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Biochemical Approaches to Biomass Measurements and Community Structure Analysis

Holly C. Pinkart[1], David B. Ringelberg[2], Yvette M. Piceno[3], Sarah J. Macnaughton[4], and David C. White[4,5]

[1] Department of Biological Sciences, Central Washington University, Ellensburg, WA 98926-7537, USA
[2] Waterways Experiment Station, CEWES-ESP, 3909 Halls Ferry Road, Vicksburg, MS, 39180-3120, USA
[3] Microbial Insights, Inc., 2340 Stock Creek Blvd., Rockford, TN 37853-3044
[4] Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575, USA
[5] Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37983, USA
Phone: 865-974-8030, FAX 865-974-8027

Rationale

Biomass measurements are of great importance in ecology because microbes not only form the base of the food web, but also mineralize biopolymers for essential reprocessing. In this review of biochemical approaches to biomass measurements, the microbes include both prokaryotes and microeukaryotes that pass through a 0.5-mm sieve. It is particularly useful to differentiate between the viable biomass, which manifests the potential for metabolic activities in the environment, and the nonviable biomass, which can still be part of the food web, but has little or no potential intrinsic metabolic activity. Classical biochemical and microbiological techniques, such as viable counts, used successfully in public health for isolating and culturing clinical specimens, have proven less than adequate for determining biomass and community structure in environmental samples. It has been documented repeatedly that viable counts of bacteria from various environmental samples may represent only a very small proportion of the extant microbial community (7, 100, 104, 109). Furthermore, microbes may be metabolically active and potentially infectious even though they are not culturable (100, 135). Finally, classical microbial tests are time-consuming and provide little indication of nutritional status or evidence of toxicity in bacterial communities, although both parameters can affect metabolic activities and can be crucial for understanding microbial ecology.

BIOMASS

Traditional Biomass Measures

Microbiologists have traditionally quantitated microbial biomass in a sample from the number of stained cells in an aliquot or cells detected by viable count with subsequent conversion of cell number to carbon content. While this is sufficient for monocultures which are readily cultured in the laboratory, it is not satisfactory for most environmental samples, such as soils and sediments, in which the culture-based assessment often represent only 0.1 to 10% of the cells detected using acridine orange direct counts (AODC) (7, 109) or biochemical methods. In soils and sediments

the often sparse and heterogeneously distributed nature of microbial communities can significantly affect the accuracy of AODC-determined microbial abundance. Also, direct microscopic counts are problematic in soils and sediments because sediment clay granules or opacities in the sediments obscure bacteria (33). This problem is often ameliorated by inducing detachment of the microbes from soil granules by using solutions containing multi-charged ions, such as polyphosphate, followed by recovery on membrane filters prior to counting (12). There is evidence, though, from lipid biomarker analysis that in subsurface sediments the detachment of the microbes is selective and often not quantitative (52, 68). When bacterial densities are less than 10^4 cells per g (dry weight) of sediment, the reproducibility of direct counts is very low and the error is large, even when counting 20 fields of view with adequate numbers of cells and replicate sub-sampling (75). Furthermore, it is important to emphasize that direct microscopic counts measure abundance and not biomass. The relationship between biomass and abundance requires insight into the biovolume, and conversion factors are not simple and can vary by a factor of 500 between different size classes of cells (88).

Biochemical Biomass Measures

An effective and quantitative way to measure microbial biomass *in situ* is to measure cellular components of the microbes. If cellular components are universally distributed, have a short (in terms of the process being studied) residence time in detrital pools after death-induced release, and are expressed at relatively constant levels among the microbial community and throughout the growth cycle, then they can be used as a measure of the biomass. Phospholipids fit these requirements well.

All intact cells contain polar lipids. Polar lipids in microbes are primarily phospholipids. Measuring lipid phosphate (LP) or phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the bacterial and eukaryotic biomass containing intact cellular membranes. With cell death, exogenous and endogenous phospholipases rapidly transform the polar lipids in the cell membranes to neutral lipid diglycerides by removing polar phosphate-containing head groups (129). Diglycerides apparently disappear in soils less rapidly than phospholipids as diglycerides are detectible in many natural environments.

Viable Biomass

The viable microbial biomass can be determined by quantifying organic phosphate from the polar lipid fraction of the lipid extract using a relatively simple colorimetric analysis (21, 33, 39, 132). The sensitivity of the classical colorimetric analysis for lipid phosphate (LP) as initially proposed for environmental samples (132) has been improved considerably with a dye-coupled reaction to sensitivities of 1 nmol LP, corresponding to about 10^7 bacteria (33). Greater sensitivities and specificities are obtainable using gas chromatography (GC)-mass spectrometry (MS) (90) and high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-mass spectrometry (MS) (29) (see Sensitivity and Limitations Section below).

