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Quantitative comparisons of *in situ* microbial biodiversity by signature biomarker analysis

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Microscopic examinations have convinced microbial ecologists that the culturable microbes recovered from environmental samples represent a tiny proportion of the extant microbiota. Methods for recovery and enzymatic amplification of nucleic acids from environmental samples have shown that a huge diversity exists *in situ*, far exceeding any expectations which were based on direct microscopy. It is now theoretically possible to extract, amplify and sequence all the nucleic acids from a community and thereby gain a comprehensive measure of the diversity as well as some insights into the phylogeny of the various elements within this community. Unfortunately, this analysis becomes economically prohibitive if applied to the multitude of niches in a single biome let alone to a diverse set of environments. It is also difficult to utilize PCR amplification on nucleic acids from some biomes because of co-extracting enzymatic inhibitors. Signature biomarker analysis which potentially combines gene probe and lipid analysis on the same sample, can serve as a complement to massive environmental genome analysis in providing quantitative comparisons between microniches in the biome under study. This analysis can also give indications of the magnitude of differences in biodiversity in the biome as well as provide insight into the phenotypic activities of each community in a rapid and cost-effective manner. Applications of signature lipid biomarker analysis to define quantitatively the microbial viable biomass of portions of an Eastern USA deciduous forest, are presented.

Keywords: microbial biodiversity; signature lipid biomarkers (SLB); phospholipid fatty acids (PLFA); *in situ* microbial biomass; community composition; nutritional/physiological status; non-culturable viable biomass

Introduction

Classical microbiological methods, that were successful with infectious disease, have severe limitations for the analysis of environmental samples. Pure-culture isolation, biochemical testing, and/or enumeration by direct microscopic counting or most probable number (MPN) are not well suited for the estimation of total viable biomass or the assessment of community composition within environmental samples. These classical methods provide little insight into the *in situ* phenotypic activity of the extant microbiota since several of these techniques are dependent on microbial growth and, thus, select against many environmental microorganisms which are non-culturable under a wide range of conditions. The classical methods provide little insight into the evolutionary phylogeny of the microbes present. It has been repeatedly documented in the literature that viable counts or direct counts of bacteria attached to sediment grains are difficult to quantify and may grossly underestimate the extent of the existing community [80,84–88,95,96]. The traditional tests provide little indication of the *in situ* nutritional/environmental status, the evidence of toxicity, or evidence of phenotypic expression of specific metabolic activities.

A quantitative direct analysis has been developed, whereby lipids and DNA are recovered from environmental samples [42,96]. The method consists of the extraction of the lipids and lysis of the cells for subsequent recovery of the DNA [42,96]. The method consists of the extraction of the lipids, their fractionation, derivatization, and subsequent quantitative analysis using gas chromatography/mass spectrometry (GC/MS) [80,84–88,95,96]. Several unique classes of lipids, including steroids, diglycerides (DG), triglycerides (TG), respiratory quinones (RQ), poly β-hydroxyalkanoates (PHA), phospholipid lipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl ethers, sphingolipids, and lipopolysaccharide hydroxy fatty acids (LPS-OHFA) can be used as signature lipid biomarkers (SLB) to characterize microorganisms or communities of microorganisms. DNA is then extracted from the lysed cells left after the lipid extraction [42].

Methods

All cells capable of independent existence are surrounded by a hydrophobic membrane. This membrane must be intact for the cell to be viable. Essential components of the limiting membrane are the lipids which are extractable from the cells with organic solvents [83]. Once extracted, the lipids can be readily fractionated into neutral lipids, glycolipids and polar lipids based on their polarity with silicic acid chromatography [28,29]. Each fraction of the intact lipids can be further fractionated into lipid components [28,29]. Each component is then hydrolyzed by transesterification to form esters of the ester-linked components followed by strong acid alcoholysis to yield the amide and vinyl ether-linked components [50]. These individual components can

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be derivatized further and quantified by capillary gas chromatography (GC) with identification by mass spectrometry (MS) [29,72]. Some of the intact lipid components such as the respiratory quinones [12,34], isoprenoid ether components [37,38,51], or phospholipids can be separated by supercritical fluid chromatography [37] and/or high performance lipid chromatography (HPLC) coupled with electrospray ionization (ES) or atmospheric pressure chemical ionization MS for detection [46]. The lipid-extracted soil residue can be re-extracted to yield cellular nucleic acids which can be enzymatically amplified and probed in many cases with sequences specific for enzymes, kingdoms, families, genera, or strains [42]. Preliminary work with the lipid extraction has resulted in increased yields of nucleic acids from spores and Gram-positive bacteria showing a substantial decrease in humics and other components which specifically inhibit enzymatic amplifications of specific sequences by polymerase chain reaction (PCR).

The lipid analysis has been applied to assessing microbial communities (bacteria, fungi, protozoa, and metazoa) in slimes, muds, soils, rhizospheres, filter retentates, bioreactors, and sediments [80,84–88,95,96]. The signature lipid biomarker methodology provides a quantitative means to measure: (1) viable microbial biomass; (2) microbial community composition; and (3) community nutritional/physiological status.

(1) Viable biomass

The determination of the total phospholipid either as ester-linked fatty acids (PLFA) or lipid phosphate (LP) provides a quantitative measure of the viable or potentially viable biomass. All intact cells contain polar lipids. Polar lipids in microbes are primarily phospholipids. Determination of LP or PLFA provides a quantitative measure of the microbial biomass that contains intact cellular membranes. Either LP or PLFA determination is a measure of the viable microbial biomass because organisms without intact cellular membranes are not viable. With cell death, exogenous and endogenous phospholipases transform the polar lipids in the cell membranes to non-polar neutral lipid diglycerides (DG) by removing polar phosphate-containing head groups within minutes to hours [83]. The resulting DG contain the same signature fatty acids as the original phospholipid, at least for some time. Consequently, a comparison of the ratio of PLFA profiles to DG fatty acid profiles provides a measure of the viable to non-viable microbial abundance and composition. The DG to PLFA ratio increases in many subsurface sediments from <0.2 at the surface to over 2.0 at >200 m [93,94].

