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Signature Lipid Biomarkers for In Situ Microbial Biomass, Community Structure
and Nutritional Status of Deep Subsurface Microbiota in Relation to Geochemical
Gradients

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INTRODUCTION

To obtain a better understanding of the microbial ecology of the deep subsurface, it was necessary to employ alternate techniques for the identification of microorganisms *in situ*. Classical microbiological techniques assay only those organisms which are culturable with bias occurring towards the media selected. Since culturable microorganisms typically represents only 0.1 to 10% of the extant microbiota, techniques for the direct assay of microorganisms *in situ* were needed. The analysis of cellular lipid biomarkers is a technique whereby microbial communities can be assayed directly in a variety of environmental matrices. Through the quantitative recovery of lipid biomarkers, estimations of cell biomass, community composition and community nutritional and/or physiological status can be obtained. Rationale for the assessment of these attributes are described in White and Ringelberg, 1997 and is summarized below.

Biomass: The recovery of ester-linked phospholipid fatty acids (PLFA) provides a quantitative measure of the viable microbial biomass. For this determination it is assumed that a viable microorganism has an intact cell membrane containing PLFA. When cells die, cell lysis occurs releasing cellular enzymes which hydrolyze the phospholipids releasing the polar head group leaving behind a lipid moiety known as a diglyceride which contains the same fatty acids as the parent phospholipid. The hydrolysis has been shown to occur within minutes to hours of cell lysis (White *et al* 1979) thus, the analysis of PLFA and diglyceride fatty acids (DGFA) provides an estimation of the total viable and non-viable biomass present in material recovered from the deep subsurface. A study of subsurface aquifer material showed that the viable biomass estimate derived from the quantification of PLFA, was equivalent to estimations based on intercellular ATP, cell wall muramic acid, and acridine orange direct counts (Balkwill *et al* 1988). Since phospholipids occur in all cellular membranes at reasonably constant amounts and have a naturally high turnover rate, the analysis of this lipid biomarker provides an accurate estimation of viable microbial biomass.

Community Composition: The analysis of lipid biomarkers provides sufficient information for the identification of specific individuals, groups and whole communities of microorganisms. Specific individuals or groups of microorganisms often contain characteristic lipid biomarkers, in particular PLFA. Examples include the sulfate reducing bacteria (Kohring *et al*. 1994) and the methanotrophic bacteria (Guckert *et al*. 1991). The lipid biomarker analysis is, however, incapable of identifying every species of microorganism in an environmental sample as many species contain over-lapping PLFA. Nevertheless, many species or physiologically similar groups of microorganisms are readily detected in environmental samples. In fact, distinctive patterns of fatty acids (largely from phospholipids and lipopolysaccharides) are currently used in the identifications of pure cultures grown under standardized conditions

(Microbial Identification System, MIDI, Newark, DE) (Welch, 1991). Patterns of lipid biomarkers can also be used in identifying a single species in an environmental matrix. The anaerobic dechlorinating bacteria, *Desulfomonile tiedjei* was detected in a community of bacteria by the identification of unique lipopolysaccharide hydroxy fatty acids (Ringelberg et al. 1993).

The analysis of other lipid biomarkers such as the hydroxy fatty acids from the Lipid A portion of a lipopolysaccharide (LPS-OHFA), glycolipids for gram-positive bacteria and phototrophs and sterols for microeukaryotes can provide a more detailed analysis of the microbial community composition. For example, genera of gram-negative bacteria, such as *Geobacter*, *Desulfomonile*, and *Legionella*, have all been readily detected and differentiated by analysis of their LPS-OHFA (Lovely et al. 1992; Ringelberg et al. 1993; Walker et al. 1993).

Application of the lipid biomarker analysis to a deep subsurface sample results in pattern of PLFA which is descriptive of the entire microbial community. By quantifying differences between PLFA patterns obtained from different deep subsurface samples, inferences can be made regarding shifts in community composition related to the hypothesis being tested, such as relations to geochemical gradients. Multivariate statistical analyses, such as hierarchical cluster and principal components analysis, have proven to be valuable tools in identifying these differences and relationships. Variance among a specific PLFA or group of PLFA within the sample set can then be interpreted based on what is currently known regarding fatty acid biosynthesis and bacterial membrane PLFA compositions.

Nutritional/Physiological Status: Specific patterns of PLFA can also reflect physiological stress. Exposures to toxic environments can lead to minicell formation and a relative increase in *trans* monoenoic PLFA. For example, Heipieper et al. 1992, showed that increasing concentrations of phenol toxicants increased the proportions of *trans* PLFA (in relation to the *cis* homologues) in the bacteria *Pseudomonas putida*. Similarly, *trans/cis* ratios and ratios of cyclopropane fatty acids to their monoenoic precursors were shown to increase in response to a change in an environmental condition, i.e. starvation and desiccation (Guckert et al 1986; Kieft et al 1994). Other lipid components have also been shown to provide insight into a microbial communities nutritional or physiological status. The accumulation of poly β -hydroxyalkanoic acids (PHA) in bacteria (Nickels et al. 1979) and triglycerides in microeukaryotes (Gehron and White, 1982) are two such compounds. When compared to estimates of biomass and community composition, the analysis of triglycerides, poly-hydroxy alkanoates and the ratios of specific PLFA (*trans/cis* and cyclopropyl to monoenoic precursor) provide valuable insights into the nutritional and physiological status of the extant microbiota.

