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Chapter 2

Signature Lipid Biomarker Analysis for Quantitative Assessment In Situ of Environmental Microbial Ecology

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Examination of the lipid components of microbes in recent sediments has provided a convenient, quantitative, and comprehensive method to define the viable biomass, community composition, and nutritional/physiological activities of the biological communities in the sediments. The lipid extraction provides both a concentration and purification of the lipids from the soils and sediments. The subsequent fractionation, purification, and derivatization, sets up the definitive separation and structural identification by capillary gas chromatography with mass spectral identification of each component. As a part of this signature lipid biomarker (SLB) analysis, the lipid extraction also lyses the cells and allows for recovery of purified nucleic acids for subsequent gene probing with and without enzymatic amplification. This polyphasic analysis adds powerful specificity to the analysis of community microbial ecology. Since the SLB analysis involves detection by mass spectrometry, rates of incorporation of non-radioactive ^{13}C and ^{15}N mass-labeled precursors into signature biomarkers can be utilized to gain insight into specific metabolic activities. Application of electrospray and other external ionization sources to ion-trap mass spectrometry will greatly increase the specificity and sensitivity of the SLB analysis.

The assessment of the microbes and their *in situ* interactions in various environments has proven to be a major problem as it has become increasingly apparent that communities of microbes act differently in geochemical cycles than the sum of the isolated individuals. This has required the application of non-traditional methodology. Classical microbiological methods, that were so successful with infectious disease, have severe limitations for the analysis of environmental samples. Pure-culture isolation,

esting, and/or enumeration by direct microscopic counting or most
ıber (MPN) destroy most of the interactions between the various
within the environment. These disruptive methods requiring isolation are
d for the estimation of total biomass or the assessment of community
within environmental samples. Moreover, these classical methods provide
ıto the *in situ* phenotypic activity of the extant microbiota because several
ues depend on microbial growth and, thus, select against many
ns which are non-culturable under a wide range of conditions. It has been
cumented in the literature that viable counts or direct counts of bacteria
diment grains are difficult to quantify and may grossly underestimate the
ommunity composition of the existing community *[1-5]*. In addition, the
ts including the new molecular biomarker technologies *[6]* provide little
he *in situ* nutritional status or evidence of toxicity within the microbial

towards the comprehensive analysis of extant microbial communities is
MIDI, Microbial Identification System (Microbial ID, Inc., Newark, DE).
permits measurement of free and ester-linked fatty acids from
ns which have been **isolated** and subsequently **cultured** from the microbial
This has been commercialized, and the MIDI system sells a comprehensive
terial isolates are identified by comparing their fatty acid profiles to the
e which contains over 8000 entries. The utilization of this system for
of clinical isolates has been remarkably successful. However, application
ystem to the analysis of environmental samples has significant drawbacks.
tem was developed to identify clinical microorganisms and requires their
ulture on trypticase soy agar at 27°C. Since many environmental isolates
grow under these restrictive growth conditions, the system does not lend
ication of some environmental organisms. Other culture conditions that
orrespond to environmental conditions in soils and sediments allow
ıter numbers of organisms, but they often require the generation of new
the MIDI system, the identification of the specific ester-linked
s methyl esters is by their gas chromatographic mobility without mass
mation. Components can be misidentified, and some specificity can be

e holistic community-based analysis that has proven to be more applicable
or biomarker analysis of sediments is based on the one-phase liquid
ctionation of the lipids, sequential hydrolysis of lipid components, and
followed by quantitative analysis/identification using gas chromatography/
ıetry (GC/MS). Research thus far has concentrated on several unique
s, including steroids, diglycerides (DG), triglycerides (TG), respiratory
, mycocerosic acids and phenol waxes in the neutral lipid fraction. The
tion contains the poly β-hydroxyalkanoate (PHA) and the complex
ides. The polar lipid fraction contains the phospholipid lipid fatty acids
mino acids, plasmalogens, acyl ethers, and sphingolipids. The lipid-
ue also contains covalently linked lipids that can be released (after acid
and extracted and derivatized. Of these the lipopolysaccharide hydroxy
S-OHFA) can be analyzed by GC/MS. The combination of these SLB
e used to characterize microorganisms or communities of microorganisms

All living cells are surrounded by a membrane containing polar lipids. Lysis of the cellular membrane results in cell death. Since the major polar lipids in sediments are phospholipids, the component fatty acids in phospholipids are one of the most important SLB classes. PLFA are essential membrane components of living cells. Unlike most other biomarkers, phospholipids are typically degraded within hours following cell death *[7]*. This rapid degradation of the phospholipids establishes the PLFA as ideal biomarkers for viable cells. Thus, the quantification of total PLFA is an accurate measurement of living biomass.

