Presented at WM'96 Tucson, AZ February 25-29, 1996

QUANTITATIVE ASSESSMENT OF IN SITU MICROBIAL COMMUNITIES AFFECTING NUCLEAR WASTE DISPOSAL

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ABSTRACT

Microbes in the environments surrounding nuclear waste depositories pose several questions regarding the protection of the surrounding communities. Microbes can facilitate microbially influenced corrosion (MIC), mobilize and facilitate the transport of nuclides as well as produce gaseous emissions which can compromise containment. We have developed an analysis of the extant microbiota that is independent of quantitative recovery and subsequent growth, based on signature biomarkers analysis (SBA). Polar lipids exist in all organisms that have intact cell membranes and intact cell membranes are a requirement for life. Extraction and measurement of polar lipids indicates the biomass of the microbes that are viable but may or may not be culturable. Phospholipid ester-linked fatty acids (PLFA) give a measure of the microbial community containing intact membranes. Phospholipids are often transformed into diglycerides by endogenous phospholipases in injured cells which retain for a time the characteristic signature profiles of the fatty acids and thus provide a measure of the viable cells with intact membranes and recently non-viable (lysed) cells. The lipid patterns of PLFA and other lipid classes also reflect exposures of the cells to nutritional imbalances, toxicity, and various stresses thereby providing phenotypic insight into the condition of the community. The lipid extraction procedure also allows recovery of the cellular DNA for probing with or without enzymatic amplification that adds extraordinary specificity for analysis for specific organisms, groups of organisms or potential enzyme activities. Specific signature lipid patterns of PLFA, steroids, respiratory quinones and

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lipopolysaccharide hydroxy fatty acids of the lipid A of gram negative bacteria allow detection of many groups of microbes with a quantitative definition of the community composition. Utilizing the SBA it has been possible to show that there are viable microbial communities in the host rock of potential subsurface waste deposit sites like Yucca Mountain. Research has shown that the viable biomass, community composition and nutritional status of the extant microbial community shifts with contamination, pollution, and disturbance. With SBA it has been possible to document that MIC is related in time and space to the corrosion process and to define which microbial communities are most likely to facilitate localized corrosion. Specific organism involved in uranium reduction can be identified by SBA. With the insight gained with SBA, predictions of potential effects microbial communities may have on the containment of nuclear wastes can be made.

INTRODUCTION

Microbes tend to be ignored because they are difficult to study. The classical methods of isolation and culture of microbes that are taught in most microbiology courses have been enormously successful in clinical medicine where isolation of specific pathogens establishes the diagnosis of disease and the *in vitro* sensitivities to antimicrobials can often predict the success of treatments. The obvious thing to do was to apply the same methods to repository system to detect the presence of microbes. Unfortunately often less than 1% of the microbes that can be detected in stained microscopic preparations can be cultured. Staining microbes in environmental samples like soils can be difficult as many are attached to soil granules and may be hidden. Agents that release attached microbes are often selective and do not release them quantitatively. The morphology of the microbes does not often reflect the function or activity so very little insight into the community structure or nutritional status is possible. Measurements of metabolic processes are complicated by the facts that most microbes in the soil are inactive, but poised for activity when nutrients appear. Adding labeled substrates to determine rates of metabolic activity induces major disturbance artifacts giving much higher rates than actually exist in the environment. This is possibly best exemplified in studies of the deep subsurface microbiota where oxygen and inorganic carbon are found in groundwater with a ground water age of greater than 1.1×10^5 years. Measurements of metabolic activity based on isotope incorporation experiments by the microbes in subsurface sediments were 10^3 to 10^6 times greater than the geochemical evidence would predict. The metabolic activities by the subsurface microbiota indicate growth rates in centuries (27).

