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Biomass Measurements: Biochemical Approaches

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RATIONALE

Determination of biomass is of great importance in microbial ecology, as microbes form the base of the food web. As used in biochemical approaches to biomass, the microbes include the 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 the metabolic activities in the environment, and the nonviable biomass, which can still be part of the food web but has little or no potential metabolic activity. Classical biochemical and microbiological techniques successfully used in public health for the isolation and culture of clinical specimens have proven less than adequate for the determination of biomass and community structure in environmental samples. It has been repeatedly documented in reviews of the literature that viable counts of bacteria from various environmental samples may represent only a very small proportion of the extant microbial community (85, 86). Furthermore, microbes may be metabolically active and potentially infectious even though they are not culturable (98). Finally, classical microbial tests are time-consuming and provide little indication of the nutritional status or evidence of toxicity which can affect metabolic activities and can be crucial in studies of microbial ecology.

TRADITIONAL BIOMASS MEASURES

Microbiologists have traditionally quantitated the biomass of microbiota in a sample to the number of viable cells detected by viable count. 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 viable counts often represent 0.1 to 10% of the cells detected by using acridine orange direct counts (AODC) or biochemical methods. In soils and sediments, the problems are intensified, as the often sparse and heterogeneously distributed microbial community can significantly interfere with the accuracy of the determination of microbial abundance biomass by AODC. 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 show variations of 500 between different size classes of cells (65). Direct microscopic counts present problems when autofluorescence of sediment clay granules or opacities in the sediments obscure detection of bacteria in situ (22). This problem is often ameliorated by inducing detachment of the microbes from soil granules by using solutions containing multicharged ions like polyphosphate followed by recovery on membrane filters for microscopic counting. We have evidence from signature lipid biomarker analysis that in subsurface sediments, the detachment of the microbes is selective and often not quantitative. With in situ direct counting of bacteria at densities of less than 10^4 cells per g (dry weight) of sediment, the reproducibility of the counts is very low and the error is large, even with counting of 20 fields with adequate numbers of cells and replicate subsampling (53).

BIOCHEMICAL BIOMASS MEASURES

An effective and quantitative way to measure the biomass of the microbiota in situ without the requirements of culture or finding each organism in microscopic fields 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 excretion by death, 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. A number of cellular components, such as muramic acid and other cell wall components (23), have been used as measures of bacterial biomass. Lipopolysaccharide (LPS) components have been used as measures of the gram-negative bacteria (71). ATP is a universal measure of metabolizing cells, provided that it does not persist in soil following cell death (42). If the assay is combined with a treatment of added extracellular ATPase, then 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 (14).

LIPID ANALYSIS

All intact cells contain polar lipids. Polar lipids in microbes are primarily phospholipids. Determination of lipid phosphate (LP) or phospholipid ester-linked fatty acids (PLFA)

provides a quantitative measure of the microbial biomass containing intact cellular membranes. Either 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 rapidly transform the polar lipids in the cell membranes to nonpolar neutral lipid diglycerides by removing polar phosphate-containing head groups (97). The diglyceride-to-PLFA ratio increases in many subsurface sediments from <0.2 at the surface to over 2.0 at >200 m (95).

BIOMASS-TO-CELL NUMBER CONVERSIONS

One of the major problems with biochemical biomass measures is that the results are in micromoles 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, 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. Problems in equating AODC measurements, with their accuracy problems (22), to estimates from the PLFA or LP derive from the lack of a universally applicable conversion factor for estimating the PLFA or LP per bacterial cell and the number of cells per gram (dry weight) of bacteria (11, 21, 22, 85, 92). 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 (31, 65). The volume of a viable cell can also 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 of LP per g of carbon for aerobic organisms, compared with contents of between 118 and 250 μmol of LP per g of carbon for anaerobic cultures (11). *Arthrobacter crystallopoietes* showed a 30% decrease in PLFA per cell after 2 weeks of starvation (48), and *Vibrio cholerae* showed up to a 99.8% decrease in PLFA per cell after 7 days of starvation, with loss of culturability but not membrane integrity (39). Brinch-Iverson and King (11) stated that the conversion factor for bacteria of 100 μmol of LP per g of carbon (100 μmol of PLFA per g [dry weight]) based on earlier work (90) was reasonably applicable to sediments with a significant proportion of anaerobes. Bacterial cell volume varies between 0.01 and 7 μm^3, with smaller bacteria having a higher dry weight-to-volume ratio than larger bacteria (65, 66). 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 show a dependence on size classes (65).