PLFA are particularly useful biomarkers because microbes contain a wide variety of structures that are readily determined by GC/MS, and this structural insight can be utilized in taxonomic identification. Different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, and PLFA are effective taxonomic markers useful for defining community composition. There is great overlap in PLFA composition amongst many species, so defining each species with a unique pattern of PLFA is impossible in a community analysis. Only about 1% of the organisms in the total sedimentary community has actually been isolated and had their PLFA analyzed. However, the patterns of PLFA when elucidated by careful structural identification of each component by mass spectrometry, and in combination with a more comprehensive analysis of the other lipid components, have proven to be very useful as a community analysis tool. The PLFA pattern of a sediment is much like the IR spectrum or a TOF-SIMS (time of flight secondary ion mass spectrometry) analysis of a complex macromolecule--all the information is there, but it is often not specifically interpretable as to which component is responsible for each spectral line. What is readily interpretable are changes in over-all patterns. Thus, similarities and differences between **communities** can be quantitatively defined. PLFA and other biomarkers have been successfully extracted from environmental matrices such as soils and sediments providing a means for direct *in situ* measurements of the microbiota. PLFA patterns recovered from different microbial communities (Figures 1, 2) illustrate the differences in the PLFA component of the SLB analysis of a subsurface soil and ground water membrane filter retentate from a different site.

Generation of electron withdrawing derivatives such as pentafluorobenzyl esters for GC/MS analysis utilizing negative ion detection gives femtomolar sensitivities that are equivalent to hundreds of bacteria the size of *E. coli [8]*. Microbes contain fatty acids with methyl branching at the *omega* (alkyl, ω) end, the iso-branched fatty acids, or 2 carbons from the ω end, the anteiso branched fatty acids, or branches at various mid chain positions. Multiple methyl branches characterize the micocerosic acids that are particularly useful in defining the pathogenic mycobactria *[9]*. Double bonds are useful as microbes contain monoenoic PLFA with the double bond usually at the ω 7 or ω 9 position which can be in the *cis* or *trans* configuration. The ω9 monoenoic PLFA are formed from the aerobic desaturase pathway common to all cells, whereas the ω7 PLFA are formed from the anaerobic desaturase pathway that is most often a prokaryotic biochemical pathway *[4]*. Other bacteria can form monoenoic PLFA with the unsaturation in unusual positions that are often characteristic of the distinct physiologic

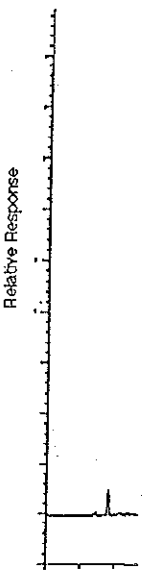

Figure 1.
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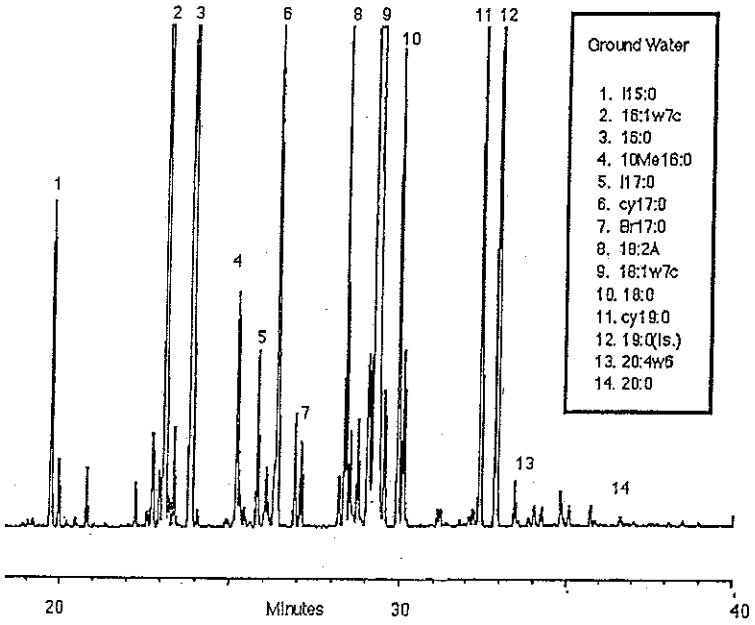