Biomass-To-Cell Number Conversions

One of the major problems with biochemical biomass measures is that the results are determined as the quantity of component (e.g. pmol PLFA or μmol LP) per gram of soil or sediment. Since some microbiologists describe biomass in terms of "cell abundance," a simple conversion could be made by determining the biomarker content of monocultured cells and then counting the cells to

determine a value per cell. There are, however, documented accuracy problems with AODCs (33), and there is no universally applicable conversion factor for estimating the PLFA or LP per bacterial cell or the number of cells per gram (dry weight) of bacteria (21, 31, 33, 120, 132). This problem results from observations that most environments harbor microbes of widely differing volumes and shapes. Bacterial biovolumes can vary over 3 orders of magnitude (0.001 and 7 μm^3) (43, 88). The volume of a viable cell also can vary with nutritional status. In bacterial enrichments and in isolates or mixed cultures from the sea, the LP content can vary between 34 and 380 μmol LP per g of carbon for aerobic organisms, compared to 118 and 250 μmol LP per g of carbon for anaerobic cultures (21). *Arthrobacter crystallopoietes* showed a 30% decrease in PLFA per cell after 2 weeks of starvation (64), and *Vibrio cholerae* showed up to a 99.8% decrease in PLFA per cell after 7 days of starvation (35), with loss of culturability but not membrane integrity (51). Brinch-Iverson and King (21) stated that the conversion factor for bacteria of 100 μmol LP per g of carbon (100 μmol PLFA per g [dry weight]) based on earlier work (130) was reasonably applicable to sediments containing a significant proportion of anaerobes. Bacterial cell volume varies between 0.001 and 7 μm^3, with smaller bacteria having a higher dry weight-to-volume ratio than larger bacteria (88, 89). These authors developed an allometric relationship between dry weight and volume, whereby biomass equals a conversion factor times the volume raised to an exponential scaling factor. Scaling factors and conversion factors are size class dependent (88). Conversion factors for eukaryotic PLFA to biomass or biomass to cell number are even more problematic. Quantifying fungal biomass based on PLFA and sterol content presents a problem since mycelia often exist as large multinucleated cells with a huge biomass, much of which is not active. For further discussion see Frostegard and Baath (38). In essence, converting LP or PLFA values to cell-based carbon content or cell numbers is problematic, and results should be interpreted cautiously.

Metabolic Activity-Based Biomass Estimates

There are also biomass measures that are based on detecting the activity of environmental microbiota. Assays dependent on enzymatic activities (112), growth, or respiration after chloroform fumigation (53) have been used. The major problem with activity-based assessments of microbial biomass is that microbial community metabolic activity does not necessarily correlate with microbial biomass (high activity does not mean an actively growing, dividing microbial community). Viable microbial biomass estimates by PLFA measurements in marine sediments recovered from the Antarctic, the deep sea, and neotropical marine mud flats were remarkably constant at approx. 10 nmol PLFA per g (dry weight) of sediment ($\sim 10^9$ equivalent cells per g [dry weight]) (122). Metabolic activities (measured with injected substrates *in situ*), however, showed neotropical sediments to be at least 300-fold more active than those in the Antarctic in terms of DNA synthesis from [^{3}H] thymidine incorporation rates (134). ATP is another universal measure of metabolizing cells, provided it does not persist in soil following cell death (56). If the assay is combined with a treatment of added extracellular ATPase, then the assay of all adenosine-containing components can be a measure of the energy charge (ATP/AMP + ADP + ATP). The ratio of adenosine to the energy charge is an exquisitely sensitive indicator of stress in bacteria, as it measures a key homeostatic mechanism to maintain the energy charge, which is essential for metabolic functioning (24). ATP concentration, however, is not readily converted to a biomass estimate. Also, great care must be taken when measuring metabolic activity to avoid generating disturbance artifacts. Lipid analysis has been used to overcome the problem of disturbance

artifacts generated in determining microbial activity (26, 34, 35, 76). The facile determination of the ratio of [^{14}C] acetate incorporation into PHA and PLFA (32) and [^{13}C] acetate into PLFA (9) has proved especially valuable in ecological studies.

Equivalence of Biomass Measures

In one specific environment, a comparison of methods for determining microbial biomass demonstrated their equivalence. Subsurface sediment samples containing sparse prokaryote communities of minicells (demonstrated microscopically) were used in these experiments. In these sediments, the viable biomass determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intracellular ATP, cell wall muramic acid, LP, and very carefully done AODC measurements (13). It was assumed there were 2.5×10^{12} cells per g (dry weight) of cells and 100 μmol PLFA per g (dry weight) of cells (13). 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 (20). It has been our experience from a wide variety of environmental samples that the relationship between the PLFA and estimated number of cells varies at least by a factor of 4.

When To Use Lipid-Based Biomass Measures

Phospholipid-based biomass measurements have been applied successfully to a multitude of environments. Since the lipid recovery procedure involves extensive solvent extraction and product concentration, there are few environments for which it cannot be applied. The assay even has been used to determine the biomass of microbes in sludges of petroleum storage tanks, although extra purification steps were included to remove the neutral lipid hydrocarbon components. Samples from an enormous variety of matrices have been analyzed for lipid content, including soils (5, 10, 18, 19, 23, 39, 53, 61, 65, 110, 112), heavy metal contaminated soil (63), rhizospheres (114, 116, 136), an ocean abyss (11), stream periphyton (45), clinical specimens (6, 90), pus (84), mummies (unpublished data), ice cores (91), mongoose anal sacs (25), sediments (2, 33, 35, 43, 76, 93, 107, 123), subsurface materials (13, 34, 37, 108, 126, 127), membrane filter retentates from ground water (67), bioprocessing units (48, 73), biofouling films (124), concrete (60), detritus (77), sponge spicule mats (134), drinking water biofilms (117), rocks (8), fungal biomass (131), grazed detritus (78), microbially influenced corrosion sites (54), anaerobic digesters (48), and indoor air (71). It is best, though, to process large samples (1-2 L water or 25 – 75 g material) if cell biomass is expected to be low; otherwise, the resulting lipid profile will not yield much useful community structure information (discussed below).