The research described in this report focuses on the application of lipid biomarker technology to the description of extant microbial communities in a variety of deep subsurface environments. Specifically, questions regarding sample quality, microbial abundance, microbial community relationships to

geochemical gradients (including sediment age), microbial transport and microbial origins were addressed.

The lipid biomarker technology was used as an inherent tracer in assessing sample quality of cores recovered from a borehole at the Thorn Hill site in VA, USA (Lehman *et al.* 1996). The technology was also used in demonstrating pitfalls related to sample storage (White and Ringelberg, 1997 and Haldeman, 1997). Relationships to geochemical gradients were addressed in boreholes drilled in SC, ID, WA and NM, USA. Two studies highlighted the ability of the lipid biomarker to identify relations to geochemical gradients. In a borehole drilled near Yakima, WA, USA, an increase in microbial biomass attributed to sulfate reducing bacteria was related to a lacustrine lithology (Fredrickson *et al.* 1995) and in a borehole drilled near Cerro Negro, NM, distinct patterns of PLFA were recovered from shales and sandstones (Ringelberg *et al.*, 1997). PLFA pattern differences were also identified in chronosequences of sediment cores recovered from boreholes drilled in WA, USA (manuscripts in preparation). By determining the distribution of PLFA and DGFA throughout two boreholes drilled near Cerro Negro, NM, USA., estimations of viable and non-viable microbial abundance were made in relation to a previous hypothesized rock sterilizing thermal impact (Ringelberg and White, 1997).

METHODS

The analysis of lipid biomarkers in deep subsurface material required that sensitivities be increased to levels below those typically used in the analysis of surface soils. This was accomplished through the minimization of background contaminants. The reduction of background noise levels resulted in enhanced signal to noise ratios allowing for the quantitative and reproducible assay of lipid biomarkers. The protocol used in the analysis of deep subsurface material is described in Tunlid *et al.* 1989 and paraphrased below.

Subsurface material collected on-site or manipulated in laboratory microcosms was placed on dry ice and shipped via overnight mail to the Center for Environmental Biotechnology. Upon receipt, the material was crushed in a sterile hood using sterilized (flamed with 70% ethanol) mortar and pestles or a rock mill. The crushed material was homogenized (but not sieved) and a 50-75 g aliquot removed to a glass centrifuge bottle for extraction in an organic solvent system consisting of chloroform, methanol and phosphate buffer (1:2:0.8, v:v:v). After sonication for 2 min. to aid in cell lysis, the mixture was allowed to stand, with periodic mixing, at room temperature for a period of 3 hr. resulting in the quantitative extraction of cellular lipids. The sample was then centrifuged and the solvent decanted into a separatory funnel where additional and equal aliquots of chloroform and water were added. After vigorous mixing, the sample was allowed to separate into two phases, the lower being a purified (of organic solvent soluble proteins and carbohydrates) total lipid extract. The lipid extract was then collected and taken to dryness under rotary evaporation for placement

onto a column of chloroform saturated silicic acid (100mg). Using sequential eluents (1 ml) of chloroform, acetone and methanol, the total lipid was fractionated into neutral-, glyco-, and polar-lipids, respectively. Both the neutral- and polar-lipid fractions were then methylated via a trans-sterification process in a mild alkaline methanolic potassium hydroxide solution. The resulting fatty acid methyl esters were then further separated and quantified/identified by capillary gas chromatography/mass spectrometry (Kieft *et al.* 1994). Sensitivities sufficient for the detection of approximately 10^3 typical subsurface bacterial cells were obtained using single ion monitoring of the predominant ions for saturated and unsaturated fatty acid methyl esters. Single ion monitoring was accomplished by detecting positive ions resulting from an electron impact ionization. Poly-hydroxy butyric acid was recovered from the glyco-lipid fraction off of the silicic acid column after the formation of an ethyl ester in an acidified solution of absolute ethanol (Findlay *et al.* 1983). Quantification and identification of PHB was accomplished in the same manner as that described for the DGFA and PLFA moieties.

As previously mentioned, in order to detect PLFA extracted from microbial cells recovered from the deep subsurface it is necessary that background levels of PLFA (those introduced in sample work-up) be kept at a consistently low level. Sample size is often restricted when working in the deep subsurface requiring that the signal to noise ratio always be optimized so that detection limits are a result of instrumentation limits and not background levels. To minimize background levels it is necessary that solvents of the highest purity available be obtained. In addition, all glassware should be muffled in a furnace at $\sim 450^\circ\text{C}$ for a minimum of four hours prior to use. As with aseptic techniques, care must be taken at all times to minimize the introduction of lipids during sample work-up. Some common sources of lipid contamination are the skin, plastics, condensation, vacuum grease, and pump oil vapors. By establishing laboratory practices which check solvent quality on a routine bases and incorporate procedural blanks with each set of samples processed, sources of contamination can be more quickly identified and eliminated minimizing damage to the research results.

