

Signature Lipid Biomarker (SLB) Analysis in Determining Changes in Community Structure of Soil Microorganisms

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Abstract

Signature lipid biomarker (SLB) analysis is a useful tool for identifying microorganisms and characterizing microbial communities in natural systems. Specific fatty acids, especially phospholipids (PLFA), are essential membrane components, make up a relatively constant proportion of the microorganisms under natural conditions and their patterns provide insight into the bacterial and fungal community structure and biomass. This method is based on direct extraction of fatty acids from cultured bacteria or environmental samples and determining the isolated methyl ester fatty acids (FAME) using gas chromatography (GC). Several PLFAs are useful markers for the detection of the specific groups, and whole cell fatty acid analysis is used for routine identification of microbial species. The fatty acid analysis has been successfully applied for the characterization of microbial communities from agricultural soils, from sites contaminated with heavy metals, aromatic compounds, alkaline dust, acid rain and from other diverse habitats.

Keywords: fatty acids analysis, PLFA, FAME, microbial community structure, microbial biomass

Introduction

The most difficult task facing microbial ecologists is the quantitative description of microbial communities. Classical microbial tests, which require the isolation and subsequent culture of microorganisms, are not adequate for the analysis of environmental samples. To overcome this difficulty, methods involving direct detection and separation of biochemical and genetic components of mixed populations to use as biomarkers have been developed. Presently, two accepted methods provide a relatively unbiased view of the structure of complex microbial communities. These methods include the examination of microbial population using ribosomal RNA or corresponding DNA sequences, methyl ester fatty acid (FAME) and phospholipid fatty acid (PLFA) analysis.

However, these techniques have limitations that should be viewed and solved in the near future.

Bacterial Membrane Lipids

The primary lipids of the bacterial membrane are the polar glycerophospholipids, although other polar lipids such as sphingolipids, sphingo- and glyceroglycolipids, as well as neutral lipids (hopanoids and carotenoids) may contribute to the structure of the membrane. In many membranes, 50% of the mass is comprised of lipids [1]. Phospholipids are amphipathic molecules containing both a hydrophilic (polar head group) and lipophilic regions (generally two acyl chains). Glycerol forms the backbone of the phospholipid molecule with two or the three hydroxy groups being replaced by fatty acids and the third by a phosphate group (Fig.1). The fatty acid can be ester or ether linked. Ether linkage is rare but has been found

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in the phospholipids of Archae [2-4]. Microbial fatty acids are typically 12-24 carbons long. The most common membrane fatty acids are 14 to 20 carbon long [5, 6]. Typically, phospholipids have one saturated and one unsaturated fatty acid. Unsaturated chains may contain up to six *cis*-double bonds [5]. However, polyunsaturated fatty acids in bacterial membrane are rare, occurring mostly in marine psychrophiles and cyanobacteria [2, 4, 7, 8]. As shown in Fig. 2, the acyl chains of bacterial membrane lipids have various structures and may contain steric features as branches or cyclopropane rings [4, 5]. Gram-positive bacteria contain a large percentage of straight and branched chain fatty acids, up to 50% of the fatty acids are branched in *Bacillus*, *Micrococcus* and *Staphylococcus* genera. However, they contain a low percentage of unsaturated fatty acids. Gram-negative bacteria lack branched chain fatty acids but contain a large proportion of cyclopropane, saturated and (mono)unsaturated fatty acids [4, 9].

Fatty acids are designated by the total number of carbon atoms followed by the total number of double bonds beginning with the position of the double bond closest to the methyl end (ω) of the molecule. The configuration of the double bond is designated by either *c* for *cis* or *t* for *trans*. For example 18:1 ω 9*c* is a PLFA with a total of 18 carbons, 1 double bond located between 9 and 10 carbons from the methyl end of the molecule in the *cis* configuration. The prefixes *a*, *i*, *cy* and *d* refer to *anteiso*, *iso*, cyclopropyl branching and dicarboxylic fatty acids, while a number followed by Me indicates the position of a methyl group (i.e., 10Me16:0). The prefixes α and β indicate that the OH groups of the hydroxyl fatty acids are located at position two or three, numbers preceded by ω indicate the position of OH groups from the aliphatic end of the fatty acids (i.e., 2-OH14:0).

