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G. STOTZKY
Department of Biology
New York University
New York, New York

JEAN-MARC BOLLAG
Department of Agronomy
The Pennsylvania State University
University Park, Pennsylvania

MARCEL DEKKER, INC. **New York • Basel • Hong Kong**

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Biochemical Analysis of Biomass, Community Structure, Nutritional Status, and Metabolic Activity of Microbial Communities in Soil

ANDERS TUNLID *Lund University, Lund, Sweden*

DAVID C. WHITE *University of Tennessee and Oak Ridge National Laboratory, Knoxville, Tennessee*

I. PROBLEMS IN ASSAYING MICROORGANISMS IN SOILS

The determination of microbial biomass and the activity of microorganisms in soil presents a complex analytical problem for assays. Numerous studies have demonstrated that classic methods, which require the isolation and subsequent culture of microorganisms, are not adequate for enumerating microorganisms in soil. Viable counts underestimate the microbial community when compared with direct count techniques [1–3] or with estimates of muramic acid in the prokaryotic cell wall [4]. This discrepancy has been attributed to the selective growth of microbes on artificial media, the formation of a single colony from bacterial aggregates, and the difficulty of quantitative removal of organisms from soil particles [4,5]. Direct counting methods are also subject to technical difficulties when applied to soil systems [5]. For example, cells may be hidden in soil particles or overlapping organisms, particularly in aggregates of organisms attached to particles, and the conversion from counts to biomass, by estimating volumes of the microorganisms, can result in large errors.

Furthermore, classic methods provide a limited insight into the metabolic function and activity of the microorganisms in soil. Specific

fluorescent dyes (e.g., fluorescein diacetate) [6,7] have been used to determine the metabolically active cells of microorganisms in soils. Active cells can also be determined by a combination of autoradiography and microscopy [8,9]. However, this methodology requires enzymatic activity in the presence of substrates and is subject to the limitations associated with the density of organisms and the thickness of the biofilm in the field of view. Moreover, when substrates are introduced to measure metabolic activity, the process of introduction can induce artificially high levels of activity, with a possible disturbance artifact.

The problems associated with the use of viable counts and microscopic examination to estimate biomass of microorganisms in soil have stimulated research on the development of new methods. One approach is to estimate the biomass of soil microorganisms by measuring the concentration of specific biochemical components of the microbial cells [5,10–12]. Components that are generally present in all cells are utilized as a measure of biomass, the components that are restricted to subsets of the microbial community are utilized to define the community structure. The validity of the concept of "signatures" for subsets of the microbial community, which is based on the limited distribution of specific components, has been shown for several groups of microorganisms [10–12]. Biochemical methods have also been utilized to indicate the nutritional status of microorganisms in natural environments, and the metabolic activity of the microbial community can be estimated by measuring the rate of isotope incorporation from labeled precursors [10–12].

The biochemical methods do not have the problems associated with the classic methods because they do not depend on growth, with the inherent problem of microbial selection, nor do they require removing cells from surfaces. Biochemical methods examine the community as a whole with the structure of the consortia left intact. In contrast with the chloroform fumigation methods, the biochemical methods give information on biomass and community structure, as well as on the metabolic activity of the microflora.

II. BIOCHEMICAL METHODS FOR BIOMASS MEASUREMENTS

When determining biomass by measuring the concentration of a cellular component, several requirements must be fulfilled [5,10–13]: (1) the measured component must only occur in living microorganisms and not exist in dead cells nor in nonliving parts of the soil organic matter; (2) it must be possible to quantitatively extract and analyze the component in soil samples with appropriate sensitivities; and (3) the component should exist in fairly uniform concentrations in the cell. Several cellular components have been used for biomass

measurements in soils. These components include adenosine triphosphate (ATP), microbial membrane components, and constituents of microbial cell walls (Table 1).

Some skepticism exists among several microbiologists concerning estimation of biomass of soil microorganisms by measuring specific cellular constituents. They assume that no cellular components fulfill the foregoing criteria [5,14]. Despite these admonitions, many papers have been published surveying the developments and applications of biochemical methods for assaying the biomass of soil microorganisms. The results of these studies are summarized below.

A. ATP

The rapid metabolism of ATP in living cells, as well as its quantification by the very sensitive luciferin–luciferase assay, have led to the widespread use of ATP analysis as a biochemical tool of biomass determination in soil [5]. The uses of ATP and other nucleotide analyses in microbial ecology have been extensively reviewed by Karl [15], and we will only briefly discuss the application of this method to soil systems.

Table 1 Biochemical Components Used as Signatures for Estimating Biomass of Microorganisms in Soil

Component[a]	Organisms	Ref.
ATP	All cells	16,17,20–25,28, 32–35
Membrane components		
Phospholipids	All cells	45,51,52,67,68,129, 130,135,145–148
LPS	Gram-negative bacteria	51,52,62,67,68
Ergosterol	Fungi	79,80
Cell wall components		
Muramic acid	Bacteria	4,52,91
D-alanine	Bacteria	92
DAP	Gram-negative bacteria	79,80,90,92–95
Glucosamine	Fungi	89,90,106–108,113–117

[a]LPS, lipopolysaccharides; DAP, diaminopimelic acid.

