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DOE-NABIR PI WORKSHOP: Abstracts

March 12-14, 2001

Warrenton, Virginia

Natural and Accelerated Bioremediation Research Program

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Table of Contents

Introduction
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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 1999-2001 by DOE's Office of Science through its Office of Biological and Environmental Research. Sixty-seven projects have been funded in the scientific program elements, two have been funded in the BASIC program, and three in the Field Research Center. Abstracts of these programs are summarized in this booklet, along with abstracts of other DOE programs related to research in the NABIR program.

NABIR Program Staff*

Office of Biological and Environmental Research (OBER)

John C. Houghton
NABIR Program Co-Coordinator
Manager: Biogeochemical Dynamics and System Engineering, Integration, Prediction and Optimization Program Elements

Anna Palmisano
NABIR Program Co-Coordinator
Manager: Community Dynamics and Microbial Ecology, Biotransformation and Biodegradation, and Assessment Program Elements

Daniel W. Drell
Manager: Biomolecular Sciences and Engineering Program Element and BASIC Program

Paul Bayer
Field Activities Manager for the Field Research Centers

Frank J. Wobber
Manager: Bacterial Transport

Jerry W. Elwood
Director, Environmental Sciences Division, OBER

NABIR Program Office

Terry C. Hazen (LBNL)
NABIR Field Research Center Coordinator

Linda Wuy (LBNL)
NABIR Program Office Team Manager

Mary Pratt (LBNL)
NABIR Program Office Team Writer/Editor

* Addresses, telephone numbers and e-mail addresses are in the Address List, page 90

Agenda
DOE–NABIR PI Workshop
March 12-14, 2001

Monday, March 12

8:30-8:45 a.m.	Welcome – Anna Palmisano, John Houghton
8:45-9 a.m.	Jerry Elwood, Director of Environmental Sciences, OBER
9-9:15 a.m.	Gerald Boyd, Deputy Assistant Secretary of Science and Technology for Environmental Management
	Field Research Overviews
9:15-9:35 a.m.	NABIR Field Research Center (Dave Watson, ORNL)
9:35-9:55 a.m.	Research at UMTRA Sites (Phil Long, PNNL)
9:55-10:15 a.m.	Research at Oyster, Va. (T.C. Onstott)
10:15-10:45 a.m.	BREAK
10:45-11:15 a.m.	Biotransformation (Bill Burgos, Penn State)
11:15-11:45 a.m.	Biotransformation (John Coates, So. Ill. U)
11:45-12:05 p.m.	Bioremediation and its Societal Implications and Concerns (Gordon Bilyard, PNNL)
12:05-1:30 p.m	LUNCH

Afternoon Session			
1:30-3 p.m.	Posters	Breakout: The use of genomic information in NABIR research (Lovely/Methe-organizers)	Breakout: Biotransformation/Biogeo-chemistry (Neu/Amonette-organizers)
3:30-5 p.m.	Roundtable with EM Customers: Research Needs (Bayer)		
5-6 p.m.	Free time		
6-7 p.m.	Dinner		
7-9 p.m.	Poster Session: Community Dynamics, BASIC, Biomolecular, Bacterial Transport. Authors must be at posters.		

Tuesday, March 13

8:30-9 a.m.	Biogeochemistry (John Zachara, PNNL)
9-9:30 a.m.	Community Dynamics (Jim Tiedje, Mich. State)
9:30-10 a.m.	Community Dynamics (Derek Lovley, U. Mass)
10-10:30 a.m.	BREAK
10:30-11 a.m.	Community Dynamics/Assessment (Craig Brandt, ORNL)
11-11:30 a.m.	Assessment (Jack Istok, Oregon State)
11:30-11:50 a.m.	BASIC (Amy Wolfe, ORNL)
Noon-1:30 p.m.	LUNCH

Afternoon Session			
1:30-3 p.m.	Posters	Breakout: Research at the FRC (Watson, Criddle, Istok)	Breakout: Bioremediation and its Societal Implications and Concerns (Wolfe/Bilyard)
3:30-5 p.m.	Discussion of Draft Strategic Plan for NABIR Program		
5-6 p.m.	Free Time		
6-7 p.m.	Dinner		
7-9 p.m.	Poster Session: Biogeochemistry, Biotransformation, Assessment, Systems Integration. Authors must be at posters.		

Wednesday, March 14

8:30-9 a.m.	Biomolecular Science and Engineering (Anne Summers, U. Ga.)
9-10 a.m.	Biomolecular Science and Engineering (Tamar Barkay, Rutgers U.)
10-10:15 a.m.	BREAK
10:15-10:45 a.m.	Bacterial Transport (Brian Wood, PNNL)
10:45-11:15 a.m.	Bacterial Transport (Bill Johnson, U. Utah)
11:15-11:30 a.m.	Wrap-up; Meeting adjourns
11:30-12:30 p.m.	LUNCH

Note: UMTRA Working Group will meet from 11:30 a.m. 4 p.m.

ABSTRACTS

PROGRAM ELEMENT 1

Biotransformation and Biodegradation

Biodegradation of PuEDTA and Impacts on Pu Mobility

Harvey Bolton, Jr.,[1] Dhanpat Rai[1] and Luying Xun[2]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]Washington State University, Pullman, Wash.

Plutonium (Pu) contamination of sediments and groundwater at many Department of Energy (DOE) sites is a long-term problem. Ethylenediaminetetraacetate (EDTA) was co-disposed with Pu, forming strong PuEDTA complexes, and enhancing Pu transport at many sites. EDTA poses a long-term problem of potentially disseminating Pu and other radionuclides (e.g., ^{60}Co) in the subsurface environment, because it is recalcitrant to biodegradation. Biodegradation of EDTA is a permanent solution to decrease chelate-assisted radionuclide transport through destruction of the PuEDTA complex and the precipitation of insoluble PuO_2. However, this biodegradation is not well understood because of the lack of information on PuEDTA aqueous species, microbial degradation (e.g., uptake into the cell and enzymology of degrading enzymes), and the effect of physicochemical factors (e.g., Pu:EDTA ratio, CO_2 partial pressure, pH, redox and other metals) on the rate and ability of microorganisms to degrade PuEDTA.

We will investigate the aerobic biodegradation of Pu(IV)EDTA and the location and mobility of the Pu, transport of EDTA complexes into the cell, and the genetics and enzymology of aerobic EDTA biodegradation. We will also enrich and isolate an anaerobic EDTA degrading bacterium to determine how the anaerobic biodegradation of PuEDTA may impact the groundwater mobility of Pu. This research will provide the necessary mechanistic understanding of how microbial biodegradation of PuEDTA will affect the groundwater mobility, fate and transport of Pu in both oxidizing and reducing groundwaters present at DOE sites.

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

William D. Burgos,[1] Richard F. Unz,[1] Brian A. Dempsey,[1] Richard A. Royer[1] and Gour-Tsyh Yeh[2]
[1]Pennsylvania State University, University Park, Penn.; [2]University of Central Florida, Orlando, Fla.

Project objectives were centered on the role of natural organic matter (NOM) in enhancing bacterial (*Shewanella putrefaciens* CN 32) solid-phase iron reduction, subsequent effects of NOM-enhanced iron reduction on the fate of metal and radionuclide contaminants, and the development and validation of reaction-based models to describe these phenomena. One of our research hypotheses is that NOM can 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, which prevents Fe(II) sorption to ferric oxide and cell surfaces.

A series of experiments were performed with well-characterized NOM and NOM fractions isolated from different environments, and chemical "functional analogs" that mimic either of the proposed NOM enhancement mechanisms. Four quinones (methyl viologen, anthraquinone-2,6-disulfonate (AQDS), methylene blue and 1,4-benzoquinone) were tested for enhancement of iron reduction via electron shuttling, and ferrozine was tested for enhancement of iron reduction via Fe(II) complexation. Combinations of AQDS and ferrozine were also tested to examine how these two mechanisms work in tandem. Nine NOM specimens were examined for their contribution to iron reduction by the two proposed mechanisms. Experiments were conducted with a suspension of hematite (2.0 g L^{-1}) in 50 mM PIPES buffer with 30 μM phosphate (pH 6.8) and H_2 as the electron donor under non-growth conditions (10^8 cells mL^{-1}) and incubated for 1 or 5 days. A concentration dependent linear relationship existed between total Fe(II) produced and ferrozine and all nine NOMs but not with quinones. The enhancement effects of both AQDS and ferrozine were additive. In an experiment in which the Fe(II) complexation capacity of the NOMs was saturated, none of these materials demonstrated any capacity for enhancing the extent of iron reduction after 5 days. Our results demonstrate that NOM can serve as both an electron shuttle and a Fe(II) complexant, however, the concentration dependence of Fe(II) production with NOM after 5 days was much more similar to ferrozine than quinones, suggesting that NOM likely enhances iron reduction initially by electron shuttling and subsequently by Fe(II) complexation.

The kinetics of reductive dissolution of hematite by *S. putrefaciens* CN32 was measured and modeled using a reaction-based biogeochemical model. Preliminary abiotic experiments revealed that Fe^{2+} sorption to hematite was a kinetic reaction. Fe(II) was not completely recoverable in the abiotic experiments after 24 hours, suggesting that additional reactions describing the formation of magnetite from hematite-sorbed Fe(II) and sorption of Fe(II) to magnetite may be required. Fe^{2+} sorption to *S. putrefaciens* CN32 was an equilibrium reaction. Two kinetic rate formulations for the bioreduction of hematite were tested: an elementary formulation, and a formulation physically-based on "free surface sites" of hematite. Both of these rate formulations captured initial bioreduction kinetics but over- or under-predicted the long term extent of bioreduction. These results suggest that reaction-based modeling of ferric oxide bioreduction is possible, however, additional reactions (e.g., "direct" and "indirect" bioreduction mechanisms, and the formation of magnetite) may need to be included and parameterized to improve model predictions.

The impact of zinc on the bioreduction of hematite was studied. The initial focus was to study the fate of free or complexed zinc under iron-reducing conditions in the presence of hematite. However, at the zinc concentrations studied (2-20 mg L^{-1}), it was found that zinc significantly inhibited bioreduction by *S. putrefaciens* CN32. Therefore, it was of interest to examine the potential toxicity of zinc. Biogenic Fe(II) did not effectively compete with zinc for adsorption sites on hematite or cell surfaces. Also, zinc did not appear to be incorporated into a substituted magnetite phase as was previously reported. Zinc may represent a problematic contaminant under iron-reducing conditions due to its apparent toxicity and reluctance to form an immobile iron-zinc mineral phase.

Bioengineering Anaerobic EDTA Degradation in a Novel (Per)Chlorate-Reducing Organism

John D. Coates,[1] Romy Chakraborty,[1] Laurie A. Achenbach,[1] Mostafa S. Elshahed,[2] Neil Wofford[2] and Michael J. McInerney[2]
[1]Department of Microbiology, Southern Illinois University, Carbondale, Ill.; [2]Department of Botany and Microbiology, University of Oklahoma, Norman, Okla.

Our previous research demonstrated that radionuclides are readily adsorbed to ferric oxides biogenically formed through the activity of (per)chlorate-reducing bacteria under anaerobic conditions. In this way, low-level contamination of these compounds is immobilized and poses little threat to groundwater supplies. However, the presence of small amounts of the metal chelator EDTA, which was often used in decontamination and nuclear fuels processing, readily remobilizes the bound metals. This is further compounded by the relative non-biodegradability of EDTA, especially under anaerobic conditions. To date, there are no known organisms capable of the anaerobic oxidation of EDTA, although there are a few aerobic organisms known, and for at least one of these, strain BNC1, it has been shown that the degradation of EDTA is mediated through the activity of an enzymatic pathway based on an oxygenase enzyme. Our recent discovery of a (per)chlorate-reducing organism, *Dechloromonas aromatica* strain RCB, which mediates the biodegradation of aromatic hydrocarbons such as benzene and toluene under strictly anaerobic conditions, offers a novel opportunity to engineer an organism capable of anaerobic EDTA degradation utilizing the aerobic pathway.

Benzene is oxidized by *D. aromatica* under aerobic conditions or anaerobically with chlorate as an electron acceptor. Similarly to all other tested (per)chlorate-reducers, *D. aromatica* dismutated chlorite into chloride and O_2. Under normal growth conditions, this O_2 is further respired by the organism. However, the O_2 produced is also potentially available to the cell for activation of the benzene ring via a dioxygenase. Benzene oxidation by *D. aromatica* is significantly stimulated by the presence of small amounts (0.1 – 1.0 mM) of an alternative electron donor such as Fe(II) or acetate. Stimulation of benzene oxidation was much greater with Fe(II) than with acetate. If strain RCB uses a dioxygenase-based pathway for the degradation of benzene under chlorate-reducing conditions, O_2 must first be produced to initiate activation of the benzene ring prior to benzene degradation. But O_2 can only be produced if ClO_3^- is reduced by electrons supplied from benzene oxidation. This explains why an additional electron donor like Fe(II) stimulates benzene degradation as it initiates ClO_3^- reduction and the O_2 produced can be fed back into the benzene dioxygenase pathway. In support of this, *D. aromatica* cannot oxidize Fe(II) aerobically, and all of the O_2 biogenically produced by *D. aromatica* coupled to Fe(II) oxidation is available for benzene degradation. In contrast, acetate can be oxidized by *D. aromatica* aerobically and would thus compete with the dioxygenase enzymes for the O_2 produced as a result of chlorate reduction. In further support of the proposed pathway, preliminary enzymatic studies of cultures *D. aromatica* grown anaerobically with acetate and benzene indicate the presence of catechol-2,3-dioxygenase, a central enzyme involved in the aerobic metabolism of benzene in many organisms, including *Pseudomonas putida*.

These studies are the first demonstration of anaerobic benzene oxidation by a pure culture and indicate that this organism has evolved a unique mechanism which couples the ability of (per)chlorate-reducing bacteria to produce O_2 as a transient intermediate in the reduction of chlorate with an O_2-dependent oxygenase-based pathway under anaerobic conditions. If this is true, then potentially any oxygenase-based enzyme pathway such as that involved in EDTA degradation could function anaerobically in this organism once successfully transformed and expressed.

Anaerobic Transformations of Pentachlorophenol-Cadmium Mixtures by Anaerobic Bacterial Consortia

Don L. Crawford
University of Idaho, Moscow, Idaho

Our objective is to understand how sediment and aquifer microorganisms can be manipulated to accelerate natural bioremediation of waters co-contaminated with organics and heavy metals. In simulated sediments, we studied anaerobic biodegradation of 50 ppm pentachlorophenol (PCP) by sulfate-reducing (SRB) and methanogenic (MET) consortia in water contaminated with PCP and cadmium (Cd). In sand sediments, removal of >90% of PCP by both consortia occurred within 82 days in the absence of Cd, versus 83 to 87% in the presence of 50 mg/L Cd. Soluble Cd initially reduced PCP degradation rates, which recovered as Cd was precipitated. HPLC, GC-MS, ^{14}C-PCP, and ^{13}C-PCP studies confirmed PCP mineralization by the consortia and incorporation of PCP carbon into consortia specific cell membrane phospholipid fatty acids (PLFAs) of PCP-degrading anaerobes. This is the first report of anaerobic biodegradation of PCP by SRB and MET consortia in the presence and with simultaneous precipitation of Cd, and of incorporation of PCP carbon into PLFAs of anaerobic bacteria.

Degradation of 50 ppm PCP in the presence of 50 ppm initially soluble Cd was further studied with consortia in low- and high-iron sand sediments. SRB enrichments from sewage sludge used lactate as electron donor. MET enrichments used acetate. Controls included untreated sediments and sediments with sludge inoculum alone. All sediments were uniformly contaminated with 50 mg/L each of PCP and soluble Cd, and were incubated (25°C) for 75 days. Samples were periodically removed at depths between 1 and 20 cm. Aqueous and solid phases of each were analyzed for Cd, PCP, PCP degradation products, redox potential and pH.

At ≥7 cm depth in all sediments except untreated controls, redox became negative within 15 to 30 days, depending on the treatment. The time for the iron poor sediment control was 60 days. Addition of lactate or acetate accelerated anaerobiosis, especially in iron-poor sediments. Except for untreated controls, PCP was degraded in the presence of Cd. Degradation was more rapid in the aerobic than in the anaerobic zones. We expected sediment PCP concentrations to equilibrate due to diffusion from the anaerobic into the aerobic zones. However, PCP degradation in the aerobic zones was faster than PCP diffusion rates from the anaerobic zones. PCP degradation proceeded faster in high-iron than in low iron sediments. SRB, followed by MET consortia, had the highest degradation rates. PCP degradation intermediates were present in the sediments at the 7-15 cm depths, except for untreated controls, which showed only PCP.

We also studied how consortia adapted to aqueous phase Cd toxicity by examining Cd effects on specific consortia originating from sewage sludge. Cultures were enriched in media developed for Cd stress studies. At inoculation, all Cd was soluble as free ion or chelated form. Physiological conditions were varied by using different electron donors/acceptors (d/a). To enrich for Cd resistance, consortia were subcultured repeatedly. All consortia adapted to higher Cd resistance. Concomitantly, growth rates and cultural diversity decreased. Initial and increased Cd tolerance were greatest under aerobic conditions (d/a: glucose/O_2), then multi-physiological (d/a: glucose/sulfate) and then fermentative (d/a: glucose/no acceptor) conditions. SRB (d/a: lactate/sulfate) and MET (d/a: acetate/CO_2) consortia exhibited lower initial Cd tolerance and lesser adaptation ability. Cd precipitation occurred under aerobic, SRB and MET conditions. Tolerance of the SRB and MET cultures was likely limited by the amount of sulfate available to form and precipitate CdS. Under all other conditions, Cd remained soluble as free ion. 16S rRNA profiles for the SRB, multi-physiological and fermentative consortia showed that all underwent succession; microbial population diversity decreased during subculturing into higher Cd media. Addition of chelators initially decreased Cd toxicity; however, the Cd eventually dissociated from the chelation molecule back into solution.

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

A.J. Francis
Environmental Sciences Department, Brookhaven National Laboratory, Upton, N.Y.

This research addresses the principal mechanisms of microbial alteration of organic radionuclide complexes and the resultant impacts on radionuclide solubility and stability under anaerobic conditions. We propose to (1) elucidate the mechanisms of biotransformation and fate of uranium complexed with organic chelating agents under anaerobic conditions; (2) identify the factors which regulate the bioreduction of complexed uranium, leading to decomplexation and precipitation of reduced uranium, and (3) enhance the reductive precipitation and stabilization of soluble complexes of uranium under anaerobic conditions in the subsurface. In this study, biotransformation of uranium-organic complexes by iron-reducing, fermentative- and sulfate-reducing bacteria, and mixed cultures isolated from NABIR field sites will be examined. The influence of soluble and particulate organic matter, pH and ionic strength on the rate and extent of biotransformation and stabilization of reduced uranium is being investigated.

Equimolar U-catechol, -protocatechuic acid, -2-ketogluconic acid -oxalate, -citrate and -EDTA complexes were prepared and characterized in solution or solid phase by using the advanced spectroscopic techniques (XPS, XANES, EXAFS, and FTIR). Initial results on the biotransformation of U(VI)-citrate by with *Clostridium sphenoides* (ATCC 19403), capable of utilizing citric acid as the sole carbon source, and *Clostridium* sp. (ATCC 53464), capable of fermenting glucose but not citrate show that *C. sphenoides* metabolized the bidentate Fe(III)-citrate complex and the released Fe(III) was reduced to Fe(II); U(VI)-citrate complex was not metabolized by the bacterium. However, addition of excess citric acid or glucose resulted in the reduction of U(VI)- to U(IV)-citrate. In contrast, the *Clostridium* sp. reduced Fe(III)- and U(VI) citrate to Fe(II)- and U(IV)-citrate, respectively, only when supplied with glucose. Basic information obtained from this study will be used in the development of in situ stabilization methods for radionuclides by enhancing the anaerobic biotransformation of organic/inorganic radionuclide complexes in the subsurface environments. This is a collaborative research involving Brookhaven National Laboratory, Colorado School of Mines (B.D. Honeyman), and State University of New York at Stony Brook (G.P. Halada).

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

James K. Fredrickson,[1] John M. Zachara[1] and Martine Duff[2]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]Savannah River Ecology Laboratory, Aiken, S.C.

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_2 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 pyrolusite (ß-MnO_2), birnessite (γ-MnO_2) and bixbyite (Mn_2O_3). In the absence of cells, the Mn oxides were able to quantitatively oxidize biogenic UO_2 to soluble U(VI) species within ~ 24 h. In suspensions with varying concentrations of U(VI) from 50 to 1000 μM, the presence of Mn oxides significantly decreased the rate and extent of U(VI) reduction relative to no Mn controls and where gibbsite at an equivalent surface area concentration. The rate and extent of Mn(II) evolution was positively correlated with the starting concentration of U(VI), indicating that U(IV) was facilitating the reduction of Mn and being recycled between the +4 and +6 oxidation states.

Thin sections of fixed and embedded cell suspensions from incubations with U(VI), with or without Mn oxides, were examined by TEM to investigate the distribution and characteristics of the bioreduced U(IV) precipitates and to potentially provide insights into mechanisms of U(VI) reduction in the presence of excess oxidant. In the absence of Mn oxide, the U was present as fine-grained precipitates external to the cell as well as in association with cell walls. This fine-grained biogenic U precipitate was previously demonstrated to be uraninite (UO_2) by x-ray diffraction and x-ray absorption near edge structure (XANES). In the CN32 cell suspensions incubated with U and birnessite, where the rate of U reduction was impeded, the distribution of U was quite different than in cell suspensions incubated without the Mn oxide. The most notable differences were the absence of fine-grained extracellular U precipitate and the presence of U exclusively in association with cells in the suspensions with bixbyite or birnessite, predominantly in the periplasm of the cells. The results from this research indicate that the presence of Mn oxides in subsurface sediments may impede in situ bioreduction of U but that accumulation of UO_2 in the periplasm of metal-reducing bacteria may afford physical protection of UO_2 against oxidation by Mn(III, IV).

Physiological Response of Metal-Reducing Bacteria to Biogenic Iron Microminerals

Yuri A Gorby,[1] Terrance J. Beveridge[2] and F. Grant Ferris[3]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]University of Guelph, Guelph, Ontario; [3]University of Toronto, Toronto, Ontario

Dissimilatory metal-reducing bacteria control the geochemistry of anoxic sedimentary and subsurface environments principally through the enzymatic reduction of Fe(III) oxide minerals. Microenvironmental conditions near the cell/mineral interface facilitate the formation of minerals that cannot be predicted by chemistry of the bulk aqueous phase. Biogenic Fe(II) can sorb to cell surface sites or form reduced mineral phases that may interfere with Fe(III) respiration. Fe(II)-induced inhibition and the physiological response of this inhibition by metal-reducing bacteria were examined in aqueous media using a variety of terminal electron acceptors.

Uncomplexed Fe(II), provided as $FeCl_2$ at concentrations in excess of the maximum sorption capacity of cells, inhibited both cell growth and iron reduction by *Shewanella putrefaciens*, strain CN32 for a period of 20 hours in a medium with ferric citrate as the terminal electron acceptor. Growth and iron reduction resumed following this lag period. $FeCl_2$ also inhibited growth of organisms in a medium with fumarate as the terminal electron acceptor. In contrast to the ferric citrate culture, fumarate cultures did not recover from Fe(II) induced inhibition. Electron microscopic observations revealed Fe precipitates distributed as small ($\leq$ 100 nm diameter) clusters over the entire cell surface. The size and distribution of precipitates corresponded to small, high contrast electronegative patches on cells before exposure to $FeCl_2$, as shown by electrostatic force microscopy. The islands of high contrast indicate that the exposed surface charge on *S. putrefaciens* is characterized by a rather heterogeneous spatial distribution. Some of the high contrast patches seemed to be associated with raised surface structures or blebs. Cells in ferric citrate medium appeared to shed Fe precipitates during the 20 hours of Fe(II)-induced inhibition by forming small blebs on their surfaces that were subsequently released as small (100 nm) membrane vesicles. Membrane vesicles also formed in aerobic cultures from which they could be isolated and examined for composition and enzymatic activity. Examination of the protein complement *S. putrefaciens* strain CN32 membrane vesicles using SDS-PAGE clearly revealed the presence of a limited number of proteins. Heme-containing proteins with molecular weights of 78.9 and 68.6 kDa were observed by staining gels using a peroxidase activity stain. Under anaerobic conditions with hydrogen as an electron donor, membrane vesicles reduced Fe(III), U(VI) and Tc(VII). This enzymatic reduction process, combined with the charged reactive surface sites provided by the membrane vesicles, initiated precipitation of mineral phases on the surface of the vesicles. This phenomenon has important implications as a physiological mechanism for removing biogenic precipitates that would otherwise suffocate metal-reducing bacteria. The biogenic mineral assemblages, termed nanofossils, are also morphologically similar to 'nanobacteria' observed on a Mars meteorite discovered in the Antarctic and may serve as paleological indicators of early life on earth and as biosignatures of life on other planets.

