Monitoring deep subsurface microbiota for assessment of safe long-term nuclear waste disposal

David C. White and David B. Ringelberg

Abstract: Microbes with their resistance to heat and radioactivity, if present and metabolically active, could have major effects on the safety of nuclear waste disposal by posing potential problems in long-term containment. This paper reviews the applicability of the signature lipid biomarker (SLB) analysis in the quantitative assessment of the viable biomass, community composition, and nutritional/physiological status of the subsurface microbiota as it exists in situ in subsurface samples. The samples described in this review are not unlike those expected to be recovered from proposed deep subsurface disposal sites. Assessment of the microbial community ecology using SLB analysis can be utilized to predict potential problems engendered by microbial metabolic activities of these communities in breaching containment by microbially facilitated corrosion and in the potential for subsequent facilitated transport of nuclides into the environment. SLB analysis of the in situ microbial ecology can be utilized to monitor the feasibility of containment options in modeling tests at the specific disposal sites.

Key words: nuclear waste, deep subsurface, microbiota, microbial corrosion, safe long-term storage, signature lipid biomarkers.

Résumé : Lorsque présents et métaboliquement actifs, les microbes résistants à la chaleur et à la radioactivité peuvent avoir des conséquences majeures sur l'élimination des déchets nucléaires à cause de problèmes potentiels lors du confinement à long terme. Cet article discute de la possibilité d'application de l'analyse des lipides caractéristiques du genre comme biomarqueurs (SLB) dans l'évaluation quantitative de la biomasse viable, la composition de la population microbienne et l'état nutritionnel/physiologique des microbiotes présents sous la surface comme cela se présente in situ dans les échantillons de sous-surface. Les échantillons décrits dans cette revue ne sont pas différents de ceux que l'on prévoit isoler de sites proposés d'enfouissement en profondeur. La détermination de l'écologie de la population microbienne par l'analyse SLB peut être utilisée pour prévoir les difficultés potentielles causées par l'activité métabolique de ces populations microbiennes comme la corrosion microbienne qui pourrait entraîner des fuites dans les aires d'entreposage ce qui pourrait favoriser subséquemment la décharge de nucléides dans l'environnement. La SLB de l'écologie microbienne in situ peut être utilisée pour évaluer la faisabilité des différentes options d'entreposage lors d'essais de modélisation à des sites spécifiques d'élimination.

Mots clés : déchets nucléaires, enfouissement en profondeur, microbiotes, corrosion microbienne, entreposage sécuritaire à long terme, lipides caractéristiques du genre comme biomarqueurs.
[Traduit par la rédaction]

Introduction

One of the major problems in the effective use of nuclear energy to provide electrical energy without the environmental consequences of fossil fuel combustion is the disposal of highly radioactive waste materials. A long-term storage of the nuclear waste where it can be effectively sequestered from the rest of environment is required. Burial in the subsurface is an attractive disposal method provided the technology employed is sufficient to adequately sequester the nuclides. There is an increasing realization that the powerful microbial metabolism that has facilitated the generation of soils and sediments from igneous rocks since the Precambrian could seriously impact the safe sequestering of nuclear waste in the subsurface. In this review we will show that utilizing nontraditional methodology provides sufficient insight into the microbial ecology of the extant subsurface microbiota to address the impact this microbiota may have on the safety of nuclear waste disposal.

Classical microbiological methods, which were successful with infectious disease, have severe limitations for the analysis of subsurface samples. Pure-culture isolation, biochemical testing, and enumeration by direct microscopic counting or most probable number (MPN) are not well suited for the estimation of total viable biomass or the assessment of community composition or in situ phenotypic activity of the microbiota in subsurface materials. Chemical analysis of signature biomarkers extracted directly from these subsurface sediments does provide a more applicable methodology for environmental microbial analysis. This paper reviews the applicability