EQUIVALENCE OF BIOMASS MEASURES

In one specific environment, a comparison of methods for determination of microbial biomass showed 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 (6). It was assumed that there were 2.5×10^{12} cells per g (dry weight) and 100 μmol of PLFA per g (dry weight) (6). 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 (10). 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.

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 (11, 22, 29, 92). The sensitivity of the classical colorimetric analysis for LP as initially proposed for environmental samples (92) has been improved considerably with a dye-coupled reaction to sensitivities of 1 nmol of LP, corresponding to about 10^7 bacteria (22). Higher sensitivities (about 10 fmol with gas chromatography [GC]-mass spectrometry [MS] and single-ion monitoring of negative ions) and specificity with a concomitant determination of the nonviable cell biomass, the community composition, and the nutritional-physiological status can be obtained through the application of the various GC-MS methods (66).

COMMUNITY COMPOSITION

Information obtained from the lipid analysis provides insight into the community composition as well. 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 the interpretation in terms of specific components may be obscured because of overlapping compositions among constituents. 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. Some specific groups of microbes contain characteristic fatty acid profiles. Several examples are listed in Table 1. However, it must be kept in mind that signature lipid biomarker analysis cannot detect every species of microorganism in an environmental sample, as many species have overlapping PLFA patterns. Further analysis of other lipids such as the sterols (for the microeukaryotes such as nematodes, algae, and protozoa) (57, 78, 91), glycolipids (phototrophs and gram-positive bacteria), or hydroxy fatty acids (OHFA) in the LPS of the lipid A of gram-negative bacteria (LPS-OHFA) (18, 21, 46, 71) can provide a more detailed community structure analysis.

EXTENSION TO NUCLEIC ACID ANALYSIS

The solvent extraction utilized in signature lipid biomarker analysis has recently been shown to liberate cellular nucleic acids which can be used for gene probing (43). Over 50% of the gene *nahA* present in intact *Pseudomonas fluorescens* cells added to soil was recovered by using the lipid extraction protocol compared with recovery by the standard techniques (68). The DNA recovered from the lipid extraction was of high quality and suitable for enzymatic amplification. The combined lipid extraction and recovery of nucleic acids can be very useful in biomass and community composition determinations. The DNA probe analysis offers powerful insights because of the exquisite specificity in the detection of genes. Concomitant DNA-lipid analysis readily provides quantitative recoveries independent of the ability to isolate or culture the microbes. The lipid analysis gives evidence of the phenotypic properties of the community that indicates ex-

TABLE 1 Examples of signature lipids and their cellular locations[a]

Genus, organism, or group	Lipid biomarker	Cellular localization	Reference(s)
Desulfovibrio	i17:1ω7c, i15:1ω7c, i19:1ω7c	PLFA	18
Desulfobacter	10Me16, cy18:0(ω7,8)	PLFA	17
Desulfobulbus	17:1ω6c, 15:1	PLFA	71
Francisella tularensis	20:1ω11, 22:1ω13, 24:1ω15, 26:1ω17	PLFA	61
Nostoc commune	i15:1ω11, br18:1, 18:3ω3	PLFA	74
Flexibacter	i5:1ω5, i15:1ω6, β-OH-i15:0, β-OH17:0	PLFA and LPS	32, 63
Vibrio cholerae	11Me19:1, 18:2ω6.9	PLFA	32
Archaea	Ether-linked lipids, diphytanyl glycerol diethers, bidiphytinyl glycerol ethers	Membrane	60
Methanotrophs, type I	16:1ω8c, 16:1ω5c	PLFA	62
Methanotrophs, type II	18:1ω8c, 18:1ω8t	PLFA and LPS	62
Thiobacillus	i17:1ω5, 10Me18:1ω6, 11Me18:1ω6, hydroxy cyclopropane, methoxy, mid-chain branched OHFA	PLFA and LPS	45, 46
Actinomycetes	Mid-chain branched fatty acids	PLFA	47, 48
Frankia	cy18:0(ω8,9), i16:1ω6	PLFA	82
Planctomyces	19:1ω10, 3-OH20:0	PLFA and LPS	44
Desulfomonile	br3-OH19:0, br3-OH21:0, br3-OH22:0	LPS	76
Legionella	3-OHi14:0, 2,3diOHi14:0, 27-oxo28:0, 27:2,3 OH22:0	LPS	83
Geobacter	14:1ω7, i17:1ω8,3-OH15:0, 9-OH16:0, 10-OH16:0, 11-OH16:0, 3-OH17:0	PLFA and LPS	50
Mycobacteria	Micocerosic acids, 2M3,3-OHFA, 2-OH alcohols	Neutral lipid	2
Bacillus or *Arthrobacter*	i15:0/a15:0, i17:0/a17:0 <0.2	PLFA	48
Fungi	18:2ω6, 18:3ω6, 18:3ω3, sterols	PLFA	57, 91
Clostridia	Plasmalogen-derived dimethyl acetals	Polar lipids	51
Diatoms	16:1ω13t, 16:2ω4, 16:3ω4, 20:5ω3	PLFA	9, 70, 96
Higher plants	18:1ω11, 18:3ω3, 20:5ω3, 26:0	PLFA	81, 99
Protozoa	20:2ω6, 20:3ω6, 20:4ω6	PLFA	85, 86