Interpretation

Viable biomass is estimated from the total amount of PLFA detected in a sample. Phospholipids are an essential part of intact cell membranes; thus, this biomass is a measure of the viable or potentially viable cells. Viable biomass also can be determined as organic LP by colorimetric methods (33, 132). The great majority of microbial phospholipids are diacyl, with a molar ratio of LP to PLFA of 1:2. Environmental samples showed LP-to PLFA ratios of 1:1.5 to 1:2 with a mean of 1:1.75 in 200 samples (unpublished data). Our laboratory uses a cell equivalent value calculated from experiments performed with subsurface bacteria. It is based on the assumptions that there are 2.5×10^{12} cells per g (dry weight) and 100 μmol of phospholipid per g (dry weight) of cells (13). This equivalent yields 2.5×10^4 cells per picomole of PLFA. Frostegard and Baath

(38) present data for cells recovered from soil from which one can calculate a value of 7.14×10^4 cells per pmol PLFA. It is important to note that the number of cells per g (dry weight) can vary by up to an order of magnitude, as summarized Findlay and Dobbs (31). As stated previously, when cells die or as cells rupture, phospholipids are attacked by enzymes, resulting in a lipid molecule called a diglyceride, which is not present in the membranes of viable cells. A rapidly growing microbial community will show a diglyceride fatty acid/PLFA ratio of $\mu \sim 0$, while in less ideal environments the ratio can exceed 3 (127). The diglyceride-to-PLFA ratio increased in a subsurface sediment from <0.2 at the surface to over 2.0 at >200 m (127).

COMMUNITY COMPOSITION AND PHYSILOGICAL STATUS

Community Composition from Lipid Analysis

In addition to biomass measurements, information obtained from lipid analysis provides insight into microbial community composition. The PLFA patterns derived from environmental microbial communities are much like the infrared spectra of complex molecules in that the PLFA patterns provide quantitative analysis, but interpretation in terms of specific components can be difficult. Quantitative comparisons of total community PLFA patterns accurately mirror shifts in community composition, but may not provide definitive analysis of shifts in specific microbial groups. Signature lipid biomarker analysis cannot detect every species of microorganism in an environmental sample because many species have similar PLFA patterns. Some specific groups of microbes, however, contain characteristic fatty acid profiles, and several examples are listed in Table 1. Further analysis of other lipids, such as sterols (for the microeukaryotes--nematodes, algae, protozoa (80, 107, 131), and glycolipids (phototrophs, Gram-positive bacteria), can provide a more detailed community structure analysis. Hydroxy fatty acids (OHFA) of the lipid A in the LPS are predominantly found in Gram-negative bacteria (28, 31, 58 - 60, 71), but are not restricted to Gram-negative bacteria (3). The recent application of HPLC/ESI/MS, or better yet HPLC/ESI/MS/MS, to analyze the intact phospholipids provides greater analytical sensitivity and specificity (discussed below).

When determining community structure using a signature lipid approach, it is crucial to consider the environment from which the sample was retrieved when interpreting results. Terminally-branched saturated PLFA are common to Gram-positive bacteria, but also are found in Gram-negative anaerobic bacteria, such as the sulfate reducing bacteria, and some Gram-negative facultative anaerobes (55). Monoenoic PLFA are found in most 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 (85). [Fatty acids designated by the total number of carbon atoms followed by the number of double bonds, with the position of the double bond indicated from the methyl end (ω) of the molecule (see footnote to Table 1)]. Polyenoic PLFA generally indicate the presence of microeukaryotes, but also are common in cyanobacteria (97, 99) and have been sparingly reported in other bacteria (46). Recently polyunsaturated PLFA have been found in some polar and deep sea microorganisms (103). Here HPLC/ESI/MS analysis would be useful because polyenoic PLFA are found predominantly in phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) in bacteria, but in eukaryotes they are found predominantly in cardiolipin in the mitochondria. The PLFA 18:2ω6 is prominent in fungi, but also is found in algae, protozoa, and humans in phosphatidylcholine (PC). Polyenoic PLFA with the first unsaturation in the ω6 position are classically considered to be of 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 typically from microeukaryotes. There are exceptions, however, to these generalizations.

Sterol types and patterns are very helpful in identifying microeukaryotes, especially when combined with PLFA results. For example, cholesterol has been found to be prominent in protozoa, such as *Cryptosporidium* species; ergosterol is found in many fungi (81); algae contain a diversity of sterols in patterns which have proved useful in describing taxonomic relationships (22) . Branched-chain monoenoic PLFA are common in the anaerobic *Desulfovibrio*-type sulfate-reducing bacteria, both in culture and in manipulated sediments (28, 93). They are also found in a large proportion of the actinomycetes (including the nocardioforms, coryneforms and mycobacteria), which contain mid-chain branched saturated PLFA, in particular 10Me18:0, with lesser amounts of other 10 methyl-branched homologs. Environments with 10Me16:0 >> 10Me18:0 often feature anaerobic Gram-negative *Desulfobacter*-type sulfate-reducing bacteria (27, 93). Although normal (straight-chain) saturated PLFA are found in both prokaryotes and eukaryotes, proportionally, bacteria generally contain more of the 16 carbon moiety (16:0), whereas the microeukaryotes contain more of the 18 carbon moiety (18:0). Methylotrophs are an exception to this rule, generally making more 18:0 than 16:0. When assessing bacterial community structure in eukaryote dominated samples, 16:0 and 18:0 should be removed prior to analysis.