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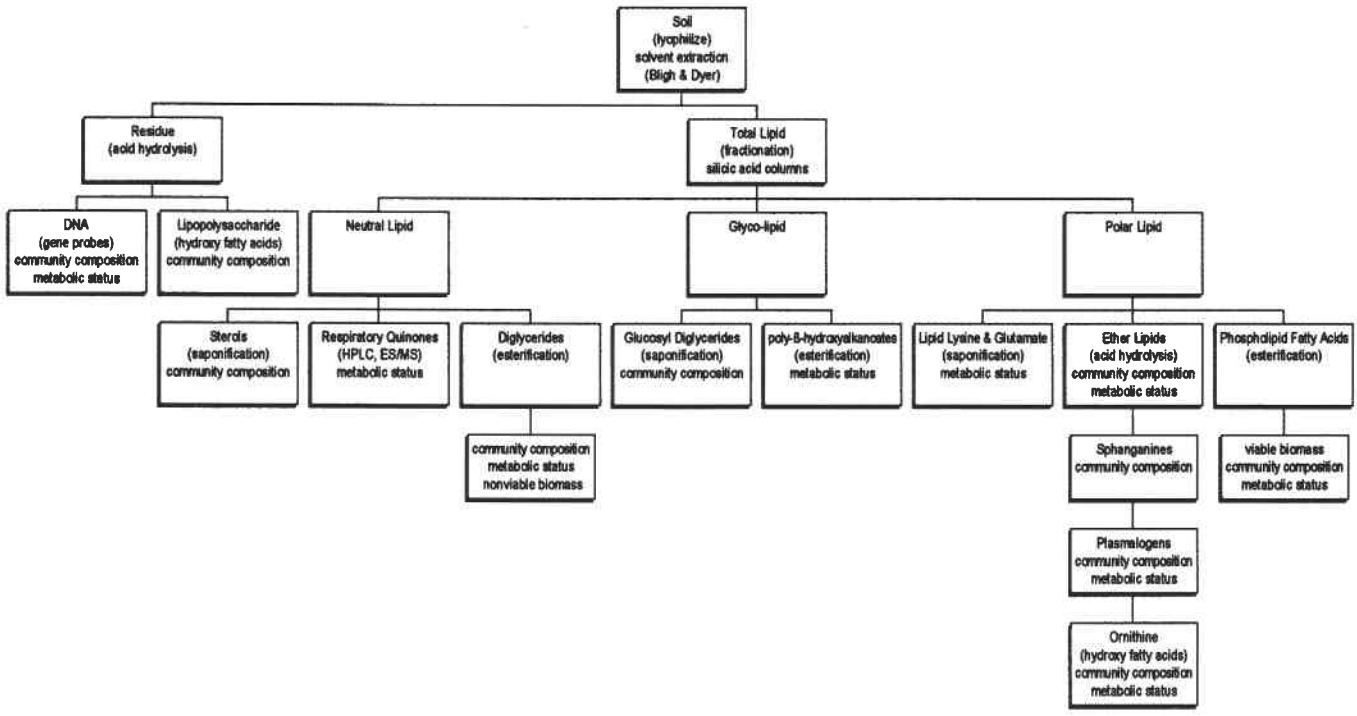
Fig. 1. Flow diagram of the SLB analysis. HPLC, high performance liquid chromatography; ES/MS, electrospray mass spectrometry.



of signature lipid biomarker (SLB) analysis, which is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography – mass spectrometry (GC–MS) (Tunlid and White 1991; White 1993; White and Ringelberg 1996). Phospholipids, one of the most important SLB classes, 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 phospholipid ester-linked fatty acids (PLFA) as ideal biomarkers for viable cells; thus, the quantification of total PLFA is an accurate measurement of living biomass (White et al. 1979). Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers. PLFA analysis can provide insight into the phylogenetic relationships between organisms, similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (Guckert et al. 1991; Kohring et al. 1994). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community, as certain fatty acids such as *trans* monoenoic and cyclopropyl PLFA provide indications of environmental stress (Guckert et al. 1986).

Methods

Sediments are typically lyophilized and then extracted in a single-phase chloroform–methanol–buffer solution (1:2:0.8 by volume). Following the formation of a two-phase solution through the addition of equal portions of chloroform and water, the organic lipid-containing phase is fractionated on silicic acid columns. Each lipid fraction is then analyzed by GC–MS following the formation of an appropriate derivative, i.e., a methyl ester (Guckert et al. 1985; Tunlid and White 1991; White 1993). A typical sample analysis scheme for the recovery of SLBs from subsurface sediments is diagrammed in Fig. 1. The origin and physical nature of the samples used in this review to illustrate the applicability of the SLB analysis are described in detail in their respective original publications.