The Role of Natural Organic Matter in Microbial Reduction of Metals: Linking Chemical Structure to Bioavailability and Redox Reactivity

Baohua Gu,[1] Jie Chen,[1] Paul G. Tratnyek,[2] James T. Nurmi[2] and David R. Boone[3]
[1]Oak Ridge National Laboratory, Oak Ridge, Tenn.; [2]Oregon Graduate Institute, Beaverton, Ore.; [3]Portland State University, Portland, Ore.

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

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

Different NOM components were found to directly reduce metals or metal oxides [such as CrO_4^{2-} and Fe(III)], although they exhibit varying ability or capacity in reducing these metals. The polyphenolic-rich NOM fraction appeared to be the most reactive in reducing CrO_4^{2-} and Fe(III) compounds to Cr(III) and Fe(II) compounds in the absence of microorganisms. The reduction of CrO_4^{2-} was confirmed by the x-ray near-edge absorption spectroscopic (XNEAS) analysis. However, in the presence of microorganisms, the humic acid component appeared to be the most effective in shuttling electrons for the microbial reduction of these metals, which was attributed to its high-molecular-weight and polycondensed aromatic structural features. Our study confirms the heterogeneous nature of NOM — different components of NOM each possess different structural and functional properties and vary in their abilities in reacting with metals and in electron-shuttling for microbial reduction of these metals. In addition, our preliminary results suggest that certain microorganisms produce their own extracellular electron shuttles that facilitate the reduction of Fe(III) oxides.

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

Ken Kemner,[1] Shelly Kelly,[1] Ed O'Loughlin,[1] Ken Nealson,[2] John Zachara,[3] Yuri Gorby,[3] Jim Fredrickson,[3] Barry Lai,[1] Joerg Maser,[1] Zhonghou Cai,[1] Dan Legnini,[1] Peter Ilinski,[1] William Rodrigues,[1] Mark Schneegurt[4] and Chuck Kulpa[5]

[1]Argonne National Laboratory, Argonne, Ill.; [2]Jet Propulsion Laboratory, Pasadena, Calif.; [3]Pacific Northwest National Laboratory, Richland, Wash.; [4]Wichita State University, Wichita, Kan.; [5]University of Notre Dame, Notre Dame, Ind.

Understanding the fate of heavy-metal contaminants in the environment is of fundamental importance in the development and evaluation of effective remediation and sequestration strategies. Bacteria and the extracellular material associated with them are thought to play a key role in determining a contaminant's speciation and mobility in the environment. Additionally, the metabolism and surface properties of bacteria can be quite different depending upon whether the bacteria exhibit a planktonic (free-floating) or biofilm (surface adhered) habit. The microenvironment at and adjacent to actively metabolizing cells also can be significantly different from the bulk environment. Thus, to understand the microscopic physical, geological, chemical and biological interfaces that determine a contaminant's macroscopic fate, the spatial distribution and chemical speciation of contaminants and elements that are key to biological processes must be characterized at micron and submicron lengthscales for bacteria in both planktonic and adhered states. Hard x-ray microimaging is a powerful technique for the element-specific investigation of complex environmental samples at the needed micron and submicron resolution. The objectives of the studies presented here are to: (1) determine the spatial distribution and chemical speciation of metals near bacteria-geosurface interfaces, and (2) use this information to identify the interactions occurring near these interfaces among the metals, mineral surfaces and bacterially produced extracellular materials under a variety of conditions.

We have used hard x-ray phase zone plates to investigate the spatial distribution of 3*d* elements in single *Pseudomonas fluorescens* cells adhered to Kapton film and those in a planktonic state. Additionally, we investigated a single hydrated *Shewanella putrefaciens* adhered to an iron oxide thin film. The zone plate used in these microscopy experiments produced a focused beam with a cross section (and hence spatial resolution) of 0.15-0.40 micron. The samples (both planktonic and biofilm) were all grown in a consistent manner in a standard growth medium. The samples investigated were the "as grown" (*P. fluorescens* and *S. putrefaciens*) or samples that were harvested and rinsed in 0.1 M $NaClO_4$ solution before being exposed to 10, 100, or 1000 ppm Cr(VI) solutions for six hours (*Pseudomonas fluorescens*) and subsequently rinsed in 0.1 M $NaClO_4$ solution.

Results from x-ray fluorescence imaging of *P. fluorescens* in an adhered state indicate that the distribution of Ca can define the location of the microbe. A comparison of the distribution of Ca with the distribution of Cr does not indicate an intimate relationship between the adhered microbe and the contaminant. Similar studies of other *P. fluorescens* cells in the *planktonic* state do show a coincident distribution of Cr and Ca, thus indicating contact of the Cr to the microbe. Results from additional studies of *P. fluorescens* as well as *S. putrefaciens* will be presented.

In summary, these studies have demonstrated the utility of x-ray microbeams, particularly those produced by hard x-ray phase zone plates, for investigating the spatial distribution as well as the speciation of biologically and environmentally relevant trace elements at the mineral-microbe interface. Specifically, we have illustrated the use of submicron hard x-ray beams for determining the spatial distribution of metals in single bacteria cells (*P. fluorescens* and *S. putrefaciens*), in both the planktonic and adhered states, after exposure to different concentrations of Cr(VI). Further development of these techniques for such applications promises to provide unique opportunities in the field of microbiology and environmental research.

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

David S. Kosson,[1] Lily Y. Young,[2] Robert M. Cowan,[2] Eric L. Hachert[3] and David J. Scala[3]
[1]Department of Civil and Environmental Engineering, Vanderbilt University, Nashville, Tenn.;
[2]Department of Environmental Sciences, Rutgers, New Brunswick, N.J.; [3]Department of Chemical and Biochemical Engineering, Rutgers, New Brunswick, N.J.

We have formalized a mathematical reactive transport model describing fluid flow through a porous soil, linked to chemical and biological reduction of Fe(III) oxides. In addition to direct contact between Fe(III) oxides and bacteria, the model incorporates contributions to Fe(III) reduction rates and extents from measurable electron shuttles and chelating agents found in soil organic matter. In this model, Fe(III) (solid phase or chelated) can be reduced directly by bacteria or indirectly by reduced electron shuttles. The model accounts for three potential fates for Fe(II) produced: sorption to Fe(III) oxides, precipitation as Fe(II) carbonate (siderite) or advection of the soluble free species.

One of the most difficult model parameters to measure is the fraction of total soil Fe(III) that is bioavailable. A method for estimating soil Fe(III) bioavailability by titrating the soil with reduced anthraquinone disulfonic acid (AQHDS) was developed and compared against hydroxylamine-HCl reduction using four pure iron oxides and six soils. For the crystalline Fe(III) oxides used in this study—high surface area goethite, low surface area goethite and hematite—AQHDS and hydroxylamine-HCl gave comparable results and both slightly underestimated the true bioavailability determined using the model bacterium *Shewanella alga* BrY. However, AQHDS titration provided a much better estimate for bioavailability of amorphous Fe(III) oxyhydroxide, which is the predominant soil form, and also showed sensitivity to occlusion of "available" Fe(III) sites by Fe(II) sorption.

In natural floodplain soils collected from the Department of Energy's Savannah River Site, AQHDS titration and hydroxylamine reduction both gave accurate Fe(III) bioavailability estimates. The AQHDS titration method has an advantage over chemical reduction methods in that it can estimate Fe(III) bioavailability in environments in which Fe(II) adsorption or deposition reduce the amount of total Fe(III) that is bioavailable.

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

Joel E. Kostka
Department of Oceanography, Florida State University, Tallahassee, Fla.

This research represents a newly funded project within the Biotransformation and Biodegradation Program Element. The project will elucidate how mineral-bacteria interactions limit the migration of uranium (U) in contaminated sediments from the NABIR-Field Research Center (FRC). We will focus on the competition between Fe(III)-reducing bacteria (FeRB) and the sulfate-reducing bacteria (SRB), their impact on Fe mineralogy in the subsurface, and how these microbially mediated mineralogical changes will govern U speciation.

The proposed work will: (1) comprehensively characterize the dominant Fe and S minerals that are likely to limit U speciation in situ; (2) directly quantify reaction rates and pathways of terminal electron-accepting processes which control subsurface sediment chemistry; and (3) identify and enumerate the organisms mediating U geochemistry using molecular biological analysis. We will focus on representative subsurface sediments which vary substantially in sediment chemistry, such as parent rock mineralogy, groundwater sulfate and nitrate concentrations. For the less studied layer silicate and sulfide mineral groups, we will quantify the impacts of terminal electron-accepting pathways and the resulting reductive dissolution processes mediated by bacteria on the sorption of U. Through determination of reaction rates of important microbial respiration pathways and an in-depth characterization of minerals likely to predominate U sorption, we will provide important inputs for reactive transport models which may be used to predict U flow in subsurface sediments.

Use of RT-PCR and In situ RT-PCR Techniques to Detect Functional Gene Expression in Dissimilatory Metal- and Sulfate-Reducing Bacteria

Timothy S. Magnuson,[1] Yuri A. Gorby[2] and Gill G. Geesey[1]
[1]Montana State University, Bozeman, Mont.; [2]Pacific Northwest National Laboratory, Richland, Wash.

Identification of metal and sulfate-reducing bacteria in environmental samples has focused on the use of 16S rDNA PCR amplification, which provides little information about gene expression events relevant to metal- and sulfate-reducing activities. We have developed PCR primers specific for a number of functional genes of interest in sulfate-reducing bacteria (SRB) and dissimilatory metal-reducing bacteria (DMRB). The genes include those encoding *Desulfovibrio* sp. hydrogenase (*hyd*), a membrane-bound metal reducing cytochrome from *Geobacter sulfurreducens* (*ferA*), and *D. desulfuricans* bisulfite reductase (*dsvA*). RNA was purified from SRB and DMRB grown under different growth conditions. RT PCR differential display experiments with SRB demonstrate that *dsvA* genes are differentially expressed when grown on sulfate.

Similar experiments demonstrate that *ferA* is differentially up expressed when the organisms are grown on metals. Hydrogenase expression appears to not be influenced by growth on metals in either SRB or DMRB. In all cases, sequence identity of the amplicons was confirmed by sequencing analysis and BLAST search. In situ RT-PCR techniques show that *dsvA* and *ferA* expression can be detected in whole cells by means of fluorescent oligonucleotide probes specific for amplicons generated by IS-RT-PCR. Expression of *dsvA* was detected in *D. desulfuricans* cells by means of IS-RT PCR techniques. Similar results were obtained for detection of *ferA* expression in *G. sulfurreducens*. The results indicate that (1) functional gene expression can be measured by RT-PCR techniques; (2) distinct differences are seen with regard to growth conditions with and without metals being present; and (3) these techniques can be extended to detection of gene expression in whole cells.

Surface Complexation of Mn Oxides: Effects on Metal Reduction as Assessed by Electron Energy-Loss Spectroscopy

Kenneth Nealson, Andrea Belz, Channing Ahn and Megan Andrews
Jet Propulsion Laboratory, California Institute of Technology, Pasadena, Calif.

Understanding the mechanisms (chemical and biological) that control the rates of metal reduction is critical to ultimately being able to manipulate such reactions in nature, and use them for bioremediation. Accordingly, the reduction of Mn oxides by *S. oneidensis* MR-1 under various conditions was monitored by studying the release of soluble Mn(II) into solution. Electron Energy-Loss Spectroscopy (EELS) was used to assess the manganese oxides during oxidation and under conditions of various anions in the solution.

Both the manganese and oxygen spectra were studied under a variety of conditions. Vernadite, a poorly crystalline Mn oxide, was shown to be highly impacted by the counter ions (phosphate or sulfate) in the medium, while highly crystalline pyrolusite showed little effect. Reduction of vernadite was rapid, consistent with it being a hydrated, more amorphous phase, while the reduction of pyrolusite was essentially zero during the time of our experiments. Vernadite reduction rates were also strongly impacted by the presence of counter-ions in solution: phosphate resulted in an inhibition of reduction rate, while sulfate strongly stimulated reduction. Analysis of the surface chemistry of the oxides suggests that the bacteria take advantage of the surface chemistry of the metal oxides for metal reduction. At the conclusion of the experiments, considerable particulate Mn remained, often closely associated with the cells. Electron diffraction of this material (nm sized) suggested that it is highly crystalline Mn oxide. A mechanism for the appearance of this product is proposed—namely, that it represents crystalline impurities initially present in the vernadite preparation.

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

Mary P. Neu, Larry E. Hersman, Christy E. Ruggiero, Mitchell T. Johnson, John H. Matonic, Seth G. John, Lee He, Sean D. Reilly, Jennifer H. Forsythe, Laura A. Vanderberg, Jason R. Fairlee, Paul D. Gladden, Matthew M. Cox, Dawn J. Chitwood, Emily P. Kenney and Greg L. Wagner
Los Alamos National Laboratory, Los Alamos, N.M.

Our overall goal is to understand the fundamental interactions of actinides and soil microbes, which can affect the mobility of actinides in the environment, and to move toward field application by studying these interactions in soil matrices. The first objectives toward achieving this goal include:

1. Characterize the chemical toxicity of actinides to bioremediating microbes and common soil aerobes.
2. Determine and characterize the binding of actinides to extracellular polymers and whole cells of microbes that produce substantially different exopolymers.
3. Determine the siderophore-mediated uptake of actinides and results of the translocation process (speciation and localization) using three different classes of soil microbes.
4. Examine the fundamental chemical interactions of siderophores and actinides under environmentally relevant conditions.

Through our previous studies, we have learned that desferrioxamine siderophores (DFO) are surprisingly slow at solubilizing Pu(IV) oxide/hydroxide solids, but eventually form Pu(IV) siderophore complexes in solution. We studied the redox properties of Pu (III, IV, V, VI) in the presence of DFO and found that at environmentally relevant solution pH each Pu species rapidly and irreversibly forms a Pu(IV)DFO complex. We also studied the solution speciation and solid state structure of Pu(IV)DFO. Once the formation and prevalence of the Pu(IV)DFO complexes were established, we studied the microbial uptake of the complex.

We have studied the uptake of Fe, Pu and U in *Microbacterium flavescens* (JG 9) mediated by the siderophore desferrioxamine B (DFB). We will show that the plutonium DFB complex can be recognized and partially taken up by the microbe in a process similar to the Fe-DFB complex via protein mediated, metabolically-dependent uptake. Models will show that it is indeed possible for the plutonium DFB complex, despite the larger size of the plutonium ion, to fit into the same protein binding pocket as Fe-DFB. The uranium DFB complex is not recognized or taken up by the same Fe-uptake system. This may indicate that Pu chelated by microbes or microbial products in the environment will exhibit similar bioavailability and bio interactions as Fe does, and therefore must be considered in bioremediation technologies for sites that contain Pu.

We have also examined the actinide binding ability of two extracellular polymers of very different composition: the well-characterized γ polyglutamic acid (PGA) polymer produced by *Bacillus licheniformis* and the polysaccharide produced by *Rhodococcus erythropolis*. The Fe(III), U(VI) and Pu(IV) binding by these exopolymers will be presented, along with thermodynamic information, such as binding strength and stability of metal complexes as a function of pH. The binding of these metals by metabolizing whole cells of *B. licheniformis* with intact polymer will be compared to the binding of the metals by the isolated exopolymer. Toxicity of these metals to the microbes will also be reported.

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

James N. Petersen,[1] Luying Xun,[1] William A. Apel,[2] Brent M. Peyton[1] and D.R. Yonge[1]
[1]Washington State University, Pullman, Wash.; [2]Idaho National Engineering and Environmental Laboratory, Idaho Falls, Idaho

Important questions concerning direct microbial reduction of Cr(VI) include: (1) which members of an environmental consortium are responsible for chromate reduction; (2) for active chromate reducers, what biochemical pathways are used for chromate reduction ; (3) what are the effects of carbon source (electron donor) and of inorganic contaminant ions 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, both bacterial consortia from the Hanford site and pure strains were examined in various ways.

A portion of the work focused on fundamental, generalized microbial mechanisms for chromate reduction. Since this activity is quite common, we hypothesized that some general reductases can reduce chromate. To test this hypothesis, an *Escherichia coli* general flavin reductase (Fre) was used to reduce chromate. The Fre was a highly effective system for chromate reduction with a specific activity of 33.9 and 103.9 $\mu mol\ mg^{-1}\ min^{-1}$ in the presence of riboflavin under aerobic and anaerobic conditions, respectively. The end product was characterized to be soluble Cr(III) complexes instead of $Cr(OH)_3$, supporting our hypothesis.

At the cellular level, we found a gradual and progressive increase in the specific Cr(VI) reduction rate with incubation time, which is gradually lost when the cultures are re exposed to oxygen, under anaerobic conditions with *Shewanella putrefaciens* MR-1, implying that Cr(VI) reduction might be due to inducible enzymes. In these cultures, nitrate inhibition of Cr(VI) reduction was observed only with denitrifying cultures and not with fumarate-reducing cultures, but nitrite inhibition is observed with both fumarate- and nitrate reducing cultures, again indicating that a non-specific mechanism may be used to accomplish Cr(VI) reduction.

Work was also focused on measuring the effects of various electron donors on cell growth and chromate reduction in anaerobic enrichments from recently acquired core samples one from 76.5 ft and the other from 85 ft from Hanford's 100-D site. These cores were chosen since they represented chromate impacted (85 ft) and non impacted (76.5 ft) sediments of similar lithology. Cultures were grown using various organic acids, alcohols, the amino acid l-asparagine and the carbohydrate d-xylose. Results suggest that maximum rates of chromate reduction occur in consortia containing a mix of denitrifying and fermenting organisms, once again indicating that a non-specific mechanism is used to accomplish Cr(VI) reduction.

Laboratory scale soil column experiments are also being performed to test our understanding of chromium (VI) reduction in an anaerobic environment simulating aquifer conditions. The column contains coarse sand inoculated with a Hanford site, subsurface bacterial consortia. The feed solution is a simulated groundwater media (SGM) amended with sucrose (150 mg/L), yeast extract (15 mg/L) and Cr(VI) (2 mg/L). In addition to monitoring column effluent for degradation products, biological markers are being used to monitor community dynamics within the column. The column data will help be used to assess the applicability of batch reactor kinetics to continuous flow systems and to expand our understanding of population dynamics in subsurface systems.

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

Brent M. Peyton,[1] Jim Amonette,[2] Gill Geesey[3] and Zbigniew Lewandowski[3]
[1]Washington State University, Pullman, Wash.; [2]Pacific Northwest National Laboratory, Richland, Wash.; [3]Montana State University, Bozeman, Mont.

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 uranium (U) and chromium (Cr) under these conditions has been shown. However, as with other possible treatments, the long-term stability of the immobilized lead and uranium, and the factors that affect the immobilization and remobilization process, must be quantified to determine whether the treatment can produce an acceptable endpoint.

In the presence of lead, we have quantified structure and activity of pure culture *D. desulfuricans* associated with hematite and quartz surfaces. Biofilms grown on hematite were more porous than the biofilms grown on quartz, both in the presence and absence of lead. H_2S concentration measured by microelectrodes in biofilms grown on hematite was lower than that in biofilms grown on quartz. Similarly, the amount of lead immobilized in biofilms on hematite was lower in the biofilm grown on quartz. These observations indicate that the mineral composition under surface-associated SRB colonies can significantly affect rates of lead precipitation and strongly supports our original proposed hypothesis.

Studies of selective colonization of minerals by SRBs were also conducted with rock thin sections subsequently treated with Ag^+ to mark reducing zones that contain either Fe^{2+} or SRBs. Mapping of surface Ag using synchrotron X-ray microscopy showed more Ag in small crevices and on Fe bearing mineral surfaces. Studies also focused on the possible inhibitory effect of Al content of the underlying mineral substratum and in solution. Batch studies showed significant effect of soluble Al on SRB populations at 1 and 10 mM and pHs 6.5, 7.2, and 8.3. Similar studies in the presence of mineral grains showed enhancement by goethite (alpha-FeOOH), slight inhibition by boehmite (gamma-AlOOH), and by 1 mM soluble Al in the presence of quartz.

In addition to lead precipitation by biogenic sulfide, SRB can enzymatically reduce U^{6+} to U^{4+}. After abiotic sorption of U^{6+} onto goethite, hematite and ferrihydrite, serum bottles were inoculated with washed cells of *D. desufuricans* G20. The bacteria grew within three days using sulfate as electron acceptor and lactate as electron donor. With an initial lactate:sulfate molar ratio of 4:1, significant SRB growth and simultaneous reduction of U^{6+} to U^{4+} was observed. Significant SRB growth was also observed at a lower lactate:sulfate molar ratio (1.5:1) however, no reduction of uranium was observed. Anion analysis indicate that *D. desulfuricans* depleted $SO_4^=$ and bioavailable Fe(III) before reducing U^{6+}. In addition, Fe^{3+} from the iron oxides precipitated as iron sulfides. In the field, the presence of iron sulfide will help maintain a low redox-potential in the treatment zone that would stop or slow the reoxidation of U^{4+} to U^{6+}.

In addition to serum bottles, ultra-low volume flow cells have been used to examine uranium reduction/precipitation processes in the presence of SRB. XPS was used to assess the valence state of uranium on hematite surfaces. Binding energy determination of the $U4f_{7/2}$ photopeak and its component bands suggests that on surfaces that were not exposed to sulfate-reducing bacteria, the $U4f_{7/2}$ photopeak results from contributions from U^{6+} (31%), U^{5+} (32%) and U^{4+} (37%). Thus in the absence of sulfate-reduction a mixed valence U-phase is present at the hematite surface probably arising from adsorption of U^{6+}. Similar analysis of the hematite surface exposed to *D. desulfuricans* G20 indicates an increased contribution from U^{4+} (58%) and U^{5+} (42%) and an absence of U^{6+}. Additionally, a strong U5f photopeak at 1 eV is consistent with a reduced U-phase. Complexed U coordination in the presence of SRB is being studied using XAS.

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

Bruce M. Thomson,[1] Larry L. Barton[2] and D. Scott Simonton[1]
[1]Department of Civil Engineering, and [2]Department of Biology, University of New Mexico, Albuquerque, N.M.

Current research on in situ remediation processes for soils and groundwater contaminated by metals, metalloids and radionuclides is focused on the use of anaerobic organisms to reduce the contaminants to insoluble phases, including oxides (e.g., Cr_2O_3, UO_2), sulfides (e.g., FeS, MnS, FeAsS, AsS_2, MoS_2) and possibly elemental forms (Se). These metals are expected to remain insoluble provided that reducing conditions are maintained in the subsurface formation. However, there is little information regarding the stability of these phases over long time periods (i.e., decades to centuries) as geochemical conditions change. This project uses a combination of laboratory research and numerical modeling to investigate the long-term stability of metals and metalloids immobilized by microbial reduction. The project has focused on the stability of arsenic (As), chromium (Cr) and selenium (Se), reduced by *Desulfovibrio desulfuricans* and *Shewanella putrefaciens*.

Electron microscopic analysis (SEM and TEM) has been used to identify the mineral phases present as a result of microbial reduction. These phases depend on whether reduction and precipitation occur in the presence of sulfate-reducing bacteria (*D. desulfuricans*) or iron-reducing bacteria (*S. putrefaciens*). Precipitates formed in the presence of high sulfide concentrations tend to be associated with sulfide phases including AsS, AsS_2, and FeS, which are not formed by cultures of iron-reducing organisms. Chrome and Se will be removed from solution in both cultures as a Cr-hyroxide and elemental Se respectively.

A series of leaching experiments has been conducted on metals immobilized by microbial reduction in packed columns containing silica sand as an inert substrate. These columns were prepared to simulate the conditions that might occur in a permeable barrier used to intercept metals from a contaminant plume. Three types of leaching experiments were done: (1) a deionized water leach, (2) an acetic acid leach as required in the Toxicity Characteristics Leaching Procedure (TCLP), and (3) a long-term leach of oxidized groundwater. The TCLP leach is especially significant because this is the regulatory test established to determine whether the contaminants would be classified as hazardous. The leachate from the TCLP showed that metals immobilized on sand by *D. desulfuricans* were not hazardous. Work is continuing on the *S. putrefaciens* system. The results of the leaching tests have been interpreted in the context of solubility predictions from geochemical modeling.

A conceptual model of metals released from a permeable barrier system has been developed. Because groundwater and drinking water regulations are based on aqueous concentrations rather than the total mass of immobilized pollutants, the implications for long-term stabilization must therefore be placed in the context of contaminant release rates and subsequent dilution from groundwater flow.

Mesoscale Biotransformation Dynamics Controlling Reactive Transport of Chromium

Jiamin Wan,[1] Tetsu Tokunaga,[1] Terry Hazen,[1] Keith Olson,[1] Mary Firestone,[2] Egbert Schwartz,[2] Steve Sutton,[3] Matt Newville[3] and Antonio Lanzirotti[3]
[1]Lawrence Berkeley National Laboratory, Berkeley, Calif.; [2]University of California, Berkeley, Calif.; [3]University of Chicago, Chicago, Ill.