Fatty Acids as Biomarkers

Fatty acids have been a great value in determining bacterial phylogeny, provide a useful set of features for characterizing strains and give important information about microbial communities present. Specific fatty acids, especially phospholipids (with the exception of archaebacteria) have useful properties to be biomarkers. They are degraded

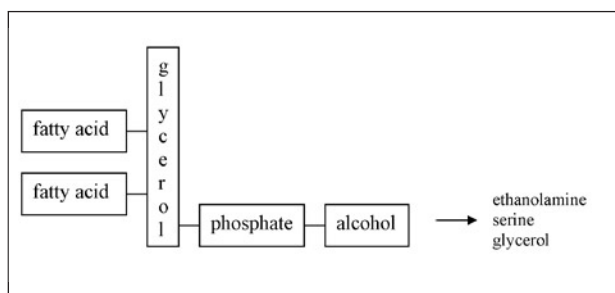


Fig. 1. Schematic structure of a glycerophospholipid with the common polar head groups.

rapidly after cell death, are not found in storage lipids or in antropogenic contaminants and have a high natural turnover rate. Bacteria contain phospholipids as a relatively constant proportion of their biomass. The different subsets of the community have various PLFA/FAME patterns. The fatty acid analysis allows measuring three important attributes of microbial communities: viable biomass, microbial community structure and nutritional/physiological status [6, 10-12]. The schematic presentation of the signature lipid biomarker analysis is shown in Fig. 3.

The total amount of PLFAs as well as ester-linked PLFAs (EL-PLFAs) have been used as indicators of microbial biomass in the environmental samples [13-15]. The viable organisms have an intact membrane containing PLFAs. After cell death or cell lysis, cellular enzymes hydrolyse phospholipids, releasing the polar head groups. The hydrolysis can occur within minutes to hours of cell lysis [16]. The lipid moiety remaining, which is called diglyceride, contains the same signature fatty acids as the phospholipids. The conversion of a phospholipid (PLFA) to a diglyceride (DGFA) as a result of the cell death is illustrated in Fig. 4. An estimation of the total nonviable and the total viable biomass can be made by measuring the diglyceride fatty acids and phospholipid fatty acids, respectively. Parker *et al.* [17] used hydroxy (OH) fatty acids of the lipopolysaccharide (LPS) for estimating the biomass of gram-negative bacteria in soil and sediments. Balkwill *et al.* [13] showed that the viable biomass determined by PLFAs was equivalent to estimations based on intercellular ATP, cell wall muramid acid and acridine orange direct counts. This means that the measurement of PLFAs provides an accurate estimation of the viable or potentially viable microbial biomass.

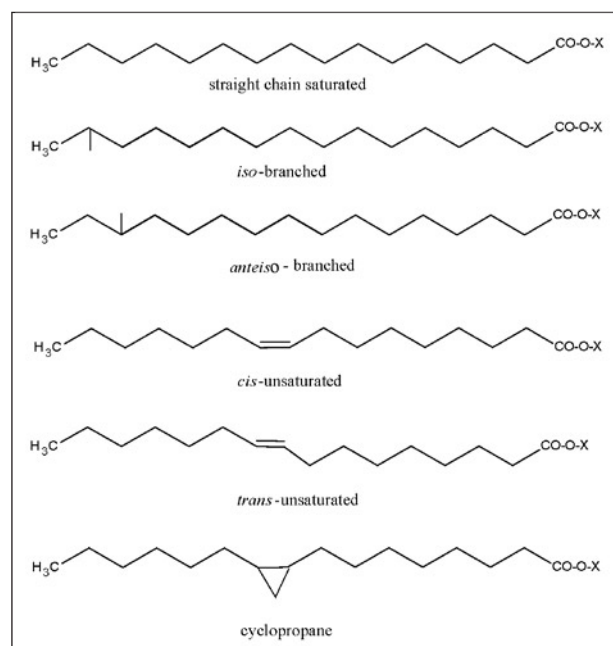


Fig. 2. Various acyl chain conformations possible in bacterial glycerophospholipids [4].

