PROGRAM ELEMENT 1
Biotransformation and Biodegradation

Biodegradation of PuEDTA and Impacts on Pu Mobility

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

Research has focused on PuEDTA aqueous speciation to allow us to predict Pu(IV)EDTA behavior in the environment and design PuEDTA biodegradation experiments. The solubility of PuO_2(am) was determined with varying EDTA concentrations and at various pHs with EDTA. The Pu(IV)EDTA species in solution and their equilibrium constants were then determined using the Pitzer ion-interaction model. EDTA greatly enhanced the solubility of Pu, with previously determined stability constants greatly underestimating the Pu(IV)EDTA in solution. The Pu(IV) forms strong $Pu(OH)_xEDTA^{x-}$ complexes (x = 1, 2 or 3), which enhance Pu solubility. Pu(IV) solubility increased with decreasing pH and increasing EDTA. Metal competition for the EDTA or its biodegradation will lower the $Pu(OH)_xEDTA^{x-}$ concentration. Studies are in progress to investigate the biodegradation of EDTA in the presence of Pu and its influence on Pu solubility.

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

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

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

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

NOM collected from a wetland pond (Georgetown NOM, GNOM), a known electron shuttling compound (anthraquinone-2,6-disulfonate, AQDS), and a strong ferrous complexing agent (Ferrozine) were employed to quantify the effects of NOM on iron reduction and to comparatively evaluate the two proposed mechanisms. Test systems consisted of serum bottles sealed with Teflon-faced butyl rubber stoppers and aluminum crimp seals containing 10 mL of medium inoculated to produce a known density of the dissimilatory iron reducing bacterium *Shewanella putrefaciens*, strain CN32. Tests were conducted under non-growth conditions with H_2 as the sole electron donor. Test medium contained 10 mM PIPES, pH = 6.8, 30 µM PO_4^{3-}, and 2.0 g_L $^{-1}$ of commercial hematite. All test vessels were incubated at 20°C on gyrarotory shakers outside of an anaerobic chamber, while all test preparation was performed in an anaerobic chamber under an N_2:H_2 (ca. 97.5:2.5) atmosphere. Specific amendments to the test medium were (final concentrations): 100 µM AQDS, 1.0 g_L $^{-1}$ Ferrozine and 250 mg_L $^{-1}$ GNOM.

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

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

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Radionuclides and other contaminating heavy metals are readily adsorbed to ferric oxides present in the environment. However, if the environment becomes reducing due to co-contamination with organic compounds, microbial activity will rapidly cause the reduction of the Fe(III)-oxides with a resulting release of the bound metals. Recent studies in our lab have identified several (per)chlorate-reducing bacteria (ClRB) that can couple the anaerobic oxidation of Fe(II) to the reduction of chlorate or nitrate. Oxidized minus reduced difference spectra of whole-cell suspensions of these organisms indicated the presence of *c*-type cytochrome(s). Difference spectra studies on anaerobic H_2-reduced whole-cells demonstrated that the *c*-type cytochrome(s) are involved in the transfer of electrons onto chlorate or perchlorate by these organisms. Furthermore, difference spectra studies of anaerobic whole-cells in the presence of Fe(II) demonstrated that Fe(II) oxidation was an enzymatic process and was not the result of abiotic reactions with highly oxidized intermediates such as chlorite, which are formed as transient intermediates during the reductive metabolism of (per)chlorate.

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

Although *D. suillus* could not grow with Fe(II) serving as the sole electron donor, it could oxidize Fe(II) while growing in basal media with acetate as a carbon and additional energy source. Growth was rapid and was directly linked to acetate concentration. However, Fe(II) oxidation continued after growth had ceased as long as there was enough electron accepting capacity in the media. Interestingly, concentrations of Fe(II) greater than 1 mM were inhibitory to growth when *D. suillus* was grown with chlorate as the electron acceptor. With nitrate as the sole electron acceptor, *D. suillus* could grow rapidly and oxidize Fe(II) concentrations as high as 25 mM. In addition, the presence of radionuclides/heavymetals such as uranium or cobalt had no significant effect on the growth of *D. suillus* at concentrations of 100 _M. ICP analyses of the soluble uranium and cobalt concentrations indicated that the soluble metal content rapidly decreased as the Fe(III)-oxides were formed and after complete oxidation of the 10 mM $FeCl_2$ added, 80% of the cobalt was removed from solution.

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

The Effects of Cadmium Toxicity on Bacterial Consortia

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The effects of cadmium toxicity on bacterial consortia originating from anaerobic sewage sludge and cultivated under a variety of physiological conditions were studied. The cultures were enriched in minimal media containing all cadmium in soluble form. To enrich for cadmium resistance, cultures were subcultured multiple times, always when an absorbance of 0.2 OD_{600nm} was reached. Physiological conditions were varied by providing differing electron donors and acceptors anaerobically, including (electron donor, electron acceptor): acetate, CO_2; lactate, sulfate; glucose, no electron acceptor; and glucose, sulfate. Aerobic cultures used glucose and O_2.