Results

Are viable microbes present in the subsurface?

Subsurface sample quality

To establish the presence of bacteria in subsurface sediments surrounding a proposed nuclear waste disposal site, it is of the utmost importance that the sample cores recovered for microbiological analysis not be contaminated with either surface or drilling mud microbiota. In 1986 the United States Department of Energy (DOE) initiated a field component to systematically investigate the microbiology of the deep subsurface (Wobber 1986). A protocol for recovering uncontaminated subsurface sediment cores resulted from this effort (Phelps et al. 1989; Russell et al. 1992; Colwell et al. 1992). In short, cores are recovered in the presence of microsphere and bromide tracers, pared of their external surfaces in glove bags saturated with argon or nitrogen at the site of recovery, and frozen for shipment overnight to the analyzing laboratory. The use of inherent tracers, SLB, and community level physiological profiles (CLPP) also provide evidence that subsurface cores are recovered uncontaminated. In a recent study (Lehman et al. 1995), significant differences in viable biomass and community composition were identified between drilling muds, makeup waters, surface soils, cuttings, and sample cores recovered from different geological horizons. Figure 2 illustrates the

Fig. 2. Results of a hierarchical cluster analysis (complete linkage) of PLFA profiles (represented as arc-sine-transformed molar percentages) showing community compositional differences between drilling muds (DM), cuttings (CU), surface soil (SS), sample cores (SC), and a combusted control (CC) recovered from the deep subsurface in Taylorsville, Va. Cluster groups are defined as having a similarity index of 0.3 or greater. Sample prefixes are followed by the depth (in meters) from which the samples were recovered.

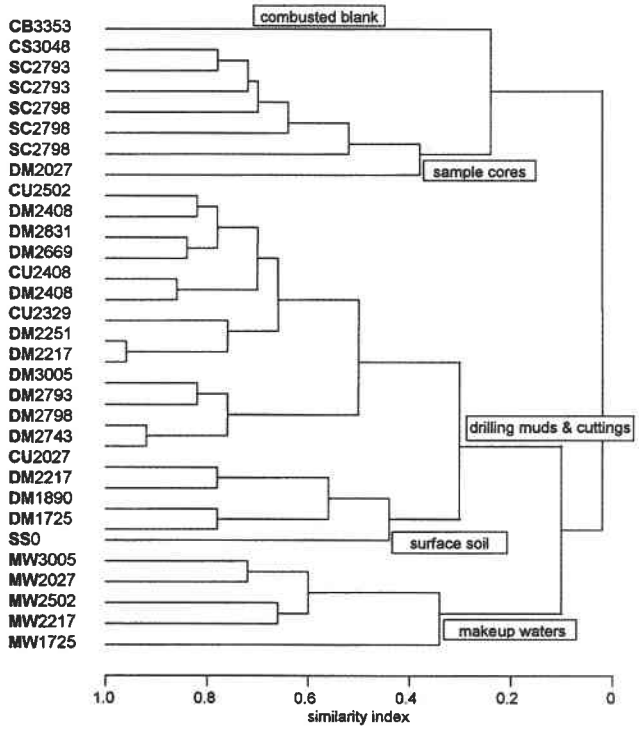
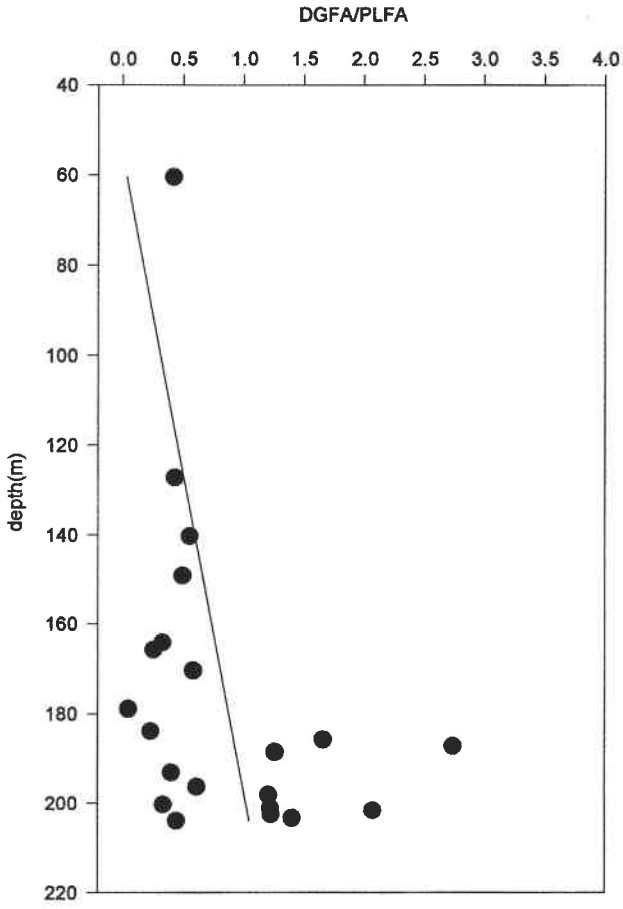


