist}^{-1} \text{ h}^{-1}$  (bacterial cell volume *ca.*  $0.23 \mu\text{m}^3$ ) and  $30.9 \text{ cells protist}^{-1} \text{ h}^{-1}$ , respectively. These rates would remove 0.5 to 2.2% of bacterial standing stock daily at the study site.
- In a field experiment ongoing at the time this abstract was submitted, we were following the time-course response of groundwater protozoans and VLPs following the injection of *Comamonas* into the flow-cell system described by Onstott et al. elsewhere in this compendium.

## Ferrographic Tracking of Bacterial Transport at Oyster, Virginia

*William P. Johnson*

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Studies investigating enhancement of bioaugmentation require novel methods to track bacterial concentrations. Tracking techniques used to monitor the concentration bacteria added to groundwater must allow selective identification of the particular microbe added to the system. In addition, high-resolution counting (e.g., quantitation down to 100 cells/mL or less) is required due to the dramatic decrease (exponential) in suspended cell concentration with distance from the injection point. Furthermore, significant information regarding the kinetics of bacterial attachment and detachment can be determined from examination of cell concentrations many orders of magnitude below the injected concentration.

Ferrographic enumeration, an innovative technique recently developed for tracking bacterial concentration, is being applied to bioaugmentation studies at the Oyster, Va., field site. The technique employs immunomagnetic tagging and ferrographic separation. The technique provides selectivity due to reliance on antibody-antigen recognition to magnetically tag the bacteria of interest. It also provides high-resolution enumeration (enumeration down to  $\sim 10$  cells/mL<sup>-1</sup> for a 1 mL sample) due to deposition of the magnetically-tagged bacteria onto an exceedingly small area on a glass slide for visual identification under an epifluorescence microscope. The method is relatively inexpensive and rapid. Visual identification of the bacteria provides information on the relative shapes, sizes and possible aggregation of bacteria that may be important characteristics in their transport behavior.

Ferrographic enumeration of bacterial concentrations was performed during recent laboratory (intact and packed core) experiments conducted with Oyster site materials, and during field transport experiments at the Oyster site. The poster shows the high resolution of bacterial cell counts achieved using ferrographic enumeration.

## The Influence of Heterogeneity and Growth on Microbial Transport in Saturated Porous Media

Ellyn M. Murphy,<sup>1</sup> Timothy R. Ginn<sup>2</sup> and Brian D. Wood<sup>1</sup>

<sup>1</sup>Pacific Northwest National Laboratory, Richland, Wash.; <sup>2</sup>University of California, Davis, Calif.

The co-disposal of organic chelating agents with radionuclides at DOE sites has often resulted in enhanced mobility of these hazardous wastes. The success of biogeochemical alterations of these complexes is ultimately controlled by the transport and distribution of bacteria in physically and chemically heterogeneous subsurface systems. Much of the work to date on bacterial transport has focused on inert biocolloids or bacteria in non-growth states. Growth, however, increases the aqueous-phase concentration of bacteria, a first step in initiating transport in groundwater. To accurately represent bacterial transport during intrinsic bioremediation, the microbial growth and transport processes must be coupled.

The relationship between microbial growth and transport is being assessed in experimental systems at various scales. These include: (1) a microflow chamber ( $\mu\text{m}$  to  $\text{mm}$ ); (2) small columns ( $\text{mm}$  to  $\text{cm}$ ); and (3) intermediate-scale flow cells ( $\text{cm}$  to  $\text{m}$ ). The microflow chambers are used to measure the attachment, detachment and residence time of bacteria to different mineral surfaces by direct observation with a confocal microscope. The residence time is used to measure the point at which irreversible adsorption to the mineral surface occurs. This process has been quantified by developing a mechanistic model that tracks the bacteria in space, time and the additional dimension of residence (or exposure) time. This model allows us to experimentally test the role of residence time on a mineral surface in adhesion processes.