PLFA patterns from a subsurface soil sample. Lipids were extracted from
l, the polar lipid transmethylated and analyzed by GC/MS. PLFA are
e number of carbon atoms: number of double bonds and position of the
l counting from the alkyl or omega (ω) end of the molecule followed by
for *trans* conformation. Prefixes indicate the position counting from the
n atoms from the carboxyl end of the molecule with OH for hydroxyl, br
hing, and cy for cyclopropane ring. Prefixes i represents iso or a for
ranching in the PLFA. Responses indicate relative proportions based on
detection with 19:0 as internal standard.

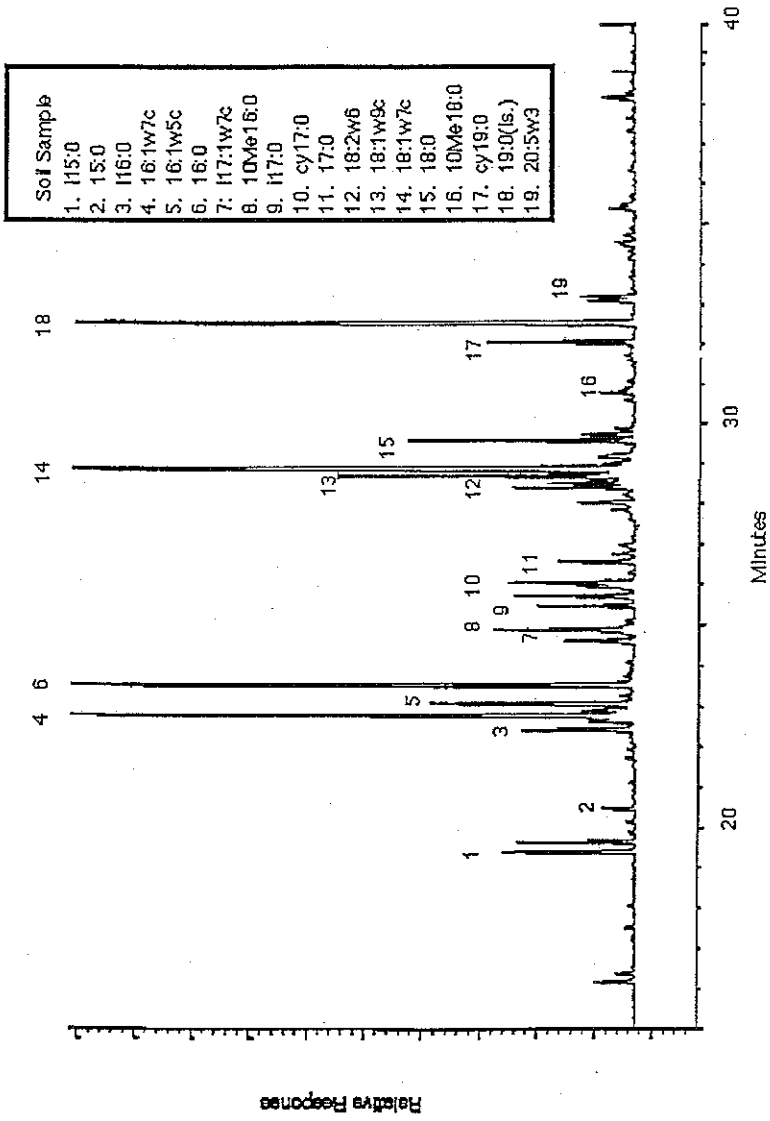

Figure 2 PLFA patterns from ground water sample analyzed as in Figure 1 from a membrane filter retentate (0.2 µm pore diameter) from a different site than Figure 1.

