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(NABIR Assessment Element, Expanded Rapid, Comprehensive, Lipid Biomarker Analysis for Subsurface, Community Composition and Nutritional/Physiological Status as Monitors of Remediation and Detoxification Effectiveness (10/01/99-9/30/04)).

Objectives:

The primary objective was to develop, update, and expand quantitative methods of assaying microbial communities in situ. The secondary objective was to provide comprehensive lipid biomarker analysis for NABIR collaborators to enhance the understanding of microbial bioprocesses and how those bioprocesses may be manipulated to achieve a desired response, as in the immobilization of metals.

Progress with lipid methods development

Respiratory Quinones

The reduction of uranium must be preceded by reduction of the high-potential electron acceptors O_2 and NO_3^- . Reduced organic compounds are added to poise the redox potential and promote reduction of U(VI) to insoluble U(IV) compounds. At the FRC and UMTRA sites, organic compounds such as acetate, ethanol, and glucose were used as electron donors to create the necessary anaerobic conditions within the portion of the saturated zone surrounding treatment wells. All respiration, including respiration of U, is mediated by quinones. When the terminal electron acceptor is oxygen or nitrate, either ubiquinones or menaquinones may be used. Under anaerobic respiration (for example iron, sulfate, or U) menaquinones are used. Fermentation, which predominates under extremely anaerobic conditions, does not require quinones. Therefore, the ratio of menaquinones to total quinones is proportional to the ratio of anaerobic respiration to total respiration, and the ratio of total quinones to PLFA is proportional to the ratio of respiration to (respiration + fermentation). Respiratory quinones are found in concentrations at least 200 times less than the PLFA or about 0.5 micro mole/g dry weight, but can be quantified at high specificity and sensitivity with LC/MS/MS. CBA's development of sensitive methods utilizing LC/MS/MS for respiratory quinones have enabled detection in environmental samples (Lytle et al, 2001a, 2001b). Determination of the isoprenologues of the respiratory quinones also provides insight into the community composition and in quinone profiling. This work has been published (Lytle et al., 2000a, Geyer et al., 2004).

Diglyceride Fatty Acids

Recently CBA has developed for routine use (via LC/MS/MS) the ability for specific analysis of diglyceride fatty acids (Lytle et al, 2000b). These compounds are polar lipid derived moieties that are breakdown products of previously intact microbial cell membranes. When the cell dies it releases endogenous lipases that cleave the phosphate head group from the glycerol backbone. The diglycerides can be selectively extracted and derivatized at a highly specific level and quantified by LC/MS/MS. By analyzing the fatty acid chain moieties of the diglycerides we can gain insight into community succession and competition quantitatively at the cellular level. The information provided by diglycerides may also be used to monitor the turnover of cellular biomass in systems under study. This may be particularly useful in sediments where only a portion of the microbial community is active.

Plasmalogen-Derived Dimethyl Acetals (DMA)

Recent work with Joel Kostka has revealed a potential biomarker for acid-tolerant Gram-positive dissimilatory iron-reducing bacteria. This laboratory analyzed the fatty acid profiles of over fifty of these organisms, and the vast majority of them contained DMAs. DMAs are found in *Clostridia* and close relatives as well as some Gram-negative bacteria. This suggests that DMA's may be used to quantitatively monitor this class of microbes *in situ*. In work at the FRC, we have detected an increase of DMA's in low pH areas (Peacock et al, 2003) and after acetate injection at the Old Rifle UMTRA site (Peacock et al, 2002).

Analysis of ¹³C Lipids

Lipid biomarker analysis of samples spiked with stable isotopes is a new approach in microbial ecology. Natural abundance isotope ratios have been used in a number of applications to study organic matter utilization in complex systems such as soil and groundwater. ¹³C- labeled substrates are incorporated into lipid and DNA biomarkers, providing direct *in situ* identification of microbes involved in specific processes and goes to the heart of microbial activity (Chang 2005). The combination of the new lipid techniques coupled with stable isotope analysis will enable investigation into microbial community dynamics heretofore not possible.

High throughput 16S rDNA analysis by DGGE

Community composition is specifically assessed by isolating DNA then amplifying specific portions of the genome with PCR. This is done by proper selection of primers and in environmental applications is usually focused on the genes for ribosomal RNA (rDNA) (Steven et al, 1999), although genes for specific functions can be utilized (Ivanova et al, 2000). The PCR amplicons are separated by denaturing gel gradient electrophoresis (DGGE). The prominent bands are excised, eluted, and sequenced. The sequences are then compared to a database and phylogenetic matching gives indications of the community diversity and dominant members (Chang et al, 2001).

Analysis of reduced metals at the micron scale

Endogenous U(IV) and associated mineralogy can now be measured by electron microscopy dark-field back scattered electron, scanning transmission electron microscopy (BE-STEM). However, in many instances, concentrations of U(IV) on surfaces are extremely low, requiring the low-level U detection and imaging available at APS (PNC-CAT). Initial analysis at PNC-CAT of both sediment and samples of amorphous FeS sludge from the bottom of injection gallery wells from the Old Rifle Biostimulation Experiment show reduced U localized in micron-sized clusters, providing direct evidence for microbial reduction of U(VI) to U(IV) with acetate addition (Long, personal communication, reported initially at the 2003 NABIR PI meeting). The next step in this development is the linkage of this type of evidence to samples where metabolic processes are also known from ¹³C-acetate labeling.