The interdependent influences that sediment structure and microbial communities have on transport and reduction of chromate are being investigated in batch systems, synthetic aggregates and natural soil aggregates of Altamont clay (Altamont, Calif.). The two main studies in this project address Cr diffusion and reduction within aggregates, and the microbial community influence on Cr reduction and its response to Cr exposure. 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. 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 in aggregates amended with TSB. Micro-XANES analysis provided direct evidence of Cr(VI) reduction to less toxic Cr(III) forms. Cr diffusion reduction profiles were modeled using spatially-dependent first-order kinetics. The extent of Cr transport into sediment blocks was far less than expected by diffusion without reduction, proportional to the boundary Cr(VI) concentration, and well correlated with locations of the reoxidation fronts. Recent measurements of Cr transport into natural soil aggregates showed results that were very similar to those from synthetic aggregates.

DNA fingerprints of soil microbial communities showed several populations that appeared only in soil sub-samples that were exposed to Cr(VI). Four sequences that only appeared in samples exposed to Cr(VI) were isolated and characterized. One sequence belonged to a *Zymomonas* sp, another to a *Synorhizobium* sp. and the last two both belonged to a *Pseudomonas cepacia* strain. We have constructed strain specific primers that target the ITS regions of these organisms in order to monitor their population dynamics in subsequent experiments. Several bacterial strains were isolated from the soil columns on media containing 500 ppm Cr(VI). Culturing attempts were only successful under aerobic conditions; no isolates were obtained in jars with gas packs or in an anaerobic glove box. The Cr(VI) tolerant aerobic isolates all were gram positive bacteria and included two *Arthrobacter* species, a *Bacillus* sp. and an *Agrococcus* sp. In order to investigate the relationship between microbial iron and Cr(VI) reduction, a dissimilatory iron reducing bacteria was isolated from the bottom of soil columns amended with 10% Tryptic Soy Broth. This strain is presently being characterized.

These results show that important microbial and chemical heterogeneity can develop from transport-limited reactions within soil aggregates, and that measurements and models with at least mm-scale spatial resolution are needed to understand such highly nonequilibrium systems.

PROGRAM ELEMENT 2

Community Dynamics and Microbial Ecology

Vadose Zone Chromium Reduction in Unsaturated Batch and Unsaturated Flow Column Experiments

Fred J. Brockman,[1] David L. Balkwill[2] and Thomas L. Kieft[3]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]Florida State University, Tallahassee, Fla.; [3]New Mexico Institute of Mining and Technology, Socorro, N.M.

A large proportion of the contaminant mass at Department of Energy sites in arid and semi-arid regions of the United States exists in deep (>10 m) vadose zone sediments, which serve as a reservoir for continued contamination of groundwater. Cr(VI) and nitrate commonly occur as vadose zone co-contaminants at DOE sites. This project used uncontaminated vadose zone sediments from an area adjacent to DOE's Hanford Site that has been exposed to artificial recharge (sprinkle irrigation) for three years, as an analog for contaminated deep vadose zone sediments exposed to anthropogenic recharge by site activities. The project goals were to: (1) determine the potential for transformation of Cr(VI) (oxidized, mobile) to Cr(III) (reduced, immobile) under unsaturated conditions as a function of different levels and combinations of (a) chromium, (b) nitrate (co-disposed w/ Cr), and (c) molasses (inexpensive bioremediation substrate); and (2) determine population structure and activity in experimental treatments by characterization of community rRNA by RT-PCR and terminal restriction fragment analysis (TRFA). Speciation of solids and centrifuged pore water was determined by HPLC, ICP-MS, XRF and XANES. Community structure and activity was examined by extraction of RNA , RT-PCR of rRNA and subsequent terminal restriction fragment analysis (TRFA).

In our initial study, we examined shifts in the microbial community in replicate columns containing vadose zone sediment exposed to no flow (5% volumetric water content, WC), unsaturated flow (15% WC), and saturated flow (39% WC) treatments. Artificial pore water lacking organic carbon, nitrogen and phosphorus was added to the columns receiving flow, and flow was maintained for four weeks before terminating the experiment. The results showed that unsaturated and saturated flow increased cell growth in the absence of added nutrients by a factor of ~ 5, and induced significant changes in community rRNA profiles.

To select a subset of treatment combinations to be evaluated in subsequent unsaturated flow column experiments containing contaminants, a five week unsaturated aerobic batch experiment was conducted using 27 treatments with varying chromate, nitrate and molasses levels. Mobile Cr(VI) was microbially reduced to immobile Cr(III) in the presence of either molasses or nitrate individually, or together. Cr(VI) concentrations in pore water decreased by 66 to 87% with the greatest reduction in the presence of both molasses and chromium. The lack of significant Cr(VI) sorption and the low level of measured abiotic reduction showed reduction was biological. The similarity dendrogram generated from TRFA data showed Cr level was primarily responsible for the higher level divisions, with nitrate and molasses levels playing some role in lower level divisions. In contrast, signature biomarker analysis separated the samples on the basis of molasses and nitrate treatments, with chromium concentrations having an impact only on the lower level divisions. To further evaluate the potential for Cr(VI) bioremediation in the deep vadose zone, the impact of unsaturated flow (7-9% WC) on Cr(VI) reduction was studied for 6.5 weeks in columns. Approximately 10% of the added Cr(VI) (13 mg/column or 32 micrograms/g sediment) was reduced to Cr(III) in columns receiving molasses and nitrate. Reduction was confirmed by a corresponding increase in solid-phase Cr in the column sediments. Cr(III) was distributed throughout the column. Chromium reduction was negligible in the molasses only, nitrate only, and blank (artificial pore water) treatments. Although the percent of Cr(VI) reduction was low relative to the batch results, columns were 15 cm long and residence times ~12 hrs. Thus, chromium loss could be significant in an actual vadose zone. TRFA showed that ~ 35% of all peaks were unique to a particular treatment. These results indicate that addition of molasses and nitrate to the vadose zone has potential to decrease the unsaturated transport of chromium into underlying aquifers.

Ecological Interactions Between Metals and Microbes that Impact Bioremediation

Allan Konopka
Department of Biological Science, Purdue University, W. Lafayette, Ind.

Our objectives are as follows:

- Determine whether spatial heterogeneity in metal distribution is a major determinant of microbial community activity and diversity in contaminated soils.
- Determine the effects of increased metal bioavailability and mobility upon microbial community activity and diversity.
- Determine the role of metal-resistant bacteria in microbial communities that contain many metal-sensitive members. These microbes might function as either "bioprotectants" through their physiological activity, or reservoirs of transferable resistance genes.
- Determine the interactions among multiple toxicants upon microbial activity in mixed-waste sites.

The correlation between bacterial biomass, population structure and the amount of lead, chromium and aromatic compounds present along a 21.6 m transect in which the concentrations of both heavy metals and aromatic compounds varied 2-3 orders of magnitude has been determined. Microbial biomass, measured as total phospholipid-P, was greatest in soils with the highest organic contamination. Both denaturing gradient gel electrophoresis of 16S rDNA gene fragments (amplified by PCR from total soil DNA) and analysis of fatty acid methyl esters derived from phospholipids (PLFA) were used to examine populations structure. Principal component analysis of fingerprint patterns from both methods showed that microbial communities were distinct from sample locations with (a) high metal and high aromatic concentrations, (b) moderate metal concentrations and no aromatic substrates, and (c) low metal and no organic concentrations.

Microbial community activity has been assessed in several ways:

- Community resistance to added Pb was tested in short-term experiments employing ^{14}C-labeled glucose or phenol. Microbial activity in a highly contaminated sample (Pb content of 8000 mg kg^{-1} soil) retained high activity up to 50 g Pb kg^{-1} soil, whereas the community previously exposed to lower contamination (<50 mg Pb kg^{-1} soil) displayed 50% inhibition of activity 10 g Pb kg^{1}.
- Experimental additions of naphthalene and/or chromium were made to soils, and the kinetics of aromatic and Cr(VI) disappearance, biomass and community composition changes were determined over a 45-day interval. There was a lag in the rate of naphthalene degradation when 160 mg Cr(VI) kg^{-1} was added to soils, but the organic was reduced by >90% by day 45. Changes in biomass amounts and community structure were relatively small. The results suggest that after long-term exposure to elevated metal concentrations, the microbial activity is more limited by available carbon than the presence of metals.
- The functional diversity of microbial communities was assessed by the short-term (<24 h) respiratory response to a suite of 15 organic substrates. Multivariate statistical analysis indicated that communities from highly contaminated locations were functionally distinct (as they were phylogenetically distinct) from sites with low metal and hydrocarbon content.

Factors Controlling the Distribution and Activity of Dissimilatory Metal-Reducing Microorganisms in Uranium-Contaminated Subsurface Environments

Derek R. Lovley
Department of Microbiology, University of Massachusetts, Amherst, Mass.

In order to learn more about the factors controlling the rate and effectiveness of U(VI) reduction in contaminated subsurface environments, U(VI) reduction was studied in sediments from the Shiprock, N.M., UMTRA site. U(VI) was only slowly reduced when uranium-contaminated sediments that were aerobic in situ were incubated under anaerobic conditions. However, the rate of U(VI) reduction could be greatly stimulated with the addition of organic electron donors, of which acetate was the most effective. In the presence of acetate, U(VI) was reduced concurrently with the reduction of Fe(III) and prior to sulfate reduction. Initial concentrations of approximately 10 μM U(VI) were reduced to less than 1 μM within 15 days. The primary mechanism for U(VI) reduction was microbial U(VI) reduction and neither sulfide, reduced AQDS, nor Fe(II) abiotically reduced U(VI) in the sediments. These studies demonstrate that controlled addition of low concentrations of electron donor, particularly acetate, to uranium-contaminated aquifers can stimulate microbial U(VI) reduction and promote the removal of soluble U(VI) from contaminated groundwater without the production of toxic sulfides.

Studies on the microbial community in the uranium-contaminated subsurface sediments demonstrated that stimulation of microbial U(VI) reduction greatly enhanced the growth of microorganisms in the *Geobacteraceae*. Other commonly studied dissimilatory metal-reducing microorganisms, including *Shewanella* species, were not important. The sediments from site 854, which have a salinity about 10-fold higher than seawater, were the exception to this finding. In these sediments, stimulation of Fe(III) and U(VI) reduction resulted in the enrichment of previously uncultured *Archaea*. Fe(III)-reducing enrichments from these sediments were established in media that mimicked the water chemistry at this unique site in order to learn more about the organisms that are likely to be responsible for metal reduction at high salinity.

The finding that *Geobacter* species are important in metal reduction in a diversity of subsurface environments led to studies to find molecular targets which can be used to quantify the activity of these organisms in the subsurface. Studies with *G sulfurreducens* and *G. metallireducens* demonstrated that they produce pili specifically in response to growth on Fe(III) oxide and that mRNA for *pilA*, the gene for an important structural pili protein, is only present during Fe(III) oxide reduction. This provides a novel potential target for determining when *Geobacter* species are using Fe(III) oxide as an electron acceptor in the subsurface. Genetic studies elucidated several other targets involved in the electron transfer to Fe(III) and U(VI) that might also be useful in monitoring the activity of these organisms. Methods for growing *G. sulfurreducens* under defined conditions in chemostats were developed in order to assess the relationship between rates of activity and the transcription of target genes. Other results on the physiology of *Geobacter* species relevant to their growth in subsurface environments will also be presented.

It was demonstrated for the first time that the addition of nitrate to anaerobic sediments can result in the oxidation of U(IV) to U(VI). This provides a mechanism for extracting uranium from the subsurface once the contaminant uranium has been concentrated into a discrete zone with microbial U(VI) reduction. The microbiology of this novel process is under investigation.

Diversity of *Cytophaga-Flexibacter-Bacteroides* Populations in a Chromium-Contaminated Superfund Site

Terence L. Marsh, Fiona Crocker, Veronica Gruntzig, Jason Schachner, Natasha Isaacs, Gary Icopini and David Long
Center for Microbial Ecology and Department of Geological Sciences, Michigan State University, East Lansing, Mich.

The Cannelton Industries Superfund Site in Michigan is primarily contaminated with chromium and other heavy metals resulting from the 50-year operation of a leather tannery. We have completed an extensive survey of the microbial community with terminal restriction fragment length polymorphism (T-RFLP). Using bacterial domain primers, unique T-RFLP profiles were observed for microbial communities in soils with high chromium concentrations and a set of unique terminal restriction fragments was repeatedly detected in these soils. The *Cytophaga-Flexibacter-Bacteriodes* (CFB) group was identified as the phylogenetic group contributing to the unique terminal fragment profiles.

These initial observations led to a more focused study directed at CFB diversity in low- and high-chromium-contaminated soils. The CFB diversity was compared using T-RFLP with CFB-specific 16S rDNA primers, phylogenetic analysis of CFB-cloned sequences, and physiological characterization of CFB strains isolated from these soils. The comparison of the T-RFLP profiles using the CFB-specific primers confirmed the presence of the unique terminal restriction fragment patterns in soils with high levels of chromium. Phylogenetic analyses of approximately 50 cloned, 16S rDNA sequences identified at least five distinct clades within the CFB line of descent. In addition, clades unique to either high- or low-chromium contaminated soils were identified. Chromium-resistant bacterial strains isolated from the contaminated soils were confirmed to belong to the CFB group on the basis of colony morphology and 16S rDNA sequencing. The mechanism of resistance to chromium as well as the interaction of chromium resistant *Cytophaga sp.* with soils contaminated with chromium are under investigation. Hence we have identified phylotypes that are unique to chromium-contaminated soils, suggesting that elevated concentrations of chromium in soils may select for different populations of CFB-related bacteria.

Microbially Induced Phosphorous Bioavailability: Effects on Community Ecology and Uranium Sequestration

Anthony V. Palumbo,[1] Scott Brooks[1] and Patricia Sobecky[2]
[1]Oak Ridge National Laboratory, Oak Ridge, Tenn.; [2]Georgia Institute of Technology, Atlanta, Ga.

Traditional approaches to the remediation of metals and radionuclides utilize dissimilatory reduction processes. However, in oxygenated environments such as the vadose zone, dissimilatory reduction often proves to be problematic. We have demonstrated an alternate approach that relies on the formation of low-solubility phosphate minerals. The key limitation in formation of phosphate minerals is delivery of phosphate, which is not mobile in many subsurface environments. We have been examining the introduction of organic phosphorous because many of the organic forms are more mobile in the subsurface.

With previous NABIR funding we have demonstrated the potential for introduction of both bacteria over-expressing phosphatase (OXP) and organic phosphorus (OP) for immobilization of U. We have introduced plasmid pJH123 into three different subsurface pseudomonads (*Pseudomonas veronii*, *Pseudomonas rhodesiae* and *Pseudomonas sp.* isolate 150), which were selected for their potential in field-scale delivery systems. Plasmid pJH123 was stable in two of the three subsurface isolates assayed during a six-day incubation period. An increase in PhoA activity, ranging from 111-fold to 860-fold was apparent in all GEMs relative to controls. Similarly, released inorganic phosphate from glycerol-3-phosphate in sterilized sediment microcosms was observed to increase concomitantly with the increase in alkaline phosphatase activity observed in each GEM. GEM-produced inorganic phosphate resulted in a range of 4% to 69% uranium precipitating as uranyl phosphate. Studies still underway are examining the impacts of this introduction on microbial communities.

We are extending our efforts to develop and use triethylphosphate (TEP)-utilizing strains and genes. Numerous strains (e.g., *Pseudomonas picketti*) capable of utilizing TEP have been isolated from several sites and characterization of their activity is ongoing. Several approaches are being used to access the genes responsible for TEP utilization (e.g., preparation of genomic libraries and expression in *E. coli*). Evidence from these studies has indicated that the genetic requirement for expression of the TEP utilization phenotype observed in transformants is large, thus suggesting a requirement for expression of multiple genes. A comparison of adsorption to Abbott's pit sediments has been completed for U and phosphorous. Much less TEP is adsorbed than inorganic P (indicating that TEP will be more mobile). U is highly sorbed in the presence of inorganic PO_4^{3-} but not in the presence of TEP (no phosphatase containing bacteria were present in this experiment).

We are now using uranium in experiments with sediments from the NABIR Field Research Center (FRC) site and will assess the influence of site geochemistry on the process. Using strains containing alkaline phosphatase and those containing genes for TEP utilization, we can further assess the bioavailability of phosphorous at the sites and immobilize U.

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

Barth F. Smets, Catalina Arango P., Olga Zelennikova, Jayne B. Morrow, Doug Marcotte, Don Stadolnik, Michelle Ng and Erzsebet Pocsi
University of Connecticut, Storrs, Conn.

The hypothesis of our research 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 examine gene transfer in soil microcosms subject to different levels of heavy metal stress and examine this for soils that are either pristine or have historic heavy metal contamination.

We aim to mimic the conditions of a semi continuous oligotrophic carbon flux that exists in the subsurface. Samples were obtained from the saturated zone of an alluvial deposit aquifer at a site contaminated with Cd and Zn adjacent to a closed metal plating waste lagoon. Each microcosm consists of subsurface soil aliquots placed in 40-ml sample vial subject to periodic (2x/week) addition and withdrawal of artificial groundwater (AGW) supplemented with a low-carbon concentration (peptone at 10 mg/L). Samples are then subject to molecular analysis and microbial enumeration. Repeated addition and withdrawal of AGW allow long term (10 to 30+ weeks) operation of the microcosm. In most experiments, Cd was studied as model heavy metal stress at different concentrations ($C_{T, Cd}$ = 0,10, 100, 1000 microM).

Several long-term experiments have been completed. In a first set, the fate of *Pseudomonas putida* KT2440 and its plasmid TOL::Tn*5*(*km*) was monitored at various degrees of Cd stress. This strain was originally proposed as the model delivery system for our work, because it contains a chromosomal copy of the $P_{A1\text{-}03/04}$-*gef*-based IPTG inducible suicide gene cassette present on a miniTn*5*. Hence, the efficacy of suicide induction in the microcosm systems was examined. Our results indicated, however, that the $P_{A1\text{-}03/04}$ –*gef* suicide miniTn*5* construct was not an effective means of selective elimination of a bacterial plasmid donor in tested soil microcosms, due to the combined effects of spontaneous loss of the mini-transposon, spontaneous mutations in the $P_{A1\text{-}03/04}$ *gef* cassette and rapid selection of escape mutants upon suicide induction. Due to the inefficacy of the suicide system for selective plasmid donor removal, several *E. coli* strains were then used for plasmid delivery in the microcosm given their presumed competitive inferiority.

Using subsurface soils historically contaminated with Zn^{2+}, Cd^{2+} and Ni^{2+}, we examined the fate of two RSF1010 plasmids (IncQ, tra mob+; pMOL 187 and pMOL 222 containing the *czc and ncc* operons, respectively) originally introduced with *E. coli* donors in semi-continuously operated microcosms at increasing Cd concentrations. We examined whether the long-term stability of the plasmid in the community could be enhanced by the simultaneous co introduction of the broad-host range plasmid RP4 (IncPα, tra+ mob+). All treatments were examined in absence or presence of RP4. While the stability of the introduced *E. coli* strain was somewhat impacted by the degree of metal contamination, the strains were typically not recoverable after 4 weeks of operation. Increases in the degree of heavy metal resistance were observed at increasing Cd additions, suggesting some community selection effects. Evidence of pMOL 222 transfer was obtained by acquisition of Ni-resistance phenotype by the indigenous community. Phenotypic evidence of pMOL 187 could not be obtained due to unexpected high background Cd-resistance.

Results to date indicate that plasmid transfer in oligotrophic and metal-impacted subsurface microbial communities occurs at detectable frequencies, and that the extent of plasmid transfer may be limited by the mobilization potential of the indigenous community. Plasmid survival in the microcosm was only slightly affected to the metal stress (Cd) applied, which was complicated by the low metal availability. Concluding experiments will provide molecular identification of transconjugants and plasmids isolated during this study.

Understanding the Roles of Spatial Isolation and Carbon in Microbial Community Structure, Dynamics and Activity for Bioremediation

James M. Tiedje,[1] David S. Treves[1] and Jizhong Zhou[1,2]
[1]Center for Microbial Ecology, Michigan State University, East Lansing, Mich.; [2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

The goal of this study is to establish a scientific foundation for improved in situ bioremediation of contaminated Department of Energy sites through understanding the mechanisms that control structure, dynamics and function of soil microbial communities. Previously, we found that microbial communities from low-carbon saturated subsurface soils showed dominance by one or a few species, whereas those from surface soils showed high diversity and a complete lack of dominance. Mathematical modeling identified spatial isolation as a key mechanism that could explain these differences. However, at a high carbon site we found that the microbial communities from several saturated samples also lacked dominance, indicating that carbon resource heterogeneity could be an additional controlling factor. To more quantitatively assess diversity in these samples and develop a common framework for assessing microbial community structure we tested the applicability of Simpson's index to our community data—with promising results. Index values for the surface soil communities were two to three orders of magnitude greater than those from the high dominance saturated soils, while index values for the high-carbon soils were similar to those from the low-carbon surface samples, and did not decrease with increasing depth or moisture content.

In the next phase of this project we will assess the combined impacts of spatial isolation and resource heterogeneity on microbial community structure by pursuing the following objectives: (1) determine the key relationships among soil texture, water content and carbon in controlling soil microbial communities; (2) determine the impacts of radioactive and mixed-waste contaminants on the structure and composition of microbial communities and the effects of spatial isolation on the responses of microbial communities to such contaminants; and (3) develop and use microarray-based genomic technologies for analyzing microbial community structure, dynamics and activities.

To achieve these objectives, we will test hypotheses about the role of spatial isolation, carbon heterogeneity and contaminants on microbial community structure and activities in the laboratory and at the NABIR Field Research Center. We are currently compiling a set of well-characterized isolates from the FRC that will serve as a model community to test our hypotheses. We are also developing and testing novel microarray-based genomic technology to more comprehensively quantify microbial community dynamics. Already, microarray fabrication and hybridization have been optimized in terms of fluorescence intensity by evaluating different glass slides, DNA deposition buffer, rehydration and renaturation times, and probe concentrations. The limit of detection in these tests was approximately 1 ng with pure genomic DNA and 25 ng with soil community DNA.

Assessment of the Impact of U(VI) Contamination on Groundwater Microbial Community Composition

David C. White,[1] Yun-Juan Chang,[1] Aaron D. Peacock,[1] Philip E. Long,[2] James P. McKinley,[2] A.K.M. Anwar Hussain,[1] Arnold M. Saxton,[1] Jonas Silva de Almeida,[3] Sarah J. Macnaughton[4] and John R. Stephen[5]

[1]University of Tennessee, Knoxville, Tenn.; [2]Environmental Technology, Pacific Northwest National Laboratory, Richland, Wash.; [3]ITQB, University Nova de Lisboa, Lisbon, Portugal; [4]National Environmental Technology Centre AEA Technology Environment, Culham, UK; [5]Crop and Weed Science, Horticulture Research International, Wellesbourne, UK

Examination of the microbial communities recovered from a U(VI)-contaminated site showed correlations between mid-chain branched PLFA, U(VI) and sulfate concentrations. PCR-DGGE analysis of rDNA using universal eubacterial primers did not show clear correlations with U(VI) concentration. PLFA analysis detected as many as 52 different mid-chain branched saturates with high proportions of 10me16:0 (22% to 72%). PCR with more specific primers showed a clear relationship between U(VI) concentration and shifts in the bacterial community composition. The distribution of sulfate-reducing bacteria (SRB) was examined using primers for [NiFe] hydrogenase genes and DSR genes. Cloning and sequencing of PCR-amplified DSR fragments followed by phylogenetic analysis showed remarkable diversity among the DSR sequences, ranging from δ-proteobacteria and Gram positives to the nitrospira division. Marked ecological differences between the sample wells were reflected in the population composition of SRB. *Desulfotomaculum* and *desulfotomaculum*-like sequences were the most dominant DSR genes detected with U(VI) contamination, and among the 70 DSR gene fragments recovered, about 76% (53/70) were affiliated with *Desulfotomaculum*. SRB within δ-proteobacteria were mainly recovered from low-U(VI) (<= 301 ppb) samples. SRB populations of sites containing >1500 ppb U(VI) were dominated by a group of *Desulfotomaculum*-like sequences genetically distant from SRB within δ-proteobacteria. A logistic regression model indicated that U(VI) concentration is significant in influencing the dominance of this clade of sequences, although other influencing factors are not excluded. Of the wells sampled #853 showed the highest proportion of i17:1w7c PLFA, a biomarker associated with *Desulfovibrio* SRB (Edlund et al., 1985), and was the one showing both rDNA and [NiFe] hydrogenase in addition to dissimilatory sulfite reductase (DSR) genes of *Desulfovibrio*, a well-known uranium reducer. In several studies conducted by this group it has proved possible to define specific microbial community responses to gradients in contaminant heavy metals.

PROGRAM ELEMENT 3

Biomolecular Sciences and Engineering

Metal Resistance Among Bacteria Isolated From Subsurface Cores

Tamar Barkay,[1] Gili Benyehuda[1] and David Balkwill[2]
[1]Department of Biochemistry and Microbiology, Cook College, Rutgers University, New Brunswick, N.J.; [2]Department of Biological Science, Florida State University, Tallahassee, Fla.