RESEARCH SUMMARY

Quality Assurance: When used as an inherent tracer, PLFA profile determinations can provide evidence of sample quality (Lehman *et al.* 1996). PLFA were used in this capacity in support of the DOE Subsurface Science Program effort in Thorn Hill, VA. PLFA profiles extracted from a sediment core and the surrounding parings collected from a depth of 10,000 meters showed no similarities to those (PLFA) profiles extracted from drilling fluids or make-up waters used in the collection of the core (fig. 1). In addition, viable biomass estimates showed an order of magnitude difference between the microbiota of the drilling fluids and that of the sediment core (table 1.). This magnitude of

difference suggests no penetration of the drilling fluid into the sample material. The results obtained with the PLFA analysis paralleled those obtained by application of a community-level physiological profile (Lehman *et al.* 1996), but provided the additional insight in the form of the biomass comparison. In addition to the cores recovered from the Thorn Hill site, the PLFA assay in the form of an inherent tracer was applied to cores recovered from the Cerro Negro borehole in NM and the Parachute Creek borehole in CO. This application of one component of the lipid biomarker analysis helped establish the fact that uncontaminated cores could be recovered from the deep subsurface for microbiological examination.

Sample Integrity: In laboratory studies addressing the origin and nature of microorganisms in deep subsurface materials, an important consideration is that those organisms cultured or manipulated in the laboratory are truly representative of the extant microbiota. In studies examining effects of storage conditions on the extant microbiota, the application of the lipid biomarker technology provided evidence of shifts in microbial community composition and abundance as well as an on community nutritional/physiological status.

Subsurface sediments recovered from an exposed landslide scarp paleosol in WA, USA were incubated in jars for 32 weeks as either intact cores or desegregated material (by mortar and pestal and passed through a 2mm sieve). The samples were incubated at 15°C under atmospheres composed of 0.5% O₂, 0.03% CO₂, and the balance as N₂. Through application of lipid biomarker techniques, differences in the responses of the extant microbiota in the intact and the homogenized sediments over the time course were observed (Table 2) (White and Ringelberg, 1997). Whole samples showed a four time increase in the total viable microbial biomass (compared to the homogenized cores) by week 15 of the incubation period whereas there was no observable increase in total viable biomass in the homogenized cores. A shift in microbial community composition was also observed in the whole cores, again, to a greater extant than those observed in the homogenized core incubations. Differences existed in the percentages of monounsaturated (mono) and terminally branched saturated (terbrsat) PLFA. Between week 3 and 32, mono unsaturated PLFA in the whole core incubations increased from 2.9 to 4.8% and terminally branched saturated PLFA from 1.9 to 35.9%. In contrast, the homogenized incubations showed a decrease in the percentage of monounsaturated PLFA (2.5 to 0.9%) and only a small increase in the percentage of terminally branched saturated PLFA (2.1 to 9.6%). Monounsaturated PLFA are typically attributed to gram-negative bacteria. This classification of bacteria reached a maximum in the whole cores at week nine of the incubation then dropped slightly by week 32. Terminally branched saturated PLFA are typically attributed to gram-positive bacteria. This classification of bacteria did not reach a maximum until week 16 of the incubation and also decreased slightly in percentage at week 32. The observed decrease in mono and terbrsat percentages at week 32 of the incubation correlated with a decrease in the

viable biomass which reached a maximum at week 16 of the incubation. These results indicated that, in intact cores, the extant microbiota will change in community composition and abundance when incubated at 15°C. And, that the community change was greatest in the gram-positive classification of bacteria.

The homogenized cores did not exhibit similar responses in community composition or biomass. Monounsaturated PLFA were measured at similar percentages for 16 weeks of the incubation period followed by an order of magnitude drop at week 32. Terminally branched saturated PLFA increased in percentage to a maximum at week 9 of the time course followed by a 2 fold decrease by week 32. Again, these observed decreases in the percentages of mono and terbrsat PLFA at week 32 correlated with a drop in the viable biomass. As already mentioned, the magnitude of the changes in PLFA percentages in the homogenized cores were far less than those observed in the intact or whole core incubations.

Data obtained from viable counts (provided by F.J. Brockman, Battelle/PNL) indicated that, initially, few organisms were culturable in either the whole or homogenized cores whereas at the end of the incubation period, culturable numbers increased by two to three orders of magnitude. The ratio of PHA/PLFA indicated that, with the increase in biomass and changes in community composition, a portion of the extant microbiota was experiencing unbalanced growth (Table 3.). This imbalance was likely due to nutrient limitations, a common characteristic to most subsurface environs.

Results of this study demonstrated that changes occur with the extant microbiota in intact sediment cores when incubated over time. These changes were identified as an increase in the viable biomass which was associated with an slight increase in the gram-negative bacterial populations and a large increase in the relative percentage of the gram-positive bacterial populations. As biomass increased and community composition shifted, the nutritional/physiological status of a portion of the extant microbiota also changed, toward a situation of unbalanced growth. These results point out that the point in time a deep subsurface sample is assayed for culturable organisms will likely dictate the results obtained.

