

2007 Environmental and Subsurface Science Symposium, featuring Biotechnology and Bioremediation

Presentation Abstracts

IN SITU DENITRIFICATION IN MINE WASTE ROCK

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Mine site risk assessments, area denitrification plans, and treatment contingency plans were developed and approved by EPA and State regulatory authorities. Water analysis and tracer studies were used to confirm flow paths from waste rock deposits to down gradient monitoring wells. Background microbial profiles were accomplished through classical microbial isolations, characterizations, quantifications, and nucleic acid profiling using terminal restriction fragment (TRF) analysis. In situ treatments consist of waters containing site and augmented denitrifying microbes supplemented with nutrients matched with site water chemistry to optimize in situ denitrification. The treatment solution is added and injected into waste rock depositories and at various other points to treat source and down gradient nitrate contamination.

In situ treatment data gathered over several years will be presented including data that show that the application of injection solution has a positive effect on increasing the numbers of denitrifying microbes at the inoculated sites and in down gradient waters. Data also indicates that over long periods of non-treatment these populations revert to native microbial populations and rates of in situ denitrification drop. Data shows seasonal variations in site nitrate levels and that nitrate levels at injection and monitored sites drop significantly with appropriate in situ treatment.

MYCOBACTERIUM: PROTEOMICS AND GENOMICS FOR BIOREMEDIATION

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Polycyclic aromatic hydrocarbons (PAHs) are associated with health risks because their incomplete breakdown products can associate with cellular DNA and initiate cancerous growth. PAHs are ubiquitous, being created by natural processes as well as by an array of industries. Degradation of PAHs includes transformation by microbes. Soil-inhabiting mycobacterium strains can completely degrade both high- and low-ring hydrocarbons yielding water and carbon dioxide as the final products. These soil mycobacterium isolates mineralize PAHs while they are growing on root surfaces so that it may be possible to augment remediation at a contaminated site by phytostimulation. Proteomics and genomics of a mycobacterium isolate KMS from a wood-preserving site have identified genes that are specifically expressed during degradation of the four-ring-PAH, pyrene. These genes are arrayed as a cluster found on the chromosome but additionally are repeated on a plasmid in KMS. Similar clusters of induced genes are detected in two other mycobacterium strains from different sources of PAH-pollution. RT-PCR is being used to understand the temporal and conducive conditions for these genes and the relevance of induction while the bacterium colonizes roots. Specific genes from these clusters are being used in development of molecular probes to determine whether PAH-degrading mycobacterium isolates are present and active at a site.

ECOPROTEOMICS: A NOVEL APPROACH FOR THE ANALYSIS OF ENVIRONMENTAL BIOFILMS

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Biofilms in natural environments contain a vast diversity of microbes housed in complex exopolysaccharide matrices. Within these biofilms a variety of metabolic processes can occur to transform natural and anthropogenic chemicals through enzyme-catalyzed processes. Some by-products of biodegradation generated by one set of microbes can then serve as carbon, nitrogen, and/or energy sources for other microorganisms within a biofilm. The metabolic rate of removal of specific compounds and their biotransformation products often can be positively correlated with the overall rate of pollutant natural attenuation. Therefore, we hypothesize that *specific proteins can be used as biomarkers for monitoring natural attenuation processes*. From this, it follows that biologically-mediated natural attenuation can be monitored using an “ecoproteomics” approach. As a model system we extracted total protein from Clearwater River (Idaho) and Snake River (Idaho/Washington) rock-derived biofilms to monitor the anthropogenic effects of industrial and waste water discharges into these rivers. Chloroform, chlorinated dioxins, furans, phenols, and adsorbable organic halides are amongst the chemicals discharged into the Snake River just below its confluence of the Clearwater River from an industrial point source upstream. We employed a novel Multidimensional Protein Identification Technology (MudPIT) to characterize the complex peptide mixtures produced by boiling biofilm biomass in either 20% formic acid or 1% Triton-X 100 and then fractionating samples every 10 minutes over a total hydrolysis time of 30 minutes. The total protein solutions produced were digested with the protease trypsin to generate peptides that were examined using Nanoacquity Ultra Performance Liquid Chromatography (UPLC) time-of-flight (QTOF Premiere) tandem mass spectrometry. The processed mass spectra were searched against the Swissprot protein database and the Mascot MS/MS ion search engine to identify the proteins most likely to be sources of individual peptides. As a result of this study we are able to detect protein signatures from specific microorganisms within the biofilms from all six hydrolysis fractionations. An approximate 10-fold increase in peptides was present in the 1% Triton-X 100 hydrolysates as compared to the 20% formic acid hydrolysates. Therefore, we have optimized a method for the extraction, digestion, and detection of proteins from environmental biofilms. In future research, we plan on using this detection method to compare up and down-stream biofilm samples for potential biomarkers that could be used to monitor natural attenuation of known pollutants.