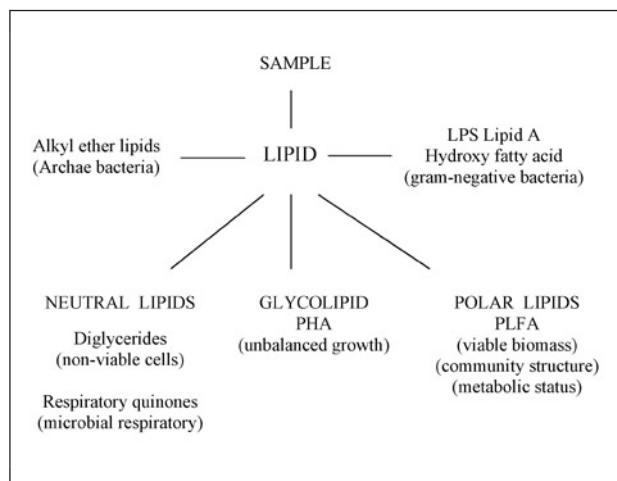


Fig. 3. Schemat of the signature lipid biomarker analysis [11].

Information obtained from the lipid analysis provides insight into the community composition as well. The fatty acids extracted from sediments allowed us to classify distinct microbial groups: microeukaryotes (polyunsaturated fatty acids), aerobic prokaryotes (monounsaturated fatty acids), gram-positive and other anaerobic bacteria (saturated and branched fatty acids in the range from C14 to C16), and sulfate-reducing bacteria including other anaerobic bacteria (saturated and branched in the range from C16 to C19) [6]. Branched fatty acids have been used as biomarkers for bacteria including anaerobic bacteria and the sulfate-reducing bacterium *Desulfovibrio*. Detection can be highly specific for a single strain. The fatty acid 10Me16:0 can be used as a signature for the presence of *Desulfovibrio* since it is not detected in other sulfate-reducing bacteria. Branched-chain fatty acids (*iso* and *anteiso*) are characteristic for gram-positive bacteria and genera *Cytophaga* and *Flavobacterium* [18], whereas cyclopropyl fatty acids are common in some gram-negative strains and some anaerobic gram-positive bacteria [19]. Methyl branching on the tenth carbon atom in the acyl chain is specific for actinomycetes [20]. Gram-negative bacteria contain unique hydroxyl fatty acids (LPS-OH) in the lipid portion of the lipopolysaccharides (LPS) in the cell wall. In the specific applications LPS-OH fatty acids were used as a general indicator of gram-negative bacteria in environmental samples [17, 21]. The monounsaturated fatty acids are characteristic for, but not unique to, eubacteria, including mainly anaerobic gram-negative microorganisms [20]. The polyunsaturated fatty acids occur only in cyanobacteria and they are considered to be signature acids for eukaryotes. High levels of the unsaturated fatty acids with low levels of the polyunsaturated fatty acids support the conclusion of bacterial dominance in the soil sample. In addition, the differences in the relative proportion of branched and monounsaturated fatty acids have been used as a marker for the proportion of gram-positive and gram-negative bacteria in marine sediments [6]. There are also some fatty acids that have been considered

as an indicator for soil fungi in the environment. Wells *et al.* [22] detected long chain unsubstituted fatty acids (24:0-26:0) in hyphal forms of fungi. Linoleic acid (18:2 ω 6) is a good indicator of the number of fungi and fungal biomass in soil [15]. Other fungal biomarkers are 16:1 ω 5 typical for arbuscular mycorrhizal fungi, ergosterol and quinones [23, 24]. The potential application of the marker fatty acids of selected genus, species and microbial groups are presented in Table 1.