There is a large body of literature, heroically compiled into a two-volume compendium edited by Ratledge and Wilkinson (99), on microbial lipid components that can greatly facilitate community lipid profile analysis when the basic physical parameters or ecology of a system is known. In effect, PLFA analysis provides a quantitative estimation of sample-to-sample or within-sample heterogeneity and community composition (30). Lipid analysis, in general, provides information about the microbial community not available from other methods. In environmental samples, though, overlapping patterns may necessitate less specific interpretations (e.g. at the functional group level). As a result, the combination of lipid biomarker analysis and nucleic acid-based analyses greatly expands the specificity and scope of community compositional determinations.

Community Composition from Nucleic Acid Analysis

DNA probe analysis has been used successfully to augment PLFA analyses (57, 65, 133). Such an approach helps define the physiological potential of a microbial community. A more complementary nucleic acid approach to PLFA analysis for community structure assessment is a combination of PCR amplification followed by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) analysis of rRNA or rDNA (see Chapter 11, this volume, for details). Using these techniques, shifts in microbial diversity can be monitored. Gradient gel electrophoresis enables DNA fragments of the same length to be separated according to their melting properties (sequences). In DGGE, double stranded DNA is separated in a linearly increasing denaturing gradient of urea and formaldehyde at elevated temperatures (79). In TGGE, the double stranded DNA is separated using a linearly increasing temperature gradient in a uniform concentration of denaturant (urea and formamide, [50]). With either technique, mixed amplified PCR products form a banding pattern. Using rRNA genes, DGGE/TGGE analysis of PCR products has proved reproducible (50, 79, 102). PCR amplification of selected bands

followed by sequencing allows approximate identification by comparison to sequence databases (110), such as the RDP (72).

Similar techniques can be used to assess functional group differences. Several investigators have targeted functional groups using primers directed toward a conserved enzyme in a pathway of interest to monitor changes in the bacterial community that could affect community function (*amoA* [111], [NiFe] hydrogenase [118], *nifH* [95]). The combination of PLFA and nucleic acid analyses can be very useful in biomass and community structure determinations (69, 110). The lipid analysis gives indications of the phenotypic properties of the community that indicates extant microbial activity by providing *in situ* indications of starvation, growth rate, exposure to toxicity, unbalanced growth, deficiencies in certain nutrients, and the aerobic/anaerobic metabolic balance (described below), while nucleic acid analysis provides more detailed structural information and can indicate the physiological potential of the microbial community (125, 133).

Biolog

The automated microbial identification system based on aerobic metabolic activities, Biolog® (17), has been used for comparing bacterial community compositions (41). The system is based on differential activity among 92 substrates and has demonstrated differences in community metabolism that paralleled those provided by lipid analysis in differentiating microbial communities in drilling fluids, makeup waters, and deep subsurface cores (66). For more detailed discussions of the advantages and disadvantages of using carbon substrate utilization patterns, consult Chapter 12 (Garland and Mills, this volume), and references 40, 62, and 106.

Community Composition Inferred from Isolated Bacteria

Distinctive ester-linked fatty acid profiles for bacteria (largely from the phospholipids and LPS of clinical isolates) are currently used to identify cultured microbes. Patterns of the prominent fatty acids of isolated microbes after growth on standardized media are used to differentiate over 2,000 species of organisms using the MIDI microbial identification system (MIDI, Newark DE) (119). Using MIDI requires isolating and culturing microbes prior to analysis. As a result, the unculturable microbes, which may represent the vast majority of the environmental microbes, are not detected. Using fatty acids from cultured microbes from a soil and sediment does not indicate *in situ* biomass, nor is it as accurate as other methods in reflecting the viable community composition.

Community Physiological Status

As already stated, it is possible to assess the physiological status of the microbial community using lipid analysis. Many subsets of the microbial community respond to specific conditions in their microenvironment by changing their lipid composition. The proportion of poly β-hydroxyalkanoic acid (PHA) in bacteria (32, 87) or triglyceride (in microeukaryotes) (42) relative to PLFA provides a measure of the nutritional/physiological status. Some bacteria undergo unbalanced growth and cannot divide when exposed to adequate carbon and terminal electron acceptors because of other limitations such as a lack of essential nutrients (e.g. phosphate, nitrate, trace metals). These bacteria form PHA, a carbon storage compound. When the essential component becomes available, these bacteria catabolize PHA and form PLFA as they grow and divide. PHA/PLFA ratios can range from 0 (dividing cells) to over 40 (carbon storage). For

example, the PHA/PLFA ratio in rhizosphere microbes from *Brassica napus* planted in sand and recovered from roots was <0.0001, compared with 6.6 for bacteria not associated with the rootlets (114). Ratios greater than 0.2 usually indicate the beginnings of unbalanced growth in at least part of the microbial community.

