PROGRAM ELEMENT 2
Community Dynamics and Microbial Ecology

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

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

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

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

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

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

Ecological Interactions Between Metals and Microbes That Impact Bioremediation

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

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

The tolerance of microbial communities to heavy metals has been tested in two ways. With the first method, culturable bacteria were recovered and the minimum inhibitory concentrations of metal for growth of individual isolates were determined. A majority of the culturable bacteria in all of the habitats appeared to be sensitive to the toxic metal. However, significant populations of resistant bacteria were also found. In the case of Pb, resistant strains could grow up to 25–70 μM Pb^{++}. In the case of Cr, bacteria resistant up to 50 mM $CrO4^{-2}$ were isolated. The second method involved measuring ^{3}H-leucine incorporation into bacteria extracted directly from soil particles. The functional response of the microbial population was modeled as $Y = 100 \times I/(IC_{50} + I)$, where Y is the percentage of microbial activity (relative to the sample with no added metal), and I is the concentration of added metal. The IC_{50} values for lead were in the low (<10) μM range for all soils tested, whereas $Cr_2O_4^{-2}$ concentrations in the low millimolar (< 5) range were necessary to reduce microbial activity by 50%.

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

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

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

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

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

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

The Structure of Microbial Communities as a Diagnostic Indicator of Ecosystems

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

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

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

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

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

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

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

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

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

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

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

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

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

A first set of microcosm experiments was completed wherein the fate of *Escherichia coli* (pMOL18 with *czc* cassette) was monitored in sterile and non-sterile soil microcosms. The results clearly revealed the competitive pressure of the indigenous soil microbial community on *E. coli* survival, the efficacy of donor-selective enumeration techniques, and the resolving ability of the ARDRA method to see time-varying community profiles. The presence of the *czc* fragment was semi-quantitatively determined using *czcD* specific primers and soil-DNA dilution series. We are, currently, adhering to an MPN-PCR technique to quantify the concentration of the introduced mobile element in the soil microcosm. Another microcosm experiment has been initiated wherein the fate of *Pseudomonas putida* KT2440 and its plasmid TOL::Tn*5*(*km*) are monitored. This strain, in addition, contains the *gef*-based IPTG inducible suicide gene cassette and we are monitoring the efficacy of suicide induction in the microcosm systems. A large set of microcosm experiments has been initiated wherein the fate of either a conjugal (Tol::Tn*5*) and non-conjugal (pMOL187) plasmid are being monitored in the presence of varying Cd concentrations (0,10,100,1000 _M).

Due to earlier limitations in the $P_{a104/03}$*gef* suicide systems, we have initiated construction of a second suicide gene cassette based on the *genE* lethal gene. In addition, using dedicated microcosm experiments we are testing the efficacy of suicide induction, and are evaluating the mechanism of the high escape mutation frequency.

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

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

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

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

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

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

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

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

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

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

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

Although total biomass showed no correlation with Cr concentration ($P>0.05$), the ordination of PLFA profiles together with sample characteristics by principal component analysis revealed associations between Cr and specific PLFA. In collaboration with PIs S. Pfiffner and C. Brandt (ORNL), the association between PLFA/PCR-DGGE profiles and Cr concentration was further pursued using both hierarchical cluster analyses and artificial neural networks (ANN), an artificial intelligence technique. At Shiprock, contaminants include a wide range of metals dominated by U, with other solutes including NO_3^-, NH_3/NH_4^+, and SO_4^{2-}. A PLFA/DGGE analysis of groundwater samples indicated the presence of diverse, active microbial communities, containing a high relative proportion of biomarkers indicating the presence of sulfate/metal reducing bacteria (SRB). There was a high degree of correlation between the PLFA and geochemical data, and DGGE revealed dominance of the bacterial communities of most samples by organisms related to known metal-metabolizing species.