[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).

tant microbial activity by providing in situ indications of starvation, growth rate, exposure to toxicity, unbalanced growth, deficiencies of specific nutrients, and the aerobic/anaerobic metabolic balance, while DNA probes define the physiological potential of the microbial community. The combined DNA-lipid analysis overcomes some deficiencies in microbial ecology studies involving only nucleic acid analysis (87).

COMMUNITY PHYSIOLOGICAL STATUS

As already mentioned, it is possible to assess the physiological status of the microbial community by using lipid analysis. Many subsets of the microbial community respond to specific conditions in their microenvironment with shifts in lipid composition. The proportion of poly-β-hydroxyalkanoic acid (PHA) in bacteria (64) or triglyceride (in the microeukaryotes) (30) relative to the PLFA provides a measure of nutritional-physiological status. Some bacteria undergo unbalanced growth and cannot divide when exposed to adequate carbon and terminal electron acceptors but lack some essential nutrient such as phosphate, nitrate, and trace metals. These bacteria form PHA. When the essential component becomes available, these bacteria catabolize PHA and form PLFA as they grow and divide. 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 (81).

Specific patterns of PLFA can also indicate physiological stress in certain bacterial species (32). 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 (77). Starvation can lead to minicell formation and a relative increase in specific *trans*-monoenoic PLFA compared with the *cis* isomers (32). It has been shown that for increasing concentrations of phenol, *Pseudomonas putida* P8 forms increasing proportions of *trans*-unsaturated fatty acids (38). Increasing the proportions of *trans*-monoenoic PLFA is not the critical feature of solvent resistance in *P. putida*. Comparison of a solvent-sensitive strain with the 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 shifts its lipid composition, decreases the proportion of monoenoic PLFA to saturated PLFA, increases the level of LPS-OHFA, and exhibits decreased permeability to the hydrophobic antibiotic difloxacin not detected in the solvent-sensitive strain (73).

Phospholipid patterns also change in response to environmental stress. Some *Pseudomonas* species form acylornithine lipids in lieu of phospholipids when growing with limited bioavailable phosphate (52). Respiratory quinone structure indicates the degree of aerobic activity in gram-negative heterotrophic facultative bacteria (37). Anaerobes are usually associated with high ratios of *iso*-branched to *anteiso*-branched PLFA which are typical of *Desulfovibrio*-type sulfate-reducing bacteria. Gram-positive aerobes like *Micrococcus* or *Arthrobacter* species have low ratios of *iso*- to *anteiso*-branched saturated PLFA.

METABOLIC ACTIVITY IN ESTIMATING BIOMASS

There are also biomass measures which are based on detecting the activity of environmental microbiota. Assays dependent on enzymatic activities (80), growth, or respiration after chloroform fumigation (40) 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). Measurements of the viable microbial biomass by PLFA determination in marine sediments recovered from the Antarctic, the deep sea, and neotropical marine mud flats are remarkably constant at about 10 nmol of PLFA per g (dry weight) of sediment (~10^9 equivalent cells per g [dry weight]) (87). 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 determined from [^{3}H]thymidine incorporation rates (96).

DISTURBANCE ARTIFACTS IN ACTIVITY MEASUREMENTS

Great care must be taken with metabolic activity measurements to avoid the generation of disturbance artifacts. Lipid analysis has been used to overcome the problem of disturbance artifacts generated in determining microbial activity (16, 24, 25, 54). The facile determination of the ratio of [^{14}C] acetate incorporation into PHA and PLFA has proven especially valuable in ecological studies (27).