Specific patterns of PLFA also can indicate physiological stress in certain bacterial species (44). Starvation and stationary-phase growth lead to conversion of monoenoic PLFA to the cyclopropane PLFA. Exposure to solvents, alcohols, and acids induces changes in PLFA (105). Starvation can lead to minicell formation and a relative increase in specific *trans*-monoenoic PLFA when compared to the *cis* isomers (44). *Trans/cis* ratios greater than 0.1 have been shown to indicate starvation in bacterial isolates (44). This value is usually 0.05 or less in healthy, non-stressed populations. It has been shown that for increasing concentrations of phenol, *Pseudomonas pudita* P8 forms increasing proportions of *trans*-unsaturated fatty acids (49). Increasing the proportions of *trans*-monoenoic PLFA, however, is not the critical feature of solvent resistance in *P. putida*. Comparison of a solvent-sensitive strain with an Idaho strain, which is resistant to saturating concentrations of solvents and surfactants, showed that although both exhibited increases in *trans*-monoenoic PLFA, the resistant strain also shifted its lipid composition, decreased the proportion of monoenoic PLFA to saturated PLFA, increased the level of LPS-OHFA, and exhibited decreased permeability to the hydrophobic antibiotic difloxacin not detected in the solvent-sensitive strain (96). Some *Pseudomonas* species form acyl-ornithine lipids in lieu of phospholipids when growing with limited bioavailable phosphate (74).

Insight into microbial community physiological status can be obtained by analyzing lipid biomarkers. The monoenoic PLFA 16:1ω7c and 18:1ω7c increasingly are converted to the cyclopropyl fatty acids cy17:0 and cy19:0, respectively, in Gram-negative bacteria as the microbes move from a logarithmic to a stationary phase of growth. This ratio varies from organism to organism or environment to environment, but usually falls within the range of 0.05 (log phase) to 2.5 or greater (stationary phase) (73, 120). An increase in cyclopropyl PLFA formation also has been associated with increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies, but Bossio and Scow (18) did not observe such a shift in agricultural fields with and without flooding.

It is sometimes useful to determine *in situ* proportions of aerobic and anaerobic metabolism in a microbial community. Benzoquinones (ubiquinones, Coenzyne Q) are produced by aerobic and facultative Gram-negative bacteria. Terminal electron acceptors in the membrane-bound electron transport chain are either oxygen or nitrate, both of which carry high potentials (47). Napthoquinones (menaquinones, dimethylmenaquinones) are produced by Gram-positive bacteria, extreme halophiles, and Gram-negative facultative or obligately anaerobic bacteria. These organisms can 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 to 100 times less abundant than PLFA. Sometimes proportions of isoprenologues of the respiratory quinones can be helpful in identifying species. Benzoquinone, with 13 isoprenolog units in the side chain, is found uniquely in *Legionella pneumophila*. When plasmologens (lipids typical of clostridia) are subjected to a mild acid methanolysis, fatty aldehydes are formed, which then can be converted into dimethyl acetals. With increasing proportions of obligate anaerobes and anaerobic metabolism, the dimethyl

acetal/PLFA ratio will increase. In certain situations, anaerobic metabolism can be estimated from the ratio of *iso*-branched to *anteiso*-branched saturated PLFA. The Gram-positive aerobes *(Arthrobacter* and *Micrococcus* species*)* have i17:0/a17:0 ratios of approximately 0.2, whereas the Gram-negative anaerobes *(Desulfovibrio*) have i17:0/a17:0 ratios of greater than 5 (27).

PRACTICAL CONSIDERATIONS AND PROTOCOLS

Sample Handling

To minimize community compositional changes, it is necessary for microbial activity to be stalled as soon as possible following sample collection. It is important to freeze (-80°C preferably) environmental samples immediately and/or to lyophilize them as quickly as possible after collection. If the samples have been grown in culture, the media should be centrifuged and the resulting cell pellet should be rinsed twice with 0.05 M phosphate buffer (pH 7.5) before lyophilization. Samples should not be held on ice (~4°C) any longer than absolutely necessary. Rock samples held at 4°C showed rapid and significant changes in biomass and community composition (8). Dry ice is satisfactory for holding frozen samples. Preserving samples with buffered formaldehyde or gluteraldehyde is not as satisfactory as frozen storage because these preservatives can damage some of the less stable lipids.

Performing the Signature Lipid Biomarker Analysis

Meticulous technique must be practiced to ensure contaminant-free analyses. Glassware must be cleaned scrupulously. Once glassware is used, it is immediately fully immersed in a washtub full of hot water and detergent. The cleaning process is sufficiently effective that phosphate-containing detergent can be used. The glassware is scrubbed with a brush and rinsed 5 times each with cold tap water and then deionized water. Glassware is dried completely before being wrapped in aluminum foil and heated in a clean muffle furnace for a minimum of four h at 450°C. Disposable glassware, such as pipettes and silicic acid columns, need not be washed but is also baked in the muffle furnace. No materials other than fired glass and acetone-rinsed Teflon may come into contact with lipid solvents. Lipids from fingers, hair, stopcock grease, oils, and hydrocarbons are potential contaminants. Plasticware cannot be used in lipid analysis.