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:teria, plants, or fungi *[10]*. The position of the unsaturation can be readily / fragmentation of dimethyldisulfide adducts of fatty acid methyl esters S *[11]*. Multiple double bonds containing (polyenoic) PLFA are rare in 'A may also have cyclopropane rings that form from monoenoic PLFA in ls. These are best assessed after mild alkaline transesterification because tive to acid conditions, and artifiacts (o-methoxy esters) may result *[12]*. xy PLFA are known in some bacteria as are PLFA with multiple methyl or ents on the carbon chain *[13]*. Hydroxy fatty acids with substitution most or 3 position, from the carboxyl, are important biomarkers. LPS-OHFA are most often 3-OH whereas sphingolipid derived OHFA are often 2-0H

arisons of PLFA patterns by hierarchical cluster analysis shows)etween individual species of methane-oxidizing bacteria that exactly)nships defined by biochemical activities and the phylogenetic relationships :equence homology of 16S ribosomal RNA *[14]*. Sequence homology of l RNA indicates close evolutionary relationships. With time these highly le sequences diverge, so differences can be related to evolutionary events. y of the PLFA patterns of sulfate-reducing bacteria showed that)ased on PLFA patterns paralleled the evolutionary phylogeny based on)omology of 16S ribosomal RNA with the exception of one species *[15]*. species could possibly establish that some substantial part of its genome rizontal gene transfer. Horizontal gene transfer is often detected as gene en different species. Hierarchical cluster analysis of the micocerosic acids alcohols of 19 isolated and culturally defined species of mycobacteria)nships that parallel the physiological properties and genetic analyses of *9]*.

r the knowledge of specific lipid biosynthetic pathways can provide insight)logical or nutritional status of the microbial community. Certain fatty *trans* and cyclopropyl PLFA, provide an indications of environmental)w).

are extracted with organic solvents and then separated into compound olumn chromatography. The effectiveness of the lipid extraction can be mparing the extraction before and after sequential alkaline and acid ch will liberate all the ester and amide linked fatty components of the cases increasing the temperature and pressure of the extraction can crease the yield of lipid and, thus, more effectively indicate the true tions containing SLBs may be further separated by thin layer or liquid ly, and individual SLB can be determined after sequential mild alkaline nild acid methanolysis and strong acid methanolysis *[10]*. Utilizing mild sterification for the ester-linked lipid components avoids confusing s free fatty acids or loss of acid-sensitive lipid components *[4,7]*. This me (Figure 3) has been successfully applied for quantitatively assessing nunities (bacteria, fungi, protozoa, and metazoa) in slimes, muds, soils, , bioreactors, and sediments *[1-5,10, 16]*. SLB methodology provides a