Extracellular ATP has been reported to rapidly decompose in soils [16,17]. However, several studies of aquatic systems have demonstrated the presence of freely dissolved ATP [15], and growth studies of *Escherichia coli* have shown that, during the death phase, viable counts decreased more rapidly than the concentration of ATP. This observation suggests that a sizable portion of the total ATP content originates from dead cells [18,19].

A major problem of ATP measurements in soil is the variability of extraction efficiency [5,20]. Several methods have been used, including cold H_2SO_4 [17,21–25], trichloroacetic acid with paraquat and phosphate [17,20], but none of these is entirely efficient for extracting ATP from all soil types. Losses during the extractions can be accounted for by using a recovery standard of authentic ATP [15].

The most common procedure for measuring ATP in environmental samples utilizes a luciferin–luciferase system, which has a sensitivity of about 10^{-14} mol of ATP [26]. It has been calculated that this sensitivity corresponds to the ATP content of approximately 10^3 bacterial cells by assuming an ATP content of 4.0 nmol mg^{-1} dry weight [15] and an average dry weight of soil bacteria of 6.4×10^{-11} cells g^{-1} dry weight [27]. The light emission from the enzymatic reaction can be measured with a spectrophotometer, a liquid scintillation counter, or a special photometer designed for ATP measurements. The reaction can be inhibited by various ions and other components in the extracted sample [28]. Procedures have been developed for eliminating ionic interference by sorption of the ATP to charcoal [29] or ion-exchange columns [21], followed by elution of the ATP for assay. However, hydrolysis of ATP can occur as a result of adsorption to charcoal [30]. Moreover, the enzyme must be purified to prevent reactivity with nonadenosine nucleotides [15]. These and other problems with the luciferin–luciferase method for measuring ATP content in environmental samples led Davis and White [31] to develop a high-performance liquid chromatography (HPLC) procedure for the isolation of ATP, which also made it possible to analyze several other adenine-containing components, including adenine, adenosine, AMP, cyclic-AMP, ADP, and NAD.

The concentrations of ATP in microbes vary, depending on the species, growth rate, and media composition. Karl [15] compiled data of ATP content in various exponentially grown microorganisms and found that the range of ATP was 0.5 to 18 nmol mg^{-1} dry weight. Furthermore, the ATP content varies with the concentrations of several nutrients. For example, growth under phosphate deficiency decreased the cellular content of ATP by more than 90%, compared with cultures grown with adequate phosphate [15,21,32]. The ATP content of microorganisms in soil increases after the addition of glucose to soil [32,33].

To establish a factor for the conversion of ATP content to biomass, the ATP concentrations in various microorganisms grown in vitro were determined. The mean values obtained from these measurements were then used as conversion factors for the soil microbiota [5]. Using such conversion factors, biomass measurements estimated from ATP contents in various soils showed relatively good agreement with other biomass measurements that are based on the chloroform fumigation—incubation method and direct counts [5,33—35]. Several stipulations have to be made for this to be a valid comparison; for example, all ATP has to be in the biomass, and the values of the carbon content of the biomass, as well as the conversion factors used in the fumigation and direct count methods, have to be defined.

B. Phospholipids

Measurements of the content of phospholipids (PL) have been used to estimate the biomass of the microorganisms, especially in aquatic sediments, but also in soil systems [10,11] (see Table 1). Phospholipids are found in the membranes of all living cells, but not in the storage products of microorganisms [36]. Phospholipids are actively metabolized during the growth of bacterial monocultures [37], and they have a relatively rapid turnover in dead bacteria added to aquatic sediments [38]. Similar studies have not been performed in soil systems, although the results from a study on the degradation of labeled phosphatidylcholine in soils suggested a rapid turnover of microbial phospholipids in soils [39].

Our laboratories have developed a suite of methods to analyze phospholipids and other lipid biomarkers of microbial biomass, as well as the community composition and nutritional activity of microorganisms in environmental samples [10,11,40]. These methods are based on an efficient one-phase chloroform:methanol:buffer extraction system, modified from Bligh and Dyer [41,42] (Fig. 1). The one-phase solvent system is then divided into two phases, with the addition of one portion of chloroform and one portion of buffer. The lipids are recovered in the organic phase. The aqueous phase can be used for the analysis of ATP, and the residue from the lipid extraction can be used for analysis of microbial cell wall signatures. The extracted lipids are separated on silicic acid columns into three fractions that contain neutral lipids, glycolipids, and phospholipids [42]. With this procedure, the phospholipids of *E. coli* cells, when added to sediments, were quantitatively recovered [38,43]. No such studies have been performed in soils.