Samples can be extracted at room temperature, but should be protected from light, especially fluorescent light, if photosensitive lipids (such as quinones) are being analyzed. The extractant consists of a single phase chloroform-methanol mixture (1:2, v/v), generally called a Bligh and Dyer (15), which can be modified to accept a phosphate buffer (132). Investigators have found that buffer modification can increase the recovery of PLFA from soils with high clay content (39). Recovery standards (e.g. 50 pmol/µl C19:0 (phosphatidylcholine nonadecanoate) or 23:0 methyl-ester, which are rarely found in natural samples) can be added to samples prior to lipid extraction to estimate lipid recovery efficiency. Solvent blanks must be processed with each sample set. Samples can be extracted in glass centrifuge bottles and then centrifuged at 6000 x g for 30 minutes with the liquid phase decanted into a separatory funnel or analyzed directly in a separatory funnel or in a test tube or other suitable container. With sandy sediments and sufficient one phase extractant volume, it is usually not necessary to wash the sediment for a quantitative recovery. To isolate the lipid fraction of the extracted sample, equal volumes of chloroform and distilled water (or buffer) are added, and the sample is shaken. A split phase develops which is then centrifuged or allowed to separate passively overnight. The lower organic phase (containing

the bacterial lipids) is collected after being filtered through a fluted Whatman 2V filter that has been preextracted with $CHCl_3$. The solvent phase is removed by rotary evaporation at 37°C.

The dried total lipid extract is dissolved in chloroform and then transferred to a silicic acid column and separated into neutral lipid, glycolipid, and polar lipid fractions (43) by elution with solvents of increasing polarity. The neutral lipid fraction is analyzed for lipids such as free fatty acids, sterols, respiratory quinones, triglycerides, and diglycerides (61, 107). The glycolipid fraction can be analyzed for PHA (87). The polar lipid fraction, containing the phospholipids, can be analyzed in one of two ways depending on the purpose of the analysis. HPLC/ESI/MS analysis of intact phospholipids (without further lipid processing) gives increased sensitivity over GC/MS analysis for detecting and quantifying whole phospholipids. Phosphatidylethanolamine (PE) is more prevalent in Gram-negative bacteria, whereas phosphatidylglycerol (PG) is more prevalent in Gram-positive bacteria (99). However, HPLC/ESI/MS analysis cannot provide the detailed structural information obtained using GC/MS, as described below, unless HPLC/ESI/MS/MS is used. For a detailed structural analysis, the phospholipid fraction can be processed to obtain fatty acids cleaved from the phosphate head group. To do this, the polar lipid fraction is subjected to a transesterification by a mild alkaline methanolysis protocol (44) resulting in fatty acid methyl esters which are then separated, quantified, and tentatively identified by capillary GC. Individual components can then be definitively identified by their mass spectra. Monoenoic PLFA double bond positions are determined by GC/MS analysis of dimethyl disulfide adducts (82). More detailed procedural information is presented in reference 128.

The LPS-OHFA from the lipid A of Gram-negative bacteria can be recovered from the lipid-extracted residue. This residue is hydrolyzed in acid, and the lipid components released by the hydrolysis are reextracted (92). After centrifugation at 6000 x *g* for 30 min., the chloroform phase is recovered, evaporated to dryness, and methylated using "magic" methanol (methanol:chloroform:concentrated HCl; 10:1:1, v/v/v) (84). The methylated OHFA are recovered, and the solvent is removed under a stream of nitrogen. The OHFA are purified by thin layer chromatography (developed in hexane:diethyl ether, [1:1, v/v]), recovered in chloroform: methanol (1:1, v/v), and then derivatized using *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) prior to GC/MS analysis.

A rapid, potentially automatable PLFA extraction technique has been developed using a Dionex pressurized accelerated hot solvent extractor (70). Macnaughton *et al.* (70) demonstrated that this technique was equivalent or superior to the modified Bligh and Dyer extraction technique for air, water, and soil samples at a substantial savings of time and solvent supplies. In addition, bacterial spore and fungal lipids were extracted more efficiently using the accelerated solvent extractor. This instrument is especially useful for low biomass samples. Another modification to the standard lipid purification procedure useful for low biomass samples is described in Tunlid *et al.* (115). Basically, much less solvent is used and the samples are fractionated using 'champagne' columns (Supelco, Bellefonte, PA). The samples were analyzed with positive ion chemical ionization mass spectrometry.

Results are reported with fatty acids designated as in the footnote to Table 1. The percent recovery of 19:0 should be calculated when a phosphatidylcholine (PC) standard is added, but generally the final amount of phospholipid in a sample is not adjusted to reflect that recovery. The reason for this is that PC is the most difficult phospholipid to recover and so correcting for it's loss would cause other phospholipid components to be overestimated. The internal standards

are best used as indicators of problems with a particular sample in a set or with a particular sample matrix.

Once compiled, PLFA and other lipid profiles can be entered into spread-sheet formats and subjected to statistical analysis. In addition to ANOVA, two multivariate statistical applications have grown in popularity to investigate similarities between PLFA profiles. In one approach, dendrograms from a hierarchical cluster analysis are constructed from arc sine-transformed PLFA mole percent values, with similarities based on modified Euclidean distances (100). In a second approach, two-dimensional plots are generated from a principal-components analysis that not only illustrate profile similarities (or differences), but also identify which PLFAs contribute to the formation of the plots and to what extent (i.e. coefficients of loadings) (6, 101, 126, 127). An adaptation of this approach is canonical correspondence analysis (113), applications of which have recently been described in references 19 and 94. Recently, artificial neural networks (4, 14) have been used to analyze signature lipid biomarker data to better effect than the linear technique of principal components analysis (4, 5). Artificial neural network analysis corrects for biases introduced by interdependencies among individual lipids (5). A neural network must be trained with a fraction of an original data set, and then the trained network can be used to analyze additional data sets provided the input variables do not change.