We found that for all cultural conditions studied, the level of cadmium resistance of the consortia increased over time. Generally, cadmium tolerance was greatest under aerobic conditions, followed by anaerobic with glucose and sulfate, and then anaerobic with lactate and sulfate. Under sulfate reducing (lactate, sulfate) and methanogenic (acetate, CO_2) conditions, the consortia exhibited the lowest level of cadmium tolerance. A significant precipitation of cadmium occurred under aerobic and sulfate reducing conditions, presumably increasing the cadmium tolerance of these cultures by lowering the bioavailability of the heavy metal. Under other enrichment conditions cadmium was transformed into a yet unidentified soluble form. In this form the cadmium was detoxified as shown by assays using E. coli as a toxicity indicator organism. The addition of iron citrate increased the amount of cadmium precipitated in some enrichments, though precipitation under sulfate reducing and aerobic conditions was decreased.

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

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

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

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

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

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

Dissimilatory iron-reducing bacteria (DIRB) can utilize ferric iron associated with aqueous or solid phases as a terminal electron acceptor coupled to the oxidation of H_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 goethite under conditions where the aqueous composition was controlled to vary U speciation and solubility. Uranium(VI) as the carbonate complexes $UO_2(CO_3)_3^{4-}{}_{(aq)}$ and $UO_2(CO_3)_2^{2-}{}_{(aq)}$, in the absence or presence of goethite [α-$FeOOH_{(s)}$], was reduced by the bacteria to U(IV). Uranium(VI) in PIPES buffer that was estimated to be initially present predominantly as the U(VI) mineral metaschoepite [$UO_3 \bullet 2H_2O_{(s)}$] was also reduced by the bacteria in the absence of presence of goethite. Anthraquinone-2,6-disulfonate (AQDS), a humic acid analog that can be reduced to dihydroanthraquinone (AH_2DS) by CN32, had a slight moderating effect on U reduction in either buffer. In contrast, only ~30% of the U(VI) associated with a synthetic metaschoepite was reduced by the organism in the presence of goethite with 1 mM lactate as the electron donor, possibly due to the formation of a layer of $UO_{2(s)}$ or $Fe(OH)_{3(s)}$ on the surface of the metaschoepite that physically obstructed further bioreduction. However, increasing the lactate to a non-limiting concentration (10 mM) increased the nominal reduction of U(VI) from metaschoepite to greater than 80%, indicating that the hypothesized surface-veneering effect was electron donor dependent. Uranium(VI) was also reduced by bacterially-reduced AQDS in the absence of cells, and by Fe(II) sorbed to goethite in abiotic control experiments. In the absence of goethite, uraninite was a major product of direct microbial reduction and reduction by AH_2DS. These results indicate that DMRB, via a combination of direct enzymatic or indirect mechanisms, can reduce U(VI) to insoluble U(IV) in the presence of solid Fe oxides. Current research is probing the microbial reduction of U(VI) in the presence of Mn oxides.

Preliminary results have demonstrated that biogenic uraninite (UO_2) is oxidized by pyrolusite (β-MnO_2) and that the presence of the oxide significantly decreases the rate of microbial reduction of U(VI). Additional experiments are underway to identify and quantify the coupled microbial and geochemical processes in these systems.

Formation and Reactivity of Biogenic Iron Microminerals

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

To evaluate the effect of pH on the products of HFO transformation, 20 mM Fe(III) as HFO was suspended in buffered, anaerobic media with pH values ranging from 5.5 to 8.5. In the absence of CO_2 and with H_2 supplied as the electron donor, *Shewanella putrefaciens* strain CN32 reduced iron in HFO at all pH values tested except 8.5. The transformation products were influenced directly by initial pH. More than 8 $mmol^{-1}$ of the 20 mmol L^{-1} Fe(III) provided as HFO were reduced at pH values below 6. The crystalline iron oxide goethite was the dominant mineral phase produced during reduction. Biogenic Fe(II) was distributed between the aqueous and mineral phases. Magnetite co-precipitated with goethite at pH 6.5, while magnetite was the dominant phase at pH values >7.0.

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

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

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

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Our first-year research has focused on the fractionation and structural characterization of natural organic matter (NOM) that may function as electron mediators for microbial reduction of contaminant metals such as chromate ($CrO_4^{=}$), pertechnetate (TcO_4^{-}) and uranyl (UO_2^{2+}). The overall goal of the project is to provide a molecular-level understanding of the roles and mechanisms of heterogeneous NOM in facilitating the reductive immobilization of metal and radionuclide contaminants by anaerobic metal-reducing bacteria. Our specific objectives are to (1) determine the bioavailability of several major organic fractions of NOM by microbes in relation to their unique structural and functional characteristics; (2) quantify the redox-active functional groups and their reaction kinetics of the NOM fractions for accepting electrons from metal-reducing bacteria (such as *Shewanella putrefaciens* and *Geobacter metallireducens*); and (3) determine the microbial reduction and immobilization of $CrO_4^{=}$, TcO_4^{-} and UO_2^{2+} in both batch and soil column flow-through systems as facilitated by each NOM fraction.