Specific pattern of PLFAs can also indicate physiological or nutritional status of bacteria. Lipids are one of the molecules that can adjust in accordance with various environmental disturbances [37]. Environmental factors such as temperature, pressure, pH, water activity, nutrients, ions and chemicals, age of culture and enzyme action can change the shift in microorganisms lipids composition [4, 38-40]. The amount of poly- β -hydroxyalkanoic acid (PHA) in bacteria or triglyceride in the microeucaryota relative to the PLFAs provides characterization of physiological/nutritional status [11]. Under unbalanced conditions, bacteria exposed to adequate carbon source but which lack some essential nutrients form PHA and cannot grow and divide. When the essential components become available these bacteria catabolize PHA and form PLFAs as they grow and divide. Lipid analysis can also indicate the physiological stress in certain bacteria species. Starvation and stationary-phase growth lead to conversion of monoenoic PLFAs (16:1 ω 7 c , 18:1 ω 7 c) to cyclopropane PLFAs (*cy*17:0, *cy*19:0), respectively [27, 41]. The ratio of monoenoic PLFAs/cyclopropane PLFAs varies from organism to organism and environment to environment, but usually falls within the range of 0.05 in log phase to 2.5 or greater in stationary phase. An increase in cyclopropyl PLFA formation has also been associated with increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies [42]. In studies conducted with *Arthrobacter protophormiae*, besides forming the cyclopropyl fatty acids, an increase in the ratio of diglyceride fatty acids to PLFAs was also observed [43]. Exposure of bacteria to organic compounds (alcohols, phenol toxicants, short-chain carboxylic

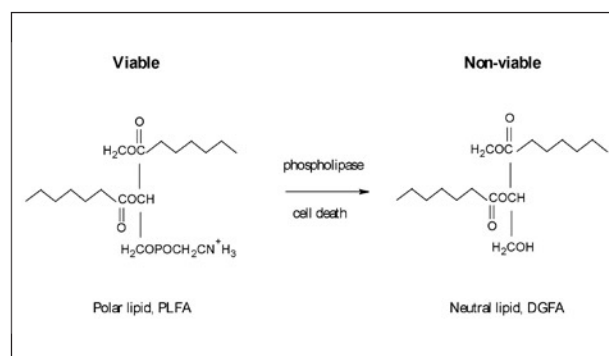


Fig. 4. The conversion of a phospholipid (PLFA) to a diglyceride (DGFA) as a result of cell death [11].

Table 1. Marker fatty acids (FAs) of selected genus, species and microbial group.

Genus, species, microbial group	Lipid biomarker	References
<i>Desulfovibrio</i>	<i>i17:1ω7c, i15:1ω7c, i19:1ω7c</i>	[25]
<i>Desulfobacter</i>	10Me16:0, <i>cy18:0 (ω7,8)</i>	[26]
<i>Vibrio cholerae</i>	11Me19:1, 18:2ω6,9	[27]
Methanotrophs	16:1ω8c, 16:1ω5c	[28]
<i>Desulfomonile tiedjei</i>	LPS-OHFA	[29]
<i>Thiobacillus</i>	<i>i17:1ω5, 10Me18:1ω6, 11Me18:1ω6</i>	[30]
<i>Pseudomonas</i>	16:0 and 16:1 (equivalent proportions), <i>18:1ω7c/ω9t/ω12t</i>	[18]
<i>Arthrobacter</i>	<i>a15:0</i> and 17:0 (high proportions)	[18]
Gram-negative bacteria	OH FAs (e.g. 3OH 16:1, 3OH18:1) monounsaturated FAs (16:1ω7c, 18:1ω7t) cyclopropane FAs (e.g. <i>cy 7:0, cy19:0</i>)	[31] [20] [32]
Gram-positive bacteria	<i>iso</i> and <i>anteiso</i> FAs (e.g. <i>i15:0, a15:0, i17:0, a17:0</i>)	[33] [20, 32]
<i>Actinomycetales</i>	10Me FAs (e.g. 10Me16:0, 10Me17:0)	[34]
Fungi	18:2ω6, 18:3ω3, 18:3ω6 24:0, 26:0 16:1ω5c	[34, 35] [22] [36]
<i>Cytophaga – Flavobacterium</i>	16:1ω5c	[34] [23]

acid) leads to increasing the proportion of *trans* PLFAs compared to the *cis* homologues [44]. For example, *Pseudomonas putida* P8 and *P. putida* S12 make 16:1ω7t or 18:1ω7t fatty acids in the presence of phenol [45, 46]. Similar results were observed in the case of *P. putida* strain mt-2 grown in a medium with the permeating or nonpermeating solute [47]. In addition, *trans/cis* ratios of greater than 0.1 have been shown to indicate starvation in bacterial isolates. This value is usually 0.05 or less in healthy, non-stressed populations.