The extraction procedure of Bligh and Dyer [41,42] has been compared with other methods for efficiency in extracting lipid-soluble phosphorus from soils [44]. Somewhat higher amounts of organic phosphorus were recovered with an acid pretreatment, followed by a hexane—acetone extraction or by a series of organic

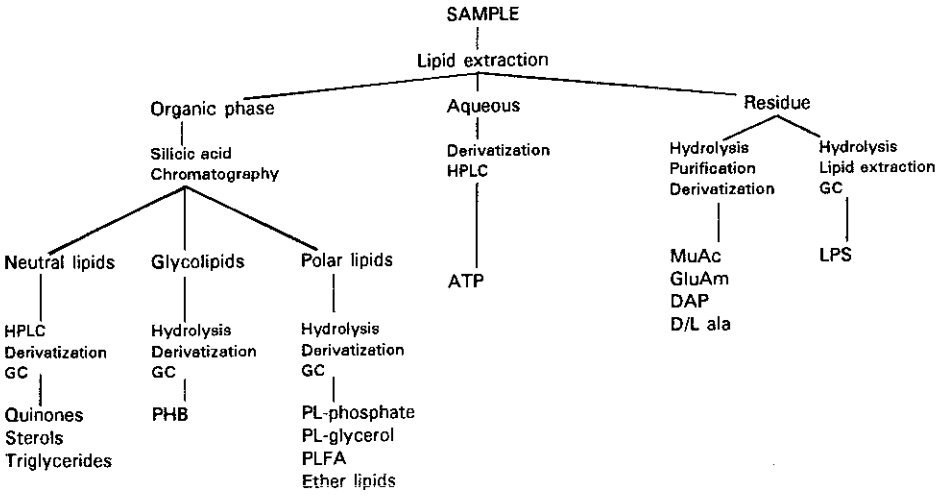

Figure 1 Scheme for the analysis of lipid fractions from soil samples.

solvents different from those used with the modified Bligh and Dyer method. However, more experiments need to be performed to establish the most reliable and accurate method for extracting phospholipids from microorganisms in soil.

Phospholipid in the polar lipid fraction from the silicic acid column is readily measured, after perchloric acid digestion, by colorimetric analysis to a sensitivity of 10^{-9} mol [38]. This enables the detection of about 10^{9} bacteria that are the size of *E. coli*. The successive hydrolysis with hydrochloric acid and hydrofluoric acid (HF) and the use of gas chromatography (GC) to assay glycerol increased the sensitivity for glycerol to 10^{-11} mol [45]. The sensitivity of the phospholipid analysis can be significantly improved by analyzing the esterified fatty acids by capillary GC [46] (see Sect. III.A).

The content of phospholipids in microorganisms varies among different taxa [36,47,48]. Kates [36] showed that the phospholipid content of various bacteria varied between 4 and 91 mg g^{-1} dry

weight. Furthermore, studies of monocultures have shown that the concentration and composition of phospholipids are affected by growth conditions, such as the temperature, pH, and the nutrient composition of the medium [49]. However, other experiments have shown that when bacteria are grown under conditions in a natural habitat, the cells contain a relatively constant proportion of their biomass as phospholipids [43,50].

Mean values of phospholipid content in various monocultures have been used to convert phospholipid measures to microbial numbers and biomass [e.g., 45,51,52]. The equivalence of microbial biomass measurements using phospholipid analysis, microscopic counts, ATP measurements, and several other chemical methods have been demonstrated in subsurface aquifer sediments (Table 2) [52]. These environments contained a sparse microflora of relatively uniform coccoid bacteria, which made the community ideal for comparing chemical measures and microscopic estimates of biomass.

C. Lipopolysaccharides

The outer cell membrane of gram-negative bacteria contain unique lipopolysaccharide (LPS) polymers consisting of a lipid (lipid A), a core polysaccharide, and an *O*-specific side chain [53]. Analysis of LPS has been used to estimate the biomass of gram-negative bacteria in soils and sediments (see Table 1) [54,58]. Studies have shown that LPS of dead bacteria are rapidly lost from sediments [54]. The decomposition rate of LPS in soils has not been examined.

The most common method used to analyze LPS in environmental samples is based on the limulus amebocyte lysate (LAL) test [59, 60]. In this test, an aqueous extract from the blood cells of the horseshoe crab (*Limulus polyphemus*) reacts specifically with LPS to form a turbid solution, with the amount of gelation being proportional to the LPS concentration. The LAL test is rapid and sensitive, with a detection limit of about 0.4 pg of LPS [60]. There are, however, several problems associated with use of this method for the quantitative analysis of LPS in samples as complex as soil. The LPS has to be quantitatively extracted from the sample, and two extraction procedures have been used: hot phenol–water [62] and trichloroacetic acid [54]. Only about 10% of the LPS in heat-killed *E. coli* cells was recovered from soils when phenol extraction was used [62]. Furthermore, the specificity of the LAL test is controversial, as there are several reports of substances, other than LPS, causing gelation [e.g., 61,64]. It has also been shown that LPS from various bacterial strains can differ in their ability to gel the lysate [66].