METHOD

A solution to the quantitative detection of microbes in the environment of nuclear storage is in signature biomarker analysis (SBA). We have concentrated on the analysis of lipids (30). Every living cell is surrounded by a lipid membrane. These lipids

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are quantitatively extracted from the microbiota *in situ* and analyzed using gas chromatography/mass spectrometry (GC/MS). Several unique classes of lipids, including steroids, diglycerides, triglycerides, respiratory quinones, ß-hydroxyalkanoate (PHA), phospholipid lipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl ethers, sphingolipids, and lipopolysaccharide hydroxy fatty acids can be used as signature lipid biomarkers to characterize microorganisms or communities of microorganisms. Recently the lipid extraction has been shown to yield DNA suitable for gene probing and enzymatic amplification (12).

Phospholipids are one of the most important SBA classes, and are essential membrane components of living cells. Unlike most other biomarkers, phospholipids are typically degraded within hours following cell death. This rapid degradation of the phospholipids establishes the PLFA as ideal biomarkers for viable cells, thus, the quantification of total PLFA is an accurate measurement of living biomass (2). Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers and can be utilized to provide insight into the community composition. PLFA analysis can provide insight into the phylogenetic relationships between organisms similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (8,15). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community as certain fatty acids, such as *trans* and cyclopropyl fatty acids, provide an indications of environmental stress. Other components indicate unbalanced growth where carbon sources and terminal electron acceptors abound but a critical nutrient prevents cell division but not growth or bioavaiable phosphate is insufficient (31). The redox level of the microbiota can be determined *in situ* by shifts in the composition of lipids in specific indicator microbes. The signature lipid biomarker techniques have been successfully applied to subsurface materials (33).

Fig. 1

RECOVERY OF MICROBES FROM SUBSURFACE

The detection of microbes from deep subsurface sediments that are possible repositories for nuclear materials requires convincing evidence that the samples recovered from drilling operations were not contaminated by the make-up water, drilling muds, or in handling. Signature lipid biomarker analysis has shown that the viable biomass, community composition, and nutritional/physiological status of the microbial communities recovered with strict sampling guidelines (3,26) were distinctive enough to assure that microbes in the pared cores were from the extant microbiota (17).

DETECTION OF *IN SITU* MICROBIAL COMMUNITY ACTIVITY

We have ample evidence that the subsurface microbial community responds rapidly to changing conditions and is thus metabolically active. Pollution readily induces shifts

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in the viable biomass, community composition, and nutritional status of subsurface microbial community with an increase in viable biomass and increased proportions of PLFA characteristic of gram-negative heterotrophs (29). Increases in the type II methane-oxidizing bacteria were detected in soil columns gassed with methane and air (23). Addition of different fatty acid substrates to anaerobic sediment cores induced marked and expected changes in the bacterial community structure (25). Subsurface sediments perfused with methane, propane, air show shifts in community structure that correlate with trichloroethylene (TCE) biodegradation (4,28). Active biodegradation of petroleum hydrocarbons in subsurface sediments results in increases in viable biomass, shifts to aerobic heterotrophic bacterial PLFA, decrease in biomarkers indicative of stationary phase growth, decrease in PHA/PLFA ratio, and increases in the proportion of benzoquinone respiratory quinones indicative of aerobic electron transport activity (28). The nutritional status of microbial consortia actively degrading petroleum differs markedly from the organisms fortuitously degrading TCE in that effective TCE biodegradation is correlated with a build-up of reducing power indicated by a high PHA/PLFA ratio (23). Clearly the subsurface microbiota responds to shifts in the environment. Unpublished experiments from the Savannah River *in situ* TCE biodegradation demonstration showed that the changes detected in the recovered sediments were reflected in the ground water microbes collected as membrane filter retentates. The recovery of specific gene probes for methane monooxygenase and the signature PLFA of the methane-oxidizing bacteria correlated well.