Fig. 3. A comparison of the ratio of diglyceride fatty acids (DGFA) to PLFA with depth in sediment cores recovered from Cerro Negro, N.Mex. The line represents a first order regression of the data on the plot.



differences between and the similarities within the different sample types as defined by a hierarchial cluster analysis (complete linkage) of the PLFA profiles (represented as arc-sine-transformed molar percentages) of each sample. The similarity in PLFA profiles between drilling muds and cuttings results from the saturation of the cuttings in the mud prior to evacuation from the borehole. The fact that the sample cores do not show as close a similarity with either of these two sample types, the makeup waters or the combusted blank, as they do with each other indicates a lack of contamination.

Presence of a viable subsurface microbiota

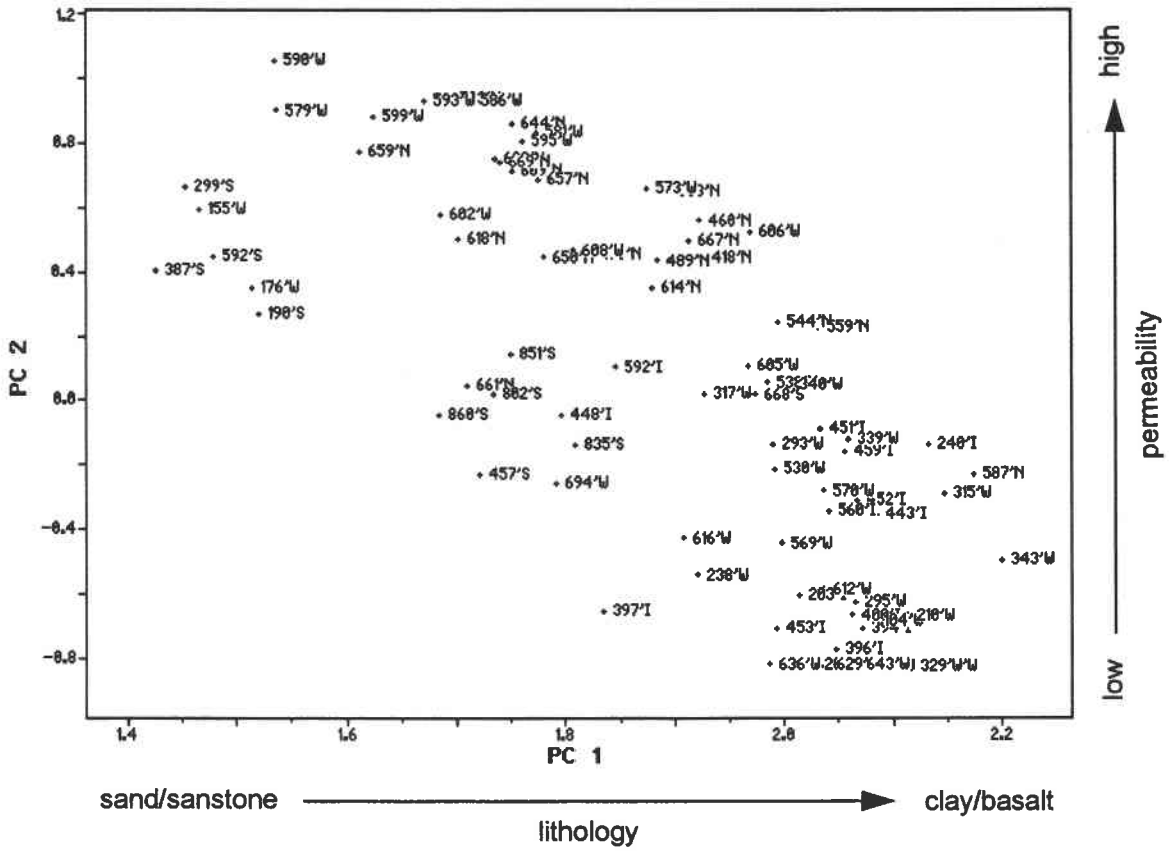
With the quality assurance criteria derived from the SLB and community level physiological activity measurements, it is possible to document the presence of an extant microbial community even from sediments 3000 m below the surface (Lehman et al. 1995). The presence of PLFA signifies the presence of cells with intact membranes. Since the presence of an intact phospholipid-containing cell membrane is essential for cell viability, PLFA represent a measure of the viable microbial biomass. Phospholipase activity hydrolyzes polar lipids producing diglycerides, which for a period of time, show the same ester-linked fatty acid pattern as did the parent phospholipid. Evidence of decreased microbial cell viability was observed in a subsurface arid sandstone of a deep vadose zone, recovered from Cerro Negro, N.Mex. These sediments showed a markedly increased proportion of diglyceride to phospholipid fatty acids with an increase in depth (Fig. 3). A study of subsurface sediment showed that viable biomass, as determined by PLFA, was equivalent (but with a much smaller standard deviation) to that estimated by intracellular ATP, cell wall muramic acid, and very carefully conducted acridine orange direct counts (AODC) (Balkwill et al. 1988).