Viable microbial biomass can be determined from quantifying organic phosphate present in the polar lipid fraction of the lipid extract using a relatively simple colorimetric analysis [10,21,27,83]. The sensitivity of the classical colorimetric analysis for LP (for environmental samples [83]) has been improved considerably with sensitivities of 1 nmole of LP (which corresponds to approximately 10^7 bacteria through the use of a dye-coupled reaction [21]). Higher sensitivities and specificity (about 10 femtomoles by GC/MS, single ion monitoring of negative ions) with a concomitant determination of the non-viable cell biomass, the community composition, and the nutritional/physiological status can be obtained through the application of the various GC/MS methods [63].

One of the major problems with biochemical biomass measures is that the results are in micromoles (μmoles) of component per gram of soil or sediment. Since microbiologists traditionally think of biomass as the number of cells in a gram of soil or sediment, supposedly a simple conversion can be made after determination of the biomarker content of monocultured cells whose numbers have been determined. Problems in equating acridine direct counts (AODC), with their accuracy problems [21,53], to PLFA or LP estimates derive from the lack of a universally applicable conversion factor [10,21,24,27,83]. The use of conversion factors has been discussed [92]. In any application of a conversion factor it is important to note that the number of cells per gram of dry weight can vary by up to an order of magnitude [24].

In one specific environment, a subsurface sediment containing a sparse prokaryote community of minicells (demonstrated microscopically), a comparison of methods for determination of microbial biomass showed equivalence. In these sediments, the viable biomass determined by PLFA was equivalent (but with a much smaller standard deviation) to the viable biomass estimated by intracellular ATP, cell wall muramic acid, LP, and very carefully done acridine orange direct counts [3]. This equivalence was based on assuming 2.5×10^{12} cells per gram of dry weight was equivalent to 100 μmoles PLFA per gram of dry weight based on experiments with subsurface isolates [3]. Generally, environmental bacteria growing in dilute media in the laboratory or as mixed bacterial populations have average volumes of 0.48 ± 0.2 μm^3 [8]. It has been our experience from a wide variety of environmental samples that the relationship between the PLFA and estimated number of cells varies by a factor of at least 4.

(2) Community composition

Lipid analysis provides insight into the community composition as well as the viable and recently lysed microbial biomass. The lipid component patterns provide a quantitative analysis but may not provide definitive interpretation of shifts in specific microbial groups because of overlapping compositions amongst constituents. However comparisons of total community PLFA and/or sterol patterns accurately mirror shifts in community composition and provide an excellent way to correlate community composition to specific metabolic properties. PLFA analysis can provide insight into the phylogenetic relationships between organisms similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA [31,47].

As an example, specific PLFA such as i17:ω7c are prominent in *Desulfovibrio* sulfate-reducing bacteria, whereas the *Desulfobacter* type of sulfate-reducing bacteria contain distinctly different PLFA [16,17]. Although normal (straight chain) saturated PLFA are found in both prokaryotes and eukaryotes, bacteria generally contain greater amounts of the 16-carbon moiety (16:0) whereas the microeukaryotes contain greater amounts of the 18-carbon moiety (18:0). Methylotrophs are an exception to this rule, generally making more 18:0 than 16:0 [59]. Terminally-branched saturated PLFA are common to Gram-positive bacteria but also

to some Gram-negative anaerobic bacteria, such as the sulfate-reducing bacteria [29,68,80]. Monoenoic PLFA are found in almost all Gram-negative microorganisms and many types of microeukaryotes. Specific groups of bacteria form monoenoic PLFA with the unsaturation in an atypical position, such as 18:1ω8c in the type II methane-oxidizing bacteria [59,61]. Polyenoic PLFA generally indicate the presence of microeukaryotes, but have also been sparingly reported in some bacteria. The PLFA 18:2ω6 is prominent in fungi but is also found in algae and protozoa. Polyenoic PLFA with first unsaturation in the ω6 position are classically considered to have an animal origin whereas organisms with the first unsaturation in the ω3 position are generally considered to be of either plant or algal origin. Normal saturated PLFA longer than 20 carbons are typical of the microeukaryotes. There are exceptions to these generalizations. Branched-chain monoenoic PLFA are common in the anaerobic *Desulfovibrio* type sulfate-reducing bacteria both in culture and in manipulated sediments [17,68]. They are also found in certain *Actinomycetes*, which, as a group contain mid-chain branched saturated PLFA, in particular 10Me18:0 with lesser amounts of other 10 methyl-branched homologues [1,9]. Environments with 10Me16:0>>10Me18:0 often feature anaerobic Gram-negative *Desulfobacter* type sulfate-reducing bacteria [16,68].

However, it must be kept in mind that signature lipid biomarker analysis cannot detect every species of microorganism in an environmental sample as many share overlapping PLFA and patterns. The analysis of other lipids such as the sterols (for the microeukaryotes—nematodes, algae, protozoa) [11,56,57,76,77,95], glycolipids (phototrophs, Gram-positive bacteria), or the hydroxy fatty acids in the lipopolysaccharide of the lipid A of Gram-negative bacteria (LPS-OHFA) [5,45,49,67,73,81,84], sphinganines from sphingolipids [25], fatty dimethyl acetals derived from vinyl ether containing plasmalogens [50,93], and alkyl ether polar lipids derived from the *Archae* [35–38,51,61] can provide a much more detailed community composition analysis than the PLFA patterns alone. Sterol types and patterns are very helpful in identifying microeukaryotes, especially when combined with PLFA results [11,56,57,76,77,95]. For example, cholesterol is prominent in protozoans such as *Cryptosporidium*, ergosterol is found in many fungi [56,57], and algae [11,83,94] contain a diversity of sterols in patterns which are useful in forming taxonomic relationships. Often, proportions of isoprenologues of the respiratory quinones can be helpful in speciation. Benzoquinone, with 13 isoprenologue units in the side chain, is found uniquely in *Legionella pneumophila*.

(3) Nutritional status

The lipid composition of microbes is the product of metabolic pathways and thus reflects the phenotypic response of the organism to the environmental niches encountered in the specific sample.

Poly β-hydroxyalkanoic acid (PHA) in bacteria [15,19,62], and triglycerides [28] in microeukaryotes are endogenous storage lipids. The relative amounts of these compounds, as compared to the PLFA, provides a measure of the nutritional status. Many bacteria form PHA under conditions of unbalanced growth when a carbon source and terminal electron acceptor(s) is present but cell division is limited by the lack of some essential nutrient [19,62]. When the essential component becomes available, these bacteria catabolize PHA and form PLFA as they grow and divide. For example the ratio of PHA/PLFA in rhizosphere microbes from *Brassica napus* planted in sand and recovered from roots was <0.0001 compared to 6.6 for bacteria not associated with the rootlets [79].

Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community as certain fatty acids, such as *trans* and cyclopropyl PLFA, provide an indication of environmental stress [30,39,43,76]. Bacteria make *trans*-monounsaturated fatty acids as a result of changes in the environment, usually as a result of stress- (ie toxicity, exposure to solvents, alcohols, or acids) induced changes in PLFA [76]. Ratios of 0.05 or less indicate healthy, non-stressed communities. Starvation can lead to minicell formation and a relative increase in specific *trans*-monoenoic PLFA when compared to the *cis*isomers [30]. *Trans/cis* ratios of greater than 0.1 generally indicate exposure to toxins or starvation [30]. It has been shown that for increasing concentrations of phenol, *Pseudomonas putida* P8 forms increasing proportions of 16:1ω7t or 18:1ω7t PLFA (*trans*-unsaturated fatty acids) [39,76]. This change occurs in the absence of bacterial growth [30,39]. Increasing the proportions of *trans*-monoenoic PLFA is not the only critical feature of solvent resistance in *Pseudomonas putida.* Comparison of a solvent-sensitive strain to the 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 [70]. These changes were not detected in the solvent-sensitive strain [70].

Other PLFA patterns also change in response to environmental stress. Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA [22,23,30,79]. The monoenoic PLFA 16:1ω7c and 18:1ω7c are increasingly converted to the cyclopropyl fatty acids cy17:0 and cy19:0, respectively, in Gram-negative bacteria as the microbes switch from an exponential to stationary growth phase. This ratio varies from organism to organism or environment to environment but usually falls within the range of 0.05 (for log phase) to 2.5 or greater (stationary phase) [29,30,85]. An increase in cyclopropyl PLFA formation has also been associated with increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies.

Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity [12,34]. 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 or Gram-positive bacteria which form naphthoquinones. Benzoquinones (ubiquinones, coenzyme Q) are produced by aerobic and

facultative Gram-negative bacteria when grown with high potential terminal electron acceptors for the membrane-bound electron transport chain such as oxygen or nitrate [34]. Napthoquinones (menaquinones, dimethylmenaquinones) are produced by aerobic Gram-positive bacteria, extreme halophiles, and Gram-negative facultative or obligately anaerobic bacteria. These organisms use succinate, CO_2 or other low potential electron acceptors in the electron transport chain. Fermentative anaerobic growth by facultative or obligate anaerobes generally produces no respiratory quinones. A ratio of total benzoquinones to total napthoquinones provides an indication of the extent of aerobic to anaerobic microbial respiration. In Gram-negative bacteria, respiratory quinones are usually 10–100 times less in content than the PLFA. The sensitivity of the determination of respiratory quinone ratios benefits greatly from the increased sensitivity of analysis by HPLC/ES/MS.

Some specific but useful insights come from analysis of organisms like the Pseudomonas species which form acyl-ornithine lipids when growing with limited bioavailable phosphate [52] while some Gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at sub-optimal acid pH levels [48].

Plasmalogen phospholipids are formed by anaerobic bacteria. When plasmologens (lipids typical of *Clostridium*) are subjected to a mild acid methanolysis, fatty aldehydes are formed which can then be converted into dimethyl acetals (DMA). With increasing proportions of obligate anaerobes and anaerobic metabolism, the DMA/PLFA ratio will increase. In certain situations, anaerobic metabolism can be estimated from the ratio of *iso* to *anteiso* branched saturated PLFA. The Gram-positive aerobes (*Arthrobacter, Micrococcus*) have i17:0/a17:0 ratios of approximately 0.2 [13] whereas the Gram-negative anaerobes (*Desulfovibrio*) have i17:0/a17:0 ratios of greater than 5 [17,68].

Validation of the signature lipid biomarker analysis

The validation of the signature lipid biomarker analysis in determining the *in situ* viable microbial biomass, community composition, and nutritional/physiological status in some specific environments has been reviewed [88]. The induction of microbial community compositional shifts by altering the microenvironment resulted in changes that were often predictable, based on past experience with microbial communities. For example, biofouling communities incubated in seawater at altered pH in the presence of antibiotics and specific nutrients resulted in a community dominated by fungi, while other conditions resulted in a community dominated almost exclusively by bacteria [84,86]. In these experiments, the morphology of the biofilm seen in scanning electron microscopy correlated with the chemical analysis. Similar experiments showed that light-induced shifts which occurred within microbial biofilms induced the expected changes in morphology and shifts in signature lipid biomarkers [6]. A second validation was the isolation of a specific organism or groups of organisms with the subsequent detection of the same organisms by signature lipid analysis in consortia under conditions where their growth was induced. It was possible to induce a 'crash' in methanogenesis in a bioreactor by inducing the growth of sulfate-reducing bacteria [36], or by adding traces of chloroform or oxygen [35]. These crashes were accompanied by shifts in the signature lipid biomarkers that were correlated to the changes in the microbial populations. Specific microbes whose signature biomarkers have been determined in isolates can be detected in biofilm communities [12,44,49,73,81], clinical specimens [1,58,63], or subsurface soils [59,72,93–96]. Specific sulfate-reducing bacterial groups can also be 'induced' in estuarine muds [68] as can methane-oxidizing populations [59,61], or propane-oxidizing actinomycetes [72] through the addition of appropriate substrates. Again, all of these community shifts were evidenced by measurable changes in lipid signatures and in lipid patterns. A third validation was the induction of shifts in microbial community nutritional status by generating conditions of unbalanced growth in which cell growth but not cell division was possible. This was accomplished by chelating trace metals in the presence of tannins on epiphytic microbiota [62], and by disturbing anaerobic sediments with oxygenated seawater [21,22]. Under these conditions the ratio of PHA to PLFA biosynthesis increased dramatically just as it does in monocultures of the appropriate bacteria under laboratory conditions. A fourth validation was the detection of specific shifts in microbial communities as a result of specific grazing by predators. The sand dollar *Mellita quinquiesperforata* was shown to remove non-photosynthetic microeukaryotes selectively from sandy sediments. Examination of the morphology of the organisms in its feeding apparatus and of the signature lipid biomarker patterns before and after grazing by the echinoderm demonstrated the specific loss of non-photosynthetic microeukaryotes [20]. Comparisons of the signature lipid biomarker demonstrated the specific loss of non-photosynthetic microeukaryotes [20]. Another example involved the amphipod *Gammarus mucronatus* which exhibited a relatively non-specific grazing of the estuarine detrital microbiota. This organism removed the microeukaryotes which were then replaced, to a large extent, by bacteria [54,55]. Results of the signature lipid biomarker analysis agreed with the cellular morphologies present as shown by scanning electron microscopy.