MINERALOGY INFLUENCES STRUCTURE AND DIVERSITY OF BACTERIAL COMMUNITIES ASSOCIATED WITH GEOLOGICAL SUBSTRATA IN A PRISTINE AQUIFER

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The subsurface displays geological heterogeneity that ranges from the kilometer to nanometer scale creating a mosaic of microhabitats for the biotic component. Previous studies have attempted to determine the influence of scale on observed microbial community structure. From these studies, it is clear that microbial populations are influenced as a result of geologic heterogeneity occurring at scales relevant to microbial systems; however, it is unclear as to what the parameters are that influence the distribution of these populations. In the present study, a combination of molecular ecological and statistical tools was used to extend our knowledge of the degree to which mineralogy influences substratum-associated microbial community structure and diversity.

Geologic substrata (quartz, hematite, and saprolite) were chosen as surrogate substrata based on the mineralogy of the background aquifer sediment at the Field Research Center at the Oak Ridge National Laboratory, Oak Ridge Reservation, Oak Ridge, TN. Geologic substrata were separated from each other with a plug of glass wool and incubated within a biofilm coupon (stainless steel mesh cylinder, 25.4 cm x 1.27 cm) for a period of eight weeks in the saturated zone of a pristine aquifer. Following incubation, the different colonized solid phases were subjected to bead beating nucleic acid extraction followed by

terminal-restriction fragment length polymorphism (T-RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S ribosomal RNA (rRNA) genes in order to semi-quantitatively estimate community structure and diversity. Community T-RFLP profiles generated for each substratum were examined using a variety of statistical algorithms to deduce ecological information for each substrata-associated microbial community and to estimate the degree of similarity between substrata-associated microbial communities. Individual differences in community composition between substrata were assessed by phylogenetic and T-RFLP analysis of a individual 16S rRNA genes derived from a clone library. To test the possibility that differences in the properties of the geological substrata created biases in the DNA extracted from the substrata-associated community, a second nucleic acid extraction technique (intact biofilm-PCR) was employed.

The results suggest that geologic substrata influence community structure, composition, richness, and diversity at biologically relevant scales in a pristine aquifer. Furthermore, each geologic substratum was dominated by a different class within the division *Proteobacteria*. Thus, the results presented underlie the importance of solid-phase mineralogy as an ecological determinant of subsurface microbial community structure and diversity. IB-PCR yielded differences in community structure among the different substrata, as well, indicating that it was not a phenomenon specific to bead beating extraction. These results suggest that the nucleic acid extraction techniques used in this study have inherent biases and that each will only reveal a subset of the true microbial diversity, thereby supporting the results of previous studies which suggest community structure biases as a result of different nucleic acid extraction techniques.

REMOVAL OF TRICHLOROETHYLENE FROM SHALLOW SUBSURFACE ENVIRONMENTS: VOLATILIZATION FROM TREES AND SOIL SURFACE VERSUS GROUNDWATER INTERCEPTION TRENCH

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Volatilization of TCE by indigenous trees (phytovolatilization) and directly from the soil surface may play a significant role in the removal of TCE from shallow groundwater plumes at some sites. In this study, we compared TCE removal via volatilization (plants and soil surface) to that removed by a 50-foot (length) groundwater interception trench installed between 22 and 35 feet below ground surface near the location of the trees. Phytovolatilization samples were collected on sorbent traps from leaves and trunks of 8 willow and polar trees, ranging in height from 6 to 12 m, for the analysis of TCE by thermal desorption GC/MS. Soil surface flux measurements were made at approximately the same locations and times. The TCE removed by each mechanism was compared to the TCE collected in the groundwater interception trench. Phytovolatilization from leaves was scaled to the amount of water transpired. Using sap flow estimates of transpiration, yearly removal of TCE at the site was in the same order of magnitude as TCE removed via the interception trench and by volatilization from the soil surface. Loss of TCE via the trunk, scaled to the area of the flux chamber, was small for the mature trees at this site.

Key Words: phytovolatilization, volatile chlorinated solvents, plant uptake, transpiration

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IMPLEMENTATION AND MONITORING OF POST-SEAR BIOAUGMENTATION AT A TCE SOURCE AREA, OU2, HILL AFB

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Background: Operable Unit 2 (OU2), is one of a number of locations at Hill AFB that has been impacted from past practices of improper disposal of waste solvents from various manufacturing and maintenance processes that resulted in the accumulation of dense, nonaqueous phase liquids (DNAPLs) below the

site. A series of remedial activities have taken place at the site over the past 16 years, including product pumping, steam flooding, surfactant and foam flooding, and surfactant enhance aquifer remediation (SEAR). Despite the cumulative recovery of over 45,000 gallons of DNAPL from the site and reductions in TCE mass flux from the site of one to two orders of magnitude using these recovery techniques, isolated areas of residual DNAPL continue to persist and contaminate groundwater downgradient of the site. A laboratory microcosm study evaluating a range of biostimulation and bioaugmentation options for biopolishing of residual source mass was conducted at the UWRL and indicated that complete TCE transformation to ethene was only possible in this source area matrix with the addition of a known TCE dechlorinating culture, Bachman Road culture, and was most effective with the use of an emulsified oil carbon donor, Newman's Zone™. Results of this laboratory microcosm study were implemented in a subsequent field bioaugmentation demonstration effort at Panel 5 within OU2. Both water quality and microbial monitoring are being carried out to track the stability and effectiveness of this bioaugmentation effort under actual field conditions at the site.