Physiological status of bacteria may be also determined by analysis of the respiratory quinone structure. Environments with high potential terminal electronic acceptors (oxygen-nitrate) induce the formation of benzoquinones in bacteria whereas bacteria respiring on organic substrates form naphthoquinones. A ratio of total benzoquinones to naphthoquinones provides an indication of the extent of aerobic versus anaerobic microbial respiration. For example, in gram-negative bacteria respiratory quinones are usually 10 to 100 times less in content than PLFAs. By determining the quinone content within a sample, the proportions of aerobic and anaerobic respiration can be estimated [11, 12].

PLFA and FAME Analysis in Microbial Ecology Studies

PLFA and FAME analyses can be used for detection of changes in soil microbial diversity in response to different soil perturbations. The whole cellular fatty acid methyl ester profiles (FAME) are applied for identification of

culturable unknown isolates from environmental mixed samples to taxonomical group and studying their phylogenetic relationships.

FAME Analysis

The ideal system for identification of microorganisms by fatty acid analysis is the Microbial Identification System (MIS) produced by Microbial ID (MIDI, Newark, USA). This method is based on isolation of whole-cell fatty acids from culture of unknown bacteria and matching the fatty acid profiles to one of the fatty acid profiles in a computer database of known microorganisms. The recognition of the fatty acids pattern allows identifying bacteria to the species or subspecies level and establishes taxonomic relationships between the species [32, 48-50]. MIS requires culturing bacteria under standardized conditions (medium, temperature, time of incubation). FAME extraction is based on a four-step procedure and consists of: a saponification, methylation, extraction of the FAMES and sample clean-up. Isolated FAMES are analyzed by gas chromatography (GC).

The FAME method was also used for whole community profile determinations. These profiles may be valuable as a marker of the degree to which communities are similar or different. Thompson *et al.* [51] due to assessment of community diversity by FAME content of bacterial isolates, revealed that the genetically modified *Pseudomonas fluorescens* had less impact on the bacterial community than the wild-type. Haack *et al.* [18] applied principal-components analysis of MIDI-FAMES

profiles in a clear separation of two different communities. They found relative similarities and differences of microbial communities that differed in taxonomic status. In other studies Cavigelli *et al.* [31] used this analysis to identify similarities and differences among soil microbial communities. They observed many differences between FAME profiles of soil and plated communities, indicating that profiles of fatty acids extracted from soil revealed a portion of the microbial community not culturable on medium. Semivariance analysis indicated that spatial distributions of soil microbial populations are maintained in a portion of the microbial community that is selected in laboratory media. This suggests that plated communities are not solely the result of selection by growth medium, but reflect the distribution of the dominant culturable soil microorganisms.

Few studies have focused on the application of the whole soil fatty acid methyl ester profiles as an indicator of changes of microbial community affected by physico-chemical factors such as temperature, pH, matric water potential, and the presence of toxic compounds. For example, Peterson and Klug [52] observed a major shift in the fatty acid profiles in soil at a near freezing temperature and 25°C, whereas Nazih *et al.* [53] did not observe changes in FAMES profiles at 22°C and 34°C. Kozdrój [54] used FAME analysis to estimate microbial community structure in technogenous wastes such as coal-mine spoil, non-ferrous metallurgical slag and coal fly-ash. He observed a high content of 18:2 ω 6,9 in the metallurgical slag, indicating the domination of fungi in this waste. In contrast, representatives of the *Cytophaga-Flavobacterium* group, for which 16:1 ω 5c fatty acid was used as a marker, dominated in the coal fly-ash.

PLFAs Analysis

Phospholipids ester-linked fatty acids have been used for the characterization of microbial community structure in sediments and soils. Rajendran *et al.* [55] analyzed the PLFAs to determine regional differences in microbial groups in sediments from Osaka bay. They indicated the predominance of prokaryotes in the sediment since the abundance of three major groups of C10 to C19 PLFA (saturated, branched and monounsaturated) comprised 84 to 97% of total PLFAs. By examining the PLFAs they distinguished two characteristics of the distribution pattern: the predominance of anaerobic bacteria and gram-positive prokaryotes characterized by a high proportion of branched PLFAs and the predominance of aerobic prokaryotes and eukaryotes as evidenced by the large amounts of monounsaturated PLFAs.