Extension to nucleic acid analysis

The solvent extraction utilized in the signature lipid biomarker analysis has recently been shown to liberate cellular nucleic acids which can be used for enzymatic amplification and gene probing [42]. Over 50% of the gene *nahA* present in intact *Pseudomonas fluorescens* cells added to soil was recovered using the lipid extraction protocol as compared to recovery by the standard techniques [64]. The DNA recovered from the lipid extraction was of high quality, and was suitable for enzymatic amplification. The combined lipid extraction and recovery of nucleic acids offers powerful insights because of the exquisite specificity in the detection of genes. The concomitant DNA/lipid analysis readily provides quantitative recoveries independent of the ability to isolate or culture the microbes. The lipid analysis gives indications of the phenotypic properties of the community that indicate extant microbial activity by providing *in situ* indications of starvation, growth rate, exposure to toxicity, unbalanced growth, deficiencies of specific nutri-

ents, and the aerobic/anaerobic metabolic balance, while DNA probes define the physiologic potential of the microbial community [91]. The combined DNA/lipid analysis overcomes some deficiencies in microbial ecology studies involving only nucleic acid analysis [90].

Specific applications of signature lipid biomarkers (SLB)

Differences in viable biomass and community composition in components of a deciduous forest biome

The viable biomass and diversity of the microorganisms in portions of an Eastern USA temporal, terrestrial, forest biome were documented with SLB analysis of the active phyllosphere, a forest litter, a foraging insect gut, fine root rhizosphere, and surface soil of white oak, *Quercus alba*, in Oak Ridge, TN and subsurface materials recovered aseptically from a borehole in Aiken, SC. The data in Figures 1 and 2 indicate there are differences in microbial abundance

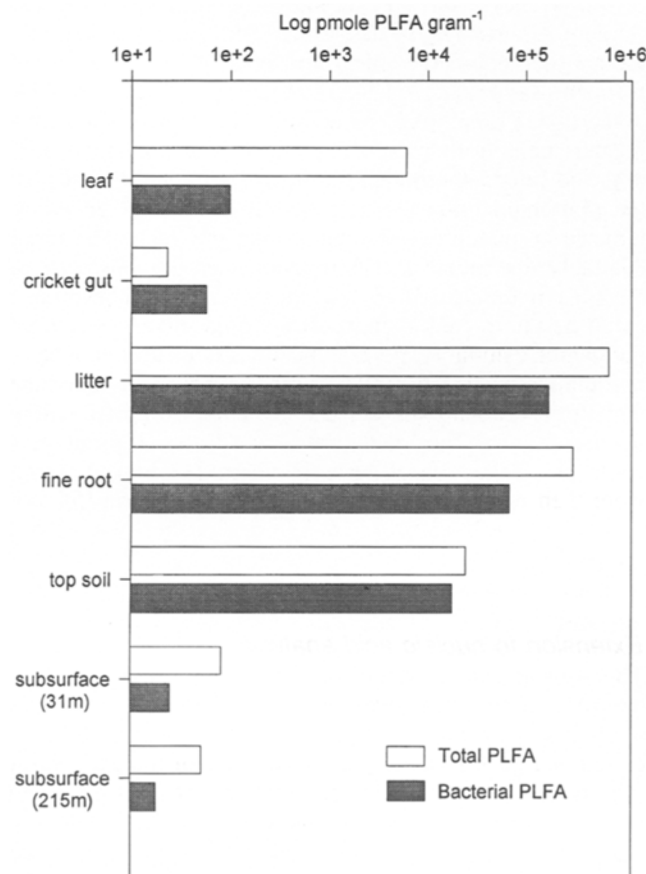

Figure 1 PLFA abundance in samples representing a vertical distribution in a deciduous forest biome. Total PLFA represent a total PLFA concentration in pmol g^{-1} and bacterial PLFA, those PLFA attributable specifically to prokaryotes. The leaf, litter, fine root (<1 mm in diameter) and topsoil samples were collected in Oak Ridge, TN from the white oak, *Quercus alba* L [66,74]. The subsurface sediments were recovered from a borehole drilled in Aiken, SC [89]. The cricket guts were provided by Dr M Kaufman at Michigan State University, East Lansing, MI [41]. The values represent an average of $n = 3$ for the leaf and topsoil, $n = 10$ for the litter, $n = 18$ for the fine root and two cricket guts corrected for the animal contribution, and five pseudoreplicates from a single bore hole for the subsurface sediments.