COMMUNITY COMPOSITION FROM ISOLATED BACTERIA

Community composition based on distinctive patterns of ester-linked fatty acids released from bacteria (largely from the phospholipids and LPS of clinical isolates) is currently used in identifying 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 by using the MIDI microbial identification system (MIDI, Newark, Del.). (84). Utilization of MIDI requires isolation and culture of the microbes prior to analysis. As a result, the unculturable microbes which may represent the vast majority of the environmental microbes are not detected. Utilization of fatty acids from cultured microbes from a soil or sediment does not indicate in situ biomass, nor is it as accurate as other methods in reflection of the viable community composition, as often the viable counts represent 0.1 to 10% of the cells detected by using AODC or biochemical measures (1, 4, 69).

BIOLOG

A new automated microbial identification system based on aerobic metabolic activities, Biolog, has been used for community microbiological composition. The system is based on differential activities among 92 various substrates and has been shown to show differences in community metabolism that paralleled those provided by the lipid analysis in differentiating microbial communities in drilling fluids, makeup waters, and deep subsurface cores (49). Unfortunately, this form of analysis requires a transparent carbon-free inoculum. Although many groundwater samples can be assayed directly, soils and subsurface sediments need to be blended, extracted with sodium pyrophosphate, and incubated without added carbon and nutrients for 24 h with agitation, and the supernatant must be flocculated with a mixture of calcium and magnesium salts before assay (49). The Biolog assay cannot be used in a quantitative determination of microbial biomass but can provide community activity comparisons between biomes. With the Biolog system, it was possible to detect considerable variation in the substrate utilization of microbial communities of soils taken from six different plant communities and compare differences in functional diversity (100). Patterns of substrate utilization were reproducible for model communities, but the extents of substrate utilization were not reflected in comparisons of responses of isolates, model communities of known composition, and soils (35). Replicate soil communities from the same pots varied considerably when the community activity analysis was used.

WHEN TO UTILIZE BIOCHEMICAL BIOMASS MEASURES

Biochemical biomass determinations have been successfully applied to a multitude of environments. Since the measurement involves extraction, concentration, purification, fractionation, derivatization, and analysis by GC-MS, with structural identification of each signature component, there are few environments to which it cannot be applied. The assay has even 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 soils (4, 29, 40, 47, 80), rhizospheres (81, 82, 99), ocean abyss (5), stream periphyton (33), clinical specimens (2, 67), pus (61), mummies (unpublished data), ice cores (70), mongoose anal sacs (15), sediments (1, 22, 25, 31, 54, 72, 78, 88), subsurface materials (6, 24, 28, 94, 95), membrane filter retentates from groundwater (50), bioprocessing (36, 51), biofouling films (89), concrete (46), detritus (55), sponge spicule mats (96), drinking water biofilms (83), rocks (3), fungal biomass (91), grazed detritus (56), substratum biodegradability (8), microbially influenced corrosion (41), predation (93), pollution (79), anaerobic digestors (36), and microcosm microbial community comparison with the field (20) have all been characterized by using lipid analysis.

COLLECTION OF SAMPLES FOR ANALYSIS

Proper sample collection techniques are essential for obtaining a representative sample from the environmental matrix. The analysis is most satisfactory when at least 10^8 bacteria are analyzed, since some of the more interesting signatures can be found as trace components in the lipid extract. It has proved possible though, to generate lipid profiles of deep

subsurface sediments which contain only 1.0 pmol of PLFA per g of sediment (equivalent to 2.5×10^4 bacteria) (94). Signature lipid biomarker analysis also can provide a nested analysis for the determination of appropriate sample sizes for experimental plots to determine the appropriate quadrat size (19). The analysis provides a quantitative estimation of sample-to-sample or within-sample heterogeneity in the determination of the microbial biomass and community composition in estuarine mud flats (19).