The problems associated with the LAL assay can be circumvented by the analysis of specific signature components in LPS. β-Hydroxymyristic acid (HMA) in lipid A and ketodeoxyoctanoate (KDO) in the core region of LPS are two such signatures. Analysis of HMA has been used to determine the LPS content in several soil studies [51, 67,68]. β-Hydroxymyristic acid can be analyzed by acid hydrolysis of the residue from the Bligh and Dyer extraction (see Fig. 1), followed by reextraction with fresh solvent, purification by thin-layer chromatography (TLC), and then separation by capillary GC [58]. This procedure yielded four to ten times more hydroxy acids than the amounts recovered in trichloroacetic acid or hot-phenol–water extracted LPS in samples of marine sediments [58]. The sensitivities of this method are approximately 10^{-12} mol using a flame ionization detector (FID), and 10^{-13} mol using electron-capture detection (ECD), which corresponds to 10^7 or 10^6 *E. coli* cells [58]. The sensitivities of the assays can be significantly improved by the use of special derivatives and mass spectrometric detection [69–71]. Ketodeoxyoctanoate can be analyzed by GC [54,72], but this method has been applied only in studies of gram-negative bacteria in estuarine sediments [54].

Some variation in the content and composition of LPS in bacterial monocultures has been demonstrated, depending on the taxa, temperature, and composition of the growth medium [53,73,74]. The lipid A part appears to be subject to less variation than the sugar parts of LPS. When conversion factors calculated from studies with bacterial monocultures were used, estimates of microbial biomass based on the content of lipid A hydroxy fatty acids showed good agreement with estimates based on direct counts and contents of ATP, phospholipid, and muramic acid in subsurface soils (see Table 2).

D. Ergosterol

Ergosterol (ergosta-5,7,22-trien-3β-ol) is the predominant sterol in most fungi [75]. Analysis of ergosterol has provided a rapid and sensitive method for the quantification of fungal invasions of plant material [76,78], and the method has been introduced by West and Grant for estimating fungal biomass in soil [79,80]. Although the turnover rate of ergosterol in fungi in soils has not been determined, it can be assumed that ergosterol, as a membrane component, is degraded at a rate similar to that of fungal cytoplasm in soil, a rate that is considerably faster than that of microbial cell walls in the same environment [81].

Ergosterol can be recovered from soils by extraction with methanol, saponification, and reextraction with hexane [79]. The ergosterol is then analyzed by HPLC using a UV detector, taking

Table 2 Comparisons Among Microscopic Cell Count, ATP, Cell Wall Components, and Membrane Lipid Biomarkers in the Determination of Subsurface Soil Biomass

Components	Concentration (nmol g^{-1} dry sediment)	Conversion factor (μmol g^{-1} dry wt. cell)	Direct count equivalence[a] (10^6 cells g^{-1} dry wt. sediment)	Dry wt. cells (μg cells g^{-1} dry wt. sediment)
Direct count			7.4 (3.5)	1.3 (1.1)[b]
Membrane				
Phospholipid fatty acids	0.35 (0.08	100	7.0 (0.2)	3.5 (0.1)
Lipid phosphate	0.11 (0.03)	50	4.4 (0.1)	2.2 (0.05)
Glycerol phosphate	0.22 (0.09)	50	8.8 (0.3)	4.4 (0.15)
LPS-OH-FAME[f]	0.09 (0.04)	15	12.0 (3.0)	6.0 (1.5)
Cell wall				
Muramic acid	0.22 (0.1)	58.5	7.5 (5.0)	3.7 (2.5)
ATP	1.39 (0.42)[c]	1.7[d]/10^7	8.2 (1.1)	1.4 (0.8)[e]

[a]Calculated with 2.0×10^{13} cells g^{-1} dry wt.
[b]Assuming a cell volume of 1.07 μm^3 and 1.72×10^{-13} g/cell.
[c]ng ATP g^{-1} dry wt. sediment.
[d]ATP given in $\mu g/10^7$ cells.
[e]Assuming 10^7 cells weigh 1.72 ng.
[f]Lipopolysaccharide hydroxy fatty acids.
Source: Data from Ref. 52.

advantage of the characteristic UV absorption of ergosterol at 282 nm, which differs significantly from the maximum adsorption of other plant and animal sterols [76]. The sensitivity of this method is approximately 10 ng ergosterol per gram of dry soil, calculated by using values of the ergosterol content in fungi of approximately 2 μg fungal dry weight per gram of soil dry weight. The physical losses in the extraction procedure for ergosterol in soil, estimated by adding [^{14}C]ergosterol, were less than 8% [79]. Ergosterol can also be extracted with the Bligh and Dyer mixture, purified with silicic acid chromatography, recovered from the neutral lipid fraction, and analyzed by capillary GC [83] (see Fig. 1).

The ergosterol content of fungal mycelium varies, depending on species and growth conditions [48,75,76]. For example, Seitz et al. [76] reported a range in ergosterol content between 2.3 and 5.9 mg g^{-1} dry weight from the analysis of three fungal species grown for various times and under different conditions. West et al. [80] demonstrated a high linear correlation between ergosterol content and fungal surface area in stored, air-dried, and substrate-amended grassland and arable soils. Furthermore, the ergosterol/biomass C ratios (estimated by microscopy) for these soils resembled ratios determined in vitro in pure cultures of fungi [80,82,84]. These data indicate that the ergosterol detected in these soils was associated with living mycelia.