Methods: Conventional water quality parameters relevant to biological activity (pH, DO, ORP, iron, nitrate, sulfate, methane) are being measured at the site over time along with TCE and its daughter products through ethene. In addition, microbial monitoring using total cell counts, standard PCR, and quantitative PCR methods are being used to track the changes in the indigenous microbial community and the amended Bachman Road culture over time. Molecular probes used in the PCR analysis include: one primer set for total Eubacteria; four DNA probes specific to dechlorinator species (three for *Dehalococcoides sp.*, one for *Desulfuromonas michiganensis*); and one primer set for the gene coding for vinyl chloride degradation (*vcrA* gene in *Dehalococcoides bacterium VS*).

Results: Prior to bioaugmentation the presence of *Dehalococcoides sp.* and *Desulfuromonas michiganensis* intermittent and *vcrA* was not detected throughout Panel 5. With the introduction of the Bachman Road culture, however, the distribution of these dechlorinators became more uniform and, the VC dechlorinating enzyme was observed shortly after the introduction of the Bachman Road culture. Cell numbers, dechlorinator distribution and dechlorination activity have reached steady-state over the 20 month monitoring period, and the rate and extent of TCE transformation to ethene has continued to increase over that time despite evidence of intermittent releases of trapped free product in response to emulsified oil addition and biologically enhanced free product dissolution.

Conclusions: Bioaugmentation of the Bachman Road culture has been successful in stimulating the reductive dechlorination of TCE to ethene in the residual source area at Panel 5 within OU2 despite continued releases of residual phase DNAPL remaining at the site following aggressive chemical free product recovery efforts. Molecular tools have been used to monitor the distribution of both specific dechlorinating organisms and functional genes associated with TCE reductive dechlorination, confirming the effectiveness of the distribution of the culture throughout the site and its maintenance and growth over time even though chemical indicators of reductive dechlorination were masked by on-going source releases for more than 12 months after bioaugmentation.

RESPONSES OF *Desulfovibrio vulgaris* TO PHYSIOLOGICAL CONSTRAINTS

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Desulfovibrio vulgaris ATCC 29579 is a well studied sulfate-reducing bacterium that has known capabilities of reducing heavy metals and radionuclides, like chromium and uranium, and recent work has observed *Desulfovibrio* spp. at field sites contaminated with different heavy metals. One of our main objectives is the identification of key genes and proteins essential during stress and survival of predominant bacterial populations under field relevant conditions. In particular, we have focused on the cellular responses to chromium exposure and the biofilm growth state, and such data will provide insight into cellular responses to heavy metals when cells are grown as surface-adhered populations. The results indicated that *Desulfovibrio* utilized lactate to reduce Cr(VI) without the reduction of sulfate, that the decline in cell viability and cell growth was most likely a consequence of Cr(III), and that an organic ligand could protect *D. vulgaris* cells from Cr(III) toxicity. Lactate consumption decoupled from sulfate

reduction in the presence of Cr(VI) could provide organic carbon for organo-Cr(III) complexes. Before elucidating biofilm responses to Cr, we compared transcriptomes between planktonic growth phases and a mature biofilm. The biofilms did not cluster with planktonic growth-phases and represented cells with a distinct expression profile. In particular, genes that encoded putative proteins for the pyruvate-acetyl-CoA-formate node showed up-expression relative to exponential- and stationary-phase cells. The results indicated that *D. vulgaris* biofilms were a unique physiological state, and genes involved in carbon and energy flow contributed to the unique expression profile. While the expression of other systems is certainly involved in biofilm physiology, the presented results indicated that biofilm cells used a unique combination of putative proteins to establish distinct energy flow different from exponential and stationary phases.

TWO DIFFERENT PATHWAYS OF TNT TRANSFORMATION - NITROGROUP AND AROMATIC RING REDUCTION - ARE INFLUENCED BY BIOTIC AND ABIOTIC PROCESSES

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TNT (2,4,6-trinitrofluorene) transformation by bacteria and yeasts in the presence and absence of humic substances, electron shuttles, iron minerals, and co-contaminants was shown to occur via two principally different pathways. Humic substances and quinone analogs, such as AQDS (anthraquinone-2,6-disulfonate), increased TNT transformation rates, the presence of reduced iron minerals increased TNT transformation kinetics and promoted nitrogroup reduction at the ortho-position, chromate as a co-contaminant also increased TNT transformation rates but resulted in the production of TNT-related dimers.

Direct aromatic ring reduction (also referred to as TNT-hydride complex formation) was observed as the predominant pathway during TNT transformation by yeast cells while nitrogroup reduction was observed to be a minor pathway. Aromatic ring reduction by yeasts resulted in the transient accumulation of eight different TNT-hydride complexes. Hydride complex formation and concomitant release of nitrite appeared to be influenced by the prevailing pH of the medium. TNT-mono- and dihydride complexes as well as protonated dihydride isomers were detected. These hydride complexes as well as other TNT metabolites were characterized using high performance liquid chromatography (HPLC), UV-visible diode array detection (DAD), and negative mode atmospheric pressure chemical ionization mass spectrometry (APCI-MS). APCI-MS analysis revealed that the eight hydride complexes can be assigned to three different groups with molecular ions at m/z 227, 228, and 230, respectively.