Sampling Devices

Solid phase samplers

Timely access to solid phase (sediment) samples can be limited by mobilization, drilling costs and possible concerns for existing well field integrity (Peacock et al., 2004). However it can be critical in the course of a biostimulation experiment to monitor changes at multiple time points. Groundwater grab samples provide a snapshot in time but are dependent on the hydrology of the day (i.e. recent rain or drought events). As such there is an associated noise factor in the data produced from such samples. Most

bacteria exist attached to surfaces, and sampling groundwater artificially excludes these communities. Additionally, in some sediment environments recovery of DNA is limited by humics, clays or other materials. In order to compensate for the chaotic signal from aqueous samples and the impediments of direct sediment sampling we developed a solid phase sampler that is deployed down well and acts as a sensitive recorder of biostimulation in the subsurface. Assessment of the extant in-situ microbial community in subsurface environments is most effectively accomplished if the solid phase samplers can be readily colonized by the resident microbiota. We have established that solvent-resistant autoclavable polyfluoralkoxy (PFA) 1.5" long, 5/8" diameter perforated tubes stuffed with glass wool (incinerated to remove organic carbon) or filled with Bio-Sep beads, are readily colonized in FRC groundwaters (Peacock, et al, 2004). Bio-Sep beads [described in U. S. patent 5,486,292] are 2-3 mm spherical beads consisting of 25% (w/w) aramid polymer (Nomex) and 75% (w/w) powdered activated carbon (PAC). The bulk density is about 0.16 g/cm³ with a porosity of 74%, and an adsorptive capacity greater than 600 m²/g. The beads are surrounded by an ultrafiltration-like membrane with a median pore diameter of 1.9 microns and with some large macropores > 20 microns. Beads can be purged of organic carbon by incubation at 350 C for at least 5 hours. A variety of solid phase samplers have been tested. Initial results indicate both the great utility of these devices in tracking changes in the microbial community, and their limitation due to differences in the well bore environment compared to the surrounding *in situ* sediment. For example, the well bore concentrations of oxidized Fe are typically low due the sparingly soluble nature of Iron III species. It is therefore likely that sulfate reduction can dominate in the well bore before sulfate reduction dominates in sediments. We propose to address this issue by providing similar levels of bioavailable oxidized Fe and other electron donor/acceptor combinations in solid phase samplers.

Provision of comprehensive biomarker analysis to collaborating NABIR investigators

Oak Ridge FRC Push-Pull

Sterile solid phase samplers loaded with glass wool or Bio-Sep biocatalyst beads were suspended down well to monitor subsurface microbial community dynamics during U and Tc bioreduction tests conducted at the Oak Ridge Field Research Center. Diverse microbial biofilms colonized these surfaces during the six-week deployment. DNA and lipid biomarkers were extracted and recovered without complications that commonly plague sediment samples such as clay or humics. Changes in viable biomass, community composition, metabolic status and respiratory state were affected by substratum composition, addition of electron donors, and the alteration in groundwater pH. The solid phase samplers provided a readily recoverable integrated microbial community that was serially monitored. This is in contrast to grab samples from the groundwater or the destructive one-time sampling of subsurface sediments each with well-documented heterogeneities in composition and activities. Compared to glass wool Bio-Sep beads induced biofilms that were 2 to 13 times greater in viable biomass, however the community was less metabolically active (higher cyclopropane/monoenoic phospholipid fatty acid (PLFA) ratios) and had a lower aerobic respiratory state (lower total respiratory quinone/PLFA ratio and ubiquinone/menaquinone ratio). The biofilms in the treated wells were anaerobic as characterized by plasmalogen phospholipids and quinones. Partial 16S rDNA sequences identified *Geobacter* and nitrate reducing organisms induced by acetate, ethanol, or glucose additions. Although microbial community composition in the groundwater or adjacent sediments may differ from those formed on down-well solid phase samplers, the metabolic activity responses of the sampler biofilms to modifications in groundwater geochemistry parallel those of the

adjacent sediments but maintain their integrative sampling and ease of recovery and analysis (Istok et al., 2004).