The ester-linked fatty acids in the phospholipids (PLFA) are currently one of the most sensitive and the most useful chemical measures of microbial community structure in the environment. Many studies concerning the changes of PLFA profiles in bacterial community affected by soil pollution have been studied extensively under field and laboratory conditions. Frostgard *et al.* [35], in studies on the influence

of Zn pollution on soil microorganisms, observed that the differences in PLFA pattern due to the Zn pollution increased gradually over time during long-term incubation. They attributed these changes in PLFA patterns to either the gradual development of a microbial community, which became increasingly tolerant over time, or to the successive breakdown of PLFAs of those cells and subsequent proliferation of the organisms feeding on them. Phospholipid fatty acids were also analyzed in a forest humus and in an arable soil experimentally polluted with Cd, Cu, Ni, Pb and Zn at different concentrations. In both soil types, there were gradual changes in the PLFA patterns for the different levels of metal contamination. Several PLFAs reacted similarly to the metal amendments in the two soil types, while others showed different responses. In both soils, the metal pollution resulted in a decrease in the *iso*-branched PLFAs *i*15:0 and *i*17:0 and in the monounsaturated 16:1 ω 5c and 16:1 ω 7c fatty acids, while increases were found *i*16:0, the branched 17:0 and 18:0, and *cy*17:0 fatty acids. In the forest soil the methyl branched PLFAs 10Me16:0, 10Me17:0 and 10Me18:0 increased in metal-polluted soils, indicating an increase in actinomycetes, while in the arable soil a decrease was found for 10Me16:0 and 10Me18:0 in response to most metals [34].

Soil bacterial biomass, pH tolerance and PLFA pattern were studied by Baath *et al.* [56] in a forest area polluted with alkaline dust. The largest proportional increase was found for the fatty acid 10Me18:0, which indicated an increase in the number of actinomycetes in the polluted sites. The levels of the fatty acids *i*14:0, 16:1 ω 5, *cy*17:0, 18:1 ω 7 and 19:1 also increased in the polluted sites while those of fatty acids 15:0, *i*15:0, 10Me16:0, 16:1 ω 7t, 18:1 ω 9 and 19:0 decreased compared with unpolluted sites.

Currently, laboratory studies are performed to provide information on the progress of bioremediation of an oil spill and aromatic hydrocarbon contaminated sites. Macnaughton *et al.* [57] revealed that the community structure of all oiled samples shifted away from that of the unoiled samples with time. The major difference in the PLFA profiles was that the microbial communities in the oiled samples contained significantly more monoenoic PLFAs, more specifically 16:1 ω 7c, 18:1 ω 7c, 16:1 ω 7t and 18:1 ω 7t indicative of gram-negative bacteria than those in the unoiled samples. The greater distance occurred between the oiled and unoiled sample profiles by weeks 4 and 8. By week 14 the microbial populations of the oil plots were becoming similar to those of the unoiled controls from the same time plot. Ringelberg *et al.* [58] observed significant negative correlation between three-ring polycyclic aromatic hydrocarbons (PAHs) and specific PLFA. For example, *i*17:0, correlated significantly with the loss of fluorene, phenanthrene and anthracene, is common to species of *Arthrobacter*, *Streptomyces* and *Rhodococcus*.

Conclusions

Soil is a complex and heterogeneous ecosystem and its characterization still imposes a significant challenge. In particular, analysis of microbial communities of contami-

nated sites is of a great interest. PLFA analyses give valuable information about microbial community structure and biomass, but interpretation of FAME profiles from environmental samples can be difficult. The problem is that many fatty acids are common to different microorganisms and many fatty acids are extracted from each soil sample. Nowadays, several approaches to microbial community profiling based on molecular technique are possible. Apart from PLFA analysis, the second strategy is the sequence information of soil nucleic acid. At the cell level *in situ* hybridization techniques targeting ribosomal RNA, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA) has been recently introduced in microbial soil ecology. The measurements of lipid biomarkers together with genetic methods allow for a more detailed investigation of soil biological features.

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