E. Muramic Acid, D-Alanine, and Diaminopimelic Acid

The peptidoglycan (PG) of the bacterial cell wall contains several unique components that are not found in other organisms. These unique components have been used as signatures for bacterial biomass in soils (see Table 1). The PG molecule consists of glycan chains of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid (MuAc). The individual chains are interconnected by short peptide bridges containing specific amino acids, including *D*-alanine and *m*-diaminopimelic acid (DAP) [85]. MuAc and *D*-alanine are present in the PG from all bacteria, whereas DAP is present in all gram-negative bacteria, but only in some gram-positive bacteria [85].

At least parts of bacterial cell walls are resistant to degradation in soils [80,81,86,87]. The half-life of microbial material synthesized from labeled precursors was calculated to be approximately 6 months in soil [86], and the cell walls are more resistant to degradation than is the cytoplasma [81]. The decomposition rate of bacterial cell walls in soil is probably the result of their stabilization by complexing with humid acid polymers [88,90].

Components of the bacterial cell wall are recovered from soil after hydrolysis with strong HCl (4,79,91–94) (see Fig. 1). They are

then purified by cation-exchange chromatography [4,96,97]. Muramic acid has been quantified in these soil hydrolysates by analysis of the lactate released from the molecule by subsequent alkaline hydrolysis [4]. The average recovery of MuAc from soils analyzed by this method was 79% [4]. A more sensitive method is to form a derivative and then analyze MuAc by GC [91,96–99] or by HPLC [100]. The sensitivity of these methods is about 10^{-11} mol of MuAc, which corresponds to about 10^{8} bacteria with a size comparable with that of *E. coli*. The detection limit of the GC analysis can be substantially improved by forming special derivatives and using electron capture devices (ECD) or mass spectrometers (MS) detection systems [96].

Grant and West [79] developed a simplified procedure for the analysis of DAP in soil samples. They combined the purification and analysis steps by using paper chromatography. The recovery of DAP added to soil using this method was, on average, 80%.

The analysis of *D*-alanine requires special methods to separate the optical isomers of alanine. Two such GC methods have been developed. In one technique, *D*- and *L*-alanine were separated on a GC column coated with an optically active stationary phase [96]. In the other method, the enantiomers were separated on an optically inactive column by forming diastereoisomeric derivatives of *D*- and *L*-alanine [101].

The contents of PG components in bacterial monocultures do not vary greatly with growth rate or medium composition [102]. However, the concentration of MuAc in gram-positive and gram-negative bacteria and cyanobacteria are different. Millar and Casida [4] analyzed the MuAc content of several bacterial isolates from soil and reported an average of 19.4 mg MuAc per gram of biomass C for gram-positive bacteria and 7.2 mg MuAc per gram of biomass C for gram-negative bacteria. Similar values were obtained by Moriarty for some marine and terrestrial bacteria [103].

Millar and Casida [4], from their values for the MuAc content in bacteria, estimated that the MuAc levels in soil were about 100- to 1000-fold higher than could be accounted for by the number of organisms found on plate counts of the examined soils. This discrepancy, in part, might be because only a fraction of the total bacterial population is recovered in plate counts. However, the higher than explainable levels of MuAc could also have resulted from dead bacterial cells and organic material [4]. West et al. [80] reported that the DAP/biomass C ratios in soils (0.32 to 2.55) were more than 100 times higher than those obtained with bacteria grown individually in pure culture, and they suggested a large proportion of the DAP in their soils was also present in nonliving organic matter [87,93]. Manipulations of the soil biomass by storage, air-drying, and glucose-amendment showed, however, that changes in

the DAP content and in the populations of bacteria determined by plating were closely related [80]. Studies in subsurface soils have demonstrated an equivalence in biomass estimations between measurements based on MuAc content, other chemical measures, and microscopic counts (see Table 2).

F. Chitin and Glucosamine

Chitin, a polymer of *N*-acetylglucosamine, is a major component of fungal cell walls [104]. Glucosamine is not unique to chitin, but occurs in the bacterial cell wall and in the exoskeleton of invertebrates [105]. Analysis of glucosamine has been used to measure mycelial biomass in wood and leaf litter [106–108], in plants infected by pathogens or mycorrhizal fungi [109–117], and to estimate the biomass of fungi in soil [79,80]. As with bacterial cell wall components, there are data that indicate the presence of glucosamine in nonliving organic material, and experiments have demonstrated a relatively slow degradation of fungal cell wall material in soils [90,98,100,122].

Glucosamine has been recovered from soil samples by strong acid (HCl) hydrolysis, followed by purification by cation-exchange chromatography [108–108]. The hexosamine has been quantified by colorimetric assays [106–108,119], or it can be a derivative formed and analyzed by GC with a sensitivity of about 3×10^{-11} mol [91–98]. The GC methods also analyze MuAc in the sample. Alternatively, glucosamine has been determined in soil hydrolysates by paper chromatography, which is a rapid and simple method that omits the cation-exchange step [79].