Two collections of aerobic heterotrophic bacteria (SMCC strains) originally isolated from Atlantic Coastal Plan sediments in the Savannah River Site (SRS, borehole P24; 783 strains) and from the Ringold formation in Hanford (borehole YB02; 275 strains), were screened for their level of resistance to the inorganic salts of Pb(II), Hg(II), and Cr(V). Resistances were estimated by disc diffusion assays and quantitated by growth in the presence of increased metal concentrations, and then were compared to resistance levels of well-characterized reference strains. Results showed the following:

- Resistances to the three metals occurred at a very high frequency among strains of both collections, suggesting that these populations evolved in the presence of metals.
- There was a higher level of resistance to Hg(II) among SRS strains as compared to Hanford strains, while both collections had similar resistances to Pb(II) and Cr(V).
- Resistant bacteria were unevenly distributed through depth profiles. In the Hanford core, bacteria resistant to all three metals were more abundant in soil sections corresponding to depth of 197 to 218 m. An enrichment of strains resistant to all metals was found at a depth of 180 to 259 m of the SRS core, while a secondary peak in resistance to Pb(II) and Cr(V) was found in depth of 45 to 118 m.
- Resistant strains belonged to the various phylogenetic groups represented in the two collections.

This study is the first comprehensive report on metal resistance among microbes from contaminated subsurface soils. As mixed wastes of radionuclei, organic contaminants and metals impact numerous Department of Energy subsurface sites, metal tolerance is a key issue determining the potential for bioremediation in the subsurface.

Engineering *Deinococcus radiodurans* for Metal Remediation in Radioactive Mixed Waste Environments

Michael J. Daly,[1] Hassan Brim,[1] Kira Makarova,[1] James K. Fredrickson[2] and Lawrence P. Wackett[3]
[1]Uniformed Services University of the Health Sciences, Bethesda, Md.; [2]Pacific Northwest National Laboratory, Richland, Wash.; University of Minnesota, St. Paul, Minn.

Seventy million cubic meters of ground and three trillion liters of groundwater have been contaminated by leaking radioactive waste generated in the United States during the Cold War. A cleanup technology is being developed based on the extremely radiation-resistant bacterium *Deinococcus radiodurans*, which is being engineered to express bioremediating functions. Research aimed at developing *D. radiodurans* for metal remediation in radioactive wastes sites was started by this group in September 1997 with support from DOE NABIR grant DE FG02-97ER62492. This grant was renewed in September 2000.

We have demonstrated that *D. radiodurans* can be genetically engineered for metal remediation using four different expression systems that were tested during growth of engineered *D. radiodurans* at 6,000 rad/hour. A variety of metal reducing/resistance functions have been cloned into *D. radiodurans* and are being studied, including genes from the following organisms that are specific for the indicated metal ions: *Escherichia coli* (*merA*), Hg(II); *Desulfovibrio vulgaris* (*cytc3*), U(VI); *Ralstonia eutrophus* CH34 (*czc*), Cd(II), Zn(II), and Co(II); and *Bacillus thuringiensis*, Cr(VI). Further, we have shown that anaerobic cultures of wildtype *D. radiodurans* can reduce U(VI) and Tc(VII) in the presence of humic acids; and Cr(VI) can be reduced in the absence of humic acids. Our development of a synthetic minimal medium for *D. radiodurans* was central to this work. This medium has also enabled us to identify the minimum nutrient requirements necessary to support growth in highly radioactive environments, and to explain how radiation resistance relates to this organism's metabolic repertoire, predicted by analysis of its recently acquired genomic sequence. The availability of such a minimal medium, in which *D. radiodurans*' growth is entirely dependent on a single carbon source, is also essential to our ongoing experimental efforts to survey its metabolic pathways and to engineer *D. radiodurans* for growth on toxic organic compounds present in most metal-contaminated radioactive waste sites. The *D. radiodurans* genomic sequence has been an important guide throughout this work and continues to be a source of inspiration in the development of new genetic technologies with which to understand and exploit this bacterium's capabilities.

The four specific goals of the next project period are to: (1) clone and express genes in *D. radiodurans* and *Deinococcus geothermalis* that can remediate radionuclides and metals, and to use and improve a novel technique to study gene expression patterns in *D. radiodurans*; (2) study and expand *D. radiodurans'* natural ability to reduce U(VI), Tc(VII), and Cr(VI); (3) develop *D. radiodurans* strains capable of utilizing toluene and related compounds as carbon and energy sources during metal remediation by metabolic pathway engineering; and (4) test engineered *D. radiodurans* for effectiveness at metal remediation in natural subsurface materials.

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

Carol S. Giometti,[1] Tripti Khare,[1] Sandra L. Tollaksen,[1] Alex Beliaev,[2] Dorothea Thompson,[2] Jizhong Zhou,[2] S. Tsapin,[3] K. Nealson,[3] Hanjo Lim[4] and John Yates[4]
[1]Argonne National Laboratory, Argonne, Ill.; [2]Oak Ridge National Laboratory, Oak Ridge, Tenn.; [3]Jet Propulsion Laboratory, Pasadena, Calif.; [4]Scripps Institute, La Jolla, Calif.

Shewanella oneidensis is a versatile microbe capable of metal reduction in both aerobic and anaerobic environments. The Department of Energy's Microbial Genome Program is funding the complete genome sequencing and the development of microarrays containing all ORFs from *S. oneidensis* MR-1. We are using the products of these Microbial Genome projects to characterize the regulatory mechanisms controlling metal reduction activity when *S. oneidensis* is grown in different environmental conditions. Two-dimensional gel electrophoresis is being used to measure the abundance of proteins expressed by *S. oneidensis* MR-1 cells grown with different electron acceptors, including iron and nitrate. Specific proteins that are observed to shift in abundance under varied growth conditions are being identified by comparing their tryptic peptide masses with the tryptic peptide masses predicted by analysis of the genome sequence. The relative abundance of the identified proteins is then being compared with the abundance of the corresponding gene transcripts on the microarray developed at Oak Ridge National Laboratory. The mRNA and protein profiles of mutant cells with altered metal reduction capability, including Fur (ferric uptake regulator) and EtrA (anaerobic regulator) mutants, are being compared with profiles from wild-type cells to confirm the relevance of observed protein differences to metal reduction activity.

Metabolic Engineering of Microorganisms for Actinide and Heavy Metal Precipitation

Jay D. Keasling,[1] Douglas S. Clark[1] and Heino Nitsche[2,3]
[1]Department of Chemical Engineering and [2]Department of Chemistry, University of California, Berkeley, Calif.; [3]The Glenn T. Seaborg Center, Lawrence Berkeley National Laboratory, Berkeley, Calif.

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

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

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

Differential Expression of *Desulfovibrio* Sp. Genes During Growth on Hydrogen Versus Lactate

Lee R. Krumholz, Jennifer L. Steger, Carr Vincent and Jimmy D. Ballard
Department of Botany and Microbiology and Institute for Energy and the Environment, University of Oklahoma, Norman, Okla.

In order to better understand gene expression of sulfate-reducing bacteria growing under different environmental conditions, RNA extracts of *Desulfovibrio desulfuricans ssp. aestuarii* and *Desulfovibrio vulgaris* were analyzed using random arbitrarily primed PCR (RAP-PCR). RNA extracts were incubated with random primers at low-stringency temperatures followed by reverse transcriptase to produce cDNA. A final PCR cycle produced banding patterns that were used to determine differential expression of genes from *Desulfovibrio* strains grown with different electron donors, hydrogen and lactate. Several putative differentially expressed genes have been identified and sequenced. BLAST sequence comparisons identified two sequences of genes from *D. desulfuricans ssp. aestuarii* expressed only during growth on hydrogen. These were identified as a dissimilatory sulfite reductase and an F_1F_0 ATPase gene. Differential expression of the sulfite reductase gene was verified by Northern blotting using an RNA probe constructed from the RAP-PCR band sequence. Several other differentially expressed genes were sequenced but did not show any homology to genes from Genebank.

RAP-PCR appears to be an effective tool in assessing differential expression of *Desulfovibrio* sp. grown with various electron donors. It also reveals that the expression of two respiratory proteins is greatly increased when *D. desulfuricans ssp. aestuarii* utilizes hydrogen as an electron donor as opposed to lactate.

Identification of Genes Regulated by *adnA* in *Pseudomonas fluorescens* Strain Pf0-1

Stuart B. Levy, Eduardo Robleto, Paul Casaz and Inmaculada Lopez-Hernandez
Center for Adaptation Genetics and Drug Resistance, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Mass.

Previously, *adnA* has been shown to affect attachment to soil particles and motility. Also, field experiments suggest that *adnA* is important for the spread and persistence of *Pseudomonas fluorescens* in natural conditions. Genetic analysis of this locus indicates that *adnA* is a homologue of *fleQ*, a transcription factor that affects flagella synthesis in *Pseudomonas aeruginosa*. This type of activator works as a two-component regulatory system, which in combination with σ^{54} activates transcription of genes necessary for nitrogen assimilation and nitrogen fixation in other bacterial spp. To validate the model that *adnA* is a transcription factor of genes involved in soil spread and persistence, we constructed two *P. fluorescens* strains with either an inducible or disrupted allele of *adnA*. For the inducible allele, we placed *adnA* under the control of the Pm promotor isolated from the meta-cleavage pathway of *P. putida*. In this system, *adnA* is expressed in the presence of 3-methyl benzoate. For the disrupted allele, we cloned an ϖ cassette, with translational terminators at each end, 515 bp from the transcriptional start of *adnA*. To exchange these *adnA* alleles into the chromosome of *P. fluorescens*, we used a suicide vector that has km-resistance as selection marker and *sacB* as a counter-selection marker. The *adnA* alleles were cloned into the suicide vector and conjugated into *P.fluorescens* by triparental matings. Km^r-tranconjugants were grown under no selection for 24 h and plated in the presence of 5% sucrose, which forces the excision of the vector DNA by a second recombination event. Presence of the inducible and disrupted alleles were confirmed by PCR. These constructs will be mutagenized with a mini Tn*5*-lacZ transposon that yields transcriptional fusions in the presence of X-gal by the presence of blue colonies. Insertions that are affected in color by either induction or conjugation of a plasmid carrying *adnA* will be genetically characterized and studied for their effect on the soil ecology of *P. fluorescens*.

Mechanisms for Uranium and Technetium Reduction in *Geobacter sulfurreducens*

Jon R. Lloyd and Derek R. Lovley
Department of Microbiology, University of Massachusetts, Amherst, Mass.

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

The protein hypothesized to reduce U(VI) in vivo, CytP a periplasmic tri heme *c*-type cytochrome, was purified to homogeneity and characterized in detail. In common with CycA, an analogous protein in closely related *Desulfovibrio* species, CytP reduced U(VI) in vitro. A mutant, prepared by PCR mediated disruption of the *cytP* gene, was constructed and was compromised in its ability to couple acetate oxidation to the reduction of U(VI) and other metals. However, the addition of hydrogen to the headspace reversed this phenotype, suggesting that the 9.6 kDa cytochrome, although involved in electron flow from acetate to U(VI), is not the terminal reductase for the radionuclide. An outer membrane cytochrome of molecular mass 41 kDa was also studied in detail. This protein was active against U(VI) in vitro, and was localized to the surface of cells using a range of biochemical techniques, consistent with a role in reductive precipitation of extracellular insoluble U(IV). However, selective removal of the cytochrome from the surface of whole cells by protease treatment had no effect on U(VI) reduction, suggesting that this protein is not involved in U(VI) reduction. Reduction of Fe(III) oxide was abolished by protease treatment, suggesting a role for the 41 kDa cytochrome in Fe(III) reduction, and confirming that Fe(III) and U(VI) are reduced via different mechanisms in *G. sulfurreducens*. Current work aims to uncover additional genes involved in U(VI) reduction in *G. sulfurreducens* using transposon mutagenesis.

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

Applications of a Multiplexed, Bead-Based Method Towards Identification of DNA Sequences in Environmental Samples

Mary Lowe and Alexander Spiro
Physics Department, Loyola College in Maryland, Baltimore, Md.

We have continued development of a multiplexed, bead-based method which utilizes nucleic acid hybridizations on the surface of microscopic polystyrene spheres to identify specific sequences in heterogeneous mixtures of DNA sequences. The method involves flow cytometry instrumentation and fluorescent labeling of beads and DNA. A quantitative analysis of its performance for measuring a mixture of three intergenic spacer region sequences appeared in Appl. Environ. Microbiol. 66, 4258-4265 (2000); excellent sequence discrimination and a lower detection limit in the femtomole range were obtained. The article also included a comparison with a DNA microarray using the same sequences. Since that work, we have shifted our attention to developing assays for detecting 16S rDNA sequences (main effort) and naphthalene biodegradation genes (side project) in environmental samples.

For the 16S rDNA work, we measured five types of sequences in TCE-contaminated groundwater at the Lawrence Livermore National Laboratory and obtained results consistent with a cloning study conducted in parallel. Capture probes were developed for *Geobacter, Geothrix, SJA-19* (a sequence similar to *Dehalobacter restrictus*), an unidentified sequence and a bacterial primer 533FA. Currently we are evaluating the quantitative accuracy of the method. We have found that the bead method is a convenient format for testing new probes, and we do not anticipate major difficulties with adding more probes to the set. For the naphthalene work, we demonstrated the ability to distinguish point mutations and obtained preliminary results on a groundwater sample consistent with independent efforts to isolate the corresponding microorganisms.

Molecular Characterization and Physiology of the Soluble Bacterial Chromate Reductases

A. Matin, Chul Ho Park, Mimi Keyhan and Claudio Gonzales
Department of Microbiology and Immunology, Stanford University, Stanford, Calif.

Cr(VI) (chromate), which is a toxic and carcinogenic compound, is wide-spread at Department of Energy sites. We are employing genetic and protein engineering approaches to construct *P. putida* strains with a superior capacity to remediate chromate. Such strains will possess an engineered chromate reductase protein with superior catalytic activity and will have the ability of expressing this protein under the stressful conditions of the DOE sites.

We previously purified to homogeneity a soluble chromate reductase from *P. putida*. Using reversed genetics we have cloned the gene that codes for this enzyme (the *chrR* gene). Blast searches of the derived amino acid sequences of this gene and that of a different chromate reductase gene cloned from *P. ambigua* (3) revealed close homologues of each. Several bacteria containing these genes reduced chromate, with the activity being induced by growth in the presence of chromate or copper. We therefore cloned additional gene homologues belonging to the two classes, overproduced the proteins using appropriate expression vectors and examined the enzyme characteristics of the pure proteins. All are soluble and possess chromate reductase activity with the null mutation in the gene resulting in impaired cellular ability to reduce chromate.

The Class I enzymes (*P. putida* gene homologues) have a low affinity for chromate but higher V_{max} and optimal temperature for chromate reduction than the Class II enzymes (homologues of the *P. ambigua* enzyme). Furthermore, the Class II enzymes, but not the Class I enzymes, can also reduce nitrocompounds. The *E. coli* nitroreductase, which has been extensively studied previously because nitrocompounds are serious pollutants in their own right, is among the Class II enzymes. The stress phenotype of the *chrR* null mutant and global regulatory pattern of these genes suggest that chromate reductase activity is an incidental property of enzymes designed to protect bacteria from oxidative stress by either preventing the formation of, or degrading reactive oxygen species. Using homologous genes of the two classes, we are currently employing the DNA shuffling approaches to engineer a super-efficient chromate reductase.

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

Margaret Romine and Jim Fredrickson
Environmental Microbiology Group, Pacific Northwest National Laboratory, Richland, Wash.

Using the near-complete genome sequence of *Shewanella oneidensis* MR-1 we have identified a number of regions that are likely to encode genes that enable this bacterium to utilize alternative energy producing pathways. By searching for heme and molybdopterin cofactor binding motifs we have identified 54 putative c-type cytochromes and 10 putative terminal respiratory reductases. Further analysis of the genome revealed that *S. oneidensis* MR-1 encodes the TAT (twin-arginine translocation) protein translocation pathway genes and that all but one (a formate dehydrogenase-like protein) of these putative terminal reductases have an N-terminal leader sequence characteristic of proteins translocated by this pathway. In other bacteria, this alternative pathway predominantly mobilizes proteins that bind a range of cofactors in the cytoplasm and that function in respiratory electron transport chains (41). Nine additional proteins were found to encode leader sequences typical to proteins translocated by this pathway and are therefore, likely to be involved in redox reactions. Work is under way to delete TAT pathway genes to determine whether the terminal reductase for nitrate or iron respiration possesses a TAT leader sequence. The genomic regions that encode proteins with these motifs have been annotated so that potential promoter-encoding regions could be identified and targeted for cloning. Several of the predicted promoter-encoding regions have been cloned.

Construction of a versatile vector for capturing additional DNA fragments that encode inducible promoters has also been initiated. The observed inherent resistance of *S. oneidensis* to ampicillin and presence of a chloramphenical resistance gene (apparently not expressed but could lead to genetic cross-over) in the genome sequence required redesigning our initial strategy for vector construction. Our new strategy entails constructing a promoter-less operon encoding GFP-*sacB Gm* (gentamycin resistance) in a broad host range vector that encodes kanamycin resistance. In addition, the low frequency of uptake of DNA propagated in *E. coli* has warranted developing an improved variant of MR-1 that can be easily selected for conjugal matings and that more readily accepts DNA from *E. coli*.

Rapid and Ultra-Sensitive Analysis of Global Protein Expression in *Shewanella putrefaciens*

Richard D. Smith, Pamela K. Jensen, Kim K. Peden, Mary S. Lipton, Gordon A. Anderson, Yuri Gorby, Margaret F. Romine and Timothy D. Veenstra
Pacific Northwest National Laboratory, Richland, Wash.

Shewanella putrefaciens is a key organism in bioremediation due to its ability to reduce and precipitate a diverse range of heavy metals and radionuclides. Developing the most efficient organisms for bioremediation requires an understanding of the key cellular pathways and proteins involved. An improved understanding of these organisms can likely be obtained from study of their proteome (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. This requires faster, more sensitive and quantitative capabilities for the characterization of cellular constituents.

We are currently developing technologies that integrate protein separation and digestion methods with advanced mass spectrometric methods. Capillary isoelectric focusing on-line with Fourier transform ion cyclotron resonance mass spectrometry (CIEF FTICR) provides a powerful tool to study the changes in expression for hundreds to potentially thousands of proteins simultaneously.

But first one must be able to identify the proteins of interest. To accomplish this, the proteins are digested with a protease and the resulting peptides are first analyzed by capillary liquid chromatography tandem mass spectrometry (LC MS/MS) on an LCQ (ion trap) mass spectrometer to obtain Accurate Mass Tags (AMTs), or "biomarkers" for each protein. The use of tandem mass spectrometry provides additional sequence information, that, when combined with the accurate mass of the parent peptide, can enable protein identification. Therefore, the same, or similar, proteomes are then analyzed using Fourier transform ion cyclotron (LC-FTICR MS). The AMTs obtained in the first step are then compared to the Accurate Masses (AMs) obtained from the FTICR. Due to the high MMA provided by FTICR, these proteolytic fragments can often be used as AMTs for identification of many proteins, which can then be used in all subsequent studies of the same organism. Although initial examination of changes in the proteome of *Shewanella putrefaciens* initially concentrated on the soluble proteins located in the periplasmic region, studies continue to characterize the entire proteome of the organism.

Optimizing the Metalloregulator MerR for Metallosequestration and Metallosensing

Anne O. Summers[1] and Michael Daly[2]
[1]Department of Microbiology, University of Georgia, Athens, Ga.; [2]Uniformed Services University of the Health Sciences, Bethesda, Md.

The focus of our research is on constructing and characterizing derivatives of the MerR metal binding domain (MBD) which have altered affinities and specificities for binding metals of interest to the NABIR program. Our objective is to construct a "single chain" tandem MerR MBD and compare its behavior to the freely associating monomers of the MBD.

Using a variant of overlap extension PCR cloning, we constructed a direct repeat of the MerR metal binding domain (MBD) in a single polypeptide chain (lab name: "Duplex") using the StrepTag affinity vector. In cells grown at 37°C the protein is ca. 75% in inclusion bodies; with 30°C growth/induction the Duplex is ca. 50:50 in inclusions. Initially we have concentrated on the soluble form to test Hg(II)-binding properties and found that it purifies very efficiently (>99% pure) on the biotin-analog column and has the expected MW (12,080 Da) by MALDI-MS.

Using equilibrium ultrafiltration, we have compared ^{203}Hg(II) binding by the soluble form of the Duplex grown at 30°C or at 37°C to wildtype full-length MerR bearing either the His-Tag or the StrepTag (both grown at 37°C). The Hg(II)-binding by both MBD preparations was indistinguishable from that of the WT MerR in a buffer containing 10 mM 2-mercaptoethanol as a competing thiol (the proteins were at 10 micromolar and the [Hg(II)] ranged from 5 to 20 micromolar). Indeed, the Duplex may be able to use both of its metal binding domains to bind Hg(II) ions, unlike the WT MerR, which can only use one of its two domains (patent application No. 60/240,465, filed Oct. 12, 2000).

We are currently comparing the solution minimal inhibitory concentrations (MICs) for WT MerR-StrepTag, the MBD Duplex construct, WT MerR His-Tag, and the single chain MBD construct (Lab name: "Delta-delta") as a His-Tag construct. These are being compared to the fully resistant *mer* operon carried by plasmid R100 and also to strains with *mer* operon mutations resulting in sensitivity and hypersensitivity to Hg(II). We do not expect the simple engineered Duplex protein to provide Hg(II) resistance at the level of the *mer* operon, which is capable of reducing Hg(II) to the relatively non-toxic Hg(0) vapor. We do expect it to enhance the protection of cells without the *mer* operon or of cells whose *mer* operon has been damaged by mutation. Thus, we have several very distinct ways to assess any contribution of the MBD Duplex to fostering Hg(II) protection.

In order to improve the yield of soluble MBD protein we will explore lower temperature and richer media (e.g., Terrific Broth) expression, both of which have worked for us to improve soluble yield of MerR protein fragments. Improved yield of soluble Duplex protein will provide material to define the actual kinetic parameters (stoichiometry, Kd, Koff, Kon) for interaction with ^{203}Hg(II) directly and by competition with Cd(II), Zn(II), Cu(II), and Pb(II) (note these are metals for which there is recent published and submitted data showing regulatory proteins of the MerR family).

Since the StrepTag system is under control of the Tet Repressor, it allows very precise tuning of gene expression (unlike the pET system which we have used previously). We will assess the influence of gene expression levels on the Hg(II) protection afforded by the MBD Duplex. This will give us optimized "expression-for *protection*" conditions. Once we have identified conditions which optimize protection in *E. coli*, we will forward this system to co-PI Michael Daly for introduction and testing in his *Deinococcus* system.

Genes for Uranium Bioremediation in the Anaerobic Sulfate-Reducing Bacteria

Judy D. Wall, Rayford Payne, Laurence Casalot, Barbara Giles and Darren Gentry
Biochemistry Department, University of Missouri-Columbia, Columbia Mo.

Cost effective bioremediation of radionuclides and metals in the subsurface will necessitate an understanding of the metabolic interactions of the anaerobic microorganisms that are found there. This knowledge will contribute to the elucidation of what is happening without intervention or what may happen with nutrient or microbial amendments. Among the bacteria inhabiting the subsurface are the sulfate-reducing bacteria, a heterogeneous group displaying a remarkable versatility in substrate utilization. Many members of this group have been shown capable of U(VI) reduction to U(IV), converting the uranium from a soluble species to a highly insoluble form that potentially could be removed from contaminated waters by precipitation or filtration.

Genetic investigation into the pathway of reductant flow to U(VI) in bacteria of the genus *Desulfovibrio* is ongoing. In *D. desulfuricans* strain G20, we have confirmed the importance of the tetraheme cytochrome c_3 by disruption of the gene encoding that cytochrome, *cycA*, and demonstration of an approximately 50% decrease in the ability of the mutant to reduce U(VI). Our studies have also revealed an unexpected decrease in detectable cytochrome c_3 content when cells were grown in the presence of U(VI). Because this decrease results in attenuation of the reduction process, an elucidation of the phenomenon is necessary to understand its prevalence and to design experiments to overcome this affect. Translational fusions of *cycA* to the *lacZ* gene have allowed a quantitative examination of the expression of *cycA* when cells were grown with uranium. The presence of uranium did not effect the expression of this tetraheme cytochrome. Therefore, alternative mechanisms for the phenomenon are being explored. The sequence of the genome of *Desulfovibrio vulgaris* has recently been made available from The Institute for Genome Research and annotation is underway. Surprisingly, genes for four tetraheme cytochromes are apparent, offering several alternative electron transport components that could potentially interact with U(VI). Results with one, *cycC*, confirms expression of the gene in wild-type and *CycA* cells.

Investigation of Reduced Flavin Channeling in Biodegradation by Biological and Single-Molecule Microscopic Analyses

Sunney Xie,[1] Tai-Man Louie,[2] Katie Drake[1] and Luying Xun[2]
[1]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Mass.;
[2]Department of Microbiology, Washington State University, Pullman, Wash.