SAMPLE HANDLING

It is important that once the environmental samples are recovered, they be frozen (at least −20°C or below) or lyophilized as quickly as possible. If this is not possible, wet weights are recorded and the sample is cooled as rapidly as possible. To minimize community compositional changes, it is necessary that microbial activity be stalled as soon as possible following sample collection. If the samples have been grown in culture, the medium should be centrifuged and the resulting cell pellet should be rinsed twice with 0.05 μM phosphate buffer (pH 7.5) before lyophilization. Samples should not be held on ice (−4°C) any longer than absolutely necessary. Dry ice is satisfactory for holding frozen samples. Rock samples held at 4°C showed rapid and significant changes in biomass and community composition (3). Preserving samples with buffered formaldehyde or gluteraldehyde is not as satisfactory, as 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. Scrupulous cleaning of all glassware is absolutely necessary. Once glassware is used, it is immediately fully immersed in a washtub full of hot water and detergent. The cleaning process is so effective that phosphate-containing detergent can be used. The glassware is scrubbed with a brush and rinsed five times each with cold tap water and then deionized water. Glassware is allowed to dry completely before being wrapped in aluminum foil and heated in a clean muffle furnace for a minimum of 4 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 all potential contaminants. Plasticware cannot be used in lipid analysis. Samples can be extracted at room temperatures but should be protected from light, especially fluorescent light, if photosensitive lipids (such as quinones) are to be analyzed. The extractant consists of a single-phase chloroform-methanol mixture (1:2, vol/vol), generally called a Bligh and Dyer (7), which can be modified to accept a phosphate buffer (88). Investigators have found that modification of the buffer can increase the recovery of PLFA from soils with high clay content (29). Samples can be extracted in glass centrifuge bottles and then centrifuged at 6,000 × g for 30 min, 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. For bacterial samples, approximately 10^8 bacterial cells are sufficient to achieve a good signal-to-noise ratio during GC or GC-MS analysis. To obtain the lipid fraction of the extracted sample, equal volumes of chloroform and distilled water (or buffer) are added and the emulsion is shaken. With time a split phase develops, which is then centrifuged or allowed to separate passively overnight. The lower organic phase (containing the bacterial lipids) is collected and filtered through a fluted Whatman 2V filter that has been preextracted with $CHCl_3$. The organic 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 (31) 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 (47, 78). The glycolipid fraction can be analyzed for PHA (64). The polar lipid fraction, containing the phospholipids, is subjected to a transesterification by a mild alkaline methanolysis protocol (32), 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 the dimethyl disulfide adducts (59).

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 (71). After centrifugation at 6,000 × g for 30 min, the chloroform phase is recovered, evaporated to dryness, and methylated by using "magic" methanol (methanol-chloroform-concentrated HCl [10:1:1, vol/vol/vol]) (61). 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, vol/vol]), recovered in chloroform-methanol (1:1, vol/vol), and then derivatized by using bis(trimethylsilyl)trifluoroacetamide prior to GC-MS analysis.

Results are reported with 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).

PLFA and other lipid profiles can be entered into spreadsheet formats and subjected to statistical analysis. In addition to analysis of variance, two multivariate statistical applications have grown in popularity in addressing similarities between PLFA profiles. Dendrograms from a hierarchical cluster analysis are generally constructed from arcsine-transformed PLFA mole percent values, with similarities based on modified Euclidean distances. The two-dimensional plots generated from a principal-components analysis not only illustrate profile similarities (or differences) but also identify which PLFA contribute to the formation of the plots and to what extent (i.e., coefficients of loadings) (2, 76, 94, 95).

INTERPRETATION

Viable biomass is estimated from the total amount of PLFA detected in a sample. Phospholipids are an essential part of the intact cell membranes; thus, this biomass is a measure of the viable or potentially viable cells. Viable biomass can also be determined as organic LP by colorimetric methods (22, 92). The great majority of microbial phospholipids are diacyl, with a molar ratio of LP to PLFA of 1:2. Environmen-

tal samples showed LP-to-PLFA ratios of 1:2 to 1:1.5 with a mean of 1:1.7 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 (6). This equivalent yields 2.5×10^{4} cells per pmol of PLFA. It is important to note that the number of cells per gram (dry weight) can vary by up to an order of magnitude, as summarized by Findlay and Dobbs (21). With cell death, or as the cell ruptures, 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 μ–0, while in less ideal environments the ratio can exceed 3 (95).

COMMUNITY COMPOSITION

Microbial community composition can be characterized from the pattern and types of PLFA identified in a sample. Some examples of signature lipids are shown in Table 1. When one is determining community structure by 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 to some gram-negative anaerobic bacteria, such as the sulfate-reducing bacteria. 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 (62). Polyenoic PLFA generally indicate the presence of microeukaryotes but have also been sparingly reported in some bacteria (34). The PLFA 18:2ω6 is prominent in fungi but is also found in algae and protozoa. 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 typical of the microeukaryotes. There are exceptions 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 protozoans such as *Cryptosporidium* species, ergosterol is found in many fungi (58), and algae (12) contain a diversity of sterols in patterns which have proven to be useful in forming taxonomic relationships. Branched-chain monoenoic PLFA are common in the anaerobic *Desulfovibrio*-type sulfate-reducing bacteria both in culture and in manipulated sediments (18, 72). 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 homologs. Environments with 10Me16:0 > > 10Me18:0 often feature anaerobic gram-negative *Desulfobacter*-type sulfate-reducing bacteria (17, 72). 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.