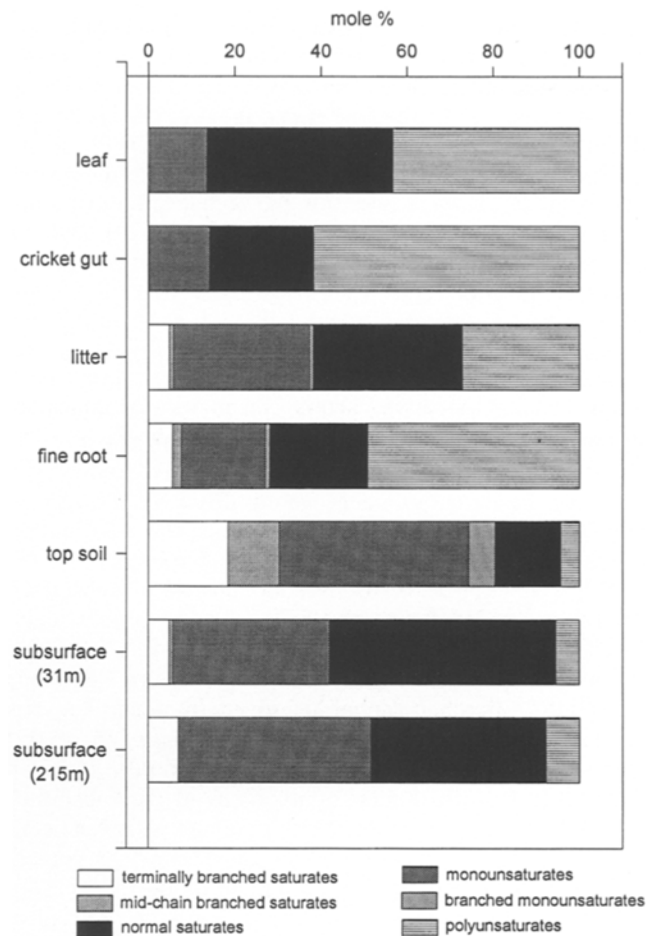

Figure 2 A comparison of the relative percentages of total PLFA functional groups in the samples described in Figure 1. Functional groups are defined according to PLFA molecular structure which is related to fatty acid biosynthesis. Normal saturates are ubiquitous, terminally branched saturates are attributed to Gram-positive bacteria, mid-chain branched saturates and branched monounsaturates to actinomycetes and sulfate-reducing bacteria, monounsaturates to Gram-negative bacteria and polyunsaturates to eukaryotes.

and community composition defined by the SLB analysis in the specific samples assessed. The details of sampling of the fine roots, top soil, and subsurface have been reported [74,87,94–98]. Ten grams (wet wt) of oven dried (65°C, 2 days) *Quercus alba* leaves were placed in square 2-mm mesh nylon bags 6 cm wide in a grid in a mesic hardwood forest in Oak Ridge, TN for 200 days [66,74]. On harvesting, exterior plant and animal matter was removed, the litter lyophilized, and the lipids extracted for GC/MS analysis. Crickets, *Acheta domesticus*, were reared in sterile boxes gnotobiotically or fed dogfood *ad libitum* and the guts dissected and sampled as described [41]. Samples were extracted with the one-phase extraction system, the lipids fractionated with silicic acid, the polar lipids transesterified and analyzed by GC/MS [74,87,94–98].

Viable biomass

The viable biomass of the host plant greatly exceeds that attributable specifically to prokaryotes in the leaves. In other niches in this forest biome, the viable biomass of the

microbes is nearly equal to that of the macroeukaryote host. Microbial abundance in terms of PLFA per g dry weight was greatest in the litter, rhizosphere, and topsoil in the specific samples. In the fine roots, it was possible to test directly the validity of the designation of which PLFA are attributable to the microorganisms and which to the plants. Microbial PLFA were based on the composition of monocultures of isolated rhizosphere microbes [79] and on experience from many isolations in the subsurface science culture collection and from subsurface [3,32,43,92,96]. Fine roots of *Populus grandidentata* trated with hypochlorite showed the PLFA attributed to the microbes on the rootlet surface disappeared in contrast to those on the roots [74,97]. The composition of the root PLFA determined as the residual PLFA after bleaching agreed with direct analysis of sterile rootlets [74,79]. Crickets reared from sterile eggs in a sterile environment were utilized for comparison with normally fed crickets [41] to define the contribution of the animal cells to the SLB of the microbiota.

The viable biomass of microorganisms in subsurface soils not associated directly with the roots varies widely. The viable biomass can vary from the equivalent of 10^3–10^9 bacteria g^{-1} dry wt in the arid Northwestern USA soils [26,93,96]. Microbial spatial heterogeneity in surface soils and subsurface sediments has been documented in several subsurface materials with classical techniques [4,7,32]. With SLB the distribution of microbes in the subsurface has been shown to be related to nitrate and phosphate concentrations [69], permeability [89] and moisture content [43]. Analyses utilizing viable cell plate counts [33] on subsurface desiccated volcanic tuff and PLFA or subtropical estuarine sediments [18] showed heterogeneity in viable microbial biomass and community composition was as great on the scale of millimeters as it was on larger scales.

The viable biomass based on prokaryotic PLFA observed in the leaves of *Quercus alba* from Oak Ridge, TN was 2–3 orders of magnitude lower than that found in the forest litter, the rhizosphere, and topsoil. Plant exudation through leaves is considerably less than the exudates through the roots [14]. Plants often transpire toxic compounds, such as phenols, to defend against predators. In addition, sunlight and desiccation inhibit microbial growth [14].

Subsurface sediments below the root zone have a significantly lower viable biomass than the topsoil or rhizosphere. In general, there is a decrease in viable cell biomass as the depth from the surface increases [74,78,93–96]. At depths below 30 m the concentration of bacteria falls below about 10^3 cells or 0.1 pmol PLFA g^{-1} dry wt. The PLFA of sediments recovered from a bore hole in South Carolina at depths of 31 and 215 m beneath the surface, illustrate this low viable biomass (Figure 1). Nutrients, in particular phosphate, are limiting in these sediments [69]. The detection of PLFA, even at depths greater than 200 meters, does, however, indicate the presence of viable organisms. PLFA analysis shows that the viable organisms in these sediments comprise a diverse microbial community. This diversity is not reflected in the cultured isolates [3] and might have gone undetected without an assay, such as for PLFA, where chemical constituents are measured *in situ* and detection does not depend on isolation and culture.

Community composition

A comparison of PLFA profiles (consisting of the total PLFA attributable to the prokaryotes plus eukaryotes) for each component of this forest biome by PLFA functional group reveals differences in microbial community composition at each level sampled (Figure 2). The two subsurface sediments and the leaves showed the greatest percentages of normal saturated PLFA which are ubiquitous in all organisms. The subsurface sediments showed a greater percentage of monosaturated PLFA which are typical of Gram-negative bacteria. The leaves and cricket gut had a greater percentage of polyunsaturated PLFA which are found almost exlusively in eukaryotes (obviously an influence from the plant and cricket gut). Hierarchical cluster analysis of the total PLFA profiles from these samples show relatedness as indicated in the dendrogram in Figure 3. Three major groups show similarities: (1) the subsurface and leaves; (2) the topsoil, litter, and rhizosphere; and (3) the cricket gut. The litter and fine roots show greater relatedness than the topsoil. This is readily seen in the ratios of polyenoic PLFA (eukaryotes) to monoenoic PLFA (largely Gram-negative bacteria). The greater eukaryotic input observed in the fine root versus the litter material from the same plant, is likely made up, in large part, by mycorrhizal fungi [9,27,65,66,80]. The topsoil showed a greater input from what are typically Gram-positive bacteria indicated as terminally-branched saturated PLFA plus the mid-chain branched saturated PLFA which generally indicate an actinomycete or anaerobe presence. The more equitably distributed PLFA functional groups observed in the topsoil (compared to the litter or fine root material), could be interpreted as indicating a more diverse bacterial community. Possibly the presence of a dominant exudative nutrient influx in the rhizosphere or the carbon source in the litter may shift the community composition to a less diverse status. The rhizosphere and litter communities could be a reflection of an enrichment. The topsoil, being