Several reduced flavin-utilizing monooxygenases, including EDTA monooxygenase and nitrilotriacetate monooxygenase, have been reported. These monooxygenases are usually reported with specific NAD(P)H:flavin oxidoreductases (flavin reductases) to supply reduced flavins despite the fact that there are other flavin reductases inside the same cells. This observation raises a fundamental question about whether the monooxygenases can couple with only their specific flavin reductases.

We examined this question with the *Escherichia coli* W (ATCC 11105) 4-hydroxyphenylacetate (4HPA) 3-monooxygenase (HpaB) and its flavin adenosine dinucleotide (FAD) reductase (HpaC). Insertional mutagensis of *hpaC* resulted in a mutant strain *E. coli* W-KO that was incapable of growing on 4HPA (Hpa^-). Thus, HpaC is absolutely required for HpaB catalysis. Expression of other flavin reductase genes in *E. coli* W-KO reverted the Hpa^- phenotype, indicating that HpaB can couple with other flavin reductases in vivo. However, the growth rates of *E. coli* W-KO cells expressing these flavin reductase genes, and oxidation of L-tyrosine to L-Dopa by HpaB in the *E. coli* W-KO recombinant cell suspensions, did not completely correlate with the FAD reductase activities in vivo. Excessive level of FAD reductase activities partially inhibited L-tyrosine oxidation. TftC, the *Burkholderia cepacia* chlorophenol 4-monooxygenase-specific FAD reductase that is most similar to HpaC in terms of function and protein sequence homology, coupled to HpaB for L-tyrosine oxidation almost as efficient as HpaC did. Thus, HpaB preferentially couples with HpaC or TftC over other flavin reductases. This observation appears to be consistent with a hypothesis of direct transfers of reduced flavins from flavin reductase to reduced flavin-utilizing monooxygenase.

The mechanism of reduced flavins transfer between HpaC and HpaB is currently under examination by steady-state kinetic analysis and single-molecule microscopy. This study will improve our understanding of the catalysis of reduced flavin-utilizing monooxygenases and their applications in bioremediation processes.

PROGRAM ELEMENT 4
Biogeochemical Dynamics

The Role of Biogenic Solids in the Reductive Stabilization of Metal Contaminants

Scott Fendorf,[1] Bruce Wielinga,[1] Colleen Hansel,[1] Shawn Benner[1] and Yuri Gorby[2]
[1]Stanford University, Stanford, Calif.; [2]Pacific Northwest National Laboratory, Richland, Wash.

Bacterial reduction of metal and radionuclide contaminants has the potential to stabilize many of these hazardous compounds in biologically unavailable forms, thus diminishing their risk. Contaminant reduction may occur by a direct, enzymatic mechanism or indirectly by geochemical reaction involving a microbial metabolic product such as Fe(II). Recent evidence has brought to light the preponderance of the potential biogenic solids formed by metal or sulfate reducing bacteria, many of which may be effective reductants of contaminants. In this phase of our NABIR research we report on (1) ferrougenous solids produced under hydrodynamic conditions and (2) the impact of ferric hydroxides on the reduction of chromate and uranyl by dissimilatory iron-reducing bacteria (DIRB).

A plethora of ferrous-bearing solids may result from the reduction of ferric (hydr)oxides by DIRB; phases such as siderite, vivianite, green rust and magnetite have all be observed in batch culture. Given the transport of Fe(II) (aq) in hydrodynamic environments, it is possible that a different distribution (or types) of phases may result. Accordingly, we conducted hydrodynamic experiments using packed mineral beds of hydrous ferric oxide (HFO)-coated sand in 2.5-cm-diameter columns that were inoculated with the bacterium *Shewanella putrefaciens*, strain CN32. A continuous pulse of 1 mM lactate buffered at pH 7 was injected, providing a velocity of 2.5 cm/h (equivalent to 0.17 pore volumes per hour). Aqueous Fe(II) increased immediately and a color gradient was established rapidly within the column, transitioning from red at the inlet to black at the outlet. Within two days, all but the first 1 cm of the flow field had graded into a black sediment. CN32 was distributed relatively evenly throughout the column—determined on material collected at the termination of the experiment—and cells were closely associated with the sand grains. In most cases, grains have bacterial coatings constituting approximately half of their surface on the basis of confocal microscopic analysis. Most importantly, a mineralogical transformation occurs along the flow field, progressing from an HFO dominated system at the inlet to one composed dominantly of magnetite and green rust—which are in nearly equal proportions—at the outlet; passivation of the HFO does not appear to occur. The transition to reactive ferrous-bearing solids has important implications on the chemical reactivity of the porous material both with regards to contaminant reduction and retention.

We also examined the reduction of uranyl by *Shewanella alga* strain BrY in the presence of environmentally relevant iron hydrous oxides having reduction potentials that vary over a range of 200 mV. At low concentrations (< 100 μM), uranyl reduction was nearly complete for aqueous phase species and for that adsorbed on goethite, but reduction was retarded when uranyl was adsorbed on ferrihydrite. However, with increased concentrations of uranyl a reverse effect is noted, particularly at longer incubation times: ferrihydrite actually increases the extent and rate of reduction. Using high-resolution transmission electron microscopy (HRTEM), we note that in the absence of an iron oxide, BrY cells are encrusted in a thick exopolymer and with uraninite deposits. In contrast, when ferrihydrite is present, a minimal glycohelix is noted and uraninite is dispersed throughout the system rather than depositing on the cells. Furthermore, addition of EDTA leads to complete reduction of uranyl independent of concentration and in all cases a lack of exopolymer. Thus, it appears that both ferrihydrite and EDTA enhance uranyl reduction at prolonged reaction times and higher concentrations by limiting, in a yet unknown manner, the formation of a dense exopolymer that diminishes BrY reactivity.

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

Peter Jaffe,[1] *Derick Brown*[1] *and Derek Lovley*[2]
[1]*Department of Civil and Environmental Engineering, Princeton University, Princeton, N.J.*
Department of Microbiology, University of Massachusetts, Amherst, Mass.

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

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

The objective of the proposed research is to gain a basic understanding of the hydrogen dynamics in an aquifer during a trace metal/radionuclide bioremediation scheme. To address this objective we have formulated the following working hypotheses:

1. Hydrogen can be used an indicator that is more accurate than redox couples or Eh measurements for identifying bacterial activities in the subsurface during a bioremediation scheme.
2. Bacteria via the combined utilization of a carbon source and hydrogen set hydrogen levels in the subsurface. These levels can be estimated for each TEAP from the biokinetic parameters of the carbon- and hydrogen-degrading organisms.
3. In TEAP transition zones, hydrogen levels will not correspond to the characteristic levels of either TEAP.

To test these hypotheses, we will conduct two different types of laboratory experiments and combine them with analytical tools to interpret and test the results in a quantitative manner. We will conduct batch experiments where we will track the simultaneous utilization of hydrogen and acetate in suspended growth experiments in order to determine if the bacterial growth on these two substrates can be explained by our conceptual model. Column experiments will be designed to manipulate a continuous-flow porous media system in which first acetate and then lactate are injected as an electron donor. We will then monitor the spatial distribution of electron donors (including hydrogen), electron acceptors and microbial populations. The columns will be operated so that the redox conditions will range from oxidizing to reduced and back to oxidizing, in order to simulate a groundwater environment from the source of a plume to its outer edge. The results from the column experiments and their numerical analysis will give us a thorough insight into how hydrogen concentrations correspond to bacterial activities in a groundwater system that is being augmented by an external carbon source.

The Role of Biogeochemical Dynamics in the Alteration of U Solid Phases Under Oxic Conditions

Heino Nitsche,[1,2] Sue B. Clark [3] and Terry C. Hazen[4]
[1]Department of Chemistry, University of California, Berkeley, Calif.; [2]The Glenn T. Seaborg Center, Chemical Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, Calif.; [3]Chemistry Department, Washington State University, Pullmann, Wash.; [4]Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Although in situ and ex situ microbial reduction have been demonstrated to reduce actinide groundwater concentrations in aerobic systems, such biological alterations must be considered temporary treatments unless long-term anoxia can be guaranteed. Under oxidizing conditions, the more mobile higher oxidation states of the actinides such as uranium (U), neptunium (Np) and plutonium (Pu) are the thermodynamically favored species. For example, in U ore deposits in which uraninite (consisting of reduced, tetravalent U as UO_{2+x}) is the parent material, exposure to oxidizing conditions results in alteration to U^{6+} minerals with the U^{6+}-phosphates frequently defining the boundaries of the ore body. While microorganisms are undoubtedly present in such systems, their role in such transformations and the ultimate precipitation of the insoluble phosphate phases is not well understood. Furthermore, the impact of wet-dry cycling on such alterations and the presence of other transuranium actinide elements has not, to our knowledge, been studied to any significant extent.

The purpose of the proposed work is to investigate the role of biogeochemical dynamics in the alteration of U solid phases under oxic conditions to form U^{6+} phosphate phases. We are investigating the role of important bacterial strains (pure cultures) and consortia of bacterial strains isolated from a Department of Energy field site on the alteration of simple U^{6+} oxide hydrates to U^{6+} phosphates and phosphate solid solutions of U^{6+} and other actinides. To address such transformations in systems that reflect both saturated and vadose zone conditions, systems of interest include those that remain constantly hydrated and those that are exposed to wet-dry cycling. The microorganisms that we are studying include pure cultures of *Bacillus sphaericus* (ATCC 14577), *Desulfovibrio desulfuricans* and *Geobacter metallireducens*. These strains of microbes were selected to reflect a variety of subsurface conditions including aerobic systems, temporarily anaerobic and/or microaerophilic, and systems with high levels of ionizing radiation. Results with pure cultures will be compared with results obtained using consortia obtained from the Field Research Center (FRC) site. We are determining transformation pathways between the initial U^{6+} oxide hydrate and the secondary solids that form, as well as rates for these transformations. We are monitoring changes in metal oxidation state and will provide coordination information about the actinides. Our research links important geochemical and microbiological aspects of this problem, and provides a fundamental basis for predicting the complex and dynamic interplay of such biological treatment strategies.

Effects of Clay Minerals on the Activity of Sulfate-Reducing Bacteria in Sediments and Pure Culture

Joseph M. Suflita, Denny Wong and Lee R. Krumholz
Institute for Energy and the Environment, University of Oklahoma, Norman, Okla.

A variety of microbial activities occur in the terrestrial subsurface, including iron-, nitrate- and sulfate reduction and methanogenesis. Studies have shown a heterogeneity in activities as one progresses down a sediment profile. Regions of low microbial activity (with sulfate-reducers in particular) are associated with regions high in clay mineral content. We have conducted surveys using sediment and pure cultures of SRB to test the effects of clay minerals and extracts (suspensions of low molecular weight components and aggregates) on sulfidogenic activity. The addition of a number of clay minerals confirmed that these can reduce the level of sulfidogenesis in sediments (ranging from 5-70% reduction of activity).

In a mixture of a washed cell suspension of *Desulfovibrio vulgaris* with a clay mineral, kaolin, a significant reduction of sulfidogenic activity (62%) was observed. Clay minerals were washed in distilled water and the resulting supernatant of the low-speed centrifugation (22,000 x g) were also applied to washed cells of *D. vulgaris* to test whether a soluble component or a small particle is involved directly in the inhibition process. Extracts from the clay minerals, attapulgite, barasym and kaolin were used. The extracts from kaolin and barasym both inhibited sulfidogenesis by 50-70%, including samples which were filtered (0.2 μm). This suggests that either very small particles or soluble materials are involved in inhibition by these clays. The attapulgite reduced the sulfate reduction activity by only 30%, but this was alleviated by the filtration, suggesting that a particle larger than 0.2 μm is involved in inhibition by this clay. These results suggest that the inhibitory effect of clays may vary between different clay minerals and that the specific particles involved may vary in size.

Stabilization of Heavy Metal Contaminants in Subsurface Environments: Microbially Mediated Precipitation of Metal Sulfides from Complexes with Organic Chelates

Murthy A. Vairavamurthy
Department of Environmental Sciences, Brookhaven National Laboratory, Upton, N.Y.

Heavy metal contaminants in subsurface environments can be immobilized in situ by precipitating them as metal sulfides by chemical or microbiological methods. Bacterial reduction of sulfate or partially oxidized inorganic sulfur species (e.g., thiosulfate) generates H_2S, but the appropriate conditions for this are not well understood because the growth of many bacteria, including sulfate reducers, is affected by the toxicity of various heavy metal pollutants. Our hypothesis is that the formation of highly stable complexes with chelates will modulate the toxicity of heavy metals, thereby allowing bacterial sulfate reduction. Ongoing studies suggest that although H_2S can react with weak metal-organic complexes to form the corresponding metal sulfides, polysulfides probably are necessary to react with highly stable metal chelates (e.g., metal-EDTA complexes). Thus, our main goal is to investigate carefully the fundamental mechanisms of biologically mediated metal sulfide precipitation under sulfate-reducing conditions in which the toxicity of two highly toxic metals, cadmium and lead, has been modulated by chelation.

Our studies will focus on the growth kinetics of different types of sulfate-reducing bacteria with the heavy metal ions present as chelates of several typical natural and synthetic ligands. For select chelates, we will compare and contrast metal sulfide formation, with sulfate and thiosulfate as sulfur precursors, to better understand the reductive pathways and the reactive species involved. Also, we will investigate the effect of pH on metal sulfide precipitation with select systems. Studies of the chemistry of polysulfide complexes with cadmium and lead will be conducted. The rate of biological conversions will be correlated with chemical equilibrium data on the metal complex. Sulfur k edge XANES spectroscopy and EXAFS spectroscopy will characterize sulfur and metal forms generated during these transformations. Transmission electron microscopy will reveal whether metal sulfide/polysulfide compounds are generated in the cytoplasm, in the periplasmic space or on the outer membrane. This research will illuminate the fundamental biological processes for precipitating heavy metals as sulfides in situ in sulfate reducing subsurface systems.

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

John M. Zachara , James K. Fredrickson, Chongxuan Liu and Raymond E. Wildung
Pacific Northwest National Laboratory, Richland, Wash.

Technetium 99 is an important Department of Energy subsurface contaminant. It is long-lived ($t^{1/2} = 2.13 \times 10^5$ y), and exists primarily as the oxidized, mobile pertechnetate anion [$Tc(VII)O_4^-$]. Pertechnetate can be microbiologically or abiotically immobilized by reduction to insoluble Tc(IV) oxide and other phases. The half-cell potential for this reaction is "intermediate." At circumneutral pH, select ferrous iron species (aqueous and solid) have thermodynamic power to reduce Tc(VII), but their kinetics are not well understood. This research supports development of an in-situ bio-barrier where sediment-bound, biogenic Fe(II) reduces and collects mobile $Tc(VII)O_4^-$. Research is investigating: (1) biogeochemical factors controlling Tc immobilization rates and long-term stability; (2) microbiologic manipulation strategies to generate reactive biogenic Fe(II) species for Tc capture; and (3) models of the involved processes supported by fundamental experimentation.

The reduction and oxidation of Tc is being studied in subsurface sediments from Hanford and the Oak Ridge-FRC and in mineral mixtures that represent their "bioavailable" Fe mineral fractions. Two dissimilatory metal-reducing bacteria (*S. putrefaciens*, *G. metallireducens*) will be used to create biomineralization products and Fe(II) phase distributions under anoxic conditions. A combination of batch and flow-cell experiments will determine how: (1) the speciation of biogenic Fe(II) controls $Tc(VII)O_4^-$ reduction rate and product identity, and (2) the speciation of the Tc(IV) phase and its mineralogic/microbiologic association controls its oxidation and solubilization rate. The influence of NO_3^- on Tc oxidation and reduction kinetics will be evaluated in later project years because it is a common co-contaminant with ^{99}Tc. Spectroscopic and microscopic techniques will be used to define Fe and Tc speciation, and linked equilibrium/kinetic biogeochemical models will be developed that incorporate new-found scientific understanding.

PROGRAM ELEMENT 5
Assessment

Field Portable Immunoassay Instruments and Reagents to Measure Chelators and Mobile Forms of Uranium

Diane A. Blake[1] and Robert C. Blake, II[2]
[1]Tulane University Health Sciences Center, New Orleans, La.; [2]Xavier University of Louisiana, New Orleans, La.

There is substantial interest in the chemical speciation of the uranium in contaminated soils, waters and sediments. This interest stems from the fact that the hexavalent form of uranium (U(VI) or UO_2^{2+}) is soluble in most natural waters, while the tetravalent form (U(IV) or UO_2) is highly insoluble. An antibody-based assay that measures hexavalent uranium in aqueous samples has been developed. This assay employs a monoclonal antibody (8A11) that binds specifically to 2,9-dicarboxyl-1,10 phenanthroline (DCP) complexes of UO_2^{2+}. The antibody was tested with a prototype handheld immunosensor based on the KinExA™ 3000 from Sapidyne Instruments, Inc. Samples containing UO_2^{2+} were mixed with buffer solutions containing 200 nM DCP; the UO_2^{2+}-DCP complex forms rapidly and spontaneously under these conditions. A fluorescently-labeled form of the 8A11 monoclonal antibody was subsequently allowed to bind to the soluble UO_2^{2+}-DCP complexes, and the incubation mixture was rapidly passed through a disposable cassette containing an immobilized version of the UO_2^{2+}-DCP complex. In this competitive immunoassay format, the accumulation of antibody in the cassette was inversely proportional to the concentration of UO_2^{2+} in the original solution. When the fluorescent signal was plotted versus time, the slope of the resulting line could be used to quantify UO_2^{2+}. The prototype immunosensor could detect soluble UO_2^{2+} at concentrations from 10 to 100 nM (2.5 to 12 ppb) in spiked samples. A smaller version of the immunosensor is presently being developed, and this technology is being extended to include cassettes and antibodies for the analysis of cadmium, lead and ethylenediamine tetraacetic acid.

Neural Network Analysis of Lipid Profiles to Determine Effects of Metal Remediation on Subsurface Microbial Communities

Craig C. Brandt,[1] Jack C. Schryver,[1] Susan M. Pfiffner,[2] Carol Smith[2] and Anthony V. Palumbo[1]
[1]Oak Ridge National Laboratory, Oak Ridge, Tenn.; [2]University of Tennessee, Knoxville, Tenn.

We conducted a laboratory experiment using soil microcosms to systematically investigate the dynamics of microbial community structure in response to metal addition and removal. The objectives of this experiment were to: (1) estimate effects of metal contamination and removal (remediation) on microbial community structure in subsurface sediment ecosystems, and (2) develop and apply nonlinear artificial neural networks (ANN) to quantitatively assess the structural changes.

The dynamics of microbial community structure were tracked using signature lipid biomarkers (SLBs) extracted from the soil microcosms. We hypothesized that (1) the addition of metals would cause changes in SLBs, producing a time evolution diverging from samples to which no metals were added, and (2) removal of metals would cause a second change in SLBs. We also expected the second change after remediation to exhibit at least some convergence toward a steady state resembling the "no metals" time evolution.

Previous work has shown that ANNs are useful tools in predicting quantities of interest from laboratory and field soil samples using derived input variables such as SLBs and terminal restriction length polymorphisms. Changes in SLBs are often complex, interactive and otherwise nonlinear, rendering traditional linear methods unsuitable for prediction. Here we further evaluate the ability of feedforward ANNs to predict experiment outcomes such as metal contamination, remediation and age by using nonlinear mappings from SLB profiles to these outcomes.

A total of 96 microcosms containing 150 g of low-iron sand from Abbott Pit, Va., were used after a pretreatment step (addition of carbon) to boost bacterial numbers. The microcosms, except for controls, received 100 ppm of cadmium, cobalt, cesium and strontium, and were destructively sampled at 0 and 2 weeks. At 4 weeks, half of the remaining microcosms were remediated using a NaCl wash followed by nutrient addition (100 ppm carbon) to all microcosms, and changes in the SLBs were followed for 24 more weeks. At 4 weeks, biomass decreased by a factor of 1.5 in microcosms with metals. The effect of washing reduced biomass by a factor of 2. Nutrient supplement following the remediation stimulated growth.

When the study was concluded, biomass for both metal- and no-metal-added microcosms were very similar to that at t=0, while biomass of remediated microcosms remained very low (3 8 times less than the non-remediated microcosms). Relative proportions of terminally branched saturate, monoenoic, cyclopropyl and mid-chain branched fatty acids varied between treatments. We trained ANNs to distinguish among treatments (metal, no-metal, removal) and age of the microcosm, and compared the performance of these networks to traditional and generalized linear models. Autoassociative ANNs were also developed to test whether SLB profiles cluster according to treatment and time.

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

Darrell P. Chandler,[1] David A. Stahl[2] and Jean Francois Gaillard[3]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]University of Washington, Seattle, Wash.; [3]Northwestern University, Evanston, Ill.

Detailed information related to changes in microbial community composition and activity as a result of metal/radionuclide contamination are needed to improve our understanding of the microbiota responsible for effecting desired bioremediation endpoints. 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 chromium (Cr), lead (Pb) and/or zinc (Zn) concentrations, and correlate microbial community structure to changes in metal speciation and mobility.

In FY00, highly reproducible voltammetric techniques were developed to measure the physicochemical speciation of metals directly in sediments and microorganisms. Extended X-ray Absorption Fine Structure (EXAFS) analysis of metal-contaminated sediments demonstrated that Zn is coordinated with a wide variety of ligands: carbonate, phosphate, sulfide and water in these sediments, with coordination varying with both location and depth in each sediment core. A significant fraction of Zn is present as an aqua-complex close to the source of contamination, suggesting that Zn is in a rather labile chemical form. Away from the source of contamination, Zn is predominantly bound to sulfide, which is a rather inert compound under the reducing conditions prevailing in these sediments. More than 20 pure cultures of metal resistant anaerobic bacteria were obtained, including fermentative, sulfate-reducing and iron-reducing bacteria, all of which tolerate up to 7 mM total Zn (dissolved and insoluble). Three of these isolates were selected for analysis using EXAFS spectroscopy following growth in media containing 500 µM zinc at neutral pH. Fitting of the first coordination shell of zinc showed that each bacterial species differed in the character of its association with this metal, varying between sulfurs to six-fold nitrogen/oxygen, with two of the isolates showing mixed coordination shells.

Microarray research during FY00 continued to focus on the specific technical challenges associated with the *direct* detection (i.e., no PCR amplification) of full-length 16S rRNA targets relative to PCR-amplified 16S rDNA. PCR-amplified products from *Geobacter* and *Desulfovibrio* species were easily and specifically detected under a range of hybridization times, temperatures and buffers. However, reproducible, specific hybridization and detection of intact rRNA could only be accomplished using genera-specific, biotin-labeled chaperones. With this knowledge, assay conditions were developed for rRNA detection with a 2-hr hybridization time at room temperature. Hybridization specificity and signal intensity was enhanced using fragmented RNA. With the chaperone-detection strategy, we were able to specifically hybridize and detect *Geobacter chapelleii* 16S rRNA directly from a total RNA sediment extract, without further purification or removal of soluble sediment constituents. Detection sensitivity for *G. chapelleii* 16S rRNA in sediment extract was nominally 0.5 µg total RNA, representing approximately 7.5×10^6 *Geobacter* cell equivalents of RNA. These results suggest that microarrays can now be applied for direct rRNA microbial community profiling in many sediments and environmental samples.

Rapid Gene Probe for Microorganism Monitoring by Novel MS Approach

Winston C.H. Chen,[1] Naranyana R. Isola,[1] Richard Hurt,[2] Jiz-hong Zhou[2] and Steve L. Allman[1]
[1]Life Sciences Division and [2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

The aims of this program are to develop new mass spectrometric assessment technology for microbial community determination. It includes two major parts. One is for new technology development. The other is to try to apply the newly developed technologies to microorganism assessment. The approach is to have new gene probe based on the coupling of hybridization, polymerase chain reaction (PCR) and mass spectrometry for DNA analysis. Special tasks include: (1) development of optical acoustic desorption for mass resolution improvement; (2) measurements and sequencing of DNA probes; (3) quantitative measurements of DNA; and (4) coupling of quantitative PCR with mass spectrometry for microbial community assessment.

Up to now, nearly all DNA analysis by a time-of-flight mass spectrometer has been based on matrix-assisted laser desorption/ionization (MALDI). However, the mass resolution and detection efficiency are low due to broad energy spread caused by matrix molecules. With optical acoustic desorption, DNA fragments are desorbed by the shaking force of acoustic wave. Thus, it is possible that mass resolution can be improved. Better mass resolution is critically important for DNA analysis for microbial application. For example, rapid measurement of the length of DNA fragments of highly variable sequences for 16S rDNA is very valuable for community assessment. Better resolution can help to achieve this goal. We have successful demonstration of optical acoustic desorption of proteins and DNAs with mass resolution improvement by optical acoustic desorption.