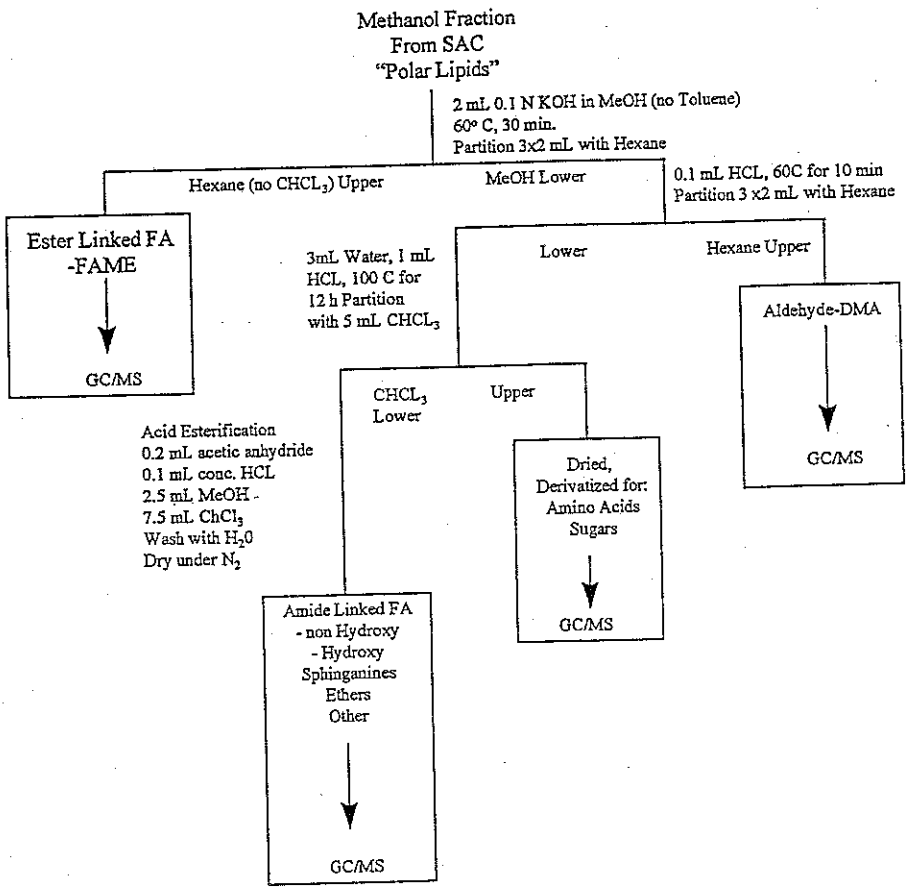

Figure 3. Sequential hydrolysis of lipid samples to define ester, vinyl ether, alkyl ether, and amide linkages in components of the lipids.

ans to measure: 1) viable microbial biomass, 2) microbial community
ıd 3) community nutritional status.

ıble Biomass The determination of the total phospholipid ester-linked
FA) provides a quantitative measure of the viable or potentially viable
viable microbes have an intact membrane which contains phospholipids
Cellular enzymes hydrolyze and release the phosphate group within
rs following cell death *[7]*. The lipid component that remains is
G). The resulting diglycerides contain the same signature fatty acids as
ospholipid, at least for days to years in the subsurface sediments.
a comparison of the ratio of phospholipid fatty acid profiles to
y acid profiles provides a measure of the viable to non-viable microbial
composition. The DG to PLFA ratio increases in many subsurface
ı <0.2 at the surface to over 2.0 at > 200 m *[17]*. A study of subsurface
ed that viable biomass as determined by PLFA was equivalent (but with a
tandard deviation) to that estimated by intracellular ATP, cell wall
and very carefully conducted acridine orange direct counts (AODC) *[18]*.
onverting viable biomass in chemical terms to numbers of microbes has
l comprehensively *[19]*, and the number of cells/gm dry weight can vary
ı an order of magnitude in different environments and for different

ımmunity Composition The presence of certain groups of microorganisms
l by the detection of unique lipids that originate from specific biosynthetic
]. Consequently, the analysis of SLB classes provides a quantitative
ıe microbial community. For example, specific PLFA are prominent in
sulfate-reducing bacteria, whereas the *Desulfobacter* type of sulfate-
ria contain distinctly different PLFA *[20,21]*. Despite the fact that that only
he sedimentary microbes in a given sample can be isolated and the SLB
SLB analysis has proven very useful in the characterization of the extant
munity. The SLB analysis of sediments provides community composition
biomarkers for groups of organisms that are reasonably expected to be in
ommunity. The induction of microbial community compositional shifts by
icroenvironment results in changes that are often predictable, based on past
th microbial communities. For example, biofouling communities incubated
altered pH in the presence of antibiotics and specific nutrients resulted in a
ominated by fungi, while other conditions resulted in a community dominated
ively by bacteria *[22]*. In these experiments, the morphology of the biofilm
ng electron microscopy correlated with the chemical analysis *[22]*. Similar
howed that light-induced shifts which occurred within microbial biofilms
xpected changes in morphology and shifts in signature lipid biomarkers.
specific organism or groups of organisms with the subsequent detection of
ınisms by signature lipid analysis in consortia under conditions where their
ıduced has been done in microcosms *[3]*. It is possible to induce a "crash" in
sis in a bioreactor by inducing the growth of sulfate-reducing bacteria, or by
of chloroform or oxygen. These crashes are accompanied by shifts in the

signature lipid biomarkers that are correlated to the changes in the microbial communities. Specific microbes whose signature biomarkers have been determined in isolates can be detected in biofilm communities, clinical specimens, or subsurface soils. Specific sulfate-reducing bacterial groups can be "induced" in estuarine muds as can methane-oxidizing populations, or propane-oxidizing actinomycetes through the addition of appropriate substrates. Again, all of these community shifts are evidenced by measurable changes in lipid signatures and in lipid patterns. An additional validation is the detection of specific shifts in microbial communities as a result of specific grazing by predators. Results of the signature lipid biomarker analysis agreed with the cellular morphologies present as shown by scanning electron microscopy. These validations for the SLB have been reviewed *[3,10].*

The analysis of other lipids such as the sterols (for the microeukaryotes--nematodes, algae, protozoa) *[22],* glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids from the lipid A component of lipopolysaccharide of gram-negative bacteria *[23,24],* sphinganines from sphingolipids *[25],* fatty dimethyl acetals derived from vinyl ether containing plasmalogens *[4],* and alkyl ether polar lipids derived from the *Archae [26]* can provide a more detailed community composition analysis.