In another study, samples of deep subsurface volcanic rock showed large changes in the numbers of viable organisms that were recoverable following one week of storage at 4°C and perturbation (Haldeman *et al.* 1994). Morphologically distinct colonies recovered before and after a period of incubation were analyzed for fatty acid content and API rapid NFT strips. The analyses showed that while recoverability increased, diversity generally decreased and that those cultures appearing post incubation showed a greater ability to utilize available carbon sources. The changes in community composition and the fact that some isolates were only recovered post incubation suggested that bacterial outgrowth had occurred. In another study, resuscitation of dormant organisms was identified as a possible explanation of the increase in culturable organisms and shifts in viable biomass and community composition over time (Haldeman *et al.* 1995). Results of these studies again point out that

the time at which a core is sampled will likely result in a different description of the extant microbiota.

Subsurface Microbial Ecology:

Application of the lipid biomarker technology to sample cores recovered from the deep subsurface has demonstrated the presence of viable microorganisms in a number of different environments. In figure 2, the vertical distribution of viable microorganisms is illustrated. Although numbers of cells can be estimated from PLFA concentrations it is important to take into consideration that the amount of PLFA synthesized by an organisms may be directly effected by environmental conditions. Nevertheless, the detection of PLFA in the deep subsurface cores indicates the presence of a viable or at least a potentially viable community of microorganisms. From the figure, two trends can be derived. One, that total biomass decreases sharply as vertical depth increases. In all but the borehole in ID, viable biomass in the surface soils was at least two orders of magnitude greater than that detected in the subsurface. The ID borehole showed only an order of magnitude decrease. And two, that total biomass is greater in the deep subsurface of the eastern coastal plains (South Carolina) than in the arid northwest (Washington).

In the borehole drilled in WA, sample core retrieval was carried out on a small enough scale to allow for the detection of biomass changes as differing geologic horizons were traversed (figure 3.). An increase in viable biomass was observed within the lacustrine lithology. The level of viable cells detected was approximately 10 fold greater in this lithology than in the fluvial gravels immediately above and the paleosol and fluvial sands immediately below. This increase in biomass was associated with the detection of sulfate reducing bacteria as indicated by PLFA profiles and DNA characteristics (Fredrickson et al. 1995).

The observed decrease in viable biomass with increase in vertical depth is sometimes associated with an increase in the non-viable biomass. Core material recovered from a borehole drilled near Cerro Negro, NM showed significant correlations to exist between vertical depth and both viable and non-viable biomass estimates (figure 4.). Viable biomass decreased with depth while non-viable biomass increased (Ringelberg and White, 1997). Application of the lipid biomarker technology to these cores provided evidence of both present and past microbial colonization. One of the hypotheses tested in this study was that microorganisms in unheated shales would be similar to modern near shore marine sediment organisms and would be absent from heated (volcanic) shales. The greatest depths attained by an angled borehole drilled at this site reached a formation, Morrison, which geological evidence indicated was subjected to volcanic heating. The heat encountered during the volcanic stage would have likely eliminated all microbial cells and cell components (PLFA and DGFA). Results of the lipid biomarker analyses (Figure 8) illustrated that a lack of PLFA abundance was encountered within this formation as compared to the three

geological horizons immediately above. Of the nine cores collected throughout the Morrison horizon, two showed PLFA abundance above background levels (both located near the top of the horizon) and no cores showed DGFA abundance above background. These results were viewed as supportive of the hypothesis that past volcanic heating adversely effected the extant microbial populations.

In a microcosm study (Keift *et al.* 1994), microbial biomass (both viable and non-viable) was shown to be effected by environmental restrictions such as starvation and desiccation. Under moist conditions and in the presence of supplemented nutrients (PTYG), PLFA abundance was greater than that observed under conditions of either desiccation or starvation. This result was observed in both a *Pseudomonas* and an *Arthrobacter* species of bacteria. However, the ratio of DGFA/PLFA (non-viable to viable cells) was found to increase significantly only in the *Arthrobacter* species under the desiccated and starved conditions. The *Pseudomonas* species appeared to compensate for the environmental restrictions by adjusting membrane fluidity, which was indicated by a change in the ratios of trans/cis monounsaturated PLFA and cyclopropyl to monounsaturated precursor PLFA. In another microcosm study, similar results were obtained using subsurface isolates of *Arthrobacter* and *Psuedomonas* when incubated in subsurface sediments (Kieft *et al.* 1997). By monitoring the lipid biomarkers over time, insight into the mechanisms of microbial adaptations to these specific environmental restrictions was gained and found to be directly related to total microbial abundance.