Nitrogroup removal from TNT as indicated by nitrite release and production of dinitrotoluene (DNT) is of particular interest since it can provide a pathway towards complete degradation and detoxification of TNT.

SORPTION AND BIODEGRADATION OF MTBE AND TBA IN HYPERHEIC ZONE SOILS

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Gasoline containing methyl *tert*-butyl ether (MTBE), a fuel oxygenate, was accidentally released from a leaking underground fuel tank (LUFT) and entered an unconfined aquifer in Ronan, Montana. The presence of *tert*-butyl alcohol (TBA) has also been observed at the site in the MTBE groundwater plume

likely resulting from *in situ* biodegradation of MTBE. Monitored natural attenuation (MNA) has been proposed as a treatment alternative for the MTBE and TBA groundwater plume at the Ronan site. This study was undertaken to measure the biological mineralization of TBA and sorption of MTBE and TBA to Ronan hyporheic zone soils in order to contribute to the feasibility evaluation of MNA as a remediation alternative at the site.

The ability of the Ronan hyporheic zone (groundwater surface water interface) to biodegrade TBA was evaluated. TBA biodegradation first order kinetic information was also observed at three different temperatures (5, 15, and 25 °C). These results were the first to demonstrate the biodegradation potential of Ronan soils for TBA and the effect of temperature on TBA biodegradation kinetics.

Sorption of MTBE and TBA was observed in the seven hyporheic zone soils. MTBE sorption was linear and significantly higher than the literature predicted and TBA sorption was highly nonlinear. The sorption distribution coefficients are the first empirically derived values reported for MTBE and TBA.

Results of this study add to the evidence collected from multiple studies on the use of MNA as a reliable remediation alternative comparable to other more active remediation methods.

HILL AIR FORCE BASE ENVIRONMENTAL RESTORATION PROGRAM – A DESCRIPTION AND DISCUSSION OF SITE MANAGEMENT APPROACH, JULY 2007

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The Hill AFB Environmental Restoration Program manages five separate areas to the east and west of the Great Salt Lake. Wendover Field and the North and South Ranges to the west include nearly 1,000,000 acres while the Little Mountain Text Annex and Hill AFB proper include more than 7000 acres. A staff of 20 engineers and scientists manage the 109 Environmental Restoration Program “sites” within these areas, primarily under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). The majority of resources are focused at Little Mountain and Hill AFB proper, just south of Ogden, Utah, which are managed as 13 Operable Units (OUs). Many of the OUs have ROD-specified remedial actions operating to control dissolved phase migration off of the Base: pump and treat, LNAPL recovery, landfill caps, permeable reactive barrier, and air sparging/ soil vapor extraction. For several plumes we are monitoring natural attenuation rates. In addition, the Restoration Program manages issues associated with contamination in the indoor air in residences located around Hill AFB. The cost to-date for restoration efforts at Hill AFB is approximately \$200,000,000 with estimated future costs through 2027 of \$350,000,000.

While historically the restoration process was tied very closely to meeting the milestones of CERCLA, over the last five years, our site management approach has moved toward tracking progress toward meeting performance objectives for our remedies and minimizing life-cycle costs, while still meeting the requirements of CERCLA. Hill AFB has developed a decision framework for evaluating treatment system performance that ties geosystem response to the evolution of the conceptual model for the site. This connection provides a basis for site management decisions that are objective and defensible. The basic premise of this framework is that the conceptual site model can be used to make predictions on how the geosystem will respond to stresses imposed by remedial actions. Appropriate monitoring of both geosystem and operational parameters allows assessment of whether the remedial action is being operated as designed AND whether the geosystem is responding as expected. If either the remedial action or the geosystem is not responding as expected, this framework provides a feedback loop to evaluate the need for updating the conceptual site model and/or making changes to operations.

Where possible, we apply this approach prior to the construction of groundwater treatment remedies so that we can demonstrate soon after start-up that these systems are impacting the sub-surface as designed. Since most of our treatment systems were constructed five to ten years ago, we are now applying this approach by comparing actual geosystem response since start-up with original design expectations, making updated predictions for remediation time frames, and improving our monitoring

approach to improve the accuracy of those predictions. The scientific method (hypothesis, test, evaluate, new hypothesis, test, ...) is the backbone of this approach; therefore, we have a defensible basis for making site management decisions and communicating our progress to Air Force headquarters, our regulators, and members of the communities surrounding Hill AFB.