3). **Nutritional/Phsiological Status** Growth without cell division can occur in microbes when carbon source(s) and terminal electron acceptors are present but some essential nutrient is missing. Under these conditions of "unbalanced growth" bacterial can accumulate poly β-hydroxyalkanoic acid (PHA) *[27-29]* and microeucaryotea accumulate triglyceride *[30].* The relative amounts of these endogenous storage lipids, as compared to the PLFA, provides a measure of the nutritional status. Specific patterns of PLFA can indicate physiological stress *[31,32].* For example exposure to toxic environments can lead to "minicell" formation and a relative increase in specific PLFA. Increased conversion of *cis* to *trans* PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol or organic solvents in the absence of bacterial growth *[33].* Increasing proportions of *trans*-monoenoic PLFA is not the only critical feature of solvent resistance in *Pseudomonas putida.* Comparison of a solvent sensitive strain of *P. pudita* to *P. pudita* (Idaho) strain which is resistant to saturating concentrations of solvents and surfactants, showed that both exhibited increases in *trans*-monoenoic PLFA. The solvent resistant strain also shifts its lipid composition by increasing the proportion of monoenoic PLFA to saturated PLFA, increasing the lipopolysaccharide hydroxy fatty acids, and decreasing permeability to the hydrophobic antibiotic difloxacin *[34].* These changes were not detected in the solvent sensitive strain *[34].* Prolonged exposure to conditions inducing stationary growth phase such as limiting concentrations of carbon substrates can induce the formation of cyclopropane PLFA *[4, 31,32].* Respiratory quinone composition can be utilized to indicate the proportion of microbial metabolism that is aerobic *[35].* Environments with high potential terminal electron acceptors (oxygen, nitrate) induce formation of benzoquinones in Gram-negative bacteria in contrast to microbes respiring on organic substrates form naphthoquinones *[35].* Some specific but useful insights come from analysis of organisms like the *Pseudomonas* species which form acyl-ornithine lipids

; with limited bioavailable phosphate *[36]* while some gram-positive
increased levels of acylamino acid phosphatidylglycerols when grown at
;id pH levels *[37]*.
:ly it has been shown that the solvent extraction utilized in the SLB lyses
/ironmental matrix which facilitates the subsequent extraction of nucleic
lipid-extracted residue *[38]*. These cellular nucleic acids can be used for
)lification and gene probing *[38]*. Over 50% of the gene *nahA* (an
)hthalene catabolism) present in intact *Pseudomonas fluorescens* cells
vas recovered using the lipid extraction protocol as compared to recovery
d techniques *[39]*. The DNA recovered from the lipid extraction was of
nd is suitable for enzymatic amplification. The combined lipid extraction
)f nucleic acids can be very useful in biomass and community composition
;. The DNA probe analysis offers powerful insights because of the
ificity in the detection of genes in the extant microbial community. The
)NA/lipid analysis readily provides quantitative recoveries independent of
)olate or culture the microbes. The lipid analysis gives indications of the
)perties of the community that indicates extant microbial activity by
tu indications of starvation, growth rate, exposure to toxicity, unbalanced
encies of specific nutrients, and the aerobic/anaerobic metabolic balance
DNA probes define the physiologic potential and highly specific
mposition of the microbial community. The combined DNA/lipid analysis
ne deficiencies in microbial ecology studies involving only nucleic acid

.t SLB methodology requires procedures that are time consuming and
and extraction procedures require extensive attention to detail in the
solvents, reagents and glassware. The interpretation of the SLB analysis
:ensive understanding of a widely dispersed database. New methods to
omate the SLB procedures and combine them with nucleic acid analysis
nder active development so this quantitative analysis can be much more

ıents

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