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

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

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

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

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

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

Modeling and Parameter Estimation of Soil Fe(III) Bioavailability

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

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

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

The interfaces between solid mineral particles and water play a crucial role in partitioning and chemical transformation of many inorganic as well as organic pollutants in environmental systems. Among environmentally significant minerals, mixed-valent oxides and hydroxides of iron (e.g., magnetite, green rusts) and manganese (hausmanite, birnessite) have been recognized as particularly strong sorbents for metal ions. In addition, minerals containing Fe(II) have recently been proven to be powerful reductants for a wide range of pollutants. Chemical properties of these minerals strongly depend on the distribution and availability of reactive sites and little is known qualitatively about the nature of these sites. We have investigated the bulk distribution of charge states of manganese (II, II, IV) and iron (II, III) in single particles of natural manganese nodules and synthetic green rusts using Scanning Transmission X-ray SpectroMicroscopy (STXM). Pixel resolved spectra (XANES) were fitted to total electron yield (TEY) spectra of single valent reference compounds. Two-dimensional maps of bulk charge state distributions clearly reveal domains of different oxidation states within single particles of Mn-nodules and green rust precipitates. Changes of oxidation states of iron were followed as a result of reductive transformation of an environmental contaminant (CCl_4) using green rust as the only reductant. In addition, similar approaches were used to follow the fine scale redox distribution of manganese oxides during biological oxidation of manganese II to Mn IV. These experiments revealed unexpected fine-scale heterogeneity in the redox states of Mn during biological oxidation of the metal oxides; oxidation differences that must be understood before pollution remediation strategies are adopted and put in place.

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

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

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

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

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

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

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

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Mixtures of metallic and radioactive contaminants, including chromate, constitute a major environmental problem at DOE facilities. Direct microbial reduction of hexavalent chromium to trivalent chromium is one potential treatment technology for such sites. While previous research has shown the potential for this treatment, important questions still remain. Such questions include: (1) which members of an environmental consortium are responsible for chromate reduction; (2) for these active chromate reducers, what biochemical pathways are responsible for chromate reduction; (3) what are the effects of carbon source (electron donor) and of inorganic contaminant ions (e.g., SO42-, TcO4- and UO22+) on the system's chromate reduction capacity; and (4) can detailed knowledge of active chromate reducers and their biochemistry be used to facilitate preferential growth of subpopulations with the greatest specific contaminant reduction rates in aquifer systems?

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

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

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

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

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

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

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

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

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

As another measure of SRB activity in mixed culture biofilms, microelectrode measurements of colony-scale H_2S profiles on surfaces will also be presented. Biofilms composed of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* were grown on inert glass surfaces. We have measured profiles of H_2S, pH and local effective diffusivity at two locations in a cell cluster: at the center and near the edge, and at three locations in interstitial voids: in a large void (about 100 microns wide), open to the flow and parallel to the flow direction, in a mid size void, open and perpendicular to the flow direction, and in a small, closed void. The pH in the biofilm was nearly constant, around 7.2, (results not shown). H_2S concentration varied significantly among locations, indicating a heterogeneity that may be significant in the presence of redox-sensitive minerals.

Specially designed flat-plate flow cells that contain pure and mixed redox-sensitive and insensitive minerals allow us to determine the spatial relationships between SRB colony location and activity, and the location and type of contaminant and sulfide mineral deposits on a hematite, or other aquifer-relevant surface. A hematite coupon surface was exposed to SRB colonization for 17 days, then it was rinsed and examined using X-ray photoelectron spectroscopy (XPS) for surface characterization. XPS data indicate the formation of reduced iron sulfide on the hematite surface and also the presence of numerous sulfur species, including S^{2-}, S_2^{2-}, S_n^{2-}, SO_3^{2-} and SO_4^{2-}; however, no elemental S or thiosulfate were detected. Our initial results indicate the possible formation of pyrrhotite ($Fe_{1-x}S$, where $0< x < 0.125$) on hematite surfaces when SRBs are grown. We are currently replicating this test and also plan to confirm the result using grazing angle XRD. If confirmed, the presence of pyrrhotite contrasts with the results of most other work in homogeneous SRB solutions where the metastable sulfide phase is mackinawite (FeS_{1-x}, where $0 < x < 0.1$). Mackinawite is a precursor to both of the thermodynamically stable high-temperature iron-sulfide phases [pyrite (FeS_2) and pyrrhotite].

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

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

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

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

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

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

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

One of the main objectives of the project is to find a bacterium that can grow in high concentrations of Cd(II) under anaerobic conditions, and transform the metal into stable products, such as cadmium sulfide (CdS). We isolated such a bacterium (strain Cd-1) from coastal sediments that grows in up to 15 mM $CdCl_2$ under minimal fermentative conditions. The isolate can grow in Cd(II) under a variety of geochemical conditions, i.e., with or without marine level NaCl, at acidic or neutral pH. It also can grow aerobically in Cd(II). Furthermore, it can grow in high levels of many other priority pollutants and co-contaminants including Cr(VI), As(V), Se(IV), Co(II), Pb(II) or Zn(II).

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

Mesoscale Biotransformation Dynamics Controlling Reactive Transport of Chromium

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

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

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