BACTERIAL COMMUNITIES STIMULATED FOR URANIUM BIO-REDUCTION DISPLAY TEMPORAL CONCORDANCE ALONG CONTROLLED FLOW PATHS

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Former radionuclide waste ponds at the ERSP-Field Research Center in Oak Ridge, TN pose several challenges for uranium bioremediation. The site is marked by acidic conditions, high concentrations of nitrate, chlorinated solvents, and heavy metals. Above-ground treatment of groundwater, including nitrate removal via a denitrifying fluidized bed reactor (FBR) pre-conditions the groundwater for subsurface uranium immobilization. A series of re-circulating wells serve to create a subsurface bioreactor to stimulate microbial growth for *in situ* U(VI) immobilization. Well FW-104 is the injection well for the electron donor (i.e., ethanol); well FW-026 is the extraction well for the recirculation loop; well FW-101 and FW-102 are the inner zones of biostimulation; and FW-024 and FW-103 are upstream and downstream wells, respectively, which are the outer protective zones. Bacterial community composition and structure of groundwater from the wells were analyzed via clone libraries of partial SSU rRNA gene. Both qualitative and quantitative methods were used to analyze the changes in bacterial diversity and distribution. LIBSHUFF analysis was used for the comparison of bacterial community population between the different clone libraries. Bacterial community from the denitrifying FBR was different from the groundwater bacterial community, which indicated that different bacterial communities were stimulated in the two separate systems. The clone libraries of the re-circulating wells showed that over each phase of manipulation for uranium immobilization, the bacterial communities of the inner zones of biostimulation were more similar to each other and than those of the outer protective zones. The outer protective zones were more similar to the injection well. Clone libraries from FW-104 (injection), FW-101 and FW-102 showed that bacterial communities of the three wells were initially similar but developed changes through time. FW-101 and FW-102 bacterial communities developed changes in parallel, while those of FW-104 showed gradual change. These results were further compared to data generated from Unifrac analysis. Preliminary results with Unifrac analyses showed that the bacterial community in each of the wells developed changes during the bioremediation process, and the changes could be attributed to the variations along temporal, spatial, and geochemical scales. Diversity indices showed that bacterial diversity tended to increase during the initial phase of uranium bioreduction and decreased toward the end of uranium bioreduction (i.e., low U(VI) levels). As uranium levels declined, increasing *Desulfovibrio* and *Geobacter*-like sequences were detected from the clone libraries, and the *Desulfovibrio*-like sequences predominated over time. The results were further confirmed via qPCR and the results correlated with OTU distributions for *Desulfovibrio*. The results indicated that the bacterial community composition and structure changed upon stimulating for uranium bioreduction conditions, and that sequences representative of sulfate-reducers and metal-reducers were detected in wells that displayed a decline in U(VI). Further analysis is underway to determine the relationships between different functional groups and site geochemistry.

ARSENIC BINDING PROTEINS FOR REMOVAL OF ARSENIC FROM SOLUTION

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Removal of soluble forms of arsenic from water is an expensive process. Alginate biopolymer bead immobilization was selected as a method to evaluate the ability of arsenic binding proteins to remove arsenic from water. Protein extracts were obtained from several microbial consortiums selected for their ability to reduce soluble metalloids. Protein material binding of arsenic was compared to live microbial arsenic binding and it was determined that protein material binds as much as 43% more arsenic than do live microbes. Immobilized protein extracts at a concentration 0.50 mg/ml yielded near maximal arsenic removal. Arsenic removal was somewhat dependent on temperature and pH, with maximum removal occurring at about 35° C and pH of 5.8. Kinetics studies show that selected protein mixtures remove ~98% of soluble arsenic in 24 hours. Immobilization of arsenic-binding proteins permits arsenic recovery and potential reuse of the immobilization biopolymer-protein complex. Assays are currently being designed in order to develop a bacterial vector that will facilitate the production and isolation of the most proteins with the highest arsenic affinity. Additional data will be presented on the arsenic-binding proteins, functional lifespan of various immobilization biopolymer-protein complexes, and arsenic removal in a bench scale bioreactor.

PURIFICATION OF REDOX-ACTIVE BIOMOLECULES FROM PURE CULTURES AND COMPLEX SAMPLES

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Mono- and polyheme c-type cytochromes and other extracellular biomolecules have been linked to ferruginous mineral respiration in a variety of bacteria, such as *Acidithiobacillus*, *Geobacter*, *Shewanella*, and *Acidiphilium*. While excellent genetic and genomic studies have been conducted, there is a paucity of information regarding the purification, manipulation, and redox behavior of these proteins *in vitro*. Many of these proteins are membrane or cell-surface associated, and preliminary evidence suggests that they can act as multiprotein electron transfer complexes. Our research goal was to identify methods for the effective purification and manipulation of these proteins and biomolecules, in order to facilitate subsequent study. Many redox active proteins are extractable by salt (NaCl, KCl) or mild detergent washing of cells, suggesting a cell surface location. Two examples are the polyheme cytochrome c OmcS from *Geobacter sulfurreducens*, and a 42 kDa cytochrome c from *Acidiphilium cryptum*. Sequential extraction of membrane fractions with incrementally stronger detergents yielded additional redox proteins, and this strategy is useful for targeted purification of particular proteins of interest. Since it is suspected that some of these redox proteins are embedded in a biofilm matrix, experiments were conducted to determine whether this material could be digested away to enable purification. Viscozyme, a mixture of polysaccharidases, was effective at depolymerizing the EPS and reducing problems associated with high levels of polysaccharide contamination. Once extracted out of the cellular and extracellular milieu, some of these proteins are extremely difficult to work with, due to hydrophobicity and subsequent aggregation. Some of these issues can be circumvented by use of non-denaturing detergents at low (sub-critical micellar concentrations) concentrations. Finally, our extraction and stabilization methods were applied to the directed proteomic detection of large-mass heme c-containing proteins directly from complex microbial communities, with the hypothesis that these proteins are similar in function to those found in pure cultures of iron reducing or iron oxidizing organisms. In all, our research efforts in protein purification of this interesting class of enzymes has revealed useful information regarding their behavior, which is critical for successful studies using spectroscopy and electrochemical techniques.