PHYSIOLOGICAL STATUS

Insight into the nutritional and physiological status of the microbial community can be determined through application of signature lipid biomarker analysis. 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 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) (51, 85). An increase in cyclopropyl PLFA formation has also been associated with increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies. Bacteria make *trans*-monounsaturated fatty acids as a result of changes in the environment, usually as a result of stress (i.e., toxicity or starvation). For example, gram-negative bacteria make 16:1ω7t or 18:1ω7t fatty acids in the presence of toxic pollutants such as phenol (38). In addition *trans/cis* ratios of greater than 0.1 have been shown to indicate starvation in bacterial isolates (32). This value is usually 0.05 or less in healthy, nonstressed populations. A ratio of storage lipid (PHA) to membrane lipid (PLFA) can be interpreted as a measure of unbalanced growth. When bacteria are in the presence of a carbon source and a terminal electron acceptor but lack an essential nutrient, they do not undergo cell division but instead form storage compounds such as PHA. When growing vigorously, they do not form PHA but instead show an increase in total PLFA. PHA/PLFA ratios can range anywhere from 0 (dividing cells) to over 40 (carbon storage). Ratios greater than 0.2 usually indicate the beginnings of unbalanced growth in at least part of the microbial community.

It is sometimes useful to determine in situ proportions of aerobic and anaerobic metabolism within a microbial community. Benzoquinones (ubiquinones, coenzyme 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 (37). Naphthoquinones (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 naphthoquinones provides an indication of the extent of aerobic versus anaerobic microbial respiration. In gram-negative bacteria, respiratory quinones are usually 10 to 100 times less in content than the PLFA. Sometimes proportions of isoprenologs 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 can then be converted into dimethyl acetals. With increasing proportions of obligate anaerobes and anaerobic metabolism, the dimethy 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 (17).

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 (86). 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 (91). 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 (9). These community compositional shifts resulting from specific perturbations have been reviewed (86). A second validation was the isolation of a specific organism or groups of organisms, with subsequent detection of the same organisms by signature lipid analysis in consortia under conditions in which their growth was induced. It was possible to induce a "crash" in methanogenesis in a bioreactor by inducing the growth of sulfate-reducing bacteria (51) or by adding traces of chloroform or oxygen (36). These crashes were accompanied by shifts in the signature lipid biomarkers that were correlated with the changes in the microbial populations. Specific sulfate-reducing bacterial groups can also be "induced" in estuarine muds (52), as can methane-oxidizing populations (60) or propane-oxidizing actinomycetes (75), 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 (58) and by disturbing anaerobic sediments with oxygenated seawater (25). 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 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 (26). Another example involved the amphipod *Gammarus mucronatus*, which exhibited a relatively nonspecific grazing of the estuarine detrital microbiota. This organism removed the microeukaryotes, which were then replaced, to a large extent, by bacteria (55, 56). Results of the signature lipid biomarker analysis agreed with the cellular morphologies present as shown by scanning electron microscopy.

LIMITATIONS

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 (92). However, this analysis is relatively insensitive, with limits of detection in the micromolar range ($\sim 10^{10}$ bacteria with the stable colorimetric analysis [92] or $\sim 10^{7}$ bacteria the size of *Escherichia coli* with the dye-coupled assay [22]).

UNITS

A major problem with 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 micromoles 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 because of the variety of shapes and sizes organisms maintained in nature. 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.

In the determination of signature lipid biomarkers in environmental samples, the lipid profiles will not 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 necessitate less specific interpretations, i.e., at the functional group level. Since DNA suitable for gene probing can be recovered with signature lipid biomarker analysis, the combination of signature lipid biomarker analysis with DNA gene probe technology greatly expands the specificity and scope of community compositional determinations.

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 1 part in 10^{14} are commonly detected by 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 too distant future, it is likely that 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.

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Phospholipid patterns also change in response to environmental stress. Some *Pseudomonas* species form acylornithine lipids in lieu of phospholipids when growing with limited bioavailable phosphate (52). Respiratory quinone structure indicates the degree of aerobic activity in gram-negative heterotrophic facultative bacteria (37). Anaerobes are usually associated with high ratios of *iso*-branched to *anteiso*-branched PLFA which are typical of *Desulfovibrio*-type sulfate-reducing bacteria. Gram-positive aerobes like *Micrococcus* or *Arthrobacter* species have low ratios of *iso*- to *anteiso*-branched saturated PLFA.