Growth and transport are also affected by the dynamic nature of a contaminant plume, which creates redox extremes that can facilitate aerobic to anaerobic respiration within relatively short distances. We have created this type of small-scale heterogeneity in the redox properties in laboratory intermediate-scale flow cells. At any point in space and time the metabolic potential of an organism is dictated by its exposure time to the electron acceptor and donor. In our experiments the electron acceptor and donor are in the aqueous phase, thus local concentrations are controlled by the flow field. The mechanistic model used to track residence time can also be used to track the exposure time of the microorganisms to the electron acceptor and donor, and therefore, accurately assess the spatial distribution of metabolic potential.

Measurements of cell-level processes are of little use to field bioremediation efforts unless this information can be used to understand, simulate, and possibly improve the processes occurring at the field scale. The use of these small-scale measurements in large field-scale applications requires a formal mathematical process known as upscaling. An upscaling method has recently been developed to derive attachment/detachment kinetics from the surface interaction potential between a microorganism and a mineral. A method for measuring the microbe-mineral surface interaction potential by atomic force microscopy is currently being developed. This allows us a unique opportunity to test the upscaling theory by using the measurements at the cell-mineral scale to predict the attachment/detachment kinetics, and then compare these results with laboratory measurements of kinetic parameters at the bulk column scale. If successful, this same approach will be extended for applications to field-scale microbial transport at the Oyster site.

# Enhancement of Bacterial Transport in Aerobic and Anaerobic Environments: Assessing the Effects of Metal-Oxide Chemical Heterogeneity

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<sup>4</sup>University of Montana, Missoula, Mont.; <sup>5</sup>Pacific Northwest National Laboratory, Richland, Wash.;  
<sup>6</sup>Old Dominion University, Norfolk, Va.; <sup>7</sup>Golder Associates Inc.; <sup>8</sup>Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Our research goals are to enhance our understanding of the fundamental processes required for successful, field-scale delivery of microorganisms to metal contaminated subsurface sites that exhibit both physical and chemical heterogeneity. The experiments being planned are designed to determine: (1) under what circumstances the preferential adsorption of bacteria to Fe, Mn and Al oxyhydroxides influences field-scale bacterial transport; (2) whether the adhesion properties of bacterial cells affect field-scale bacterial transport; (3) whether microbial Fe(III) reduction can enhance field-scale transport of Fe-reducing bacteria (IRB) and other microorganisms; (4) what level of site characterization, laboratory-scale experimentation and scaling-up approaches is required to accurately model field-scale bacterial transport; (5) which bacterial tracking methods yield the most reliable data for field-scale transport of living, viable bacteria; and (6) which other methods can be employed at the field-scale to enhance bacterial transport.

In the past year, two flow cells have been installed in a surficial aquifer located just south of Oyster, Va., on the DelMarVa peninsula. One flow cell was installed in an aerobic portion of the aquifer, and the other in a suboxic portion of the same aquifer. At the aerobic flow cell, 24 multilevel samplers (each with 12 sampling ports distributed over a depth of 3 m) were designed and installed by Oak Ridge National Laboratory and Golder Associates. The strata in both flow cells are comprised of quartz-rich fine-grained sands with significant grain size variations occurring over the cm to meter scale; significant variations in the concentrations of Fe, Mn, and Al oxyhydroxides also occur over the same scale. Higher levels of Fe(II) are present in the groundwater and sediment at the suboxic flow cell relative to the aerobic flow cell.

Five different strains of IRB have been recovered from groundwater and sediment samples by Florida State University and Pacific Northwest National Laboratory (funded independently by DOE/OBER) that satisfy the antibiotic resistance profiles and are currently being tested for field injection next spring by Envirogen, Inc. A 3-D hydrodynamic model of the aerobic flow cell was constructed based upon surface geophysical data gathered by Lawrence Berkeley National Laboratory and geological data gathered by Old Dominion University and pump tests and modeling performed by Golder Associates. Bacterial transport experiments confirm the existence of a dependency of the bacterial adhesion upon pH as expected for sediment containing Fe, Mn and Al oxyhydroxides. Bacterial transport models were applied to the intact core results to test hypotheses regarding bacterial transport and fate and to develop model parameterizations that were incorporated into the 3-D hydrodynamic model of the aerobic flow cell. An intact core experiment was also performed that tested the various bacterial tracking methods to be utilized during the bacterial field transport experiment. The *Comamonas* strain was labeled with both <sup>13</sup>C and a viable protein stain (CFDA) and injected into an intact core under conditions designed to simulate the field injection. The bacterial breakthrough was monitored using plate counts and CFDA direct counts and plate reader measurements (Envirogen), by ferrographic separation and counting (Univ. of Utah), by stable isotope analyses, DAPI counts and quantitative PCR (University of Montana) and by PLFA analyses (University of Tennessee). The success of the various tracking methods will be presented along with other results from a simultaneous injection of Br and <sup>13</sup>C/CFDA labeled *Comamonas* into the aerobic flow cell.