COUPLED BIOGEOCHEMICAL PROCESS EVALUATION FOR CONCEPTUALIZING TRICHLOROETHYLENE CO-METABOLISM

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Our research focuses on the coupled biogeochemical processes that dictate the rate of methane-driven co-metabolism of trichloroethylene (TCE) in the Snake River Plain aquifer at the Test Area North (TAN) site of the Idaho National Laboratory. Natural attenuation (NA) has been accepted as a remediation strategy at this location and our study seeks to quantify the contribution of methanotrophs to NA, and access the coupling between biotic and abiotic processes involved in TCE degradation. Aquifer microbial communities and chemistry from within the TCE plume were characterized using water samples from wells and using biofilm communities from in situ incubation of basalt chips. Methanotrophic microbes, or evidence of their activity, were detected in numerous wells in the “medial zone” of the TCE plume where TCE concentrations ranged up to 500 ppb. Combined analyses using fluorescent in situ hybridization (FISH), enzyme activity probes, Phylochip community characterization and community proteomics targeting the methanotroph-specific soluble methane monooxygenase (sMMO) enzyme all detected evidence of methanotrophs in the groundwater and in biomass obtained from basalt chips. Phylochip analyses also indicated the presence of several methanogenic genera in the wells suggesting that biogenic methane may contribute to methanotroph sustenance in the aquifer. Additionally, the catalytic subunit of sMMO, *mmoX*, has been detected in concentrated groundwater by conventional PCR. Currently, flow-through in situ reactors (FTISR) are incubating at two distinct aquifer flow rates in order to determine the affect of hydraulic flow on the microbial communities capable of TCE co-metabolism. Subsequent studies will assess the contribution of methane in TCE co-metabolism carried out by the reactor communities. Determination of the rate of TCE co-metabolism at different methane concentrations and groundwater flow velocities will yield key modeling parameters for the computational simulations that describe the attenuation, and accordingly improve the predictive capability of the models. Accurate assessment of NA rates under different aquifer conditions will further justify the use of NA at the INL and at other DOE sites.

BIOLOGICAL URANIUM REDUCTION AND SUBSEQUENT STABILITY

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Because of its toxicity, which is primarily due to chemical rather than radiological action, U is a priority pollutant for the US Department of Energy. Uranium-contaminated groundwater threatens ecosystem health, and poses a real threat to human health when sensitive receptors are affected. The mobility and bioavailability of subsurface U is dependent on abiotic, biotic, and coupled reactions that determine oxidation state, aqueous complexation, and interactions with mineral surfaces. *In situ* bioreductive immobilization may be an attractive option at many sites for removal of U from the aqueous phase because of low cost and minimal secondary waste generation. Recent evidence indicates, however, that bioreduced U is susceptible to reoxidation and remobilization.

Because SRB are present in nearly all contaminated subsurface field sites, stimulation of anaerobic bacteria by injection of substrate to promote SRB activity may be a viable treatment option for immobilizing a variety of heavy metals and radionuclides, including uranium. The primary goal of in situ bio-immobilization of radionuclides is to prevent their transport to sensitive receptors (e.g., surface or drinking water). Success in reaching this goal will largely be determined by the extent to which the fundamental microbial/mineral processes that occur at a contaminated site are understood. Results will be presented that show the presence of a mineral surface can significantly change the system chemistry, and the resulting secondary stability of mineral precipitates.

Our recent work is focused on microbial U reduction and reoxidation at hematite, goethite, and ferrihydrite surfaces under sulfate reducing conditions. We have shown that *Desulfovibrio desulfuricans* G20 (hereafter simply written as G20) reduced U(VI) to nanocrystals of uraninite which were extracellular as well as associated with cells. The stoichiometry of lactate and sulfate, and their products acetate and sulfide suggested that G20 reduced soluble sulfate and U(VI) concomitantly; and that most reduction of Fe(III)-(hydr)oxide was indirect via biogenic hydrogen sulfide. In addition to U reduction with hematite, goethite, and ferrihydrite, we showed that once lactate was depleted, residual Fe(III) of Fe(III)-(hydr)oxides oxidized uraninite nanocrystals. Subsequent addition of hematite to stationary phase cultures containing microbially reduced uraninite crystals also resulted in reoxidation to U(VI). At this time it is unclear whether nanocrystalline uraninite directly reduces Fe(III) at the mineral surface or if an electron-shuttle or Fe(III) siderophore compound is involved. Hematite added to heat-killed cell controls, however, reoxidized only a very small amount of U, thus indicating that the reoxidation was bio-catalyzed. Under abiotic conditions, hematite did not reoxidize uraninite nanocrystals. The stoichiometry of uraninite consumption and Fe(II) production in a ferric chloride treatment was consistent with $U(VI)+2Fe(III) \rightarrow U(VI)+2Fe(II)$.

