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Biochemical Markers for *In Situ* Microbial Community Structure

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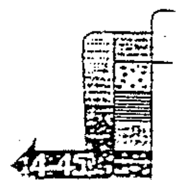
Abstract

Signature biomarker techniques based on phospholipid esterlinked fatty acid pattern analysis (PLFA) provide data on the total viable or potentially viable communities without the necessity of quantitative recovery from the sediments or the ability to culture the organisms. The technique provides evidence for the nutritional status (starvation or unbalanced growth) *in situ*. To perform these analyses on deep subsurface sediments, increasing the sensitivity of the analysis was required. Inverse sequential extraction was coupled with selected ion monitoring, which together provided the necessary sensitivity. Recoveries of PLFA significantly above background and the patterns of PLFA proportions indicated insignificant drilling mud contamination. Ratios of total cell counts to the extractable PLFA, show three degrees of cultural recoverability relative to potential viability based on the presence of PLFA. The degree of cultural recoverability correlates with the permeability of the sediment. The similarities of the total community structure of the deep subsurface show clustering of the most similar PLFA patterns in the permeable sedimentary horizons with distinct differences in the surface soil and impermeable clay aquicludes. The PLFA patterns of the microbiota in recovered drilling muds show no similarity with those from the sediments. Preliminary indications show the PLFA clustering of isolates from the Subsurface Microbiology Culture Collection (SMCC) into four major classes that correspond to distinct fatty acid biosynthetic pathways. The four major types of organisms with specific PLFA patterns are not uniformly distributed throughout the sedimentary column.



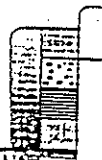
solvents was decreased and the number of the serially extracted sample subsets increased, the number of typical bacterial PLFA patterns increased dramatically at the expense of the contamination patterns. Utilization of several key ions from the mass spectrometric detection system with the "extracted ion chromatography program" increased the sensitivity from 10^{-9} to 10^{-12} molar, so each sample could be run with the GC/MS directly without a preliminary GC analysis. The sensitivity was increased to 10^{-15} molar with the application of smaller extraction volumes and chemical ionization mass spectrometry as the detection system.

Polar lipid analysis. Polar lipids are a part of every cellular membrane. In bacteria and nearly all living cells, the major polar lipids are phospholipids. These polar lipids actively turnover and with cell death they are transformed into neutral lipids. In other words, petroleum contains no phospholipids. Consequently, phospholipids are a measure of the viable or potentially viable cells in a sample. Phospholipids are an excellent measure of the biomass of the subsurface microbiota.[5,6] The estimate of bacterial numbers and biomass from acridine orange direct cell counts (AODC), muramic acid (a unique component of the bacterial cell wall), the polar lipids, and the cellular adenosine triphosphate of a subsurface sediment gave identical values.[1]

Community structure distribution. The permeability, rather than the proportions of clay, sand, and silt, correlated best with the ability of the bacteria to be grown on artificial medium (Table 1) and the similarity of PLFA patterns (Table 2). The hierarchical analysis of PLFA from the subsurface illustrated in Table 2 is independent of isolating or culturing microorganisms from the sediments or the drilling muds, or of artificial groupings of fatty acids as defined by the investigators. The clear differentiation of the microbiota from the surface and all the subsurface sediments from the drilling muds is powerful evidence that there was essentially no significant contamination of the samples by the drilling muds used in sample recovery. The sample that was most suspect of contamination by drilling muds by various investigators was the highly permeable sandstone found in G-16. G-16 clearly contained a microbiota more like the rest of the subsurface active microbiota than the drilling muds. Cluster analysis of the data indicated that the microbiota in G-16 was more closely related to the surface sands that were collected with a flamed shovel, than any drilling muds (Table 2).

Community nutritional status. The parallel detection of high ratios of PLFA to culturable cells and the *in situ* detection of high proportions of cyclopropane or <u>trans</u> monoenoic PLFA in the least permeable sedimentary horizons, clearly indicates these are areas of high stress to the resident microbiota.

PLFA of isolated microbiota from the deep subsurface. The pooled PLFA from 17 isolates collected from different sedimentary horizons were three factored into four major groups by hierarchical cluster analysis. Cluster Group 1 (major PLFA, 18:1*w7c*) represents a pathway of anaerobic desaturase unique to eubacteria, which forms both this PLFA and 16:1*w7c*. Cluster Group 2 shows the other predominant product of the anaerobic desaturase (16:1*w7c*), along with the saturated analogue (16:0). Palmitic acid (16:0) is the most common PLFA found in Gram-negative rods



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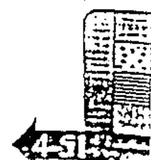
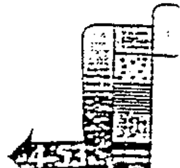


Table 2. Hierarchial Cluster Analysis of Deep Subsurface Field Samples by PLFA Profiles

Sample	Depth[a]	Premeability[b]	Rescaled Distance Cluster Value	
G-5	71	8	4	
G-9	213	7	4	
G-22	526	High	4	8
G-3	120	36	5	14
G-10	239	<0.1	5	16
G-16	406	56		
Surface	0	23		23
G-13	303	<0.1	13	Aquicludes
G-20	437	<0.1	13	25
DM-10	-	-	11	
DM-11	-	-	11	Drilling Muds
DM-12	-	-	11	

[a] Depth in meters.
[b] Permeability in Darcy's.



Q and A

J. Wiegel: How fast do you get the hydrolysis of the substrate?

D. C. White: When they die?

J. Wiegel: Yes. What kind of stage of death do you get?

D. C. White: It is hard to define death, because one must irradiate them so they will not grow. One can detect the lipids of hydrolysis in an hour or two. That was done a long time ago, and it is very fast. The turnover of phospatidydl glycerol, for example, is a very good measure of the bacterial growth rate. As the phosphate is lost in phosphate turnover, there are some lipids such as the phosphatidyl choline that do not turn over very fast. A lot of them, however, do turnover. With the growth rate, they are very active. In growing organisms, if one does a pulse-chase of ^{32}P, one can get a 20-minute half-life. A 10-minute half-life is obtained for phosphate.

J. Wiegel: What happens to those organisms that are down there? Do they really grow down there or are they just remnants? What kind of rate of hydrolysis do you get then? Do you compare those two different stages of being alive?

D. C. White: This has only been done with marine sediments. This is an unfair way to do it, but a whole system was used. I think the question is a very good one. Let's put a little ^{33}P down the well and see.

N. Tonso: Bacteria change their cell walls with their environment. Can you tell me how much you think that growing them on laboratory media will change them from environmental surface stresses?

D.C. White: This happens with every grant I write, in that comments are made to the effect that one can not tell anything about a bacteria by its fatty acids. If one takes a monoculture and puts it in a chemostat, one can really change it. Taking it from its optimum temperature and moving it up 20 degrees can really make it change. One can make some bacteria change, but some will not change at all. What one has to do is look at the conditions in which they exist in nature. For instance, we had some experience, sort of by mistake, with the organism that causes tularaemia. That organism makes a characteristic pattern of ester-linked fatty acid, which we found in soil, in infected rabbits, in pus from humans, and also in pieces of lung. It is obvious that the organism was there; therefore, it does not change. It can not grow under conditions that would change its fatty acid. Now there are organisms that, certainly Lactobacilli for example, can pick up whatever is in the media. Treponema pallidum, as far as we know, picks up human fatty acids. With that one, there is a problem. Every time (in at least eight times) that methane has been added with air to the soil, a Type II methane oxidizer has always been obtained. So its absolutely