## Vibration-Accelerated Transport of Microbes in Subsurface Media

*Tommy J. Phelps and Barry Kinsall*

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The low transport rates of microorganisms through porous subsurface media pose severe limitations on the impact and applicability of biological processes for in-situ remediation of subsurface contaminants. We are investigating the use of vibrational energies as a tool for accelerating the transport of microorganisms and nutrients in subsurface media. This basic research focuses on evaluating the applicability of vibration-induced transport through detailed hypothesis testing in laboratory experiments to be complemented by field-scale verification. We hypothesize that vibrational energies will increase microbial transport as a result of changes in subsurface porosity, increased dispersion and increased desorption.

Our results indicate that vibrational energies do indeed increase dispersion and transport in sediments. Comparisons between vibrated and non-vibrated control columns revealed stark differences in the flow, distribution and dispersion of tracers and microspheres. Current results suggest frequencies between 40-200 Hz with power levels of several Kilowatts per cubic meter may be best suited for microbial transport. Modeling by colleague C. Santamarina of Georgia Tech revealed that vibrations of these frequencies and powers should transmit more than 10 meters in radius within shallow sandy sediments. Our column experiments revealed that vibrations of 40-200 hertz typically increased aqueous flow in sediments by 60-100%. In addition to the increased flow, we observed faster breakthrough of iodide conservative tracers, although the fraction of the tracer recovered at the height of the peak ( $C/C_0$ ) was ~30% of that observed in non-vibrated controls.

These results, when combined with the dramatically increased tailing observed in vibrated columns, are indicative of increased dispersion with vibration. Although the flow dramatically increases with the onset of vibration, there is a drop in the velocity of water flow over time. In experiments using Abbott's Pit intact columns, the water velocity decreased ~5% per hour of vibration, and after long periods of vibration water flow through the columns dropped below levels in non-vibrated controls as a result of plugging within the column. These observations are consistent with the ~10% reduction column volume after vibration, resulting from the repacking of grains. As expected, upon cessation of excessive (>10 hr) vibration, water flow is always much less than in non-vibrated controls.

Transport of fluorescent microspheres as determined by flow cytometry is orders of magnitude greater than in non-vibrated columns (typically below detection limits after 0.5 m). Although the fraction of transported microspheres remains low, microsphere breakthrough appeared faster than the conservative iodide tracer. The faster breakthroughs are followed by long tails indicative of dispersion followed by secondary peaks corresponding to the iodide breakthrough. Analyzing slices of columns by confocal microscopy or UV light reveals greater transport and lateral dispersion of all sizes and colors of fluorescent microspheres in the vibrated columns. Current experiments are expanding to microbial transport, dissecting mechanisms of increased transport facilitated by vibration as well as field-testing procedures (e.g., multilevel samplers, sampling protocols and drive-point well installations) for a field-scale verification of vibration-facilitated microbial transport.

# Enhanced Quantitative Methods as Integrating Elements of Multidisciplinary Bacterial Transport Research at the Oyster Site

*Timothy D. Scheibe*

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Experiments being conducted under NABIR at a field site near Oyster, Va., are identifying and quantifying microbial transport processes in sandy aquifers under varying biogeochemical conditions. At the field scale, multiple hydrologic and biogeochemical processes interact in a heterogeneous subsurface environment to complicate the interpretation of experimental results. In this complex environment, a well-designed suite of quantitative models can effectively serve as a focal point for the design and interpretation of microbial transport experiments, quantitative testing of research hypotheses, management and integration of data and transfer of information between different scales.