What types of bacteria are present in the deep subsurface?

Presence of specific groups of microbes in the subsurface

Microbes have been isolated from rock sections of the volcanic tuft of Yucca Mountain, Nev., and characterized by fatty acid analysis to show their heterogeneous distribution in terms of both biomass and community composition on the rock surface (Amy et al. 1992; Haldeman et al. 1993, 1995). Specific groups or, in some cases, specific species of microbes have sufficiently unique lipid profiles to be utilized as signatures (Tunlid and White 1991; White 1993). For example, subsurface isolated *Sphingomonas* species contained distinctive sphanganine profiles compared with surface isolated *Sphingomonas* species

Fig. 4. Results of a nationwide comparison of subsurface sediment PLFA profiles of arc-sine-transformed molar percentages expressed as eigenvalues (expressing variance of principal components analysis) as a principal components analysis. PLFA profiles were recovered from subsurface sediments collected across United States (W, Washington state; S, South Carolina; N, New Mexico; I, Idaho). All samples represented were recovered from a depth of greater than 100 feet. Principal component 1 (PC1) explains 78% of the variance within the data set and principal component 2 (PC2) explains an additional 7%.



(Fredrickson et al. 1995). Sulfate-reducing bacteria and methane-producing *Archae* contained SLB, which allowed for the detection of shifts in community composition in concretion-forming marine sediments (Coleman et al. 1993) and in a Miocene lacustrine subsurface environment (Fredrickson et al. 1996). Thermophilic sulfate-reducing bacteria have been identified in petroleum reservoirs (Brink et al. 1994), while acetogens, mineral acid producing *Thiobacilli*, methane-oxidizing bacteria, and propane-utilizing *Actinomycetes* all contained signature biomarkers that allowed for their detection in the environment (Phelps et al. 1991; Kerger et al. 1987; Ringelberg et al. 1988). Iron-reducing bacteria, such as *Geobacter metallireducens* which also reduces uranium, have been detected by the examination of signature lipopolysaccharide hydroxy fatty acids (Lovely et al. 1992). Figure 4 illustrates how the PLFA profiles (expressed as arc-sine-transformed mole percentages) from a nationwide cross section of subsurface sediments are related when subjected to a principal components analysis. Although site-specific similarities could be identified, preliminary studies that looked at correlations between PLFA abundance and composition with geologic parameters have hinted that parameters such as lithology and permeability (White et al. 1991) result in the best correlations.