QUANTIFICATION OF AMMONIA OXIDIZING BACTERIA AND ARCHAEA FROM GROUNDWATER AND BASALT FOLLOWING UREA TREATMENT TO PROMOTE CALCITE PRECIPITATION FROM STRONTIUM IMMOBILIZATION

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Accelerating calcite precipitation by stimulating microbial urea hydrolysis is the basis of a novel method for remediating trace metal and radionuclide contaminants in groundwater. Urea hydrolysis causes an increase in pH and carbonate alkalinity, which promotes calcite precipitation and the co-precipitation of divalent metal ions. It results in the production of ammonium ions however, which could potentially stimulate the activity of ammonia oxidizing microorganisms. Ammonia oxidation can lead to the formation of nitrate, a regulated contaminant, and also increased acidity, which could be detrimental to calcite stability. To gauge the likelihood for these potential drawbacks we have developed real-time PCR assays to quantify the ammonia oxidizing bacteria and archaea. The assays were applied to groundwater and basalt samples collected during field experiments where dilute molasses and urea were added to stimulate microbial growth and promote calcite precipitation.

The bacterial assay developed was specific to ammonia oxidizing bacteria with a detection limit of <20 cells with the predominant organisms most closely related to *Nitrosomonas*. The ammonia oxidizing bacterial cell numbers statistically increased >10 fold in the groundwater and slightly in the basalt samples following treatment. These results suggest that ammonia oxidizing bacteria were stimulated by the treatment and may play a role in the remediation strategy.

To determine the extent of ammonia oxidation by archaea, samples were assayed by targeting conserved regions of archaeal ammonia oxidation genes. Preliminary analysis of the samples by quantitative PCR suggests that archaeal ammonia oxidizers may predominate in ammonia oxidation. Ongoing quantification and characterization experiments will help determine the extent of the archaeal contribution.

BACTERIA IN IRON REDUCER ENRICHMENTS FROM TCE CONTAMINATED AQUIFER MATERIAL AT HILL AIR FORCE BASE

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Biostimulation may be used as a relatively low cost treatment to enhance reductive dehalogenation of chlorinated ethenes that contaminate ground water. Biostimulation has been proposed as a treatment method for a trichloroethene (TCE) contaminated aquifer on and near Hill Air Force Base (HAFB), Utah. Dehalorespiration may be less effective because the energy transformation of the respiring portion of the microbial community is dominated by dissimilatory iron reducing bacteria (DIRB) while dehalorespiratory activity does not increase. Biostimulation treatability-study microcosms made with the target aquifer material failed to produce TCE dehalogenation products but a large fraction of the ferric-iron minerals were reduced. A clone library of 16S rDNA from an aquifer material sample suggested that *Rhodoferax ferrireducens* and closely related bacteria may be important iron reducing bacteria while *Geobacter*, and other well studied iron reducing bacteria, appeared to be present in relatively low concentrations or were not cloned. Dilution-to-extinction enrichments of HAFB aquifer material samples, targeting *Rhodoferax ferrireducens*, that provided lactate as the electron donor and poorly crystalline iron oxide (PCIO) as the electron acceptor were made. Higher dilutions showing Fe reduction were streaked onto solid media containing Fe(NTA), PCIO, or goethite as electron acceptors. DNA was extracted from these same dilutions and clone libraries prepared. Clone plasmid insert sequences indicated that the enrichment community from aquifer material from one site collected 60 m deep was dominated by *Pseudomonas* sp., possibly *P. stutzeri*, but included *Anaerobacter* sp. and *Rhodoferax* sp., possibly *R. antarcticus*. Enrichments from one water table sample, ~ 6 m deep, from another site were dominated by *Bacillus* sp. but included *Tissierella* sp., *Herbispirillum* sp., and *Cellulomonas* sp. Another water table sample enrichment series was dominated by *P. stutzeri* but included *Tessaracoccus* sp., *Geobacter* sp., *R. antarcticus*, *Geothrix* sp., and *Trichlorobacter* sp. Bacteria growing on solid media included members of the *Cellulomonadaceae*, the *Propionibacteriaceae*, *Acinetobacter* sp., *Sanguibacter* sp., and *Burkholderia* sp. A similar analysis using glucose and acetate as electron donors is in progress. Iron reducing bacteria in HAFB aquifer materials that responded to carbon and energy source enrichments appear to be dominated by organisms often considered to be “fermenters.”

EVALUATION OF MONITORED NATURAL ATTENUATION OF MTBE AT OU11, HILL AFB, UT

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This presentation provides an overview of the ongoing investigation and evaluation of monitored natural attenuation (MNA) as the remedial alternative for groundwater contamination at Operable Unit (OU) 11 at Hill AFB, UT. MNA has been evaluated using traditional groundwater sampling and analysis techniques, as well as carbon and hydrogen stable isotope analysis.