This project is developing and applying a series of advanced hydrogeological models of tracer and bacterial transport, drawing on and integrating data provided by collaborators (e.g., geophysical data from E. Majer/LBNL, hydrologic data from T. Griffin/Golder Assoc., and geological data from D. Swift/Old Dominion Univ.). Several levels of model complexity and various length scales are addressed through multiple linked models ranging from one-dimensional core-scale models of laboratory experiments to high-resolution heterogeneous models of field-scale transport. These models have been used for experimental design (e.g., location of multi-level samplers) and interpretation (e.g., testing of hypotheses regarding scaling of laboratory experiments for field-scale prediction).

Most recently, we have developed a novel approach to the simulation of microbial exclusion phenomena, based on a modified particle-tracking method. The application of these models in the areas of data management and integration, parameter and process scaling, collaborative interaction, and experimental design is the focus of several specific research elements. The scaling element will develop three-dimensional core-scale flow and transport models to quantify microbe/solid surface interactions and obtain field-scale process representations. Tracer test inversion techniques will be evaluated in terms of their ability to enhance model predictions relative to other types of characterization data. The experimental design element will employ a collaborative tool for identifying, guiding and documenting design decisions, and will integrate quantitative pre-modeling results with qualitative investigator input and practical considerations. The data management element will populate a web-based data and information repository with linkages to the numerical model framework, experimental design tools and collaborative databases.

This research will lead to specific results of relevance to the subsurface microbiological sciences, will increase the overall value of data and information collected at the Oyster site, and will develop a systematic approach and knowledge base applicable to future research at other sites (and ultimately to bioremediation applications).

## Heterogeneity of Sedimentary Aquifers: Role in Microbial Dynamics Assessed by Radar Imaging and by Acoustic and Radar Tomography

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The Heterogeneity Program is an integrated, sedimentological, geochemical, geophysical and microbiological study designed to identify scales of physical heterogeneity that affect the biodynamics of natural subsurface environments. At the Oyster, Va., site, a regional grid of Ground-Penetrating Radar (GPR) lines have been constrained by Cone Penetrometer Test profiles (CPT profiles) in order to resolve the sub-regional stratigraphy. Special study areas have been cored and trenched. They have been subjected to more closely spaced radar lines in order to provide higher resolution stratigraphic information, and to compare with radar and seismic tomography. Cores have been split and subjected to infrared imagery and air minipermeameter analysis. More than 1,000 samples from cores and excavations have been analyzed for grain size. Of these, 300 have been analyzed for permeability, and a further subset has been analyzed for iron oxides.

The deposits of the Oyster site are highly permeable, with values on the order 0.1 to 50 Darcys. These deposits have been grouped into hydrofacies on the basis of grain size, permeability and stratal architecture, as observed in cores and GPR records. In order to estimate microbial activity, hydrogen uptake rates were measured under aerobic and anaerobic conditions across three of the five hydrofacies identified at the Oyster site. Activity was low and not highly variable, ranging from 6 to 60 nmol of hydrogen used per g per day. Microbial abundance has been measured using most probable number (MPN) analysis for aerobic and anaerobic heterotrophs and sulfate-reducing bacteria. MPN estimates for both heterotroph groups vary by 3 to 4 orders of magnitude. Phospholipid analyses and MPN determinations agree fairly well and indicate that microbial abundances ranged from 10<sup>5</sup> to 10<sup>6</sup> cells per gram. Phospholipid fatty acid analysis showed that all samples contained a relatively diverse microbial community structure. Principle component analysis showed that most samples clustered together in agreement with the low variability observed for microbial activity measurements.

Preliminary studies indicate a much closer relationship than heretofore envisaged between large-scale stratigraphy as resolved by Ground-Penetrating Radar and CPT profiles, and the meso- and small-scale physical heterogeneity seen in cores and excavations. In order to determine small-scale heterogeneity, a preliminary analysis of spatial correlation has been undertaken. It reveals a dominant log-permeability integral scale in the vertical direction of ~30-40 cm and a horizontal integral scale of ~1.5 m. It is apparent that the scale of spatial organization of microbial populations is significantly shorter (1-10 vertical cm; 10-40 horizontal cm). However grain size and permeability variations are apparent at this finer scale also, and appear to be significant controls of microbial population distribution. Present work focuses on relating these fine-scale physical parameters to physical parameters observed at larger spatial scales, and ultimately, to non-invasive geophysical images.