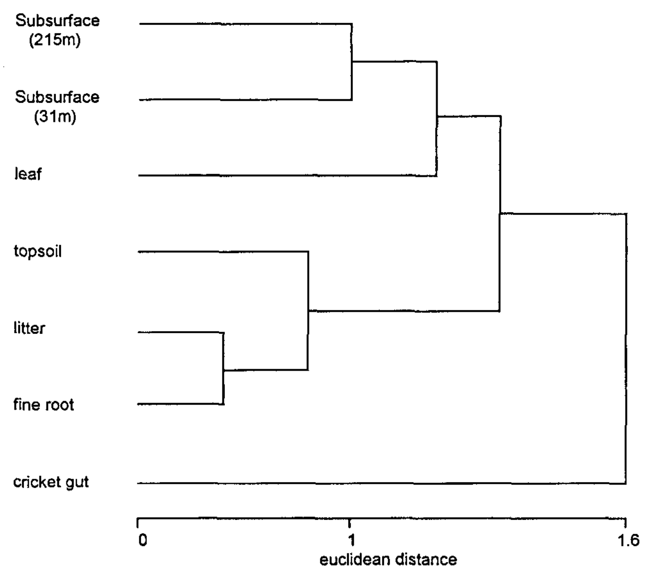

Figure 3 A dendrogram representation of a hierarchical cluster analysis (complete linkage based on euclidean distances) for the bacterial PLFA profiles described in Figure 1.

influenced to a much smaller degree probably does not show the same enrichment characteristics.

The microbiota observed in the cricket gut is by far the most unique community of the biome (Figure 3). The very large presence of polyunsaturated PLFA is a reflection of the cricket digestive apparatus itself and not the microbiota of the gut. Both the cricket gut and the fine roots show percentages of polyunsaturated PLFA greater than 40 mole percent, but individual PLFA are of different chemical structures. Both these communities lack mid-chain branched saturated or branched monounsaturated PLFA suggesting a microfauna of limited diversity in comparison to the other samples collected from this forest biome.

A comparison by principal components analysis of PLFA profiles in this part of the forest biome (adjusted to reflect only prokaryotic PLFA and excluding the cricket gut sample) resulted in components of the forest biome showing the same basic similarities seen in the cluster analysis (Figure 4a). The fine root and litter samples form a group closest to the topsoil and the two subsurface sediments form a closer group that shows a relationship to the phyllosphere microbial community. The adjustment to reflect only prokaryotic PLFA and excluding the cricket gut sample allowed a more detailed analysis of the PLFA most important in differentiating the communities. Two principal components were derived which accounted for, sequentially, 84 and 9% of the variance inherent in the data set. The first principal component was most strongly influenced by 18:1ω7c and to a somewhat lesser extent by 16:1ω7c PLFA (Figure 4b). These PLFA are primarily the end product of the bacterial anaerobic desaturase pathway that is prominent in Gram-negative heterotrophic bacteria. The second principal component is heavily influenced by the mole percentages of 16:1ω5c, 10me16:0, i17:1ω7c, and 16:1ω7t. The monounsaturate 16:1ω5c in addition to being a component of specific Gram-negative bacterial membranes is also a component of certain arbuscular mycorrhizal species [65]. The mid-chain branched saturate (10me18:0) and branched monosaturate (i17:1ω7c) are components of obligate anaerobe membranes, specifically the sulfate-reducing bacteria (*Desulfobacter* and *Desulfovibrio*, respectively [16,17]) as well as the membranes of certain actinomycete species, specifically the genus *Streptomyces* [9]. The 16:1ω7t is an indicator of metabolic or toxin-induced stress in Gram-negative bacteria [30,39,70,76]. By using the multivariate analysis, variance between the microbial communities in the portions of the forest biome examined, were defined in terms of specific bacterial types, genera, and/or species. In Table 1 the relative distribution of the PLFA whose proportions affect the differences in the prokaryote diversity amongst the microbiota of the phillosphere, litter, topsoil, rhizosphere and subsurface are illustrated. The greater diversity of the prokaryote microbiota in the rhizosphere, litter, and topsoil are indicated by the larger proportions in each bacterial type. The high levels of organic polymer food sources in the litter, rhizosphere, and topsoil coincide with the presence of celluloytic bacteria like *Cytophaga* sp, *Vitroscilla*, *Flexibacter*, and *Filibacter*, and arbuscular mycorrhiza as indicated by a significant proportion of 16:1ω5c [40,60,65,82]. The actinomycetes, with 10 Me 18:0, predominate in the rhizosphere and topsoil.