METABOLIC ACTIVITY IN ESTIMATING BIOMASS

There are also biomass measures which are based on detecting the activity of environmental microbiota. Assays dependent on enzymatic activities (80), growth, or respiration after chloroform fumigation (40) 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). Measurements of the viable microbial biomass by PLFA determination in marine sediments recovered from the Antarctic, the deep sea, and neotropical marine mud flats are remarkably constant at about 10 nmol of PLFA per g (dry weight) of sediment ($\sim 10^9$ equivalent cells per g [dry weight]) (87). 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 determined from [^{3}H]thymidine incorporation rates (96).

DISTURBANCE ARTIFACTS IN ACTIVITY MEASUREMENTS

Great care must be taken with metabolic activity measurements to avoid the generation of disturbance artifacts. Lipid analysis has been used to overcome the problem of disturbance artifacts generated in determining microbial activity (16, 24, 25, 54). The facile determination of the ratio of [^{14}C] acetate incorporation into PHA and PLFA has proven especially valuable in ecological studies (27).

COMMUNITY COMPOSITION FROM ISOLATED BACTERIA

Community composition based on distinctive patterns of ester-linked fatty acids released from bacteria (largely from the phospholipids and LPS of clinical isolates) is currently used in identifying 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 by using the MIDI microbial identification system (MIDI, Newark, Del.). (84). Utilization of MIDI requires isolation and culture of the microbes prior to analysis. As a result, the unculturable microbes which may represent the vast majority of the environmental microbes are not detected. Utilization of fatty acids from cultured microbes from a soil or sediment does not indicate in situ biomass, nor is it as accurate as other methods in reflection of the viable community composition, as often the viable counts represent 0.1 to 10% of the cells detected by using AODC or biochemical measures (1, 4, 69).

BIOLOG

A new automated microbial identification system based on aerobic metabolic activities, Biolog, has been used for community microbiological composition. The system is based on differential activities among 92 various substrates and has been shown to show differences in community metabolism that paralleled those provided by the lipid analysis in differentiating microbial communities in drilling fluids, makeup waters, and deep subsurface cores (49). Unfortunately, this form of analysis requires a transparent carbon-free inoculum. Although many groundwater samples can be assayed directly, soils and subsurface sediments need to be blended, extracted with sodium pyrophosphate, and incubated without added carbon and nutrients for 24 h with agitation, and the supernatant must be flocculated with a mixture of calcium and magnesium salts before assay (49). The Biolog assay cannot be used in a quantitative determination of microbial biomass but can provide community activity comparisons between biomes. With the Biolog system, it was possible to detect considerable variation in the substrate utilization of microbial communities of soils taken from six different plant communities and compare differences in functional diversity (100). Patterns of substrate utilization were reproducible for model communities, but the extents of substrate utilization were not reflected in comparisons of responses of isolates, model communities of known composition, and soils (35). Replicate soil communities from the same pots varied considerably when the community activity analysis was used.

WHEN TO UTILIZE BIOCHEMICAL BIOMASS MEASURES

Biochemical biomass determinations have been successfully applied to a multitude of environments. Since the measurement involves extraction, concentration, purification, fractionation, derivatization, and analysis by GC-MS, with structural identification of each signature component, there are few environments to which it cannot be applied. The assay has even 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 soils (4, 29, 40, 47, 80), rhizospheres (81, 82, 99), ocean abyss (5), stream periphyton (33), clinical specimens (2, 67), pus (61), mummies (unpublished data), ice cores (70), mongoose anal sacs (15), sediments (1, 22, 25, 31, 54, 72, 78, 88), subsurface materials (6, 24, 28, 94, 95), membrane filter retentates from groundwater (50), bioprocessing (36, 51), biofouling films (89), concrete (46), detritus (55), sponge spicule mats (96), drinking water biofilms (83), rocks (3), fungal biomass (91), grazed detritus (56), substratum biodegradability (8), microbially influenced corrosion (41), predation (93), pollution (79), anaerobic digestors (36), and microcosm microbial community comparison with the field (20) have all been characterized by using lipid analysis.