The differences in microbial abundance observed in the number of deep subsurface environments sampled were sometimes associated with changes in microbial community composition. A multivariate statistical comparison of the PLFA profiles extracted from the borehole cores described in figure 2, identified three community types (Figure 5) (White and Ringelberg, 1997). One group was comprised of surface samples collected from all three sites. The prominent PLFA in these samples are also known to be characteristic of soil Actinomycetes (i.e. 10me18:0 or tuberculosteric acid). Associated with the surface samples, were the higher biomass (> 8 pmol PLFA gram⁻¹) vadose zone samples collected from the ID and WA sites. Loadings from the principal components analysis identified 16:1w7c and 18:1w7c as significant PLFA in describing the similarity among the PLFA profiles of this group. The two PLFA are end products of the bacterial anaerobic desaturase fatty acid biosynthetic pathway utilized by most gram-negative bacteria and thus are descriptive of this component of the microbial community. Both the high biomass vadose samples and the surface soils contained microbial communities influenced by the presence of either aerobic or anaerobic gram-negative bacteria. Another group was found to be comprised of the majority of deep subsurface vadose zone sediments from the two western sites which were also the samples to show the lowest abundance of viable microbial biomass. An interesting observation was the detection of evidence of toxicity in the samples collected from around the water table (indicated by the *). Trans/cis ratios in these samples was found to be greater

than the 0.1 which is a threshold level identified by Guckert *et al.* 1986 as an indication of environmental stress within *Vibrio cholera*. This observation suggested that the organisms near the water table were in a stage of growth where they were experiencing some form of environmental stress, such as starvation. The final group of samples was comprised largely of the SC site samples. The principal components analysis identified two PLFA, 14:0 and 18:2w6, as being significant in defining similarities among the PLFA profiles of this group. The dienoic, 18:2w6, is known to be a prominent fatty acid in the cell membranes of fungal species whereas the normal saturate, 14:0, has been shown to be prominent in a number of species of micro-algae. Although neither fatty acid alone verifies the presence of either of these functional groups, taken together they do suggest a micro-eukaryotic presence in these sediments. In summary, differences in microbial community composition were apparent among the core materials recovered from the various sites. These differences could then be related to geological characteristics (semi arid western vadose zone vs eastern coastal plain saturated materials).

Specific relationships between microbial community composition and geological parameters were also identified. As described above, a biomass increase related to a sulfate reducing bacterial presence was identified in the lacustrine sediments recovered from the borehole drilled in WA. Sulfate reducing bacteria contain both monounsaturated and terminally branched saturated PLFA in their cell membranes. Of the terminally branched saturated PLFA, the *iso* configuration is typically more abundant distinguishing this group of bacteria from the majority of gram-positive bacteria. In figure 6, the relative percentages of the monounsaturated, terminally branched saturated and *iso* and *anteiso* 17 carbon PLFA are illustrated. The observed increase in the percentages of these PLFA groups and types across the lacustrine horizon was evidence of an sulfate reducing presence. This evidence was corroborated by DNA gene probe analysis (Fredrickson *et al.* 1995).

Evidence was also obtained which suggested that the extant microbiota of shales differed from those of sandstones. It was hypothesized that different microbial communities would be detected in shales and sandstones as a result of environmental influences and restrictions. An analysis of PLFA profiles recovered from the core material taken from a borehole near Cerro Negro, NM showed that different patterns of PLFA were identifiable in the two geological forms (Ringelberg and White, 1997). In figure 7, differences in the PLFA profiles obtained from the Mancos shale and Two wells sandstone horizons are illustrated. The sandstone showed no detectable levels of the terminally branched saturates i16:0, i17:0 and a17:0 or the monounsaturates i17:1w7c, 16:1w7c, cy17:0 or 18:1w7c, while their presence was readily detected in the shale horizon. The presence of *iso* terminally branched saturates and the terminally branched monounsaturate i17:1w7c is typically associated with *Desulfovibrio* sp. (Kohring *et al.*, 1994). In general, shales throughout the borehole showed greater percentages of this and other monounsaturated PLFA. As already described, monounsaturated PLFA are typically found in Gram-

negative cell membranes but include organisms such as the sulfate reducing bacteria.

The sandstones showed significantly greater percentages of all the normal saturates, in particular 16:0 and 18:0 which are prominent PLFA in most all viable cell membranes, prokaryotic and eukaryotic (Vestal and White, 1989). An increase in the saturation of a cell membrane can reflect a decrease in membrane fluidity suggesting that this observation may be related to cell physiology as well as community composition. By applying the lipid biomarker technology to these cores, evidence was obtained of differing extant deep subsurface microbial communities directly related to differing geological lithology.

Subsurface material collected along a chronosequence provided evidence of community differences with respect to sediment age. In figure 9, results of another principal components analysis are illustrated. PLFA profiles recovered from sediments dated as ~10 ka years old formed a distinct cluster group as did profiles recovered from sediments dated as >34 ka years old and >74 ka years old. Viable biomass within the three respective age groups was also found to decrease with increase in sediment age. In a more recent examination of another chronosequence, non-viable biomass estimates were observed to increase with sediment age whereas viable biomass estimates correlated positively with prolonged assessments of potential *in situ* activities (Kieft *et al.* Manuscript in preparation). In both studies, sediment age appeared to exert a greater influence on microbial community composition than did soil or sediment type.

The lipid biomarker technology was also utilized to address questions of spatial heterogeneity in the subsurface. At a site located near Oyster, VA core material was collected along a 1km transect. The transect crossed fields where soybean and corn had previously been cultivated. The cores taken from beneath these fields showed striking differences in PLFA patterns (figure 10.) This site differed in many respects from the boreholes previously described in that the subsurface geochemistry was believed to be uniform. Although, the differences in community composition identified with the lipid biomarker techniques may be attributable to man's influence, the results demonstrated that environmental restrictions do effect the composition of extant microbial communities. The pattern of PLFA observed in the soybean field sediments suggested a greater prevalence of gram-positive and/or sulfate reducing bacteria as indicated by the greater percentage of terminally branched saturated PLFA detected. The corn field sediments showed a relatively high percentage of long chain normal saturated PLFA which have been associated with some species of oligotrophic bacteria. Although it was not possible to define the precise make-up of either community, the detection of differing PLFA patterns indicated that spatial heterogeneity could exist within a geologically uniform subsurface environment.