Validation

The use of signature lipid biomarker analysis in determining the *in situ* viable microbial biomass, community composition, and nutritional-physiological status has been validated in a series of experiments (121). The induction of microbial community compositional shifts by altering the microenvironment resulted in changes that were often predictable, given 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 (131). Similar experiments showed that light-induced shifts which occurred within microbial communities were matched by expected shifts in signature lipid biomarkers and in terminal electron acceptors (16). These community compositional shifts resulting from specific perturbations have been reviewed (121). A second type of validation came from 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 (73) or by adding traces of chloroform or oxygen (48). These crashes were accompanied by shifts in the signature lipid biomarkers that were correlated to the changes in the microbial populations. Specific sulfate reducing bacterial groups also can be "induced" in estuarine muds (74) as can methane-oxidizing populations (83), or propane-oxidizing actinomycetes (100) 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 type of validation came from 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 (81) and by disturbing anaerobic sediments with oxygenated seawater (35). Under these conditions, the ratio of PHA to PLFA biosynthesis increased dramatically, just as it does in monocultures of appropriate bacteria under laboratory conditions. A fourth type of

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 selectively remove nonphotosynthetic microeukaryotes 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 nonphotosynthetic microeukaryotes (36). Another example involved the amphipod *Gammarus mucronatus* that exhibited a relatively nonspecific grazing of the estuarine detrital microbiota. This organism removed the microeukaryotes that were then replaced, to a large extent, by bacteria (77, 78). Results of the signature lipid biomarker analysis agreed with the cellular morphologies present as shown by scanning electron microscopy.

Analytical Sensitivity and Limitations for Lipid Analyses

Limits of detection and quantification depend on lipid recovery, the amount of background lipid contamination (in solvents, on glassware, etc), and on the sensitivity of the analytical equipment. Measuring microbial biomass with a colorimetric analysis of phospholipid derived organic phosphate is straightforward and requires little specialized equipment other than a spectrophotometer (132). However, this analysis is relatively insensitive, having limits of detection in the micromolar range (~10^{10} bacteria with the stable colorimetric analysis [132] or ~10^{7} bacteria the size of *Escherichia coli* with the dye-coupled assay [33]).

Conditions, under which the sensitivity of biomass detection is defined, especially when the chromatographic detector is a mass spectrometer, must be considered carefully. The first consideration is that the signal to noise ratio must be > 3 for the response to be acceptable. Sensitivity is defined in terms of limits of detection (LOD), which is the mean value of the background/ three times the standard deviation, and limits of quantitation (LOQ), which is the mean value of the background/ ten times the standard deviation (1). With MS, the full scan of target m/z (mass/charge) values gives less sensitivity (but a greater number of ions detected) than selected ion monitoring (SIM) of one or a few selected ions. The longer the MS can have to analyze at a given m/z (dwell time), the more accurate the response becomes and the greater is the sensitivity in the signal response. Full scanning decreases the duty cycle time devoted to each ion. The time for MS analysis of a component is limited by the width of the chromatographic peak. Usually, only the peak is analyzed, and the shoulders of that peak are used to correct for the baseline drift or to detect overlapping components eluting at the same time.

Sample ionization during MS analysis also affects sensitivity. Electron impact ionization produces many fragments, which decreases the sensitivity as the biomass component is divided into many fragments. A full scan with ions from m/z = 50 to above the molecular ion of m/z = 312 has an LOQ at least 50 times greater than the SIM. PLFA analysis using GC/chemical ionization mass spectrometry (GC/CIMS), which is a much softer ionization, produces far fewer ion fragments, thereby increasing the LOQ. In-source collisionally activated dissociation (CAD) leads to the formation of fatty acid fragment ions in the detector in negative ion mode. With HPLC/ESI/MS/MS, the precursor ions of each phospholipid molecule species can be selected in the first quadrupole for fragmentation in the CAD and then the product ions of that specific progenitor scanned in the third quadrupole. With tandem quadrupole mass spectrometry (TQMS), the LOQ can be 50 times lower than with the single quadrupole system.

For practical reasons, though, LODs and LOQs for environmental samples will necessarily be much greater than for purified (authentic) lipid sources used for establishing analytical

equipment LODs and LOQs. Firstly, lipid recovery from environmental samples depends on the sample matrix. Secondly, Tunlid *et al.* (115) demonstrated that recovering lipids from low microbe-containing samples is inherently difficult. This is, in part, due to lipids adsorbing onto glassware and the silica gel during sample processing. Thirdly, if the environmental sample has a very sparse bacterial content, then a large amount of matrix needs to be processed and that requires a large volume of extractant solvents. This large volume must be concentrated prior to any chromatography. The concentration step concentrates contaminants in the solvents. Contaminants in purified solvents for GC/MS analysis procedures can be between 4 and 20 pmol of PLFA (115). Also, PLFA analysis requires mild alkaline transmethylation which produces methyl esters of all the fatty acids. One of the chief culprits for contamination is in the sodium hydroxide used to make the methanolic sodium hydroxide. The contamination is almost exclusively in the PC which is relatively rare in bacteria. One of the advantages in terms of sensitivity of HPLC/ESI/MS is that the fatty acids can be examined in specific lipids so PE and PG, which are much less likely to be from eukaryotic contamination of the solvents, can be measured. In addition, there is more specificity in analysis of each phospholipid than in the total patterns for all the phospholipids as the GC/MS method requires. Also, the HPLC based methods do not require derivatization by transmethylation. HPLC/ESI/MS/MS will magnify these advantages of specificity and reduction of chemical noise for even greater sensitivity.