**PROGRAM ELEMENT 7**  
**System Engineering, Integration,  
Prediction and Optimization**



# Simulating Bioremediation of Uranium Contaminated Aquifers; A Global Uncertainty Assessment of Model Parameters

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Mobility of trace metals and radioisotopes in groundwater can be controlled to a significant extent via in-situ manipulation of key biogeochemical reactions that directly or indirectly alter their solubility. For this purpose, specified redox profiles need to be established in the groundwater. These redox profiles develop, in response to biostimulation schemes, from the availability and transport of different electron acceptors and their utilization by different bacteria during the degradation of an organic substrate. The objective of this research is to assess modeling techniques that are capable of simulating the fate of trace metals and radionuclides in the subsurface in response to different biostimulation schemes.

A time-dependent one-dimensional reactive transport model has been developed. The model consists of a set of coupled, steady state mass balance equations, accounting for advection, diffusion, dispersion and a kinetic formulation of the transformations affecting an organic substrate, electron acceptors, corresponding reduced species and contaminant metals of interest. This set of equations is solved numerically, using a finite element scheme. The redox conditions of the domain are characterized by estimating the pE, based on the concentrations of the dominant terminal electron acceptor and its corresponding reduced specie. This pE and the concentrations of relevant species are passed to a modified version of MINTEQA2, which calculates the speciation and solubilities of the species of interest. Kinetics of abiotic reactions are described as being proportional to the difference between the actual and equilibrium concentration. Simulations are performed to illustrate the effect of biostimulation on the transport of uranium in the subsurface.

In the model, the relationship between the various biological chemical variables and the model output may be nonlinear, and various interdependencies are expected to exist. It is important to identify all of these variable interactions, especially for the key model parameters, and use this information to: (1) develop more robust models; (2) identify the key coefficients that need to be specified with the highest precision; and (3) develop reliable bioremediation schemes.

A recently developed nonlinear analysis tool, RS-HDMR (Random Sampling-High Dimensional Model Representation) is employed to determine the relationship between the various model inputs and outputs. The RS-HDMR formulation provides a highly efficient and thorough sampling of the overall space of variables to identify those that are important and are acting either independently or cooperatively. An initial analysis has been performed to investigate the effect of 20 rate constants and transport parameters on the transport of four chemical species, including uranium. The global output uncertainties in the whole domain of 20 input variables was quantified by RS-HDMR, and the key model parameters were identified. A significantly higher variance was associated with outputs representing a given concentration in space and time than for outputs integrated over space and/or time such as cumulative flux at a given location.

***BASIC PROGRAM ELEMENT:***  
**Bioremediation and its Societal  
Implications and Concerns**

## Communicating Effectively with NABIR Stakeholders

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Based on preliminary work in FY98, public engagement events were tested to attempt to reduce suspicion and forestall conflict in the conduct of bioremediation research and the siting of Field Research Centers. Many public engagement forums are known for the tendency to communicate in hostile, polarizing or problem-centered ways. In contrast, the design we tested brought together three groups not often exposed to one another: NABIR scientists, seasoned stakeholders and previously uninvolved stakeholders. We focused interactions in micro-community systems of three to 20 people rather than a presentational or expert-based forum that depended on one-to-many communication. Our goal was to place public science in a context that encouraged discussion of a range of issues associated with bioremediation and Field Research Centers. We developed a design that encouraged citizens and scientists to interact and included scientists as stakeholders. From previous work, we were interested in (1) the development of trust; (2) identifying scientific and programmatic information needs; (3) the process of communication; (4) identifying issues and concerns; (5) discovering ways for citizens and scientists to engage in productive dialogue; and (6) identifying the strengths and limitations of dialogue methods for use in public engagement.

Our events were designed to explore one general research question and a number of derivative questions. The general question was: Can dialogue be used in an engagement event to enable scientists, policy makers, internal stakeholders, and external stakeholders to discuss basic science in productive ways? Other questions included: Can the way NABIR scientists communicate about NABIR science enhance trust? Can scientific information be conveyed interactively using dialogue design and techniques? Does dialogue provide a clear alternative to expert delivery models that currently prevail? What kinds of scientific information are required to initiate a productive dialogue about science? What kinds of information needs must be met for productive dialogue? What kinds of communication skills are used in productive dialogue? What does productive dialogue look like? Does productive dialogue look the same to all participants? Can event design be used to construct a context in which productive dialogue can occur?