Hybridization on chips is becoming an important tool for sequence identification. It can be used for microbial community analysis. With the present hybridization approach, the detection is mostly based on laser-induced fluorescence or radioactivity. Thus, dye or radioactive material tagging is needed, which increases the cost and produces hazardous wastes that require disposal. For each site, only single hybridization reaction can be pursued. Furthermore, there are no convenient methods to ensure the sequence of DNA probes. We tried to develop mass spectrometry to detect and/or sequence DNA probes for hybridization. With MS measurements of DNA probes, the above disadvantages can be eliminated. Major achievements include: (1) successful measurements of DNA probes by mass spectrometry, and (2) rapid sequencing of DNA probes to assure the accuracy of hybridization.

In order to achieve population determination for microbial community, it is important to be able to measure DNA quantitatively. However, it is extremely difficult to achieve quantitative measurements of DNA by mass spectrometry. We have developed an internal calibration method to obtain quantitative measurement of DNA samples. Major tasks that have been pursued include: (1) successful demonstration of quantitative DNA measurements by mass spectrometry using internal standards, and (2) simultaneous extraction of DNA and RNA for quantitative analysis.

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

Mark E. Fuller,[1] Tullis C. Onstott,[2] Brian J. Mailloux,[2] James A. Hall,[2] William P. Johnson[3] and Pengfei Zhang[3]
[1]Envirogen, Inc., Princeton Research Center, Lawrenceville, N.J.; [2]Princeton University, Princeton, N.J.; [3]University of Utah, Salt Lake City, Utah

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. 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 spectrofluorometry will be determined. The fluorescent tracking method will be evaluated during transport experiments using intact sediment cores and in the field, and compared with several other detection/enumeration methods.

During the past year, the vital fluorescent stain-based tracking methodology has been evaluated further. The green CFDA/SE stain (5-(and-6-)-carboxyfluorescein diacetate, succinimidyl ester) and a new red stain, TAMRA/SE (5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester), were used to label two bacterial strains for a dual-injection field experiment. *Comamonas* sp. strain DA001 and the facultative iron reducer *Acidovorax* sp. strain OY-107 were stained with TAMRA/SE and CFDA/SE, respectively. A total of 1800 L of cells (~5 x 10^7 cells/ml each strain) and bromide (~100 mg/L) were introduced under anoxic conditions into the sub-oxic flow cell at the South Oyster field site. Both CFDA- and TAMRA stained cells were enumerated simultaneously in real-time in the field during the experiment using microplate spectrofluorometry; approximately 1,200 samples were collected and processed over the course of the experiment. Samples were also analyzed in the laboratory using spread plating, epifluorescent microscopy, flow cytometry and ferrographic capture. The detection limit using microplate spectrofluorometry was on the order of 10^5 cells/ml for both organisms, and was in good agreement with results from the other methods. Flow cytometry was not able to effectively enumerate TAMRA/SE-stained DA001 cells. Both strains were transported a significant distance through the flow cell, and the two stains performed very well. Sediments were also collected from 10 boreholes within the flow cell after the transport phase of the experiment was complete. Injected cells that were retained in the sediments are currently being extracted and enumerated by spread plating, epifluorescent microscopy, flow cytometry and ferrographic capture.

The use of multiple enumeration methods allows the collection of real-time, high-density datasets, as well as the detection of the labeled organisms over seven to eight orders of magnitude. These results have confirmed that the use of vital fluorescent stains is a robust, field-applicable method for monitoring bacterial transport during field-scale injections.

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

Kirk Hatfield,[1] Andrew Ogram,[1] Michael Annable,[1] Tim Campbell[2] and Tom Stauffer[2]
[1]University of Florida, Gainesville, Fla.; [2]Armstrong Laboratory, Tyndall AFB, Fla.

Microbially-mediated reduction of heavy metals has been proposed as a possible in situ method for remediating subsurface contamination by heavy metals, organic chemicals and radionuclides. However, many of the remediation methods under study have been hampered by a lack of information concerning presence, distribution and activity of subsurface microbes responsible for catalyzing the reduction reaction. The object of this study has been to establish nondestructive in situ tracer methods for detecting the presence, distribution and activity of subsurface heavy metal-reducing microorganisms. Recent research efforts have focused on two areas, the first being the development of in situ methods for measuring biotracer fluxes in porous media, and the second being the development of mathematical tools to characterize subsurface microbial distribution and activity through the fate and transport of biotracers.

Under the first area of focus, in situ methods for measuring biotracer fluxes in porous media are being developed and tested in one-, two- and three-dimensional model aquifers. The idea is to measure changes in constituent mass fluxes for electron acceptors [i.e., Cr(VI)], electron shuttles, (i.e., AQDS), and electron donors and then use this information to quantify microbial activity in three-dimensional porous flow systems. Experiments have been conducted with Cr(VI), AQDS, and various nonreactive tracers. Various branched alcohols have been used as nonreactive tracers to assess water flux. The results of these experiments follow theoretical expectations, and it is expected that the methods tested will facilitate efforts to extend biotracer technologies to the field.

Under the second area of developing of mathematical tools, one , two- and three-dimensional finite-element models were developed to interpret subsurface microbial activity and distribution through the fate and transport of biotracers. The model simulates microbial growth and the dissolved transport of electron donors and acceptors [i.e., Cr(VI)], sorption and biological processes (i.e., electron donor/acceptor utilization, substrate inhibition and cell death). Currently, the model is being used to develop new methods for assessing field scale Monod kinetic parameters (i.e., microbial yield coefficients, maximum specific growth coefficients and substrate half-saturation coefficients) from field samples.

In Situ Determination of Microbial Metabolic Activity

Jonathan D. Istok[1] and Jennifer A. Field[2]
[1]Department of Civil Engineering and [2]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Ore.

In this project we are evaluating the capabilities of the single-well "push-pull" test for quantifying in situ microbial metabolic activity in the subsurface. Objective 1: We are examining the use of a suite of substituted-nitrophenyl substrates to determine if the rate of hydrolysis of these substrates can be used as a simple quantitative indicator of the levels of metabolically-active microbial biomass in the subsurface. In laboratory incubations, rates of PNP production are determined at several different initial substrate concentrations to obtain estimates for the maximum rate of substrate utilization (V_{max}) and the Michaelis constant, K_m. In field push-pull tests, site groundwater is amended with the selected substrate and nonreactive tracer and then injected into the aquifer using an existing monitoring well. During the extraction phase, groundwater samples are collected and analyzed for concentrations of injected substrate and tracer and PNP formed in situ. These data are then interpreted to obtain in situ estimates for the enzyme kinetic parameters. In particular, we have developed a method for obtaining the rate of PNP production as a function of substrate concentration by increasing the substrate concentration during the injection phase of the test to create a range of substrate concentrations in the subsurface. Using this approach, we have demonstrated that it is possible to obtain values for V_{max} and K_m using data from only a single push-pull test.

Objectives 2 and 3: An extensive series of field push-pull tests is being conducted at a field site in Norman, Okla., to measure rates of consumption of injected electron acceptors and electron donors in a portion of the site undergoing active sulfate-reduction. Tests aimed at quantifying in situ rates of sulfate reduction are being conducted by injecting site groundwater amended with a tracer and either SO_4^{2-} (in the absence of background SO_4^{2-}) or $^{35}SO_4^{2-}$ (in the presence of background SO_4^{2-}. In these tests, in situ "intrinsic" rates of sulfate reduction obtained with push-pull tests agree closely with measured rates of sulfate reduction obtained from laboratory microcosm studies. Tests aimed at quantifying in situ rates of electron donor utilization are also being conducted to compare rates of utilization of injected hydrogen, formate, lactate and acetate. Preliminary results indicate rapid hydrogen utilization and variable and site-specific utilization of formate, lactate and acetate.

Recently, an extensive series of field tests was initiated to measure in situ rates of U(VI) reduction in this aquifer under various conditions of added electron acceptors and electron donors. In these tests, ~50 L of site groundwater amended with a Br^- tracer, 0.25 mg/L U, and various combinations of electron acceptors (nitrate and sulfate) and electron donors (lactate, formate and acetate) are injected into drive-point wells and then sampled over time. Although these tests are still in progress, preliminary results indicate that U(VI) concentrations initially decrease rapidly and then decrease more slowly. The initial rapid decrease in U(VI) concentrations is attributed to abiotic processes involving either sorption/ion exchange of injected U(VI) or reduction by reduced mineral phases (e.g., Fe(II)). (Note that this hypothesis is being tested in a separate series of short-term (~hrs) tests conducted under conditions that minimize the potential for microbially mediated U(VI) reduction). The subsequent slow decrease in U(VI) concentrations is attributed to microbial activity. Observed rates of U(VI) reduction during this phase appear to be unaffected by the presence or type of exogenous electron donors but are strongly influenced by the type of electron acceptor (nitrate or sulfate).

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

Philip E. Long,[1] Timothy D. Scheibe[1] and John L. Wilson[2]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]New Mexico Institute of Mining and Technology, Socorro, N.M.

The complex interrelation between biogeochemical and physical heterogeneity in the subsurface presents a significant limitation to the successful use of in situ bioremediation. This project seeks to characterize the key geochemical and physical heterogeneities that influence bacterial transport and thus provide information that will improve our understanding of both biostimulation and bioaugmentation approaches to in situ bioremediation. The approach used in this project is to obtain detailed descriptions of small-scale heterogeneity and observations of their relationship to bacterial attachment as a key input to quantitative modeling and theoretical analysis of bacterial transport. We have focused on integration of innovative, core-scale imaging technologies to enhance detailed assessment of physical and biogeochemical heterogeneity at sub core scales.

Ultra-sensitive infrared (IR) images (256X256 pixels), high-resolution color scanner images and air permeability data were collected this year from approximately 80 m of core from the South Oyster Focus Area (SOFA) at Oyster, Virginia. The cores were drilled after a bacterial injection experiment at SOFA. Results will be linked to observed attached microbial population distributions and used in post-test modeling. Efforts to integrate 20 μm resolution X-ray Microtomography (XMT) data on core segments and 3 μm resolution synchrotron light source XMT on subcores are continuing. When the physical parameter measurements are integrated with microbial distribution and transport data for SOFA, we expect to identify specific controls on microbial attachment in heterogeneous porous media. Moreover, these latest results bring us closer to the ultimate goal of developing a detailed data set describing millimeter- to centimeter-scale joint physical and biogeochemical heterogeneity. Once available, this data set should significantly enhance the accuracy of assessments of in situ field-scale bioremediation.

Techniques developed in this project have been successfully transferred to a Department of Energy Weapons Complex Site (the Hanford Site), where IR imaging and air permeability measurements are being used to characterize potential fast pathways associated with clastic dikes. Clastic dikes are natural heterogeneities that cross-cut horizontal sedimentary layers and are known to occur beneath high-level radioactive waste tanks at the Hanford Site. The relationship among clastic dikes, contaminant transport in the vadose zone and subsurface microbiology may be a relevant area for future research.

Biogeochemistry and Bacterial Diversity of the Shiprock Uranium Mill Tailings Site, Shiprock, New Mexico, USA

Philip E. Long,[1] Todd Anderson,[2] David Balkwill,[3] Yun-Juan Chang,[4] Dwayne Elias,[5] Jennifer Field,[6] Kevin Finneran,[2] Craig Goodknight,[7] Veronica Gruntzig,[8] Jonathan Istok,[6] Lee R. Krumholz,[5] Derek R. Lovley,[2] James P. Mckinley,[1] Sarah Macnaughton,[9] Stan Morrison,[7] Aaron Peacock,[4] John R. Stephen,[10] Joseph M. Suflita,[5] James Tiedje[8] and D. C. White[4]

[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]University of Massachusetts, Amherst, Mass.; [3]Florida State University, Tallahassee, Fla.; [4]University of Tennessee, Knoxville, Tenn.; [5]University of Oklahoma, Norman, Okla.; [6]Oregon State University, Corvallis, Ore.; [7]MACTEC ERS, Grand Junction, Colo.; [8]Michigan State University, E. Lansing, Mich.; [9]National Environment Technology Centre AEA Technology Environment Culham, Abingdon, UK; [10]Horticulture Research International, Wellesbourne, Warwick, UK.

The NABIR Program is conducting research at selected Uranium Mill Tailings Remedial Action (UMTRA) sites with the goals of identifying: (1) the dominant terminal electron accepting processes, and (2) the biotransformations of metals in a field-scale system. Based on data from groundwater and sediment samples, the subsurface microbial community within the contaminant plume at the Shiprock, N.M., UMTRA site is active and diverse. For example, denaturing gradient gel electrophoresis (DGGE) results indicate that dominant sediment bacterial communities consist mostly of bacterial groups associated with metal metabolism or resistance (including the genera *Bacillus, Vibrio, Geobacter, Shewanella, Marinomonas, Pseudomonas* and *Pedomicrobium*). The dominant bacterial populations of groundwater samples were commonly only distantly related to any cultured organisms, but organisms related to the genera *Sphingomonas*, *Pedomicrobium* and *Geobacter*, organisms similar to δ-subgroup proteobacterial sulphate reducers, actinomycetes, cyanobacteria and ε-subgroup proteobacteria were detected. Samples outside the plume exhibit much less diversity in community structure.

Enrichments and microcosm studies demonstrate that microbial communities in the subsurface are capable of reducing U(VI) to insoluble U(IV). Where nitrate is present, its removal is a prerequisite to sulfate and U reduction but this is rapidly accomplished under laboratory conditions by adding dilute solutions of electron donor (e.g., acetate). These results suggest that, given appropriate consideration of lateral and vertical variations of terminal electron accepting processes, it may be possible to decrease concentrations of dissolved U, nitrate and sulfate at uranium mill sites by stimulating growth of subsurface microbial communities capable of metal reduction. Moreover, detailed multi-level sampling profiles indicate that U(VI) reduction occurs locally without biostimulation, demonstrating a microbial component of natural attenuation of U(VI) in at least some portions of the Shiprock site.

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

Chris Murray,[1] Eric Roden,[2] Ken Overstreet,[2] Yi-Ju Chien,[1] Jinsong Chen,[3] Susan Hubbard[4] and YuLong Xie[1]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]University of Alabama, Tuscaloosa, Ala.; [3]University of California, Berkeley, Calif.; [4]Lawrence Berkeley National Laboratory, Berkeley, Calif.

We are addressing the spatial distribution of subsurface microbial iron oxide reduction potential at the field scale, which will provide significant information required to understand the heterogeneity of iron-reducing bacterial activity in physically and chemically heterogeneous subsurface environments. The spatial distribution of metal-reducing bacteria may have a significant impact on their ability to immobilize metal and radionuclide contaminants.

We have sampled several boreholes at the South Oyster Focus Area. The site is near Oyster, Virginia, and serves as an analog to contaminated Department of Energy sites that are in sandy sediments. High-resolution crosshole radar and seismic tomographic data were recorded between each pair of boreholes. The sediments at the site are coastal plain sediments that include fine-grained lagoonal and back-bay sediments as well as sand rich sediment layers, and are similar to those found at DOE's Savannah River Site. Two additional boreholes were cored and sampled during the 2000 field season. The samples from all boreholes were analyzed for microbial iron reduction potential (MIRP) using a low-cost batch measurement method. We have also measured the extractable iron oxyhydroxide content, hydraulic conductivity, bulk density, porosity, organic matter concentration and grain size of the samples. In parallel with the activity-based assays of MIRP, we plan to use a molecular detection procedure (MPN-PCR) on samples from the most recent boreholes to estimate the abundance of three different groups of Fe(III)-reducing bacteria (FeRB) across different geologic strata in Oyster sediments. We are also pursuing additional wet-chemical and spectroscopic techniques for characterizing the sedimentary iron mineralogy of the sediments, which has an important bearing on the distribution and activity of metal-reducing bacteria. The application of these techniques will allow us to infer relationships between FeRB abundance and activity in the context of geological and geochemical heterogeneity. Geostatistical methods will be used to provide a quantitative model of the distribution of FeRB at the site in the near future.

Results to date indicate that FeRB are present in all sediment types at the South Oyster site, but have a patchy distribution. More than one-third of the samples did not show any microbial iron reduction potential. These results suggest that it might be necessary to augment existing metal-reducing bacterial populations, either by injection of additional bacteria, or by stimulation and transport of indigenous bacteria, in order to effectively remediate a contaminated analog site with comparable heterogeneity in the distribution of FeRB.

The results of this research project will provide a model for the distribution of FeRB and microbial iron reduction potential on the Atlantic Coastal Plain, and a methodology that can be applied to develop similar models for other locations where metal-reducing bacteria may be utilized for contaminant remediation.

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

Robert W. Smith, Carl Palmer and Jonathan Ferris
Idaho National Engineering and Environmental Laboratory, Idaho Falls, Idaho

The characteristics and abundance of reactive surfaces in aquifer media have long been recognized as key factors controlling the migration of dissolved constituents in groundwater. In principle, the reactivity within an aquifer can be estimated using geostatistical techniques and laboratory-measured parameters for individual samples; however, this approach is fraught with difficulties associated with inadequate sample coverage and lack of appropriate methods for scaling laboratory measurements. An alternative approach to the difficult process of estimating important geochemical parameters such as effective surface area is to use both conservative and slightly reactive tracers. This multiple-tracer approach sacrifices detailed understanding of the fine-scale heterogeneity but can provide integrated large-scale estimates of effective geochemical parameters that can be used for the prediction of reactive transport.

The relationship between effective reactive surface area of heterogeneous porous media and advective groundwater is being evaluated using reactive tracer experiments on materials collected at the NABIR Field Sites at Oyster, Va., and at Abbott Pit in Mappsville, Va. Batch adsorption experiments have been conducted using these materials to determine the adsorption behavior for fluoride (a weakly interacting anionic tracer), strontium (a weakly interactive cationic tracer and DOE-relevant contaminant) and uranium (a DOE-relevant contaminant that exhibits anionic or cationic behavior depending on pH). Fluoride and strontium yield retardation factors of approximately 5 for Oyster sands.

Transport experiments using Oyster sands have been initiated in columns and a small (15 x 15 x 3 cm) two-dimensional flow cell has been constructed. This flow cell is being used to evaluate the role of centimeter-scale geochemical heterogeneity in controlling the magnitude and anisotropy of larger-scale adsorption parameters in preparation for intermediate-scale (IS) reactive tracer experiments that will be conducted using the Oyster site. The use of a conservative tracer with the reactive tracer allows retardation factors to be estimated from the first moments of the concentration breakthrough curves. The effective geochemical parameters (e.g., reactive surface area) are evaluated from the retardation factors and the batch adsorption experiments. Using the method of moments to estimate retardation factors requires neither making a priori assumptions about the nature of the porous media nor solving the advection dispersion transport equations. Furthermore, the method is applicable to both laboratory studies and field tests.

Analytical and numerical modeling of solute transport in heterogeneous porous media is being conducted to provide insight into the averaging processes, to identify geochemical parameters that will exhibit anisotropic behavior and to estimate the change in average properties with increasing scale.

Progress on Expanded Rapid, Comprehensive, Lipid Biomarker Analysis for Subsurface, Community Composition and Nutritional/ Physiological Status as Monitors of Remediation and Detoxification Effectiveness

David C. White, Cory A. Lytle, Aaron D. Peacock and Yun-Juan Chang
CEB Pellissippi Laboratory, University of Tennessee, Knoxville, Tenn.

The predictability of the concentration of bioavailable Cr(III) in soils was investigated using the in situ microbial community composition at the 75-acre Cannelton Tannery Superfund Site, on the Saint Marie River near Sault St. Marie, Upper Peninsula, Mich. Phospholipid ester-linked fatty acid (PLFA) analysis was more sensitive and accurate at predicting the pollutant concentration than by using any other property of the soil, or biomarker of the microbial community. These experiments were performed in collaboration with the T.L. Marsh team from Michigan State University, with statistical and artificial neural net analysis provided by J. Almeida and the ORNL team. Repeatedly the PLFA analysis has proven to be the most comprehensive, rapid, single analysis of the viable biomass, community composition and nutritional status of the microbial community. When combined with DNA analysis of rDNA and functional genes, PLFA becomes even more potent.Our current research program is primarily focused on increasing the speed, sensitivity and specificity of the lipid biomarker analysis, while decreasing the cost. This will be integrated with DNA array technology as both lipids and DNA can be recovered from the same sample. We have focused on application of high-performance liquid chromatography (HPLC) coupled to electrospray (ES) ionization and tandem mass spectrometry (MS/MS) for detection. HPLC greatly expands the mass range available for analysis, as the constraints of requiring volatility necessary for Gas Chromatography (GC) are not required. From a neutral lipid fraction, respiratory ubiquinones (UQ) can be detected with LOD of 4 pmols/uL (equivalent to 10^6 *E. coli*) three orders of magnitude better than previous techniques and the isoprenologues (which can be diagnostic for some species) identified by MS/MS. The presence of UQ indicates the organisms were in the presence of a high potential electron acceptor (oxygen or to a lesser extent nitrate). This is an in situ biosensor the size of bacteria. A rapid potentially automatable derivatization generating charged pyridinium derivatives for mono hydroxy sterols and diglycerides have been applied with sub femto moles/uL sensitivity, capable of defining microeukaryotes (sterols) or cellular lysis (diglycerides) in the environmental microniches. The intact phospholipids (PL), whose charge is induced, are readily separated isocratically and without derivatization, undergo collision-induced dissociation (CID) in the MS/MS, and fragments for the *sn*1 and *sn*2 PLFA and the polar head group are readily identifiable utilizing multiple reaction monitoring (MRM). Sub femto moles/uL sensitivities are achieved and it is possible to count all the phospholipid molecular species in a sample, providing heretofore-unprecedented PLFA resolution. Pre-labeling tracer bacteria in ^{13}C acetate has allowed detection of specific strains by their PL in transport studies. The rate-limiting step in lipid biomarker analysis is the extraction and fractionation. High pressure/temperature extraction apparatus capable of multiple, parallel, sequential extractions has been assembled for testing and processing selected UMTRA and FRC NABIR samples.

Development and Use of 16S rRNA Gene-Based Oligonucleotide Microarrays for Assessing Microbial Community Composition and Dynamics

Jizhong Zhou, Dorothea K. Thompson, Richard A. Hurt, Dong Xu and Ying Xu
Oak Ridge National Laboratory, Oak Ridge, Tenn.

Rapid, parallel and cost-effective detection tools that can be operated in real time and in field scale heterogeneous environments are needed for assessing microbial communities that impact the in situ bioremediation of radionuclides and metals. Conventional nucleic acid-based approaches to characterizing microbial populations are currently too labor-intensive and time-consuming for high output and real-time data analysis. Thus, the goal of this study is to optimize and validate 16S ribosomal RNA (rRNA) gene-based oligonucleotide microarrays for analyzing microbial community composition and dynamics at contaminated DOE sites. The project objectives are to: (1) optimize and validate 16S rRNA gene-based oligonucleotide microarrays for assessing microbial community composition and dynamics at radioactive and mixed waste sites; and (2) create and implement new computer algorithms for designing oligonucleotide probes that are specific for different taxonomic groups of targeted organisms.

Although we have successfully used DNA microarrays for analyzing microbial communities in soils and marine sediments, oligonucleotide-based arrays present special challenges in terms of probe design and immobilization, hybridization specificity and sensitivity. Our strategy is to address these challenges by (1) optimizing hybridization with small-scale model oligonucleotide microarrays in terms of sensitivity, specificity and quantitation; (2) validating larger prototype oligonucleotide arrays using environmental samples from the proposed FRC; and (3) devising new bioinformatics programs that facilitate the probe design process for microarray applications. In the past two months, we have begun to optimize the conditions for oligonucleotide microarray construction and develop bioinformatic tools for oligonucleotide primer designing.

PROGRAM ELEMENT 6
Bacterial Transport

Top-down Controls on Bacterial Transport in Oxic and Suboxic Subsurface Environments

Fred C. Dobbs, Keun-Hyung Choi and Doug W. Smith
Department of Ocean, Earth and Atmospheric Sciences, Old Dominion University, Norfolk, Va.

The purpose of this investigation was to assess the impact of top-down processes (protistan grazing and viral infection) on bacterial transport through a shallow, unconfined, sandy aquifer at the Department of Energy study site in Oyster, Virginia. A cultured, adhesion deficient, viably stained, indigenous bacterial strain (DA001) was injected during a field experiment performed at an oxic site in October 1999, while DA001 and iron-reducing bacteria were coinjected at a nearby suboxic site in August 2000. Groundwater samples were collected before and after injection and the abundance of protistans and virus-like particles was determined using epifluorescence microscopy. Predation apparently was more pronounced at the oxic site, given that post-injection protistan abundance, relative to the biomass of injected bacteria, was greater there than at the suboxic site. The effect of protistan grazing on bacteria injected in the field experiment, however, was significantly less than that determined in a laboratory predation experiment. The abundance of virus-like particles increased as much as six-fold in the month following injection of DA001 at the oxic site, yet plaque assays revealed no evidence supporting lytic infection of the bacteria. We will discuss the ramifications of these results with respect to the influence of top down controls in the field experiments and more generally, bacterial transport through aquifers.