The chitin and glucosamine contents of fungi vary over a wide range, depending on species, growth conditions, and age. Aaronson [120] reported that the concentration of chitin ranged between 10 and 250 mg g^{-1} dry weight in various fungal species. The range of glucosamine in three species of salt marsh fungi measured at different ages was 8.5 to 92.8 mg g^{-1} dry weight [121]. In general, the chitin content increased with the age of the mycelium, an observation that has been made in other studies [122,123]. The chitin content in *Ciriolus versicolor*, which was used in Swift's earlier work [106], was 2.4 mg g^{-1} dry weight when grown in wood, but 12.4 mg g^{-1} dry weight when grown in vivo.

Another problem that occurs when using glucosamine analysis to estimate fungal biomass is that the contribution of glucosamine from invertebrates and bacteria must be eliminated or accounted for. Glucosamine from prokaryotes can be accounted for by measuring the amount of MuAc in the sample, and assuming an MuAc/glucosamine molar ratio of 1:1 [85]. However, the main problem of the glucosamine method is, as for the MuAc and DAP analyses,

the presence of glucosamine in nonliving organic material. West et al. [80] found that the glucosamine/biomass C ratio was about 100 times above the values reported from the analysis of fungal cultures.

III. ANALYSIS OF COMMUNITY COMPOSITION

Analysis of lipid composition is an important tool for taxonomic and phylogenetic classification of microorganisms, particularly of prokaryotes [48,124,125]. Many lipid compounds are relatively easily extracted and analyzed in environmental samples. Hence, lipids are the most often used signature components for determining the community composition of microorganisms in ecological studies [126–128].

A. Phospholipid Fatty Acids

The ester-linked fatty acids in the phospholipids are currently the most sensitive and the most useful chemical measures of microbial community structure [128]. Analyses of monocultures and consortia of microorganisms isolated from the environment have shown that subsets of a microbial community can be identified by specific "signature" phospholipid fatty acids (PLFA; Table 3). For example, bacteria characteristically contain odd-chain, methyl-branched (e.g., iso- and anteiso-branched), and cyclopropane fatty acids [36,49, 126–130]. Fungi, on the other hand, typically synthesize saturated even-chained and polyenoic fatty acids [48,129]. Many actinomycetes contain methyl-branched tuberculostearic acid (10 Me 18:0) [131]. Signature PLFA have also been identified for methane-oxidizing bacteria [132], sulfate-reducing bacteria [133,134], the soil bacterium, *Flavobacterium balustinum* [135], and for *Francisella tularensis* [136]. By utilizing fatty acid patterns of bacterial monocultures, Myron Sasser of the University of Delaware, in cooperation with Hewlett Packard, has been able to distinguish over 8000 strains of bacteria [137].

Techniques to Analyze Phospholipid Fatty Acids

Phospholipids are extracted and purified from soils using the Bligh and Dyer extraction procedure and silicic acid chromatography, as described in Section II.C (see Fig. 1). The ester-linked fatty acids are then transesterified to methyl esters by mild alkaline methanolysis, and the fatty acid methyl esters (FAME) are analyzed by capillary GC using FID [128,135,138]. Environmental samples, including soils, usually contain a very complex mixture of PLFA; for example, 30 to 50 different fatty acids can be identified in soils and sediments.

Table 3 Examples of Signature Phospholipid Fatty Acids[a] for Microorganisms

Eubacteria	
Common signatures:	i15:0, a15:0, 15:0, 16:1w5, i17:0, 17:0, 18:1w7
Sulfate reducer:	10Me 16:0, Br17:1, 17:1w6
Methane-oxidizing bacteria, type 1:	16:1w8c, 16:1w8t, 16:1w5c
type 2:	18:1w8c, 18:1w8t, 18:1w6c
Flavobacterium balustinum:	i17:1w7, Br 2OH-15:0
Francisella tularensis:	24:1w5c, 22:1w13c, 20:1w11c
Actinomycetes:	10Me18:0
Fungi:	18:2w6, 18:3w6

[a]Fatty acids are designated as total number of carbon atoms: number of double bonds, with the position closest to the aliphatic (w) end indicated and using *c* for *cis* and *t* for *trans*. The prefixes "i," "a," and "Br" refer to *iso*, *anteiso*, and (ms) methyl-branching in unconfirmed positions. Cyclopropyl fatty acids are indicated with the prefix "cy."
Source: From Refs. 126–136.

A long (50-m × 0.2-mm id) fused silica column, coated with a cross-linked nonpolar stationary phase has a satisfactory separation efficiency and is stable and reproducible for long-term analysis of environmental samples [139]. However, for special applications, such as determining *cis*- and *trans*-isomers in complex mixtures of fatty acids, columns with more polar stationary phases are needed [140]. The GC retention times give valuable information on the structure of the FAME. However, MS is needed for verification of the chemical structures. Methyl esters of fatty acids have electron impact (EI) mass spectra which, in most cases, give information on the presence of methyl branches, hydroxy groups, unsaturations, and cyclopropyl groups [141,142]. Special techniques are needed to determine the configurations and localizations of double bonds and the positions of cyclopropyl rings [143,144].