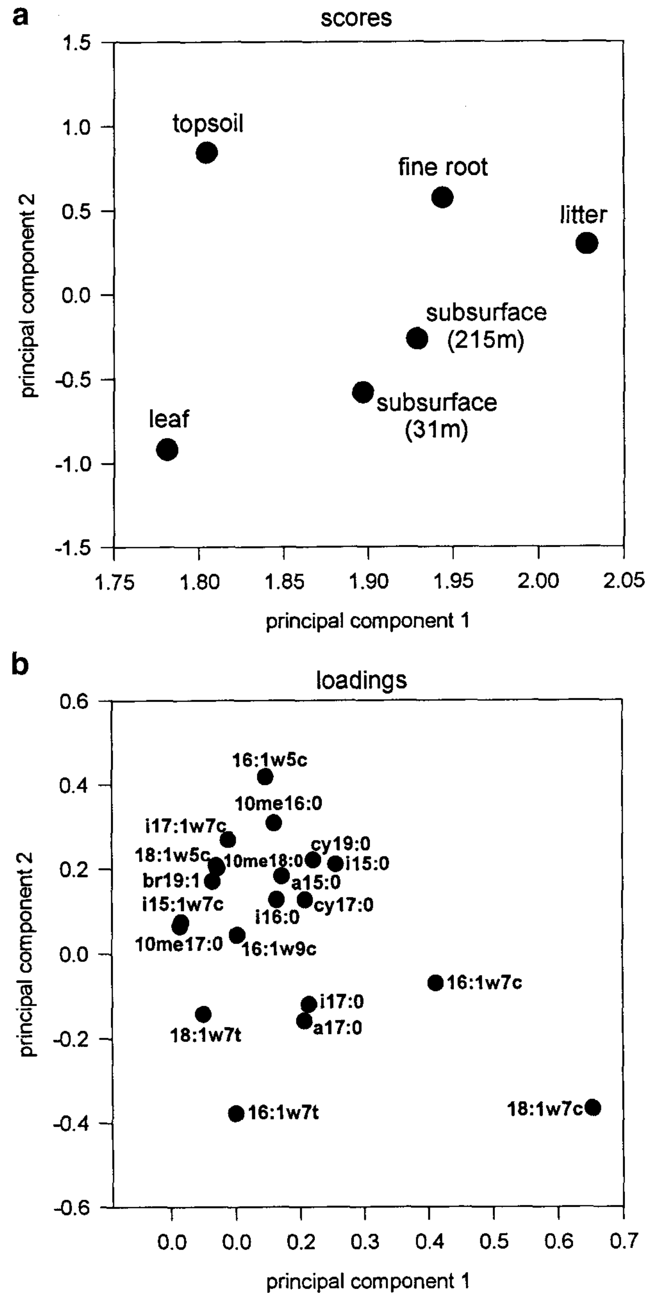

Figure 4 Principal components analysis. (a) A scatter plot of the scores results from a principal components analysis of the bacterial PLFA profiles described in Figure 1. Principal component 1 described 84% of the variance in arcsin-transformed PLFA mole percentages and principal component 2, 9% of the variance. (b) A scatter plot of the coefficient of loading derived from the principal components analysis described in (a).

The topsoil has the highest proportion of PLFA indicating anaerobic microniches exist, based on the biomarkers for sulfate-reducing bacteria [68,95]. The phyllosphere and the subsurface show the stress of exposure in the ratio of 16:1ω7t/16:1ω7c. Starvation of the Gram-negative bacteria is evident in the subsurface with the higher proportions of cylopropane PLFA relative to the monoenoic PLFA precursors except in the 31-m subsurface sample.

Table 1 Comparison of specific prokaryotic PLFA (mole %) in samples collected from portions of a forest biome

Bacterial type	Litter	Fine root	Subsurface sediments		Top soil	Leaf
			(31 m)	(215 m)		
Gram-positive						
(a15:0 and a17:0)	7.3	8.7	3.2	3.4	9.4	20.6
(i15:0/a15:0)	2.4	1.9	nd	1.3	1.6	0.9
Gram-negative						
(16:1ω7c and 18:1ω7c)	52.9	38.0	54.4	65.1	25.2	59.1
(16:1ω7t/16:1ω7c)	nd	nd	0.6	0.0	nd	0.8
(cy17:0/16:1ω7c)	0.3	0.4	0.0	0.3	0.4	nd
(16:1ω5c)	5.0	9.5	0.0	0.0	8.7	0.0
Actinomycete						
(10me18:0)	0.9	2.3	nd	nd	2.4	nd
Sulfate-reducing bacteria						
(10me16:0 for *Desulfobacter* sp)	3.2	5.9	2.8	nd	10.0	nd
(i17:1ω7c for *Desulfovibrio* sp)	1.7	2.7	0.0	nd	4.8	nd
Total SRB	4.8	8.5	2.8	nd	14.8	nd

nd, not detected.

Influences on the rhizosphere microbiota

The rhizosphere microbiota can be influenced by factors influencing the host plants. In a nitrogen-limited deciduous forest ecosystem, the fine roots of white oaks support a constant level of microbial biomass per fine root when grown under conditions of elevated atmospheric CO_2 [74]. This constant level of microbial cells per unit area of rootlets suggests that the supply of nutrients coming from the fine roots sustains a specific number of microorganisms and that a symbiotic equilibrium is reached. The level of bacteria found associated with the root was similar to that found associated with the plant litter (Figure 1). The litter was primarily oak leaves, however, the composition of a litter microbial community was quite distinct from that of the rhizosphere microbiota (Figures 2 and 3).

PLFA profiles from the microbiota of white oak leaves differed between two consecutive years reflecting a response to a drought year (unpublished data). The differences in PLFA could be atrributed to either community compositional changes or physiological responses (Table 2). The *trans/cis* monoenoic PLFA ratio has been discussed as indicating a physiological response of Gram-negative bacteria to conditions of stress and toxin exposure [30,39,70,76,91]. In the dry year there was an increase in the *trans/cis* ratio in the monoenoic PLFA. Dryness also induced a decrease in the proportion of Gram-positive relative to Gram-negative bacteria in the litter microbiota. The decreases in terminally branched saturated and monounsaturated PLFA and increase in normal saturated PLFA might indicate a shift toward oligotrophic bacteria during the drought year [71,91].

Table 2 Comparison of specific bacterial PLFA abundance (pmol PLFA g^{-1}) from white oak leaf material during wet (1994) and dry (1993) growing seasons

PLFA (pmol g^{-1})	White oak	
	Wet	Dry
Gram-positive		
a15:0	3.8 ± 1.2	1.9 ± 0.4
Gram-negative		
16:1ω7c	12.4 ± 3.0	11.5 ± 3.3
18:1ω7c	48.2 ± 7.1	46.3 ± 1.9
Physiological status		
16:1ω7t/16:1ω7c	10.58 ± 0.14	0.91 ± 0.45

Values represent an average ($n = 3$) ± one standard deviation.