Extended Tailing of Bacteria Following Breakthrough at the Narrow Channel Focus Area, Oyster, Virginia

William P. Johnson,[1] Pengfei Zhang,[1] Timothy D. Scheibe,[2] Keun-Hyung Choi,[3] Fred C. Dobbs[3] and Brian J. Mailloux[4]

[1]Department of Geology and Geophysics, University of Utah, Salt Lake City, Utah; [2]Pacific Northwest National Laboratory, Richland, Wash.; [3]Department of Ocean, Earth and Atmospheric Sciences, Old Dominion University, Norfolk, Va.; [4]Department of Geosciences, Princeton University, Princeton, N.J.

Extended tailing of low bacterial concentrations following breakthrough at the Narrow Channel focus area was observed for four months. Bacterial attachment and detachment kinetics associated with breakthrough and extended tailing were determined by fitting a 1-D transport model to the field breakthrough-tailing data. Spatial variations in attachment rate constant (k_f) were observed under forced gradient conditions (i.e., k_f decreased as travel distance increased), possibly due to decreased bacterial adhesion with increased transport distance. When pore water velocity decreased by an order of magnitude at 9 days following injection, apparent bacterial attachment rates did not decrease with velocity as expected from filtration theory, but instead increased greatly for most of the wells. The coincidence of the increase in apparent attachment rate with the occurrence of protist blooms suggested that the loss of bacteria from the aqueous phase during the protist blooms was not governed by filtration, but rather by predation.

Detachment of Indigenous Bacteria in Response to Breakthrough of Injected Bacteria

William P. Johnson,[1] Pengfei Zhang,[1] Philip M. Gardner,[1] Mark E. Fuller[2] and Mary F. DeFlaun[2]
[1]Department of Geology and Geophysics, University of Utah, Salt Lake City, Utah; [2]Envirogen, Inc., Lawrenceville, N.J.

Detachment of indigenous bacteria in response to injected bacteria was observed during a field injection experiment. Injected and indigenous counterparts of the same bacterial strain were distinguished based on the presence of an internal fluorescent stain used to label the introduced cells prior to injection. Use of fluorophore conjugated antibodies in ferrographic capture analyses allowed enumeration of the total number of cells (injected and indigenous) of the target bacterial strain. The analytical method allowed quantification of cell concentrations ranging from less than 100 cells/mL to a few tens of thousands of cells per mL, with analytical errors of 10 to 20%.

Equivalent enumeration of the same cell suspension was obtained using the fluorophore-conjugated and non-conjugated antibodies, indicating no differences in the reactivity of the two antibodies. Mixing of two different target strains in the same sample, and mixing of the two antibodies to the two target strains, did not affect cell recovery of either strain. Breakthrough of injected bacteria at some sampling ports showed simultaneous breakthrough of indigenous bacteria that often outnumbered the injected bacteria by a factor of 2 to 100. Breakthrough of indigenous bacteria was observed only at relatively low concentrations of injected cells, and breakthrough of indigenous cells did not always accompany breakthrough of injected cells.

The breakthrough behavior of indigenous cells was not consistent with selective concentration of an unstained fraction of the injectate, but was consistent with detachment of indigenous bacteria in response to breakthrough of the injected cells. A laboratory column experiment yielded results consistent with detachment of attached indigenous bacteria in response to hydrodynamic interaction or "collision" with mobile injected bacteria. The results indicate that bacterial attachment and detachment processes are highly dynamic even in the absence of physiologically-driven mechanisms of detachment.

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

Ellyn M. Murphy,[1] Timothy R. Ginn[2] and Brian D. Wood[1]
[1]Pacific Northwest National Laboratory, Richland, Wash.; [2]University of California, Davis, Calif.

The success of biogeochemical alterations of metals and metal chelate complexes is ultimately controlled by the distribution of stimulated bacteria relative to the location of the contaminant in subsurface systems. The subsurface distribution of metal-reducing bacteria continually evolves through transport processes; therefore, an understanding of the subsurface transport behavior of bacteria is crucial to our ability to predict and control subsurface remediation. Currently, the ability to accurately control and predict these transport processes is far behind our desire to implement field bioremediation projects. Microbial transport is dramatically influenced by the physical properties of the bacteria, which may change as a function of phenotype. To accurately represent bacterial transport during intrinsic bioremediation, the microbial growth and transport processes must be coupled. Linking fundamental physiological properties of bacteria to field bacterial transport behavior remains a scientific challenge and is the focus of this project.

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 cell-scale measurements in field-scale applications requires a formal mathematical process known as upscaling. We are currently developing a method to describe attachment/detachment kinetics from such a cell-level process, the surface interaction potential between a microorganism and a mineral, and we have successfully measured the interaction potential between a microorganism and mineral surface using atomic force microscopy (AFM). Measurement of the interfacial potential allows us to test our upscaling theory by comparing the attachment/detachment kinetic parameters predicted from AFM measurements with the kinetic parameters measured at the bulk column scale.

To examine the effects of physical and chemical heterogeneity at field-relevant scales, we are conducting microbial transport experiments in intermediate scale flow cell systems. The physical and chemical heterogeneity of the sediments, groundwater chemistry and bacterial species used in intermediate-scale experiments are all patterned after the Oyster, Va., field site. In the initial intermediate scale experiment, the system has been simplified to include only physical heterogeneity. Subsequent intermediate-scale experiments will use the same physical heterogeneity while also adding chemical heterogeneity in the form of iron (oxy)hydroxide coated sediments. This sequential approach allows rigorous testing and comparison of the relative influence of physical versus chemical heterogeneity on bacterial transport. Therefore, the information collected will have direct relevance to ongoing field efforts at this site and will allow us to test theoretical approaches for upscaling laboratory data to field applications. The intermediate scale flow cell experiment is underway and preliminary results will be presented at the NABIR investigator's meeting.

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

Tullis C. Onstott,[1] Mary F. DeFlaun,[2] David L. Balkwill,[3] William E Holben[4] and Donald Swift[5]
[1]Princeton University, Princeton, N.J.; [2]Envirogen, Inc.; [3]Florida State University, Tallahassee, Fla.; [4]University of Montana, Missoula, Mont.; [5]Old Dominion University, Norfolk, Va.

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 conducted are designed to determine: (1) under what circumstances the preferential adsorption of bacteria to Fe, Mn and Al oxyhydroxides influence field-scale bacterial transport; (2) whether the adhesion properties of bacterial cells affect field-scale bacterial transport; and (3) whether microbial Fe(III) reduction can enhance field-scale transport of Fe reducing bacteria (IRB) and other microorganisms, and the effect of field-scale physical and chemical heterogeneity on all three processes.

During the past year an anaerobic, dual injection was successfully completed at the sub-oxic focus area (SOFA) of the South Oyster Bacterial Transport Field Site while analyses were completed on the aerobic injection performed at Narrow Channel Focus Area in November 1999. The goal of the SOFA injection was to compare the transport of a Fe(III)-reducing bacteria to adhesion deficient DA001 and to determine whether either were preferentially adsorbed to Fe, Mn, Al oxyhydroxides by collecting cores from the SOFA flow cell following injection. A new multi-level sampler (MLS) was designed by Princeton University that could be deployed in existing screened wells, that could be removable and that enabled the introduction of well-characterized solid substrates, e.g. Fe-coated sand, into the flow cell. The latter, referred to as sediment implants, are used to test the adsorption of the bacteria under in situ conditions. An injection zone between 6 and 9 meters below the surface was selected based upon core data, geophysical observations and groundwater geochemistry. A 3 D hydrodynamic model of the sub-oxic flow cell was constructed by PNNL based upon surface geophysical and tomographic data gathered by LBNL and geological data gathered by Old Dominion University, and pump tests and modeling performed by Golder Associates. Envirogen Inc. and Princeton selected Fe(III)-reducing bacterial strains based upon their adhesion assay and upon their transport through using 7-cm long, horizontal intact and repacked cores collected from vertical, rotosonic cores of the sub-oxic flow cell. The latter cores were run in the field using site groundwater pumped from the MLSs at SOFA. Based upon these experiments a low adhesion, gram-negative, facultative Fe(III)-reducing bacteria (OY107) was selected. Envirogen determined the type of viable stains required to track DA001 and OY107 simultaneously, while other investigators (U Utah and U Montana) developed additional tracking tools for OY107. Envirogen and Princeton designed an anaerobic injection system for the SOFA flow cell. During July/August 2000, the anaerobic injection of Br, DA001 and OY107 and subsequent coring were completed at SOFA. Breakthrough of both bacteria was detected 10 meters down gradient from the injection well. The bacterial breakthrough was measured off site using plate counts, direct counts and plate reader measurements, by ferrographic separation and counting, quantitative PCR and by flow cytometer analyses. Bacterial cells, including DA001 and OY107 were removed from the sediment cores and sediment implants and their concentrations estimated by various techniques. Analyses of the bacterial breakthrough data for the November 2000 injection of DA001 at Narrow Channel Focus Area indicated that DA001 migrated further than predicted from the intact sediment core experiments. The greater than expected transport is best explained by the distribution in attachment efficiency, α that exists within a population of DA001.

Vibration-Accelerated Transport of Microbes in Subsurface Media

Tommy J. Phelps and Barry R. Kinsall
Oak Ridge National Laboratory, Oak Ridge, Tenn.

Low transport rates of microorganisms and particulates through porous subsurface media pose severe limitations on the applicability of bioaugmentation for in situ remediation. This research investigates the use of vibration-based energy to increase microbial and colloidal transport in subsurface media. This basic research focuses on evaluating the applicability of vibration-induced transport through detailed hypothesis testing in laboratory experiments which will be complemented by field scale verification. We hypothesized that vibrational energies would result in increased particulate and microbial transport because of changes in subsurface porosity, increased dispersion and reduced sorption. Our results confirm that vibrational energies effectively increase bacterial transport and increase dispersion, presumably by mixing and opening pore spaces. Results of controlled laboratory experiments also indicate that water flow and particulate transport are increased when vibration energies are added. Comparisons of vibrated columns and non-vibrated columns revealed stark differences in the flow, distribution and dispersion of the particulates. Experiments quantifying vibrational energies in terms of frequency (hertz) and power (watts) suggest that frequencies between 40 and 200 Hz at power levels of several kilowatts per cubic meter of sediment may most effectively increase microbial transport.

The impact of frequency and power on flow behavior dramatically differed in columns containing different subsurface media. Sand columns from Abbott's Pit exhibited maximal water flow when vibrated between 70 and 120 Hz with energies of less than 5 watts, and average flow increases ranged from 7 to 60% above the flow in non-vibrated columns. In the sandy columns, frequency appeared to be a major determinant of vibration-facilitated transport processes. In stark contrast, saprolite cores from Oak Ridge National Laboratory (ORNL) exhibited maximal flow at vibration frequencies of 20 to 40 Hz. At frequencies typical for increased flow in sands, flow reverted to non vibrated levels in saprolite cores. Furthermore, the saprolite cores exhibited considerable sensitivity to power input. For example, aqueous flow through one saprolite core increased ~sixfold with high-power vibrational energies (>15 W at ~30 Hz). These contrasting sedimentary materials may provide ideal tests for the influence of vibration on flow in field-scale test plots.

Vibrational impact on spatial dispersion of microspheres was determined by using multivariate statistical clustering on data from column sections. At transport distances of 15 and 25 cm, the dispersion of 1 · and 2 micron-sized microspheres in vibrated columns was nearly an order of magnitude greater than dispersion in non-vibrated columns. Results indicate that:

1. the experimental apparatus can be used in rigorous hypothesis testing;
2. major impacts of vibration include increased dispersion at the macro-scale (centimeter vs pore throats), increased flow;
3. vibrational energies that increase water flow and microbial flow can be propagated in columns.

Furthermore, literature review and our analysis suggest that vibrations will affect dispersions in a >5-m radius in field tests.

Efforts in FY 2001 will focus on small field-scale test plot examinations, development of publications, and technology assessment.

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

Timothy D. Scheibe
Pacific Northwest National Laboratory, Richland, Wash.

Experiments being conducted under NABIR at a field site near Oyster, Virginia, 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 W.T. Griffin/Golder, and geological data from D. Swift/ODU). 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, the model suite has been applied to interpret the results of tracer and bacterial transport experiments conducted at the Narrow Channel (NC) focus area at the Oyster Site. Specific questions being addressed include: (1) how to appropriately assign spatially-variable bacterial attachment rates; (2) what types and amounts of characterization data are most useful for predicting field-scale transport; and (3) how to utilize the results of laboratory experiments to effectively predict field-scale transport.

A comparison of pre-model predictions (used for experimental design) and experimental results is also presented, demonstrating the effectiveness of the experimental design approach that was utilized. The results of modeling, site characterization and field experimentation are documented and shared by use of two websites developed by this project. The private website, used by project investigators, includes data libraries and detailed information on experimental design plans.

This research provides specific insights into field-scale bacterial transport processes of relevance to the subsurface microbiological sciences, increases the overall value of data and information collected at the Oyster site, and is developing a systematic approach and knowledge base applicable to future research at other sites (and ultimately to bioremediation applications).

Facies, Hydrofacies and Microbiofacies at the Oyster, Virginia, Experimental Site: Observations of Physical, Chemical and Microbial Heterogeneity Compared with Geophysical Imaging

Donald Swift,[1] Maria Green,[1] Ernest Majer,[2] Susan Hubbard,[2] M. McInerney,[3] Christopher Murray[4] and Tullus C. Onstott[5]
[1]Department of Ocean Sciences, Old Dominion University, Norfolk, Va.; [2]Lawrence Berkeley National Laboratory, Berkeley, Calif.; [3]Department of Microbiology, University of Oklahoma, [4]Pacific Northwest National Laboratory, Richland, Wash.; [5]Department of Geosciences, Princeton University, Princeton, N.J.

We have directly measured physical, chemical and biological heterogeneity at successive spatial scales at the Oyster, Virginia, site so that we might compare these values with geophysical images and establish optimum combinations of invasive and non-invasive sampling.

At Oyster, cores from Narrow Channel flow cell and South Oyster flow cell were sampled from 0 to 10 meters depth and a sampling trench was opened in the vadose zone of the Narrow Channel site. Cross-well radar tomography data sets were recorded between the series of boreholes. The sediments consist of quartz sand deposited in a shoreface to back barrier setting and locally interfingered with peat. Sedimentary facies have been quantitatively redefined by a combination of multivariate statistical methods. The textural parameters (mean grain size, sorting, percent of gravel and percent of silt and percent of organic matter) have been grouped by cluster analysis, and the validity of the group assignments has been tested with analysis of variance. Seven lithofacies have been identified. For the sandy lithofacies the hydraulic conductivity range is restricted, lying between 10^{-4} and 10^{-5} cm/s. Nevertheless, well defined permeable zones occur. Hydraulic conductivity variations are closely related to lithofacies. Bivariate plots show that the seven lithofacies reduce to four hydrofacies: an impermeable hydrofacies (peat and muddy sand), and low-, medium- and high-permeability sands. Microbiological samples were collected both aerobically and anaerobically at intervals of 2-30 cm from the Narrow Channel trench to determine the relationship between hydrogen uptake rates and grain size, moisture content, bioavailable iron and total carbon, nitrogen, sulfur and hydrogen. Microbial community structure was assessed by phospholipid fatty acid analysis (PLFA). A weak but significant correlation was detected between hydrogen uptake rates and moisture content, with the highest uptake rates associated with high moisture contents. Samples with high gravel content had less bioavailable iron and moisture and had low hydrogen uptake rates. PLFA analysis showed that all samples contained a relatively diverse microbial community and principle component analysis showed little community variation consistent with the small variation in grain size observed in this region.

The variations in physical and chemical properties determined in the laboratory appear to be detectable on the geophysical data recorded at both sites and provide clues to co-varying microbial populations. Tomographic images of radar velocity show a well-defined pattern of hydraulic conductivity, which matches the pattern of falling head measurements obtained from the laboratory. Due to the differing scales of measurement, there is a difference of order of magnitude between the two sets of results. Nevertheless, both indicate higher hydraulic conductivity along zones of pebbly sand in both sites, while the "impermeable" zones imaged at the South Oyster site correspond to the impermeable hydrofacies. Further comparison of laboratory granulometry with geophysical imaging will reveal how grain size variation and its spatial organization control continuum flow properties and determine, in turn, large-scale transport patterns.

Field Research in Bacterial Transport

Raymond E. Wildung
Pacific Northwest National Laboratory, Richland, Wash.

Bioaugmentation, the delivery of injected microorganisms to the subsurface to effect long-term contaminant immobilization or mobilization for pumping and treating, has been identified as a key requirement in bioremediation. However, the unique requirements for effective bacterial injection at Department of Energy sites (e.g., the needs for uncompromised microbiological samples, assessment of heterogeneity in situ, and computational tools for design and evaluation of injection experiments in complex environments) provide formidable challenges to implementation of bioremediation at DOE sites. Multi-institutional, interdisciplinary field experiments were therefore undertaken at Oyster, Virginia, and at satellite sites to develop an understanding of the effects of interdependent geochemical, microbial and hydrogeologic processes and spatial and temporal heterogeneities on microbial transport and remediation strategies. This project, in collaboration with Golder Associates Inc. (W.T. Griffin, PI), helps provide the innovative research tools and site and regulatory liaisons needed to implement the DOE scientific research plan for field research at these sites and assists in transferring new tools and concepts to DOE sites for use in remediation of subsurface environments.

Rotosonic coring methods, groundwater flow meters and tracer injection systems were designed specifically for characterization and modeling of the hydrogeology in both oxic and suboxic flowing sand environments at the Oyster site. This information was used by the NABIR research team in designing bacterial injection experiments, including injection/extraction rates needed to achieve target groundwater flow rates and velocities, and optimum sampling schemes. Closed-loop extraction/injection systems were designed, constructed and shown to maintain oxygen in groundwaters at natural levels in both oxic and suboxic systems. Down-hole data loggers were installed to monitor water levels, pH, conductivity and dissolved oxygen levels in both oxic and suboxic environments; this information was incorporated into a database available to all investigators.

Documentation was prepared and an Environmental Monitoring Plan was implemented to meet rigorous administrative and regulatory requirements stipulated by the Nature Conservancy, U.S. Environmental Protection Agency, Virginia Department of Environmental Quality and DOE. An outreach program was also initiated to communicate the nature of the field experiments and their importance in bioremediation to regulators at the county, state and federal level. An informational brochure was prepared to facilitate understanding of the projects and help stakeholders, including local citizens, formulate questions that were addressed at a public meeting held at the site.

Future efforts will be directed toward completion of field experimentation, ensuring that all regulatory requirements related to site closure have been met, and transferring the results of research to the user community for application to DOE sites nationwide.

PROGRAM ELEMENT 7

System Engineering, Integration, Prediction and Optimization

Simulating Bioremediation of Uranium Contaminated Aquifers: Uncertainty Assessments of Model Parameters and its Relation to Model Implementation

Peter R. Jaffe, Sookyun Wang, Genyuan Li, Sheng-Wei Wang and Herschel A. Rabitz
Department of Civil and Environmental Engineering, Princeton University, Princeton, N.J.

Bioremediation of trace metals and radionuclides in groundwater may require the manipulation of redox conditions via the injection of a carbon source. For example, bacteria can reduce soluble U(VI) to U(IV), which is a solid (uraninite), after the system is more reduced than during denitrification. Since uranium contamination is often found in the presence of high nitrate concentrations, the first step in the biological immobilization of uranium is to add a carbon source that will induce biological denitrification. After nitrate has been consumed, uranium can be reduced simultaneously with other electron acceptors, such as Fe(III).

The objective of this research was to identify some of the key processes that a modeling effort of the bioremediation of uranium-contaminated aquifers needs to consider. For this purpose, and to gain an understanding of the interactions among the numerous biogeochemical processes that will occur during such a bioremediation scheme, a time-dependent reactive transport model has been developed and tested. 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 uranium. 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.

In this and similar models, the relationship between the various biological chemical variables is non linear, and various interdependencies are expected to exist. It is important to identify all 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) identify what parameters have to be measured in the field, and where, in order to most effectively test the model against field data.

A recently developed nonlinear analysis tool, RS-HDMR (Random Sampling-High Dimensional Model Representation) was employed to determine the relationship between the various model inputs and outputs. An analysis was performed to investigate the effect of 20 rate constants and transport parameters on the transport of four chemical species, including uranium. Results showed that a clear description of the hydrogeology is key for the successful modeling of the biogeochemical transformations. Furthermore, because of the link to nitrates described above, of the different biological processes considered, denitrification has by far the largest contribution to the overall variance of uranium-bioremediation-model outputs. This indicates that an accurate simulation of denitrification is essential to describe bioremediation of uranium. Finally, results also showed that a significantly higher variance is 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

Words and Images as Data: Analyzing Dialog Interactions to Understand More About Science Communication

Gordon R. Bilyard,[1] Charlotte J. Word[2] and James R. Weber[3]
[1]Environmental Technology Division, Pacific Northwest National Laboratory, Richland, Wash.; [2]Department of Communication, Portland State University, Portland, Ore.; [3]Communication Directorate, Pacific Northwest National Laboratory, Richland, Wash.

The roles of scientists appear to be changing with respect to communicating with the public. There is a greater demand by the public to hear directly from scientists rather than from spokespeople. Conversely, some scientists would like a greater voice in administration and policy formation. Although many regard communication activities as transparent, they can be treated as natural phenomena. Human communication studies identify communication activities and their variations, trace influences on communication behaviors, and examine and test patterns of words, images and concepts that make up much of the evidence of communication behavior. In this research, we pursue systematic studies of communication among scientists and non-scientists in order to identify communication patterns and possibly develop strategies for more effective science communication.

Communication data consists of taped and transcribed discussions about science (facilitated and unfacilitated) in groups and between individuals, videos and documents (email, websites, published and unpublished reports, and notes of interactions). Data are analyzed and interpreted through triangulation and established quantitative and qualitative research tools, including discourse and network analysis, which provide insight into specific communication dynamics: evident and less-evident patterns of words, images and interactions, in varying degrees of resolution (larger scope or more local), influence of sustained and familiar relationships, salient topics and issues.

Two examples of our findings are presented. In the first, different usages and meanings are revealed for scientists' and non-scientists' use of the word "complexity." In the second, citizens, regulators and scientists are seen to revert to linguistic/conceptual scripts that appear to inhibit open discussion of scientific issues. In both examples, the participants' need to negotiate meanings becomes evident.

During this project, we have discovered ways in which the informational needs of scientists, stakeholders and members of the general public differ, and some influences arising from differing self-interests, world-views and uses of information. All groups need basic, albeit different information about the physical properties of contamination, remediation and the prospects of bioremediation, about their own roles and interests (as groups and collectively), and about how they can cooperate. This research points to the importance of taking a systemic perspective to communication that involves analyzing ongoing communication events and integrating findings to enhance effective communication practices.

Integrating BASIC into the NABIR Field Research Center

Amy K. Wolfe and David J. Bjornstad
Oak Ridge National Laboratory, Oak Ridge, Tenn.

This presentation discusses efforts to integrate BASIC (Bioremediation and its Societal Implications and Concerns) into the Department of Energy's NABIR (Natural and Accelerated Bioremediation Research) Program Field Research Center (FRC). This integration will occur through both research and application activities.

Research goals are to enhance understanding of (a) the interactive social and organizational processes that influence bioremediation field research, and (b) community responses to field-level bioremediation research in Oak Ridge and other communities. To achieve these goals, we will conduct three primary research activities, as follows:

- a longitudinal case study of the FRC scientific endeavor;
- a longitudinal case study of responses to the FRC and its separate field experiments; and
- a series of small-scale bioremediation case studies, developing a compendium of cases.

Application goals are to enhance communication within the FRC-scientific arena and between that arena and the non-scientific arena. Accomplishing this goal will strengthen FRC research and improve public understanding of bioremediation in general and the FRC in particular. To achieve these goals, we will undertake three primary application activities:

- facilitate communication among FRC-related scientists, which we informally label "scientists talk";
- promote listening among FRC related scientists—listening both to scientists from other disciplines and with different perspectives and to the issues of interest and concern among non-scientists (our informal label is "scientists listen"); and
- promote dialogue about bioremediation, and particularly the FRC, among non-scientists ("non-scientists talk and listen").

Together, these research and application activities will provide the substance that allows us to achieve our overarching goal, which is to integrate BASIC into the regular functioning of the FRC.

Public Acceptability of Controversial Bioremediation Technologies

Amy K. Wolfe and David J. Bjornstad
Oak Ridge National Laboratory, Oak Ridge, Tenn.

Our research focuses on the issue of social acceptability of bioremediation technologies, particularly genetically engineered microorganisms (GEMs). Experience has shown that some technologies, such as incineration or those involving things nuclear, frequently are dismissed out of hand as non-options. Therefore, the fundamental question underlying our work is, "under what circumstances would a technology such as genetically engineered microorganisms (GEMs) be considered seriously as a remediation alternative?" We have emphasized a site-specific, decision-making context in which involved parties—technology sponsors, regulators, local government, researchers, civic and environmental groups—deliberate about remediation options.