COLLECTION OF SAMPLES FOR ANALYSIS

Proper sample collection techniques are essential for obtaining a representative sample from the environmental matrix. The analysis is most satisfactory when at least 10^8 bacteria are analyzed, since some of the more interesting signatures can be found as trace components in the lipid extract. It has proved possible though, to generate lipid profiles of deep

subsurface sediments which contain only 1.0 pmol of PLFA per g of sediment (equivalent to 2.5×10^4 bacteria) (94). Signature lipid biomarker analysis also can provide a nested analysis for the determination of appropriate sample sizes for experimental plots to determine the appropriate quadrat size (19). The analysis provides a quantitative estimation of sample-to-sample or within-sample heterogeneity in the determination of the microbial biomass and community composition in estuarine mud flats (19).

SAMPLE HANDLING

It is important that once the environmental samples are recovered, they be frozen (at least −20°C or below) or lyophilized as quickly as possible. If this is not possible, wet weights are recorded and the sample is cooled as rapidly as possible. To minimize community compositional changes, it is necessary that microbial activity be stalled as soon as possible following sample collection. If the samples have been grown in culture, the medium should be centrifuged and the resulting cell pellet should be rinsed twice with 0.05 μM phosphate buffer (pH 7.5) before lyophilization. Samples should not be held on ice (+4°C) any longer than absolutely necessary. Dry ice is satisfactory for holding frozen samples. Rock samples held at 4°C showed rapid and significant changes in biomass and community composition (3). Preserving samples with buffered formaldehyde or gluteraldehyde is not as satisfactory, as 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. Scrupulous cleaning of all glassware is absolutely necessary. Once glassware is used, it is immediately fully immersed in a washtub full of hot water and detergent. The cleaning process is so effective that phosphate-containing detergent can be used. The glassware is scrubbed with a brush and rinsed five times each with cold tap water and then deionized water. Glassware is allowed to dry completely before being wrapped in aluminum foil and heated in a clean muffle furnace for a minimum of 4 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 all potential contaminants. Plasticware cannot be used in lipid analysis. Samples can be extracted at room temperatures but should be protected from light, especially fluorescent light, if photosensitive lipids (such as quinones) are to be analyzed. The extractant consists of a single-phase chloroform-methanol mixture (1:2, vol/vol), generally called a Bligh and Dyer (7), which can be modified to accept a phosphate buffer (88). Investigators have found that modification of the buffer can increase the recovery of PLFA from soils with high clay content (29). Samples can be extracted in glass centrifuge bottles and then centrifuged at $6,000 \times g$ for 30 min, 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. For bacterial samples, approximately 10^8 bacterial cells are sufficient to achieve a good signal-to-noise ratio during GC or GC-MS analysis. To obtain the lipid fraction of the extracted sample, equal volumes of chloroform and distilled water (or buffer) are added and the emulsion is shaken. With time a split phase develops, which is then centrifuged or allowed to separate passively overnight. The lower organic phase (containing the bacterial lipids) is collected and filtered through a fluted Whatman 2V filter that has been preextracted with $CHCl_3$. The organic 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 (31) 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 (47, 78). The glycolipid fraction can be analyzed for PHA (64). The polar lipid fraction, containing the phospholipids, is subjected to a transesterification by a mild alkaline methanolysis protocol (32), 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 the dimethyl disulfide adducts (59).

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 (71). After centrifugation at $6,000 \times g$ for 30 min, the chloroform phase is recovered, evaporated to dryness, and methylated by using "magic" methanol (methanol-chloroform-concentrated HCl [10:1:1, vol/vol/vol]) (61). 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, vol/vol]), recovered in chloroform-methanol (1:1, vol/vol), and then derivatized by using bis(trimethylsilyl)trifluoroacetamide prior to GC-MS analysis.

Results are reported with 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).

PLFA and other lipid profiles can be entered into spreadsheet formats and subjected to statistical analysis. In addition to analysis of variance, two multivariate statistical applications have grown in popularity in addressing similarities between PLFA profiles. Dendrograms from a hierarchical cluster analysis are generally constructed from arcsine-transformed PLFA mole percent values, with similarities based on modified Euclidean distances. The two-dimensional plots generated from a principal-components analysis not only illustrate profile similarities (or differences) but also identify which PLFA contribute to the formation of the plots and to what extent (i.e., coefficients of loadings) (2, 76, 94, 95).

INTERPRETATION

Viable biomass is estimated from the total amount of PLFA detected in a sample. Phospholipids are an essential part of the intact cell membranes; thus, this biomass is a measure of the viable or potentially viable cells. Viable biomass can also be determined as organic LP by colorimetric methods (22, 92). The great majority of microbial phospholipids are diacyl, with a molar ratio of LP to PLFA of 1:2. Environmen-

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