UMTRA Old Rifle Biostimulation

The Old Rifle Uranium Mill Tailings Remedial Action (UMTRA) site is a former ore-processing facility located approximately 0.3 mile east of the city of Rifle in Garfield County, CO. The site is situated on a relatively low-lying alluvial terrace created by a flood-plain meander of the Colorado River. Uranium is the most prevalent site-related contaminant occurring in the alluvial ground water. Concentrations up to 0.35 mg/L present beneath the site exceed the UMTRA maximum contaminant level of 0.044 mg/L, but steadily decrease to background levels near the downgradient edge of the site. During the first month of acetate addition in situ, U(VI) in groundwater decreased by more than 70%. During the last 2 months of the experiment, the rate of U(VI) reduction decreased, allowing U(VI) concentrations in groundwater in the treatment zone to increase (Well M-03, samples collected on 8/21/02 and 9/19/02). Background (control) samples (Well B-02) showed little change during the same time period. Other data indicate that Fe and U reduction dominated during the first 1.5 months of the experiment followed by sulfate reduction after bio-available oxidized Fe in the subsurface was consumed. The decreased rate of U(VI) reduction in the latter half of the experiment is thus explained by the onset of sulfate reduction dominated by acetate oxidizing sulfate reducers, typically not effective at reducing U(VI).

The addition of acetate to the groundwater increased the microbial biomass on solid phase samplers by an order of magnitude within twenty-four feet of the injection gallery. By forty-eight feet downgradient, the microbial biomass returned to background levels. The starvation index (cyclopropyl to monounsaturate precursor ratio) was highest in the background and forty-eight feet downgradient of the treatment area, showing the biostimulation of the microbes in the immediate zone of effect. Analysis of 16S rDNA showed a diverse microbial community that included sulfate and metal reducers. *Geobacter sp.* (a known U reducer) was detected in the treatment area 12 feet from the injection gallery. Injection of the highly bioavailable acetate increased viable microbial biomass and decreased the starvation biomarker at both the shallow and deep wells immediately downgradient from the point of injection. Acetate addition created anaerobic conditions and stimulated the activity of Fe and U reducers resulting in reduction of soluble U(VI) to insoluble U(IV). The microbial community change associated with the acetate addition was readily detected by DNA and lipid biomarkers extracted from the traps. Specific rDNA analysis identified known U and sulfate reducers whose quantitative proportions were reflected in the lipid biomarkers. The response of the biofilm communities in the BioSep beads to the acetate addition indicated that bioimmobilization could be induced and its perpetuation monitored. (Anderson et al., 2005).

In situ microcosm experiment using ¹³C Benzene and Toluene

Bio Sep beads loaded with ¹³C-labeled benzene and toluene (approx. 45mg/50beads) were exposed *in-situ* in a highly BTEX contaminated aquifer. There was essentially no exchange of label with the surrounding environment as indicated by gas chromatography/isotope ratio mass spectrometry (GC/IRMS). The isotopic signature of the contamination plume in the aquifer was ¹³C -25 atom% . The isotopic signature of ¹³C-U-benzene and ¹³C-1-toluene utilized in the Bio Sep beads remained at 98 atom% and 14 atom%, respectively after 4 weeks. In the 4 week exposure more than 80% of the xenobiotics in the traps were degraded. The ¹³C-label could be detected in bacterial PLFA recovered from the Bio Sep beads and showed (up to delta +13,000 ¹³C, which

establishes the involvement of the bacteria in the biodegradation processes. PLFA recovered from unbaited control Bio Sep beads showed the natural ^{13}C abundance of around $\delta -30$ ^{13}C . Bio Sep beads have a high affinity for organic compounds which serve as substrates for bacteria as indicated by the incorporation of ^{13}C into PLFA in the baited beads. There was preferential utilization of the ^{13}C -labeled benzene and toluene in the baited beads. The unchanged isotopic ratio in the ^{13}C -benzene and ^{13}C -toluene solid phase samplers establishes that an exchange with the aquifer BTEX contamination by diffusion is very limited (not measureable) and the ^{13}C -labeled baited material does not leak in the 4 week exposure. This exposure was sufficient for generation of a biofilm that incorporated ^{13}C -into bacterial PLFA (Geyer et al., 2005).

Janet Chang of our laboratory has developed methods involving incubation of Bio Sep beads containing ^{13}C acetate in microcosms then isolating the DNA and subjecting the isolated DNA to density gradient centrifugation (Chang, 2005). She has been able to recover the ^{13}C labeled DNA and from the gradient then amplify with PCR to show that a subset of the microbial community was metabolically active. This work will form her PhD thesis. The three case studies indicate the technology of multi-compartment (MLS technology) sampling including solid phase samplers and newly expanded analyses of lipid and rDNA biomarkers, together with the effective baiting with ^{13}C -labeled substrates and local incorporation of ^{13}C into biomarkers are an effective field-tested monitoring system. This system can now be developed for validation as a cost-effective means to determine if bio-immobilization can work and provide a monitoring system to assess post-remediation performance.

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Peer-reviewed papers supported by DE-FC02-96ER62278 P.I., D.C. White 1999-2004. In this period CBA produced in one patent disclosure, 42-peer reviewed publications, 1 PhD thesis, and 62 presentations with abstracts (not listed), 14 of which were international and there are currently 2 papers in press:

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Patent disclosure Feb. 17, 2003 on behalf D. C. White, A. D. Peacock, & G. A. Davis, for the University of Tennessee Research Foundation and Microbial Insights, Inc., Rockford, TN "Use of stable isotope enriched solid phase samplers for defining specific microbial Activities".