Bacterial PHA and microeucaryotic triglyceride (6) are endogenous storage lipids. The relative amounts of these compounds compared to the PLFA, provides a measure of the nutritional status of specific components of the microbial community. Many bacteria form PHA under conditions of unbalanced growth such as when a carbon source and terminal electron acceptor(s) are present but cell division is limited by the lack of some essential nutrient (5,24). The determination of the ratio of PHA/PLFA has proved useful in monitoring the effectiveness of bioremediation in the subsurface--effective biodegradation of petroleum hydrocarbons correlates with a low ratio of PHA/PLFA (28) whereas the fortuitous metabolism of trichloroethylene correlates with a high ratio of PHA/PLFA (23,4). Specific ratios of PLFA acids have been shown to correlate with physiological stress (7). Exposure to toxic environments can lead to minicell formation and a relative increase in PLFA specific to the exposures. For example, increased conversion from *cis* to *trans* PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol in the absence of bacterial growth (11). Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA (7). The respiratory quinones, the detection of plasmalogen lipids and other biomarkers can be utilized to indicate the degree of microbial aerobic activity (10,31). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce the formation of benzoquinones in bacteria

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in contrast to microbes respiring on organic substrates which form naphthoquinones. There are other lipid biomarkers such as lipid amino acids liberated after hydrolysis of the lipids can yield further insights into the conditions of the subsurface microbial microniches (31).

POTENTIAL FOR MICROBIAL INFLUENCED CORROSION

The application of SBA lipid technology to subsurface sediments recovered with the quality assurance that the communities sampled represent the extant microbiota clearly show the presence of a diverse and viable microbiota that responds to geochemical and hydrologic gradients. The metabolic activities of bacteria found in the subsurface can produce significant risks to the containment of nuclear waste over extended periods of time. Microbes are associated with pitting corrosion (14). The ready destruction of concrete sewers by *Thiobacilli* (13) or buried gas transmission pipelines by acetogens (27) attest to the destructive power microbes. Microbially influenced corrosion (MIC) poses potentially serious problems in the maintenance of containment barriers (18). Microbes have been shown to particularly attack weldments (20) and are quite capable of localized MIC on "noble" substrata such as stainless steel (1). Because of these microbial potentials, it becomes necessary that a thorough understanding of the extant subsurface microbiota be known prior to the deposition of nuclear wastes.

TRANSPORT

Microbes have considerably higher hydraulic transmissivity than conservative tracers like Bromine (16). Microbes readily bind nuclides (21) and can thus transport nuclides very effectively. SBA of lipids has shown that subsurface microbial communities can respond to differences in the hydraulic conditions. The viable microbial biomass, based on total extractable PLFA, decreases with depth in sediments from the arid northwest. This viable biomass decrease with depth is slightly less pronounced in areas where there is a high recharge rate. An analysis of microbial community composition throughout the tested depth interval shows that PLFA indicative of *Actinomycetes*, in particular tuberculostearic acid (10me18:0), continue to represent a constant or increasing percentage of the total in the area of high recharge which was in sharp contrast to the decrease in percentage of these PLFA in the area of low recharge (33).

POTENTIAL EFFECTS

Microbial contamination and metabolic activity in the subsurface if controlled and monitored not be a major threat to the containment of buried nuclear waste. Microbes have a remarkable capacity to immobilize nuclides through the formation of insoluble compounds (19). Bacterial metabolic activities may have been responsible for the original concentration of uranium nuclides in sedimentary deposits and for their

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containment over the past billions of years at sites like Cigar Lake, Saskatchewan or in fission products like those at Oklo, South Africa.

ORGANISMS AT PROPOSED SITES (YUCCA)

Application of SBA lipids to potential sites has shown that microbes are present in the uncontaminated volcanic tuft of the Yucca Mountain, NV site. Concentrations recovered from the aseptic (in so far as possible) carefully prepared sites show bacteria in concentrations equivalent to 10^2-10^4/gm. Some are cuturable and show diversity and heterogeneity in their distribution (9). There are large amounts of as yet uncharacterized glycolipids and terminally branched saturated PLFA characteristic of gram-positive bacteria. No samples have been assessed after the tunnel preparation which would be expected to increase the diversity, activity and biomass of the microbes. Simply aseptically grinding the tuft in the absence of any added nutrients or water significantly stimulates microbial growth and activity (P. Amy and D. Haldeman personal communication).