Recently, the solvent extractions utilized for the recovery of lipids have been shown to liberate DNA as well and in a form suitable for gene probing (Kerhmeyer et al. 1996). DNA gene probing substantially increases the specificity of this in situ analysis and allows for detections at the strain, species, genus, or kingdom levels. DNA gene probes can also be utilized in the detection of specific metabolic activities or for the potential for these activities. The utilization of combined SLB and DNA probing technologies on the same sample will invalidate some limitations with nucleic acid analysis of subsurface microbiota, as the SLB provides evidence of phenotypic expression, not just genetic potential (White 1994).

Can the activity or health of the subsurface community be determined?

Community nutritional/physiological status

Bacterial poly β-hydroxyalkanoic acid (PHA) and micro-eucaryotic triglyceride (Gehron et al. 1982) are endogenous storage lipids. The relative amounts of these compounds, as compared with 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 (Nickels et al. 1979; Findlay and White 1983). 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 (Ringelberg and White 1992), whereas the fortuitous metabolism of trichloroethylene correlates with a high ratio of PHA/PLFA (Nichols and White 1989; Cox et al. 1994). Within a PLFA profile, specific ratios of fatty acids have been shown to correlate with physiological stress (Guckert et al. 1986). 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 (Heipieper et al. 1992). Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA (Guckert et al. 1986). Another lipid class that is biochemically related to microbial physiology are the respiratory quinones. Quinone composition can be utilized to indicate the degree of microbial aerobic activity (Hedrick and White 1986). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce the formation of benzoquinones in bacteria, in contrast to microbes respiring on organic substrates which form naphthoquinones. There are other lipid biomarkers that can yield further insights into the conditions of the subsurface microniches as outlined in White (1995).

Can the transport of bacteria through the subsurface facilitate the escape of nuclides?

Shifts in subsurface microbial community composition associated with transport

Microbes have considerably higher hydraulic transmissivity than conservative tracers like bromine (Lawrence 1996). Microbes readily bind nuclides (McLean and Beveridge 1990) and can thus transport these nuclides very effectively. SLB analysis 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 (Table 1). An analysis of microbial community composition throughout this depth interval shows that PLFA indicative of *Actinomycetes*, in particular tuberculostearic acid (10-methyl-18:0), continue to represent a constant or increasing percentage of the total in the area of high recharge, which is in sharp contrast to the decrease in percentage of these PLFA in the area of low recharge (Table 1).

Table 1. A comparison of viable microbial biomass and a signature ester-linked phospholipid fatty acid for *Actinomycetes* over depth intervals from low and high recharge sites located near Yakima, Wash.

Depth (feet)	Low recharge[a]		High recharge[d]	
	Biomass[b]	10-Methyl-18:0[c]	Biomass	10-Methyl-18:0
2	280	3.3	730	1.7
10	153	3.6	100	1.9
15	443	2.3	6	3.6
20	0	0	2	4.4
30	5	4.1	1	7.9
45	0	0	1	2.2

[a] <150 Cl^- g/m^2.
[b] pmol ester-linked phospholipid fatty acids/g.
[c] Mol% tuberculostearic acid.
[d] >500 Cl^- g/m^2.

Discussion

The application of SLB 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. Lessons learned from the destruction of concrete sewers by *Thiobacilli* (Kerger et al. 1987) or buried gas transmission pipelines by acetogens (Phelps et al. 1991) attest to the destructive power microbes. Microbially influenced corrosion (MIC) poses potentially serious problems in the maintenance of containment barriers (Little 1996). Microbes have been shown to particularly attack weldments (Luo et al. 1994) and are quite capable of localized MIC on "noble" substrata such as stainless steel (Angell et al. 1994). 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.

Microbial contamination and metabolic activity in the subsurface may not be totally deleterious to the containment of buried nuclear waste. Microbes have a remarkable capacity to immobilize nuclides through the formation of insoluble compounds (Lovely 1995). Biotechnology could possibly provide the ultimate in a secure containment barrier. Bacterial metabolic activities may have been responsible for the original concentration of uranium nuclides in sedimentary deposits and for their containment over the past billions of years at sites like Cigar Lake, Sask., or in fission products like those at Oklo, South Africa. The possibility of introducing these microbes into backfill material surrounding the nuclear waste deposits and maintaining them in a proper physiological status could provide an unbreachable living containment wall.

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