The presence of a xenobiotic chemical has also been shown to affect both microbial community composition and physiological status in the subsurface [78] and the rhizosphere [2]. The presence of about 30 ppm trichloroethylene (TCE) in topsoils where both a legume *Lespedeza intermidis* (bush clover) and *Pinus taeda* (Loblolly pine) were growing, corresponded to a shift in the rhizosphere soil communities of both plants (Table 3). In addition to a significant increase in the biomass of the legume rhizosphere microbiota, a significant increase in the proportion of terminally branched saturated PLFA, or Gram-positive bacteria, was apparent. Although no significant increase in the biomass of the pine rhizosphere microbiota was detected, a significant increase in the percentage of monounsaturated PLFA, or Gram-negative bacteria, was measured. The two plants and their associated below-ground rhizosphere soil microbiota showed different responses to the presence of the contaminant. Both plants did, however, show a similar physiological response to the TCE presence (Figure 5). An increase in the ratio of PHB/PLFA was observed in both rhizosphere soils in the presence of the contaminant at a depth of 61 cm. The lack of any observable effect above this depth is likely due to surface transport of nutrients down, allowing for a utilization of the contaminant as a carbon source. The increase in the ratio at the 61-cm depth suggests carbon from the contaminant was used in storage due to nutrient limitations. By analyzing the microbiota *in situ* by application of the PLFA technology, it was possible to quantify the effect of the contamination on the viable microbial populations. Methods utilized in these experiments have been reported [2].

Table 3 Rhizosphere microbial community differences between uncontaminated and contaminated surface soils

	Legume		Pine	
	Control	TCE	Control	TCE
Biomass				
nmol PLFA g^{-1}	4.18 ± 3.47	16.97 ± 3.35*	11.15 ± 4.55	7.80 ± 2.57
cell equivalence	1.0E ± 09	4.2E ± 09	2.8E ± 09	2.0E ± 09
Community composition (mole %)				
normal saturates	21.68 ± 6.36	22.39 ± 1.97	22.26 ± 3.44	21.26 ± 1.33
terminally branched saturates	8.11 ± 3.89	14.16 ± 1.80**	15.27 ± 3.30	12.24 ± 1.79
mid-chain branched saturates	10.40 ± 3.52	7.01 ± 1.17	10.06 ± 2.34	8.89 ± 1.17
monounsaturates	39.13 ± 6.60	41.35 ± 7.58	36.84 ± 4.85	46.30 ± 4.51**
branched monounsaturates	3.75 ± 0.78	4.40 ± 0.56	4.03 ± 0.83	4.68 ± 0.33
polyunsaturates	16.92 ± 5.10	10.70 ± 3.71	11.53 ± 3.94	6.63 ± 1.20

*Cell equivalent assumes 2.5×10^4 cells per pmole PLFA.
**Indicates significance at 95%, $n = 3$.

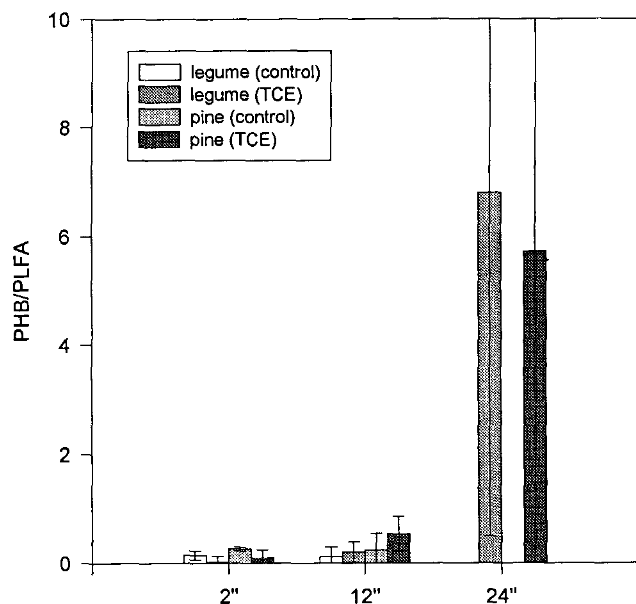

Figure 5 A comparison of PHB/PLFA (poly-hydroxy butyric acid/ester-linked phospholipid fatty acids) ratios in rhizosphere soils from test plots located in Aiken, SC [2]. The control samples showed no detectable levels of TCE and the contaminated plots showed TCE concentrations on the average of 35 ppm. The legume was *Lespedeza intermedia* and the pine, *Pinus taeda* (Loblolly).

Conclusions

Estimation of microbial biodiversity by signature lipid biomarkers in environmental samples cannot result in the definition of each individual species present. Some species are readily defined since they contain either unique lipid components or unique lipid patterns. However, in environmental analyses, overlapping patterns may make less-specific interpretations necessary, at the functional group level. Since DNA suitable for gene probing can be recovered with the signature lipid analysis, the combination of the signature lipid biomarker analysis with DNA gene probe technology should greatly expand the specificity and scope of community compositional determinations. We have illustrated by analysis of various components of the Eastern USA forest biome, that the diversity of microbial communities is readily and quantitatively compared with this technology. The SLB technique is probably the most efficacious comprehensive microbial biodiversity analysis currently available.

A major problem with the signature lipid biomarker analysis in determining environmental microbial biomass is that the results are not presented in the traditional units. Biomass is measured as picomoles of PLFA or picomoles of LP per sample instead of cells per sample. Although this value can be related to the number of specific organisms present, the conversion is problematic due to the variety of shapes and sizes of organisms found in environmental samples. Determination of microbial biomass with a colorimetric analysis of the organic phosphate of the phospholipids is straightforward and requires little specialized equipment other than a spectrophotometer [21,24,83]. However, this analysis is relatively insensitive with limits of detection in the micromolar range of about 10^{10} bacteria with the stable colorimetric analysis [83], or about 10^7 bacteria the size of *E. coli* with the dye-coupled assay [21]. The analysis of fungi based on sterol content also presents a problem since mycelia often exist as large multinucleated cells with a huge biomass, much of which is not active.

Analysis of lipid components requires special analytical skills and entails expenses for extractions, processing, and GC/MS equipment for analysis. Scrupulous attention must be paid to the purity of solvents, reagents, and glassware since signatures at one part in 10^{14} are commonly detected using these analyses. Once any difficulties in performing the analyses have been overcome, the interpretation of community composition and nutritional/physiological status requires an extensive familiarity with widely scattered literature. Research toward automating and accelerating the speed of the analysis has been initiated in a number of laboratories. In the not so distant future, it is likely that the signature lipid biomarker analysis will be fully automated and accomplished in a matter of hours instead of the current time frame of days. In the meantime, lipid analysis provides significant insight into the microbial biomass, community structure, and physiological status of environmental samples, and provides a quantitative means for obtaining this type of information that has the potential to be coupled with DNA extraction and genetic analysis.

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