Currently, GC/MS LODs are approx. 10^5 bacterial cells and HPLC/MS LODs are approx. 10^4 bacterial cells (29). HPLC/MS LOQs are approximately 10^5 cells. For bacterial PLFA analysis, approx. 10^{7-9} bacterial cells are sufficient to obtain results that include some rare PLFA with good signal-to-noise ratio during GC or GC/MS analysis.

Lipid analysis requires special analytical skills and entails expenses for extractions, processing, and GC/MS or LC/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 are overcome, the interpretation of community composition and nutritional/physiological status requires an extensive familiarity with widely dispersed literature. Research toward automating and accelerating the speed of the analysis demonstrated the following benefits: required less solvent, labor, and time, and more efficiently extracted bacterial and fungal spore lipids (70). Currently, 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 information.

Acknowledgments: We acknowledge Cory A. Lytle, Aaron Peacock, and Margaret Gan (Center for Environmental Biotechnology, University of Tennessee) for providing the LOQ and LOD data reported herein. This work was supported by National Science Foundation grant DEB 9814813 and the Department of Energy, Office of Science, grant number DE-FC02-96ER62278 (Assessment Component of the Natural and Accelerated Bioremediation Research Program, NABIR).

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Table 1 Examples of signature lipids and their cellular location[a]

Genus, organism, or group	Lipid Biomarker	Cellular localization	Reference (s)
Desulfovibrio	i17:1ω7c, i15:1ω7c, i19:01ω7c	PLFA	28
Desulfobacter	10Me16, cy18:0(ω7,8)	PLFA	27
Desulfobulbus	17:1ω6c, 15:1	PLFA	92
Francisella tularensis	20:1ω11, 22:1ω13, 24:1ω15, 26:1ω17	PLFA	84
Nostoc commune	i15:1ω11, br18:1, 18:3ω3	PLFA	97
Flexibacter	i15:1ω5, i15:1ω6, β-OH-i15:0, β-OH-17:0	PLFA and LPS	44, 86
Vibrio cholerae	11Me19:1, 18:2ω6,9	PLFA	44
Archaea	Ether-linked lipids, diphytanyl glycerol diethers, Bidiphytinyl glycerol ethers	Membrane	83
Methanotrophs, type I	16:1ω8c, 16:1ω5c	PLFA	85
Methanotrophs, type II	18:1ω8c, 18:1ω8t	PLFA and LPS	85
Thiobacillus	i17:1ω5, 10Me18:1ω6, 11Me18:1ω6, hydroxy Cyclopropane, methoxy, mid-chain branched	PLFA and LPS	59, 60

	OHFA		
Actinomycetes	Mid-chain branched fatty acids	PLFA	61, 64
Frankia	cy18:0(ω8,9), i16:1ω6	PLFA	116
Planctomyces	19:1ω10, 3-OH20:0	PLFA and LPS	58
Desulfomonile	br3-OH19:0, br3-OH21:0, br3-OH22:0	LPS	101
Legionella	3-OHi14:0, 2,3 diOHi14:0, 27-oxo28:0, 27:2,3 OH22:0	LPS	117
Geobacter	14:1ω7, i17:1ω8, 3-OH15:0, 9-OH16:0, 10-OH16:0, 11-OH16:0, 3-OH17:0	PLFA and LPS	67
Mycobacteria	Micocerosic acids, 2M3,3-OHFA, 2-OH Alcohols	Neutral lipid	6
Bacillus or *Arthrobacter*	i15:0/a15:0, i17:0/a17:0 <0.2	PLFA	64
Fungi	18:2ω6, 18:3ω6, 18:3ω3, sterols	PLFA	80, 131
Clostridia	Plasmalogen-derived dimethyl acetals	Polar lipids	73
Diatoms	16:1ω13t, 16:2ω4, 16:3ω4, 20:5ω3	PLFA	16, 91, 134

Higher plants	18:1ω11, 18:3ω3, 20:5ω3, 26:0	PLFA	114, 136
Protozoa	20:2ω6, 20:3ω6, 20:4ω6	PLFA	120, 121

[a]Fatty acids are designated by the total number of carbon atoms followed by the number of double bonds, with the position of the double bond indicated from the methyl end (ω) of the molecule. Configuration of the double bonds is indicated as either *cis* (c) or *trans* (t). For example, 16:1ω7c is a PLFA with 16 total carbons with one double bond located seven carbons from the ω end in the *cis* configuration. Branched fatty acids are designated *iso* (i) or *anteiso* (a) if the methyl branch is one or two carbons, respectively, from the ω end (e.g. i15:0) or by the position of the methyl group from the carboxylic end of the molecule (e.g. 10Me16:0). Methyl branching at undetermined positions in the molecule is indicated by the prefix "br". Cyclopropyl fatty acids are designated by the prefix "cy" followed by the total number of carbons (e.g. cy17:0). The position of a hydroxyl group is numbered from the carboxyl end of the fatty acid, with OH as a prefix (e.g. 3-OH16:0).