The Public Dialogue Consortium of Albuquerque, N.M., was chosen to conduct the events. Their facilitators are experienced in handling contexts with widely varying histories and degrees of conflict, and are familiar with DOE's issues and interested parties. Preliminary results identified five categories of concerns: environmental and economic impacts; allowing scientists to do their jobs; public understanding of the problems, techniques and results of NABIR science; programmatic strategies and policy issues; the current state of knowledge about bioremediation; the problems to which bioremediation is being applied; and the micro-organisms being studied. During the events, all three participating groups contributed to each category, all of which overlap. However, given the interests of the three groups, the first three areas of concern seem particularly appropriate to group-specific interests. Although it is clear that a dialogue method of public involvement supplements rather than replaces other methods of information-gathering, the use of small groups and the participants' mixed levels of expertise made it possible for novices and experts to interact without fear of ensuing conflict.

# The Determinants of Social Acceptability of Genetically Engineered Microorganisms for Remediation: Applying the Public Acceptability of Controversial Technologies (PACT) Framework to Improve Communication

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This project builds upon a previous NABIR/BASIC effort that produced a conceptual framework—PACT (Public Acceptability of Controversial Technologies)—that addresses the social acceptability of the site-specific use of genetically engineered microorganisms (GEMs) for remediating hazardous waste. “Acceptability” is defined as serious consideration, as opposed to out-of-hand refusal to consider, and is distinct from technology selection or deployment. We focused on GEMs as a member of a class of controversial technologies for which society regularly faces public policy choices about technology adoption and implementation.

PACT has the following three overarching goals: (1) enhance understanding of the interactions among stakeholder groups that lead to context-specific, formalized decisions about the acceptability of controversial technologies; (2) provide insights into the dynamics of that decision-oriented dialog process; and (3) establish a basis for recommendations to improve the ability of stakeholder groups to engage in productive dialogs that lead to deliberate decisions about the use of technologies. The model is structured along four dimensions. The first dimension, the decision-rule continuum, describes the dynamics of multi-constituency group dialog over time. The second dimension consists of constituency groups’ values, motivations and strategies, within the general context of bioremediation. The third dimension encompasses specific attributes of bioremediation strategies relative to other remediation technologies. The fourth dimension consists of the physical, social and institutional context within which cleanup decisions are made.

In the past year we have worked to refine PACT—primarily its dialog and constituency group dimensions—by examining empirical evidence of cleanup-related dialog. Specifically, we focused on one institutional structure for public participation promoted by the Department of Energy, site-specific advisory boards (SSABs). We analyzed audio or video recordings of SSAB meetings held during the course of one year at three of the 12 DOE sites that have these formally chartered advisory groups. Because GEMs were not discussed and other types of bioremediation rarely were mentioned, we looked to the SSAB meetings mainly to detect patterns evident in cleanup-oriented dialogs. As examples, we investigated the kinds of technological issues raised and the issues that seemed particularly controversial. We also examined the kinds of constituency groups represented at the SSAB meetings, how they presented their positions and how they shifted their positions over time.

Among our preliminary findings are the following: SSABs function very differently at the various DOE sites, affecting who participates, the process of interaction and the kinds of issues discussed. Further, the issues discussed reflect the sites’ social, physical and institutional attributes—though there are similarities across sites, there also are striking differences. Thirdly, the SSAB meetings contain relatively little discussion of the technological and risk-related attributes of various cleanup options.

## **NABIR RELATED**

## NABIR Data and Information Management System (NADIMS)

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The NABIR Data and Information Management System (NADIMS) is designed to provide cost-effective sharing of data among the NABIR investigators. NADIMS has been developed with input by NABIR researchers, science team leaders and NABIR program managers. The web-based object-oriented structure of NADIMS is modular, flexible and expandable. NADIMS reflects the interdisciplinary nature of the NABIR program and provides functionality to enable NABIR researchers to:

- browse and query "metadata"—information about datasets, projects, NABIR PIs and publications;
- search the metadata by microbes, biochemicals, field sites, rock materials, measurement methods, etc.;
- display, query and navigate spatial data representing NABIR field research sites;
- retrieve data from the NADIMS data repository in multiple formats (i.e., tables, graphics and text);
- perform spreadsheet and charting functions on tabular data retrieved from the repository.