A study of microbial isolates recovered from volcanic tuff in tunnels at Rainer Mesa, Nevada Test Site (Haldeman *et al.* 1993) used membrane lipids to characterize isolates recovered from a 21 m³ section of volcanic tuff. A comparison of the fatty acid profiles, by hierarchical cluster analysis, obtained for

each isolate was then interpreted in terms of microbial diversity. Twenty nine genera were identified within the section of the volcanic tuft. A comparison of the fatty acid profiles with those contained in a bacterial library database (Microbial Identification, Inc., Newark, DE) indicated the genera *Arthrobacter*, *Gordona*, and *Acinetobacter* to be the most prevalent. There appeared to be no clear pattern to the distribution of the bacteria within the rock cube. Most of the isolates were, however, recovered from three specific sites within the rock cube highlighting the fact that spatial distribution in the deep subsurface is a concern when attempting to recover viable organisms. Two other genera were identified which did not show any similarity to the ~650 organisms contained within the database. The detection of classically unidentifiable organisms such as these represent a potential for the discovery of unique bacteria. These bacteria would have likely gone undetected if only standard microbiological assays were applied. The potential for other unique organisms is in reality greater, since direct bacterial counts were several orders of magnitude higher than viable counts.

Other unique bacteria have been cultivated from the deep subsurface. Lipid biomarker techniques have played a role in the classification of a number of these organisms. Yitai *et al.* submitted on *Desulfotomaculum* species, Fredrickson *et al.* 1995 on *Sphingomonas* species and Balkwill *et al.* submitted on *Sphingomonas* species.

SUMMARY

Application of the signature lipid biomarker technique to deep subsurface material provided insight into the abundance, nature and distribution of subsurface microorganisms in this unique environment. Microbial abundance was quantified through the recovery of membrane lipid moieties, specifically PLFA which indicated the presence of viable populations. An estimation of non-viable populations was accomplished through the measurement of DGFA. Both assays are independent of the bias inherent in classical culturing techniques providing a more accurate estimation of *in situ* microbial populations. The lipid biomarker analyses also provided a description of the nature of the microorganisms present in the variety of deep subsurface material examined. This description, based on the relative patterns of PLFA recovered, identified the composition of the extant microbial communities. Distribution of microorganisms in the deep subsurface in relation to geochemical gradients were reflected in both the abundance and patterns of lipid biomarkers recovered. Comparisons between patterns of PLFA recovered from different geologic horizons or with respect to storage conditions highlighted the impact the environment has on influencing microbial ecology of the deep subsurface.

REFERENCES

- Balkwill, D. L., Leach, F. R., Wilson, J. T., McNabb, J. F., and White, D. C., Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. *Microbial Ecology*, 16, p. 73, 1988
- Balkwill, D.L., G.R. Drake, R.H. Reeves, J.K. Fredrickson, D.C. White, D.B. Ringelberg, D.P. Chandler, M.F. Romine, D. Kennedy and C.M. Spadoni, Taxonomic study of aromatic-degrading bacteria from deep saturated terrestrial subsurface sediments: proposal of *Sphingomonas aromavorans* sp. Nov., *Sphingomonas subterrestris*, sp. Nov., and *Sphingomonas aquiferans* sp. Nov., *J. Bacteriol.*, submitted.
- Findlay, R. H., and White, D. C., Polymeric beta-hydroxy alkanooates from environmental samples and *Bacillus megaterium*, *Appl. Environ. Microbiol.*, 45, p. 71, 1983.
- Fredrickson, J. K., Mckinley, J. P., Nierzwicki-Bauer, S. A., White, D. C., Ringelberg, D. B., Rawson, S. A., Shu-mei Li, and Brockman, F. J., Microbial community structure and biogeochemistry of Miocene subsurface sediments, *Molecular Ecology*, 4, p. 619, 1995.
- Fredrickson, J.K., D.L. Balkwill, G.R. Drake, M.F. Romine, D.B. Ringelberg and D.C. White, Aromatic-degrading *Sphingomonas* isolates from the deep subsurface, *Appl. Environ. Microbiol.*, 61(5): 1917-1922, 1995.
- Gehron, M. J., and White D. C., Quantitative determination of the nutritional status of detrital microbiota and the grazing fauna by triglyceride glycerol analysis, *J. Exp. Mar. Biol.*, 64, p. 145, 1982.
- Guckert, J. B., Hood, M. A., and White, D. C., Phospholipid, ester-linked fatty acid profile changes in during nutrient deprivation of *Vibrio cholera*: Increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids, *Appl. Environ. Microbiol.*, 52, p. 794, 1986.
- Guckert, J. B., Ringelberg, D. B., White, D. C., Henson, R. S., and Bratina, B. J., Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the proteobacteria, *J. Gen. Microbiol.*, 137 p. 2631, 1991.
- Haldeman, D. L., Amy, P. S., Ringelberg, D. B., and White, D. C., Characterization of the microbiology within a 21 m³ section of rock from the deep subsurface, *Microbial Ecology*, 26, p. 145, 1993.
- Haldeman, D. L., Amy, P. S., Ringelberg, D. B., and White, D. C., Changes in bacteria recoverable from subsurface volcanic rock samples during storage at 4°C, *Appl. Environ. Microbiol.*, 60, p.2697, 1994.
- Haldeman, D.L., P.S. Amy, D. Ringelberg, D.C. White, R.E. Garen, and W.C. Ghiorse, Microbial growth and resuscitation alter community structure after perturbation, *FEMS Microbiol. Ecol.* 17:27-38.
- Haldeman, D.L., The storage-related phenomenon: implications for handling and analysis of subsurface samples, In: *The microbiology of the terrestrial subsurface* (P. Amy and D. Haldeman, eds.), CRC Press, Boca Raton, FL, Chpt. 5. 1997
- Heipieper, H. J., Diffenbach, R. Keweloh, H., Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol degrading *Pseudomonas putida* P8 from substrate toxicity, *Appl. Environ. Microbiol.*, 58, p. 1847, 1992.
- Kieft, T. L., Ringelberg, D. B., White, D. C., Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium, *Appl. Environ. Microbiol.*, 60, p. 3292, 1994.
- Kieft, T.L., E. Wilch, K.O. Conner, D.B. Ringelberg and D.C. White, Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms, *Appl. Environ. Microbiol.*, submitted.
- Kohring, L. L., Ringelberg, D. B., Devereux, D., Stahl, M., Mittleman, M. W., and White, D. C., Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA

sequence similarities among dissimilatory sulfate-reducing bacteria, *F.E.M.S. Microbiol. Letters*, 119, p. 303, 1994.

Lehman, R. M., Colwell, F. S., Ringelberg, D. B., and White, D. C., Drilling mud microbial communities as fortuitous tracers for sample collection in deep terrestrial habitats, *J. Microbiol. Methods*, in press.

Lovely, D. R., Giovannoni, S. J., White, D. C., Champine, J. E., Phillipis, E. J. P., Gorby, Y. A., and Goodwin, S., *Geobacter metallireducans* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals, *Arch. Microbiol.*, 159, p. 363, 1992.

Nickels, J. S., King, J. D., and White, D. C., Poly-beta-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota, *Appl. Environ. Microbiol.*, 17, p. 459, 1979.

Ringelberg, D. B., Townsend, T., Dewaard, K. A., Suflita, J. M., and White, D. C., Detection of the anaerobic dechlorinator *Desulfomonile tiedjei* in soil by its signature lipopolysaccharide branched-long-chain hydroxy fatty acids, *F.E.M.S. Microbiol. Ecology*, 14, p. 9, 1993.

Ringelberg, D.B., S. Sutton, D.C. White, Microbial ecology of the deep subsurface: analysis of ester-linked phospholipid fatty acids, *Microbiol. Ecol.*, submitted.

Tunlid, A., Ringelberg, D. B., Phelps, T. J., Low, C., and White, D. C., Measurement of phospholipid fatty acids at picomolar concentrations in biofilms and deep subsurface sediments using gas chromatography and chemical ionization mass spectroscopy, *J. Microbiol. Methods*, 10, p. 139, 1989.

Vestal, R.J. and D.C. White, Lipid Analysis in microbial ecology; quantitative approaches to the study of microbial communities. *Bioscience* 39(8):535-541, 1989.

Walker, J. T., Sonesson, A., Keevil, C. W., and White, D. C., Detection of *Legionella pneumophila* in biofilms containing a complex microbial consortium by gas chromatography-mass spectrometric analysis of genus-specific hydroxy fatty acids, *F.E.M.S. Microbiol. Letters*, 113, p. 139, 1993.

Welch, D. F., Applications of cellular fatty acid analysis, *Clinical Microbiology Reviews*, 4, p. 422, 1991.

White, D. C., Davis, W. M., Nickels, J. S., King, J. D., and Bobbie, R. J., Determination of the sedimentary microbial biomass by extractable lipid phosphate, *Oecologia*, 40, p. 51, 1979.

White, D.C. and D.B. Ringelberg, Utility of the signature lipid biomarker analysis in determining the in situ viable biomass, community structure and nutritional/physiological status of deep subsurface microbiota, In: *The microbiology of the terrestrial subsurface* (P. Amy and D. Haldeman, eds.), CRC Press, Boca Raton, FL, Chpt. 8. 1997

Yitai Liu, K.F. Jarrell, T.M. Karnauchow, D.L. Balkwill, D. B. Ringelberg and D. R. Boone, Description of two new thermophilic species of *Desulfotomaculum*, *Desulfotomaculum putei* sp. Nov, from a deep terrestrial subsurface and *Desulfotomaculum luciae* sp. Nov, from a hot spring, *J. Bacteriol.*, submitted.