The sensitivity of these analyses is at the picomolar level, which corresponds to the content of PLFA in 5×10^6 bacterial cells, such as *E. coli*. Some applications require a substantially higher sensitivity

of PLFA analysis than the GC-FID procedure. Introduction of new mass spectrometric methods have made such analyses possible [40]. One method, called selective ion monitoring (SIM), uses the MS instrument as a highly sensitive and specific detector by measuring only preselected ions that are specific for the analyte. By forming special derivatives and using chemical ionization techniques with negative ion detection, SIM has sensitivities to a few femtomoles (10^{-15}) of fatty acids [69].

The SIM techniques have been used for analyzing PLFA profiles of microorganisms in deep subsurface sediments. When analyzing these samples, it became obvious that special care and techniques have to be used to avoid introducing fatty acid contaminants during the handling and preparation of the samples [146]. The content of PLFA in these samples, was about 6 pmol g^{-1} dry weight, Which corresponds to 10^5 to 10^6 bacterial cells for each gram of dry weight. The application of SIM techniques for the analyses of bacterial signature components have been described in more detail [40].

Applications to Soil Systems

In soil systems, analysis of PLFA has been used to study the dynamics of bacteria associated with the roots of rape plants [146] and to characterize bacteria that suppress damping-off caused by *Rhizoctonia* [135]. The method has also been utilized to examine the biomass and structure of microbial communities in subsurface soils, including soils contaminated with organic pollutants [67,68, 129,130,147,148]. For example, analysis of PLFA demonstrated that degradation of trichloroethylene (TCE) was correlated with the presence of type II methane-oxidizing bacteria [51].

Potential problems with defining microbial community structure by analysis of PLFA could result from a shift in fatty acid composition of some monocultures with changes in temperature and media composition [58]. However, there have been no studies that show that such shifts in PLFA composition substantially changed the interpretation of the community structure during natural growth conditions.

Despite that the analysis of PLFA cannot provide an exact determination of such species or physiological type of microorganism in a given environment, the analyses provide a quantitative description of the overall microbiota in the particular environment sampled. By use of statistical analysis, it is possible to obtain an estimate by PLFA analysis of the differences among various samples and treatments [128].

B. Lipopolysaccharides, Ether Lipids, Sphingolipids and Quinones

Analysis of lipid components other than PLFA can provide further insight into the composition of the microbial communities. The composition of hydroxy acids in LPS varies among different groups of gram-negative bacteria [53], and this composition has been used for classifying clinical bacteria and for indicating community composition in marine sediments [57]. This analysis has not been tested in soils.

Archaebacteria are characterized by their unique biphytanyl and di-biphytanyl glycerol ether lipids which are not found in other organisms [152]. These lipids have been used as biomarkers for archaebacteria in sediments, hot spring mats, and fermenters [153–155]. Phytanyl glycerol ether lipids can be analyzed by HPLC after appropriate derivatization [156,157], or by supercritical fluid chromatography [158].

The occurrence of plasmalogens (mono-alk-1-enyl monoacyl glycerophosphatides) in microbes is restricted to specific groups of anaerobic bacteria [150,151]. Plasmalogens can be assayed by their resistance to mild alkaline methanolysis and extreme sensitivity to mild acid [38].

Analysis of respiratory quinones has been used as a sensitive biomarker of aerobic versus anaerobic metabolism in environmental samples [159]. The redox potential of the respiratory quinones suggests that the terminal electron acceptor of those bacteria containing ubiquinones (benzoquinones) should be of higher potential when compared with those of bacteria containing naphthoquinones. Bacteria capable of forming both types of respiratory quinones form ubiquinones when grown aerobically and naphthoquinones when grown anaerobically [160]. Aerobes contain benzoquinones, and some, but not all anaerobes, contain naphthoquinones [161,162]. Hedrick and White [160] analyzed the respiratory quinones from the neutral fraction of the silicic acid-purified lipid extracts (see Fig. 1) with HPLC using electrochemical detection. Manipulation of sediments between aerobic and anaerobic conditions shifted the naphthoquinones/benzoquinones ratio from 0.03 for the aerated consortia to 3.0 for the fermenters, as expected from studies with monocultures.

IV. NUTRITIONAL STATUS

Microoranisms in soil are subjected to the stress of fluxes in nutrients, which may take the form of either the partial or near-complete

absence of nutrients [163,164]. Such fluctuations may lead to a transition from balanced to either unbalanced or complete cessation of growth. Unbalanced growth in microorganisms is commonly associated with the accumulation of energy-reserve polymers [165]. Numerous studies have shown that the synthesis of such polymers is induced when the nitrogen, oxygen, phosphorus, potassium, or sulfur supply (depending on the organism) becomes limiting in the presence of an excess of the carbon source [165]. Chemical methods have been utilized to indicate unbalanced growth conditions by measuring the ratio of lipid storage polymers to cellular biomass [166–169].

Microeukaryotes, such as protozoa and fungi, use triglycerides as storage polymers, and the nutritional status of these organisms has been monitored by measuring the triacylglycerols/phospholipid glycerols ratio [166].

Some bacteria form the endogenous lipid storage polymer, poly-β-hydroxybutyrate (PHB), under unbalanced growth conditions [165]. Detailed analyses of PHB extracted from bacterial isolates and environmental samples have shown that this polymer can be a mixture of polymers containing a number of various short-chain fatty acids [167]. This "mixed" polymer of PHB was called poly-β-hydroxyalkanoate (PHA) [167].