NADIMS is organized into two components; one component manages the metadata (developed at Lawrence Berkeley National Laboratory), and the other manages the spatial organization of the datasets and the datasets content, usually represented as spreadsheets (developed at Argonne National Laboratory). Recently, the two components supporting the metadata and the actual datasets have been linked. One can browse the datasets based on spatial navigation and link to the metadata about a dataset of interest. Conversely, one can start by searching the metadata and link to the actual datasets.

Over the last year several datasets derived from sampling at the Gunnison and Shiprock UMTRA sites were entered to the database and are now on line. More datasets will be added, especially from the Field Research Center (FRC). Because many of the datasets collected by NABIR researchers are associated with field sites and are inherently locational, NADIMS was developed to include a map-based query system. Users can get to datasets by clicking on-line maps. In addition to the metadata associated with specific datasets, the metadatabase was also enriched by indexing numerous progress reports and publications relevant to NABIR.

NADIMS is an evolving system based on NABIR community needs. The current version of NADIMS is available now through the Web. The entry point to the metadata is: <http://gizmo.lbl.gov/NADIMS/metadata.html> and the entry point to the spatial search and datasets content is: <http://nabir.dis.anl.gov> (a password is required; contact Terry Hazen, (510)486-6223, [TCHazen@lbl.gov](mailto:TCHazen@lbl.gov), to receive password approval).

## Proposed NABIR Field Research Center at Oak Ridge, Tennessee

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The Environmental Sciences Division at Oak Ridge National Laboratory (ORNL) proposes to establish a Field Research Center (FRC) on DOE's Oak Ridge Reservation (ORR) in Tennessee for the DOE Office of Biological and Environmental Research. The proposed FRC would provide a site for NABIR investigators to conduct research and obtain samples related to in situ bioremediation.

The proposed FRC would include a contaminated area to be used for conducting experiments on a plume of contaminated groundwater, a background area that would provide for comparison studies in an uncontaminated area, and ancillary structures that would be located within a 3.2-mile (5.2-km) radius of each other on the ORR. The contaminated and background areas would be located on DOE land in Bear Creek Valley, which is within the Y-12 Plant area. Both of these sites are well characterized, well instrumented and should be available for the duration of the proposed NABIR FRC (5 to 10 years). The water table resides at 0 to 3 m below the surface and would be readily accessible to the rapid instrumentation of multilevel groundwater monitoring wells. ORNL's unique track-mounted pneumatic hammer has the capability of installing drive-point wells deep within the unconsolidated zone and transition zone and offers an effective and cheap alternative to traditional drilling technologies.

The proposed FRC contaminated field site would include the co-mingled groundwater plume found in the shallow unconsolidated sediments (<10 m depth), Nolichucky Shale and Maynardville Limestone that originated from a combination of the S-3 Waste Disposal Ponds and the Bone Yard/Burn Yard. However, the primary focus of NABIR investigations would be on the easily accessible shallow unconsolidated sediments that overlie the Nolichucky Shale. Contaminants in this plume and in the shallow saturated and unsaturated soils include uranium, Tc-99, strontium metal, nitrate, barium, cadmium, volatile organic contaminants (VOCs) and other inorganics and radionuclides believed to be of interest to the NABIR investigators.

Injection of non-toxic tracers into groundwater has been conducted as part of numerous research projects on the ORR and does not require any state permits. Based on recent discussions with the state of Tennessee, injection of indigenous and non-indigenous naturally occurring bacteria should be possible at the proposed FRC. ORNL staff have experience with hydrocarbons, methane, air, nutrients, gases, volatile acids, various ionic and inert tracers, and microbial injections into the subsurface either on the ORR or in nearby environments. The adjacent soil lysimeters (OBER-funded research) would also be available to NABIR researchers for bioaugmentation, biostimulation and other studies.

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