OU 11 is a former gasoline station located in the southwest portion of Hill AFB. Historic releases of gasoline at this site resulted in a significant groundwater contamination plume containing BTEX and MTBE. A separate release of TCE has resulted in a co-mingled MTBE/TCE plume downgradient from the initial release site. A soil vapor extraction (SVE) system was installed at the site in 1995 and has since removed approximately 3,500 gallons of free product from the source area. The BTEX plume extends approximately 300 feet from the source area, and historical groundwater sampling indicates the plume is shrinking. The MTBE plume extends approximately 1200 feet downgradient and appears to be expanding. The plume boundary is still under investigation. Groundwater analyses have indicated that the groundwater is slightly aerobic. However, specific regions of the plume are anaerobic due to biodegradation of BTEX compounds. TBA, a breakdown product of MTBE, has also been measured at several monitoring wells at the site.

In addition to measurement of traditional MNA parameters, carbon and hydrogen stable isotopes were evaluated to aid in determining if biodegradation of MTBE is occurring at the site. Carbon and hydrogen stable isotope analysis has been used at several MTBE-contaminated sites throughout the United States to provide unequivocal evidence of MTBE biodegradation. Ten wells at OU11 were sampled for stable isotopes, and the groundwater was sent to the University of Oklahoma Environmental Forensics Laboratory for analysis. The samples were analyzed using GC-IRMS. One well showed slight enrichment of ¹³C compared to non-degraded MTBE. However, additional carbon and hydrogen stable isotope sampling and analysis is planned for summer 2007. These results, combined with the evaluation of traditional MNA parameters, will aid in determining the appropriate remedial alternative for contaminated groundwater at OU11.

A MODULAR SRB BIOREACTOR FOR ACID ROCK DRAINAGE TREATMENT

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Abstract. The presentation focuses on the design, construction and early performance of a modular sulfate-reducing bacteria (SRB) bioreactor system for remediation of acid rock drainage (ARD). This passive remedial technology takes advantage of the ability of SRB that, if supplied with a source of organic carbon, can increase pH and alkalinity of the water and immobilize metals by precipitating them as metal sulfides or hydroxides.

The remoteness of ARD sites and their abundance require that the design of an SRB bioreactor is simple and inexpensive. Therefore, bioreactors need to be designed to a size that complies with the US Department of Transportation regulations and allows for transportation using primitive roads. To satisfy these requirements a design for a modular treatment system was developed using reactive cartridges (RC) that are prefabricated as 1,100-gallon capacity, 6-ft diameter vessels. The RC has been designed so it supports the prime functional aspects of a bioreactor such as high permeability, ample supply of organic carbon, ability to maintain anaerobic conditions, and capacity to accumulate precipitated metals and means for their periodical removal, as needed. In addition, the configuration of the RC allows for an easy replacement of the organic carbon. The RCs can be transported to an ARD site and assembled into a treatment system with a number of modules as required by the ARD flow rate and the metals load. A bioreactor system consisting of four RCs was installed in Black Hawk, Colorado to treat ARD of pH 5 and a significant load of metals. The bioreactor system uses reactive medium, a mix of walnut shells and corn stover, which was developed by MSE Technology Applications (MSE) and Colorado School of Mines.

The RC design was developed by the Mine Waste Technology Program (MWTP) at MSE, Butte, Montana, USA. The work was funded by the U.S. Environmental Protection Agency (EPA) and was jointly administered by the EPA and the U.S. Department of Energy (DOE) National Energy Technology Laboratory and performed at the Western Environmental Technology Office under DOE contract number DE-AC09-96EW96405.

COMPARISON ANALYSIS ON CELL SURFACES OF GRAM-NEGATIVE BACTERIA AND GRAM-POSITIVE BACTERIA BY ATOMIC FORCE MICROSCOPE AND RAMAN MICROSPECTROSCOPY

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Abstract

The difference of the cell wall structures is one of the most distinguished variances between gram-positive and gram-negative bacteria. Gram-positive *Mycobacteria* KMS (M. KMS) and gram-negative *Pseudomonas Putida* KT2440 have been demonstrated great potential in bioremediation due to their capability of degradation of toxic polycyclic aromatic hydrocarbons (PAHs). The surface properties of the bacteria are considered to play important role in implementing of the interaction between the bacteria and the surrounding microenvironment. With the use of Atomic Force Microscope, an exquisitely sensitive tool, we investigate the surface mechanical properties of both M. KMS and *P. putida* KT2440 through probing their interaction with the tip of AFM. The cell surface topographic features of the two bacteria are imaged at liquid or air at nanoscale resolution and the surface force profiles at single cell level of the both organisms are compared by evaluating the elastic properties and the outer layer structures. The cell surface of the KMS exhibits a flat and smooth appearance while *P. putida* KT2440 is covered by a net-like structure with average depth of 10 nm. The bacterial spring constant of the M.KMS was measured to be about 0.07N/m, and the probe indentation into surface of M.KMS is much shorter (12nm), compared with that of *P. putida* KT2440. The combination of surface topography and the force curve analysis implies the existence of a much thicker layer of exopolymers outside *P. putida* KT2440, compared to M. KMS, and the effect of these extracellular biopolymer on the surface adhesion and elastic properties for both microorganisms. As a contract, the interaction of bacteria M.KMS with the tip of AFM is primarily associated with a thinner layer of exopolymers and lipopolysacchride. The Raman images and spectra confirmed the existence of such exopolymeric structures on both bacterial surfaces, even different characteristic vibrational spectroscopic peaks are observed on both microorganisms.