Initially, we developed a conceptual framework called Public Acceptability of Controversial Technologies (PACT) that identified critical dimensions affecting acceptability. Then, we collected data. In particular, we analyzed tapes of DOE Site-Specific Advisory Board (SSAB) meetings as a venue through which we could observe a real-world, formal deliberative process unobtrusively, using PACT to structure our analyses. This presentation reports on a continuation of our research. We present preliminary results from a simulation exercise designed to gather empirical data in a relatively controlled way. This exercise involved presenting small groups of role-playing "involved parties" with a series of scenarios, each of which incrementally adds pieces of information that have the potential to alter their willingness to consider GEMs seriously. It combined two information-gathering methods—a highly structured approach that derives from experimental economics with an observational approach that derives from ethnographic analysis.

FIELD RESEARCH CENTER

Field-Scale Evaluation of Biostimulation for Remediation of Uranium-Contaminated Groundwater at a Proposed NABIR Field Research Center in Oak, Ridge, Tenn.

Craig Criddle and Peter Kitanidis
Department of Civil and Environmental Engineering, Stanford University, Stanford, Calif.

Microbial reduction of uranium may prevent its migration to receptor streams. However, application of this technology to field sites is untested, and future site remediation will require improved understanding of basic processes and implementation strategies in heterogeneous environments.

The objectives of this study are to: (1) develop a predictive capability for the rates and mechanisms controlling microbial reduction of U in heterogeneous field settings, and (2) develop a system capable of delivering electron donor to a highly heterogeneous subsurface environment enabling spatially uniform in situ immobilization of U in groundwater upon passage through a subsurface biocurtain.

To meet these objectives, we have proposed a field study in a near-surface aquifer southeast of the S-3 pond in the Bear Creek hydrogeologic regime at Oak Ridge, Tenn. This regime is characterized by gram per liter levels of NO_3^-; part per million levels of U(VI); acidic groundwater (pH 3-5); extreme heterogeneity in contaminant distribution; and fractured saprolite of low hydraulic conductivity. Nitrate must be removed because it prevents reduction of U, and, if the NO_3^- is reduced to N_2, the resulting gas could further reduce aquifer permeability.

Modeling studies are on-going to evaluate flow recirculation schemes that will enable reliable delivery of chemical agents needed to remove nitrate and uranium and excess nitrogen gas resulting from denitrification. Laboratory studies are underway to evaluate the effects of flow, changes in U adsorption as a function of pH, and the effect of low pH on extent and rates of denitrification. For nitrogen removal, studies are underway to evaluate the use of a vacuum stripper. We are also in the process of designing a system that will impose hydrological and geochemical controls on the U source permitting reliable determination of U reduction rates. We envision use of tracers to quantify hydrological and geochemical processes and to develop mass balances. Field-scale and companion bench-scale studies will evaluate hypotheses on dissimilatory metal-reducing activity.

Determination of In-Situ Uranium Immobilization using the Push-Pull Test

Jonathan D. Istok,[1] John M. Senko,[2] Lee R. Krumholz[2] and Joseph M. Suflita[2]
[1]Department of Civil Engineering, Oregon State University, Corvallis, Ore.; [2]Department of Botany and Microbiology, Institute for Energy and the Environment, University of Oklahoma, Norman, Okla.

For this project, we deploy the single well, "push-pull" test in determining the kinetics of microbially-mediated uranium reduction in unconsolidated sediments during preliminary experiments at an aquifer contaminated with landfill leachate, and then at the proposed Bear Creek Valley Field Research Center (FRC). A push-pull test consists of the controlled injection of a prepared aqueous test solution into the saturated zone, followed by the extraction of the test solution/groundwater mixture from the same location.

At the FRC, we will use laboratory microcosm studies to select electron donors to use in field manipulation experiments. Short-term (~ hrs) push-pull tests will be conducted in each monitoring well to determine site-specific values for aquifer hydraulic conductivity and dispersivity, and retardation factors for uranium and other potentially reactive groundwater constituents. Field manipulation experiments will be conducted to test the effect of electron donor additions, in-situ. Finally, the effect of electron donor additions on sediment geochemistry will be determined from analyses made on groundwater and sediment samples collected from within the zone of influence of each control and treatment well.

We have completed a preliminary study of uranium precipitation in an aquifer contaminated with municipal landfill leachate. Complete immobilization (precipitation) of 0.85 mM U(VI) was observed in nonsterile sediment slurries incubated in the laboratory for 14 d. Abiotic control incubations showed some U(VI) loss, but complete immobilization required microbial activity. In-situ microbial activity was evaluated with single-well push-pull tests using groundwater amended with bromide as a conservative tracer, an electron acceptor (either NO_3^- or SO_4^-), and 1.5 µM U(VI). Similar patterns of soluble U(VI) loss were observed in the field experiments as in the laboratory incubations, with complete U(VI) immobilization after 8 d. Amendment with 0.5 mM SO_4^- slowed U(VI) immobilization and yielded a higher U(VI) recovery (10%) than the unamended treatment (4%). Nitrate amendment (5 mM) further inhibited U(VI) immobilization, and appeared to "remobilize" uranium that had been previously immobilized. U(VI) recovery from wells amended with NO_3^- were 40% or greater. Experiments using heat-killed sediments and oxides of nitrogen suggest that denitrification intermediates can potentially account for the remobilization activity. These experiments indicate that while in-situ subsurface U(VI) immobilization can be expected to take place through the concerted action of geochemical and microbiological processes, the presence of SO_4^- and/or NO_3^- as microbial electron acceptors may detrimentally influence this process.

NABIR Field Research Center - Oak Ridge, Tennessee

David Watson
Oak Ridge National Laboratory, Oak Ridge, Tenn.

The Environmental Sciences Division at Oak Ridge National Laboratory has established a Field Research Center (FRC) on the Department of Energy's Oak Ridge Reservation in Tennessee for DOE's Office of Biological and Environmental Research. The FRC provides a unique site for investigators in the Natural and Accelerated Bioremediation Research (NABIR) program to conduct field-scale research and obtain samples related to in situ bioremediation of metals and radionuclides.

The FRC includes a contaminated area that can be used for conducting experiments on a plume of contaminated groundwater and sediment, and a background area that provides for comparison studies in an uncontaminated area. The contaminated and background areas are located on DOE land in Bear Creek Valley (BCV), which is within the Y-12 Plant area. Contaminants include uranium, Tc-99, strontium, nitrate, barium, cadmium, volatile organic compounds (VOCs) and other inorganics and radionuclides believed to be of interest to NABIR investigators. The water table resides at between 0 to 3 m below the surface and is 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.

FRC activities that are ongoing or completed include the following:

- Preparation of FRC Management documents and Site Characterization Plan;
- Setting up of FRC field trailers and laboratories;
- Completion of Phase 1 site characterization activities;
- Initiation of Phase 2 site characterization activities;
- Collection of groundwater and sediment samples (cores and composites) from the background and contaminated site for use by NABIR PI's in laboratory studies;
- Site selection and initiation of two NABIR Investigator field studies (i.e., OSU/UO team and Stanford/ORNL team);
- Setting up of groundwater computer model grid;
- Establishment of FRC library and implemetation of data management tasks.

ADDRESS LIST

Tamar Barkay
Department of Biochemistry and Microbiology
Cook College, Rutgers University
76 Lipman Dr.
New Brunswick, NJ 08901-8525
Phone: 732-932-9763
E-mail: barkay@aesop.rutgers.edu

Paul E. Bayer
Environmental Sciences Division
Office of Biological and Environmental Remediation
Office of Science, U.S. Department of Energy
19901 Germantown Road, MS F-240
Germantown, MD 20874-1290
Phone: 301-903-5324
E-mail: paul.bayer@science.doe.gov

Sally M. Benson
Earth Sciences Divison
Lawrence Berkeley National Laboratory
One Cyclotron Road, MS 90-1116
Berkeley, CA 94720
Phone: 510-486-7071
E-mail: SMBenson@lbl.gov

Gordon R. Bilyard
Pacific Northwest National Laboratory
Battelle
P.O. Box 999, MS K3-54
Richland, WA 99352
Phone: 509-372-4219
Fax: 50-9-372-3618
E-mail: gordon.bilyard@pnl.gov

Diane A. Blake
Department of Ophthamology
Tulane University School of Medicine
1430 Tulare Ave.
New Orleans, LA 70112
Phone: 504-584-2478
E-mail: blake@tulane.edu

Harvey Bolton, Jr.
Pacific Northwest National Laboratory
P.O. Box 999
Richland, WA 99352
Phone: 509-376-3950,
Fax: 509-375-1321
E-mail: harvey.bolton@pnl.gov.

Craig C. Brandt
Environmental Sciences Division
Oak Ridge National Laboratory
P.O.Box 2008, MS6038
Oak Ridge, TN 37831-6038
Phone: 865-574-1921
Fax: 865-576-8646
E-mail: fcb@ornl.gov

Fred Brockman
Pacific Northwest National Laboratory
P.O. Box 999, Mailstop P7-50
Richland, WA 99352
Phone 509-376-3300
Fax 509-376-1321
fred.brockman@pnl.gov

Scott C. Brooks
Environmental Sciences Division
Oak Ridge National Laboratory
P.O. Box 2008, MS 6038
Oak Ridge, TN 37831-6038
Phone: 865-574-6398
Fax: 865-576-8646
E-mail: brookssc@ornl.gov

William D. Burgos
Pennsylvania State University
212 Sacket Building
University Park, PA 16802
Phone: 814-863-0578
Fax: 814 863-7305
E-mail: bburgos@psu.edu

Darrell P. Chandler
Environmental Microbiology Group
Pacific Northwest National Laboratory
P.O. Box 999, Mail Stop P7-50
Richland, WA 99352
Phone: 509-376-8644
Fax: 509-376-1321
E-mail: dp.chandler@pnl.gov

Winston C. H. Chen
Oak Ridge National Laboratory
Bldg. 5500, MS6378
Oak Ridge, TN 37831-6378
Phone: 865-574-5895
Fax: 865-576-1225
E-mail: chenc@ornl.gov

John D. Coates
Department of Microbiology
Southern Illinois University
Carbondale, IL 62901
Phone: 618-453-6132
Fax: 618-453-8036
E-mail: jcoates@micro.siu.edu

Don L. Crawford
Department of Microbiology, Molecular Biology and Biochemistry
University of Idaho
Moscow, ID 83844-3052.
Phone: 208-885-6001
Fax: 208-885-6518
E-mail: donc@uidaho.edu

Craig Criddle
Department of Civil and Environmental Engineering
Stanford University
Stanford, CA 94305 4020
Phone: 650-726-9032
E-mail: criddle@ce.stanford.edu

Michael J. Daly
Uniformed Services University of the Health Sciences
4301 James Bridge Road
Bethesda, MD 20814
Phone: 301-295-3750
E-mail: mdaly@usuhs.mil

Fred C. Dobbs
Old Dominion University
4600 Elkhorn Avenue
Norfolk, VA 23529
Phone: 757-683 5329
Fax: 757-689-5303
E-mail: fdobbs@odu.edu

Daniel W. Drell
Life Sciences Division
Office of Biological and Environmental Remediation
Office of Science, U.S. Department of Energy
19901 Germantown Road
Germantown, MD 20874-1290
Phone: 301-903-4742
E-mail: daniel.drell@oer.doe.gov

Jerry W. Elwood
Ecologist
U.S. Department of Energy
Headquarters Germantown
MS F-240
19901 Germantown Road
Germantown, MD 20874-1290
Phone: 301 903-3281
E-mail: jerry.elwood@science.doe.gov

Scott Fendorf
Stanford University
GES-Braun Hall
Stanford, CA 94305-2115
Phone: 650-723-5238
Email: fendor@stanford.edu

A.J. Francis
Brookhaven National Laboratory
P.O. Box 5000, Bldg. 490A
Department of Applied Science
Upton, NY 11973
Phone: 631 344-4534
Email: francis1@bnl.gov

James K. Fredrickson
Pacific Northwest National Laboratory
P.O. Box 999
Richland, WA 99338
Phone: 509-376 7063
Fax: 509-376 1321
E-mail jim.fredrickson@pnl.gov

Mark E. Fuller
Envirogen, Inc.
Princeton Research Center
4100 Quakerbridge Road
Lawrenceville, NJ 08648
Phone: 609-9369300
Fax: 609 936-9221
E-mail: fuller@envirogen.com

Carol Giometti
Argonne National Laboratory
Biosciences Division
9700 South Cass Avenue
Argonne, IL 60439
Phone: 630-252 3839
FAX: 630-252-5517
E-mail: csgiometti@anl.gov

Yuri A. Gorby
Pacific Northwest National Laboratory
P.O. Box 999, MS P7-50
Richland, WA 99352
Phone: 509-373-6177
Fax: 509 376-1321
E mail: yuri.gorby@pnl.gov

Baohua Gu
Oak Ridge National Laboratory
P.O. Box 2008, MS 6036
Oak Ridge, TN 37831-6036
Phone: 865-574-7286
E-mail: b26@ornl.gov

Kirk Hatfield
University of Florida
P.O. Box 116580
Gainesville, FL 32611-6580
Phone: 352 392 0956
E-mail: khatf@ce.ufl.edu

Terry C. Hazen
Environmental Remediation Technology Department
Earth Sciences Division
Lawrence Berkeley National Laboratory
One Cyclotron Road, MS 70A-3317
Berkeley, CA 94720
Phone: 510 486 6223
E-mail: TCHazen@lbl.gov

John C. Houghton
Environmental Sciences Division
Office of Biological and Environmental Remediation
Office of Science, U.S. Department of Energy
19901 Germantown Road, MS F-240
Germantown, MD 20874 1290
Phone: 301-903 8288
E-mail: john.houghton@oer.doe.gov

Jonathan Istok
Department of Civil Engineering
Oregon State University
Apperson Hall 202
Corvallis, OR 97331-4501
Phone: 541 737-8547
E-mail: jack.istokj@.orst.edu

Peter R. Jaffe
Department of Civil Engineering
Princeton University
Princeton, NJ 08544
phone: 609-258 4653
Fax: 609-258-2799
E-mail: jaffe@princeton.edu

William P. Johnson
Dept. of Geology and Geophysics
University of Utah
135 South 1460 East
Salt Lake City, Utah 84112
Phone: 801 581-5033
Fax: 801-581-7062
E-mail: wjohnson@mines.utah.edu

Jay Keasling
Chemical Engineering Department
University of California, Berkeley
201 Gilman
Berkeley, CA 94720
Phone: 510-642-4862
Fax: 510 643-1228
Email: keasling@socrates.berkeley.edu

Kenneth M. Kemner
Environmental Research Division
Argonne National Laboratory
9700 South Cass Avenue
Argonne, IL 60439-4843
Phone: 630-252-1163
Fax: 630-252 2959
E-mail: kemner@anl.gov

Allan Konopka
Department of Biological Science
Purdue University
W. Lafayette, IN 47907
Phone: 765-494-8152
Fax: 765-494-0876
E-mail: akonopka@purdue.edu

David S. Kosson
Department of Civil and Environmental Engineering
Vanderbilt University
Box 1831 Station B
Nashville, TN 37245
Phone: 615 322 1064
Fax: 615-322-3365
E-mail: DavidKosson@Vanderbilt.edu

Joel Kostka
Department of Oceanography
Florida State University
317 OSB, Call Street
Tallahassee, FL 32306-4320
Phone: 850-645-3334
Fax: 850-644-2581
Email: jkostka@ocean.fsu.edu

Lee Krumholtz
Department of Botany and Microbiology
The University of Oklahoma
770 Van Vleet Oval, Room 135
Norman, OK 73019
Phone: 405-325-0437
Fax: 405-325-7619
E-mail: krumholz@ou.edu

Stuart B. Levy
Tufts University School of Medicine
136 Harrison Avenue, SC704
Boston, MA 02111
Phone: 617 636-3651
E-mail: stuart.levy@tufts.edu

Jon Lloyd
University of Massachusetts
Department of Microbiology
Morrill Science Centre
Amherst, MA 01003
Phone: 413-545-0339
Fax: 413 545-1578
E-mail: jrlloyd@microbio.umass.edu

Philip E. Long
Pacific Northwest National Laboratory
PO Box 999/ MS K9 33
Richland, WA 98816
Phone: 509-376-6090
Fax: 509-372-6089
E-mail: philip.long@pnl.gov

Derek R. Lovley
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
Phone: 413-545-9651
Fax: 413-545-1578
E-mail: dlovley@microbio.umass.edu

Mary Lowe
Loyola College in Maryland
Physics Department
4501 N. Charles St.Baltimore, MD 21210
Phone: 410-617-2709
Fax: 410-617-2646
E mail: mlowe@loyola.edu

Timothy Magnuson
Center for Biofilm Engineering
Montana State University
366 EPS Building
Bozeman, MT 59717
Phone: 406-994-4564
Fax: 406-994-6098
Email: tim m@erc.montana

Terence L. Marsh
Center for Microbial Ecology and Department of Geology
Michigan State University
41 Giltner Hall
East Lansing, MI 48824
Phone: 517-432-1365
Fax: 517-432-3770
E-mail: marsht@pilot.msu.edu

A. Matin
Department of Microbiology and Immunology
Stanford University School of Medicine
Sherman Fairchild Science Building
Stanford, CA 94305
Phone: 650-725-4745
Fax: 650-725-6757
E mail: a.matin@stanford.edu

Ellyn M. Murphy
Pacific Northwest National Laboratory
P.O. Box 999, MSIN K9-76
Richland, WA 99352
Phone: 509-375-5914
Fax: 509 375-4343
E mail: ellyn.murphy@pnl.gov

Christopher J. Murray
Applied Geology and Geochemistry
Pacific Northwest National Laboratory
P.O. Box 999, MS K6-81
Richland, WA 99352
Phone: 509-376-5848
Fax: 509 376-5368
E mail: Chris.Murray@pnl.gov

Kenneth H. Nealson
California Institute of Technology, and
Jet Propulsion Laboratory
4800 Oak Grove Drive, MS 183-301
Pasadena, CA 91109
Phone: 818 354-9219
E mail: knealson@jpl.nasa.gov

Mary Neu
Los Alamos National Laboratory
MS G739
Los Alamos, NM 87545
Phone: 505-667-9313
E-mail: mneu@lanl.gov

Heino Nitsche
Chemical Sciences Division
Lawrence Berkeley National Laboratory
MS 70A 1150
One Cyclotron Rd.
Berkeley, CA 94720
Phone: 510-486 5615
Fax: 510-486-7444
Email: HNitsche@lbl.gov

Tullis C. Onstott
Deptartment of Geosciences
Princeton University
Princeton, NJ 08544
Phone: 609-258-4100
Fax: 609-258 1274
E mail: tullis@princeton.edu
Website: http://geo.princeton.edu/geomicrobio/

Anna C. Palmisano
Environmental Sciences Division
Office of Biological and Enviornmental
Remediation
Office of Science, U.S. Department of Energy
19901 Germantown Road, MS F-240
Germantown, MD 20874-1290
Phone: 301 903-9963
E-mail: anna.palmisano@science.doe.gov

Anthony V. Palumbo
Oak Ridge National Laboratory
P.O. Box 2008, MS 6038
Oak Ridge, TN 37831-6038
Phone: 423-576-8002
Fax: 423-576 8646
E-mail: palumboav@ornl.gov

James N. Petersen
Chemical Engineering Department
Washington State University
P.O. Box 64210
Pullman, WA 99164-2710
Phone: 509-335-1003
Fax: 509 335-4806
E-mail: jnp@mail.wsu.edu

Brent M. Peyton
Chemical Engineering Department
Center for Multiphase Environmental Research
Washington State University
P.O. Box 642710
Pullman, WA 99164-2710
Phone: 509-335-4002
Fax: 509-335-4806
E-mail: bmpeyton@che.wsu.edu

T.J. Phelps
Oak Ridge National Laboratory
P.O. Box 2008, MS 6036
Oak Ridge, TN 37831
Phone: 423-574-7290
Fax: 423-576-8543
E mail: tkp@ornl.gov

Margaret Romine
Pacific Northwest National Laboratory
PO Box 999, MS K4-06
Richland, WA 99352
Phone: 509-376 8287
Fax: 509-376-1321
E mail: margie.romine@pnl.gov

Tim Scheibe
Pacific Northwest National Laboratory
PO Box 999 MSIN K9-36
Richland, WA 99352
Phone: 509-372 6065
Fax: 509-372-6089
E-mail: tim.scheibe@pnl.gov

Barth F. Smets
University of Connecticut
261 Glebrook R., U-37
Storrs, CT 06269
Phone: 860 486 2270
E-mail: bath.smets@uconn.edu

Richard Smith
Pacific Northwest National Laboratory
PO Box 999
EMSL MS K8-98
Richland, WA 99352
Phone: 509-376-0723
Fax: 509-376-2303
E-mail: rd_smith@pnl.gov

Robert W. Smith
Biotechnologies Department
Idaho National Engineering and Environmental Laboratory
P.O. Box 1625, MS 2107
Idaho Falls, Idaho 83415
Phone: 208-526-9345
Fax: 208-526-9822
E-mail: rqs@inel.gov

Joseph M. Suflita
Department of Botany/Microbiology
University of Oklahoma
Norman, OK 73019-3032
Phone: 405-325-5761
Fax: 405-325-7541
E-mail: jsuflita@ou.edu

Anne O. Summers
Department of Microbiology
527 Biological Sciences Building
University of Georgia
Athens, GA 30602-2605
Phone: 706-542-2669
Fax: 706-542-6140
E-mail: summers@uga.edu

Donald J. P. Swift
Department of Oceanography
Old Dominion University
Hampton Boulevard
Norfolk, VA 23629
Phone: 757-683 4937
Fax: 757-683-5303
E-mail: dswift@odu.edu

Bruce M. Thomson
Dept. of Civil Engineering
University of New Mexico
Tapy Hall
Albuquerque, NM 87131
Phone: 505-277-4729
Fax: 505-277-1988
E-mail: bthomson@unm.edu

James M. Tiedje
Center for Microbial Ecology
Michigan State University
540 Plant and Soil Science Bldg.
East Lansing, MI 48824
Phone: 517-353-9021
Fax: 517-353-2917
E-mail: tiedjej@pilot.msu.edu

Murthy A. Vairavamurthy
Brookhaven National Laboratory
Building 815
Upton, NY 11973-5000
Phone: 516-344-5337
Fax: 516-344-5526
E-mail: vmurthy@bnl.gov

Judy D. Wall
Biochemistry Department
University of Missouri-Columbia
117 Schweitzer Hall
Columbia, MO 65211
Phone: 573-882-8726
Fax: 573-882-5635
E-mail: wallj@missouri.edu

Jiamin Wan
Earth Sciences Division
Lawrence Berkeley National Laboratory
One Cyclotron Road, MS 90-1116
Berkeley, CA 94720
Phone: 510-486-6004
Fax: 510-486-7797
E-mail: jmwan@lbl.gov

David Watson
Oak Ridge National Laboratory
PO Box 2008, MS-6038
Oak Ridge, TN 37831-6038
Phone: 865-241-4749
Fax: 865-574-7420
E-mail: watsondb@ornl.gov

David C. White
Center for Environmental Biotechnology
University of Tennessee
10515 Research Drive, Suite 300
Knoxville, TN 37932
Phone: 423-974-8001
Fax: 423-974-8027
E-mail: milipids@aol.com

R. E. Wildung
Environmental Science Research Center
Pacific Northwest National Laboratory
P.O. Box 999, MS P7-54
Richland, WA 99352
Phone: 590 376-5680
Fax: 509-376-9650
E mail: r.wildung@pnl.gov

Amy K. Wolfe
Oak Ridge National Laboratory
P.O. Box 2008
Oak Ridge, TN 37831-6205
Phone: 865-574 5944
Fax: 865-574-8884
E-mail: wolfeak@ornl.gov

Frank J. Wobber
Environmental Sciences Division
Office of Biological and Environmental
Remediation
Office of Science, U.S. Department of Energy
19901 Germantown Road, MS F-240
Germantown, MD 20874-1290
Phone: 301-903-5549
E mail: frank.wobber@science.doe.gov

Linda Wuy
Earth Sciences Division
Lawrence Berkeley National Laboratory
One Cyclotron Road, MS 90-1116
Berkeley, CA 94720
Phone: 510-486-7418
Fax: 510-486-7714
E mail: LDWuy@lbl.gov

Sunney Xie
Department of Chemistry and Chemical
Biology
Harvard University
12 Oxford St.
Cambridge, MA 02138
Phone: 617-496-9925
Fax: 617-495-8709
Email: xie@chemistry.harvard.edu

John M. Zachara
Pacific Northwest National Laboratory
P.O. Box 999, MS K8-96
Richland, WA 99352
Phone: 509-376-3254
Fax: 509-376-3650
E-mail: john.zachara@pnl.gov

Jizhong Zhou
Environmental Sciences Divison
Oak Ridge National Laboratory
P.O. Box 2008, MS 3038
Oak Ridge, TN 37831-3038
Phone: 865-576 7544
Fax: 865 576-8646
Email: zhouj@ornl.gov

U.S. Department of Energy
Office of Science
Office of Biological and Environmental Research