ACKNOWLEDGMENT

This work has been supported by grant DE-FG05-90ER60988 from the Subsurface Science Program, administered by F. J. Wobber, from the U. S. Department of Energy.

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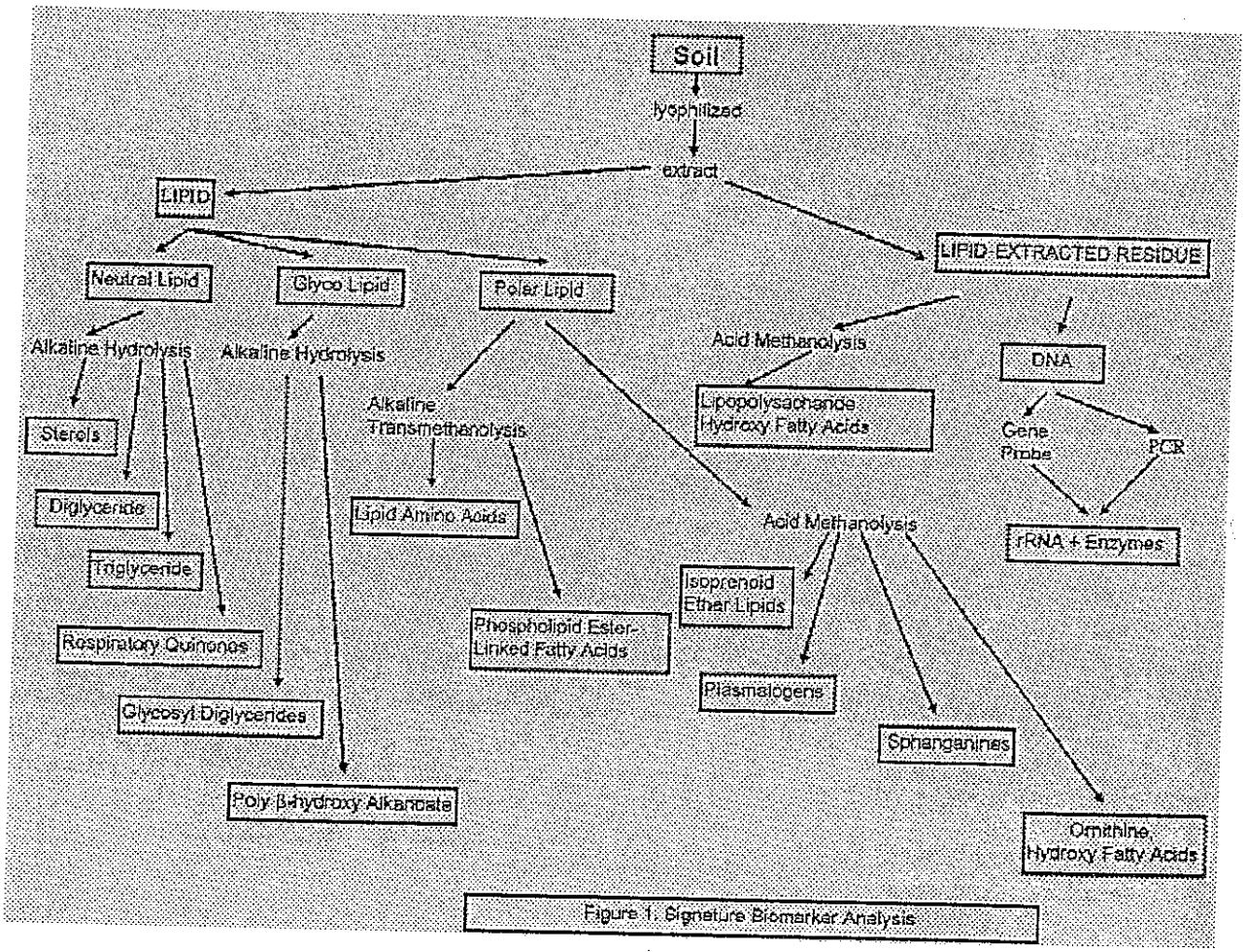

Figure 1. Signature Biomarker Analysis

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