The PHB-PHA polymer has been extracted from environmental samples with boiling chloroform and sodium hypochlorite [167–170]. Findlay and White [171], however, demonstrated that PHB can be quantitatively extracted by using the modified Bligh and Dyer lipid extraction method (see Fig. 1). The extracted PHB is further purified by silicic acid chromatography, hydrolyzed, derivatized, and analyzed by GC. The detection limit of this assay using a FID detector is 100×10^{-15} mol of PHB. The sensitivity of this analysis can be significantly improved by using special derivatives and MS detection [172].

PHB analyses, in combination with lipid biomass measurements, have been used in soil systems, for example, to examine the nutritional status of bacteria associated with plant roots [146], to compare the nutritional status of bacteria in uncontaminated and contaminated subsurface soils [67,68], and to monitor bacteria degrading halogenated hydrocarbons in methane-enriched soil columns [172].

Starvation of some bacteria induces the formation of minicells. An examination of PLFA profiles from starved marine bacteria has shown that there is a marked increase in the proportion of PLFA with double bonds in the trans configuration during the formation of such minicells induced by nutrient deprivation [174]. It has been suggested that the nutritional status of some bacteria can be monitored by measuring the ratio of *cis/trans*-PLFA [174].

V. METABOLIC ACTIVITY

The analyses of the biochemical signatures described in the foregoing involve the isolation of the biological components from microbial communities. Inasmuch as each of the components are isolated, the incorporation of labeled isotopes from precursors can be utilized to provide rates of synthesis or turnover.

Measurements of the rates of DNA synthesis with [^{3}H]thymidine provides an estimate of the rates of heterotrophic bacterial growth. When short incubation times are utilized, isotope dilution experiments can be used to estimate precursor concentrations, and DNA can be purified [175] as long as the thymidine is not catabolized significantly. This method was first applied to soil systems by Thomas et al. [176]. The basic assumptions underlying this method were recently examined in greater detail [177]. Soil DNA was extracted and separated from other macromolecules, such as proteins, which contained a significant amount of labeling, by using an acid–base hydrolysis method. The recovery of added [^{14}C]DNA was 58% in humus soil and 75% in sandy loam soil. Isotope dilution experiments were utilized to estimate the pool of exogenous thymidine; the uptake of added [^{3}H]thymidine was linear with time for 60 min [177]. The thymidine method has also been utilized to estimate the growth rates of bacteria in the rhizosphere [178] and in subsurface soils [179–181]. In estuarine sediments, thymidine was shown to be rapidly catabolized, with [^{14}C]carbon dioxide appearing 10 min after exposure to [^{14}C]thymidine [182]. This finding suggests that thymidine catabolism could complicate the accuracy of using thymidine incorporation to estimate bacterial growth in soils.

The incorporation rates of $H_3{}^{32}PO_4$ and [^{14}C]acetate into PL have been utilized to measure the activity of the total microbiota [183]. The contribution of the microeukaryotic portion to PL synthesis has been estimated by measuring synthesis in the presence of cycloheximide [184]. Incorporation of [^{14}C]acetate has been utilized to examine the synthetic activity of subsurface soils and sediments [180,181]. The technique enables measurements of microbial activities that span more than five orders of magnitude.

Labeling experiments with [^{14}C]acetate have also been used to measure the formation rate of PHB and PLFA [185]. This ratio was shown to be a sensitive measure of the nutritional status of the bacterial habitat, and the technique made it possible to measure the "disturbance artifact" involved in the application of labeled precursors in the environment [185].

Mass spectrographic analysis enables the use of precursors labeled with stable isotopes to study the rate of synthesis of biochemical signature compounds in microbial communities. Stable isotopes are superior to radioactive isotopes in that stable isotopes have

higher specific activities, include isotope markers for nitrogen, and can be efficiently detected using SIM techniques with the MS. The high specific activity enables the assay of critical reactions at substrate concentrations that are similar to the levels present in natural environments. Methods have been developed to measure ^{15}N incorporation into *D*-alanine in bacterial cell walls and to determine ^{13}C environment in PLFA [186,187].

VI. CONCLUSION

Chemical measures for the biomass, structure, nutritional status, and metabolic activity of microbial communities, based on the analyses of specific cell components, represent a quantitative and sensitive method for the assay of microorganisms in soils. Several studies have demonstrated that membrane lipids, such as phospholipids and ergosterol, are particularly useful as signatures for biomass. They are comparatively easy to extract, they have a rapid turnover, and estimates of biomass based on their content corresponds well with classic methods. The analysis of the ester-linked fatty acids in the phospholipids enables the detection of specific subsets of the microbiota. With this technique, shifts in the structure of the microbial community can be quantitatively assayed. Rates of formation of membrane lipids and turnover of endogenous storage polymers, such as PHB, provide insights into the nutritional status and metabolic activities of the community. The validation of these techniques has been reviewed [11]. Further applications of these techniques will provide both insights into the ecology of microorganisms in soil as well as further validations of the methods.

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