Table 1. Comparison of viable biomass estimates (PLFA) of samples recovered from the drilling of a borehole.

depth ¹	drilling mud	make-up water	cutting	sample core
1725	147 ²	2		
1890	121			
2027	1	15	66	
2217	186	24		
2251	234			
2329			38	
2408	18		8	
2502		12		
2669	33		19	
2743	65			
2793	297			
2798	136			16
2831	98			
3005	188	7		
avg±sd ³	124±88	12±8	33±25	16

¹ depth expressed in meters

² values expressed as pmole PLFA gram⁻¹ of sample except for make-up waters which are expressed as pmole PLFA ml⁻¹ of water filtered

³ values expressed as the average ± a standard deviation

Table 2. Viable microbial biomass and community composition shifts with time in whole (intact) and homogenized sediment cores incubated at 15°C.

incubation ^a	Biomass ^b		Monounsaturates		Terminally Branched Saturates	
	whole ^c	homogenized ^d	whole	homogenized	whole	homogenized
3	0.5±0.4	1.1±1.4	2.9±1.4	2.5±1.8	1.9±2.4	2.1±2.0
5	0.6±0.1	0.6±0.6	5.8±1.4	1.8±1.9	13.6±11.2	3.3±4.0
9	0.6±0.3	.7±0.6	7.4±1.4	2.2±3.1	25.6±7.2	17.2±20.6
16	4.3±3.7	1.5±0.6	6.6±0.8	2.3±2.1	43.3±8.0	5.2±5.5
32	2.0±1.8	0.3±0.2	4.8±1.7	0.9±1.5	35.9±19.4	9.6±12.2

Note: values are expressed as an average ± a standard deviation (n=3)

^a incubation time in weeks at 15°C

^b biomass expressed as picomole PLFA per gram

^c whole indicates an intact core

^d homogenized indicates a core after grinding by mortar and pestle and sieving (2 mm)

Table 3. Comparison of PHA/PLFA ratios in whole and homogenized subsurface sediments over a 32 week incubation period.

time ¹	whole ²	homogenized ³
3	.05±.09	.02±.03
5	0	0
9	.01±.00	0
16	10.7±13.5	5.45±9.45
32	66.7±112	175±275

¹ time expressed in weeks

² intact sediment cores

³ sediment cores homogenized with mortar and pestal and sieved (2mm)

⁴ values expressed as a ratio (PHA/PLFA)

Figure 1. A dendrogram illustration of the results of a hierarchical cluster analysis relating sample core (including core parings), drilling muds and borehole cuttings and make-up waters based on the arcsin transformed mole % of the PLFA profiles. Sample abbreviations are as follows: RP=inner sample paring, CS=outer sample paring, RS=sample core, CU=corehole cutting, DM=drilling mud and MW=make-up water followed by the depth (in meters) from which each sample was recovered.

Figure 2. A plot of the estimated numbers of viable microbial cells (based on PLFA pmole gram⁻¹ concentrations) present in deep subsurface core material recovered from boreholes drilled in four states across the US: WA Washington, SC South Carolina, NM New Mexico and ID Idaho. Numbers of cells were calculated by assuming 1 pmole of PLFA is equivalent to 2.5×10^4 cells (Balkwill *et al.* 1988).

Figure 3. An estimation of the viable microbial biomass distribution across 3 different geologic horizons in cores recovered from a borehole drilled near Yakima, WA.

Figure 4. An estimation of the viable (PLFA) and non-viable (DGFA) biomass present in core material recovered from a borehole drilled near Cerro Negro, NM. Linear correlation are described by r^2 values.

Figure 5. Results of a principal components analysis of the arcsin transformed mole percentages of PLFA the PLFA profiles extracted from deep subsurface core material collected from 4 sites across the US: WA Washington, SC South Carolina, NM New Mexico and ID Idaho. Group #1 was defined to contain the surface samples and the greater vadose zone biomass samples, Group #2 the low biomass vadose zone samples and Group #3 the eastern coastal plain samples.

Figure 6. A plot of the mole % of monounsaturated (mono), terminally branched saturated (terbrsat), i17:0 and a17:0 PLFA in core material recovered from a borehole drilled near Yakima, WA.

Figure 7. A plot illustrating differences in PLFA profiles observed between sediment cores collected from the Mancos shale horizon and the Two Wells sandstone horizon. Error bars indicate one standard deviation.

Figure 8. Viable (PLFA) and non-viable (DGFA) microbial biomass estimates observed throughout the lower portion of an angled borehole drilled near Cerro Negro, NM. The solid line indicates the background level of PLFA detected in procedural controls and the dashed line the background for the DGFA assay. The geologic horizons penetrated by the borehole are indicated in the box to the left of the plot.

Figure 9. Results of a principal components analysis of the arcsin transformed mole % profiles extracted from sediments of ages ranging from present to >740 ka years. Soil types extracted included types C, Bkm and A/Bw. Biomass estimates are based on the assumption that 1 pmole PLFA is equivalent to 2.5×10^4 cells of a typical subsurface bacteria (Balkwill *et al.* 1988).

Figure 10. An illustration of the mean mole % of PLFA extracted from sediments recovered from beneath a soybean field (n=18) and a corn field (n=11). Error bars indicate one standard deviation.

Figure 1.

Figure 3.

Figure 2.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.