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Lipid Analysis in Microbial Ecology

Quantitative approaches to the study of microbial communities

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In nature, microorganisms rarely exist as monocultures, but live in communities with other microbes. These communities play an important role in the biosphere, primarily in recycling biologically important elements. All the essential biochemical cycles of carbon, hydrogen, nitrogen, oxygen, and sulfur are mediated by communities of microorganisms. Consequently, understanding what microbes in a natural environment are doing, rather than simply which microbes are present, is important to understanding their role within ecosystems. Analytical techniques developed in the last decade offer insights into the nature of these important ecosytem components.

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Microbial communities include viruses, eubacteria, archaebacteria, fungi, protozoa, micrometazoa, and algae (Margulis et al. 1986). The communities exist throughout the biosphere and even occupy such extreme environments as boiling-hot springs (Brock 1978); the deep sea at high hydrostatic pressure (ca. 1200 atmospheres; Jannasch and Taylor 1984); the cold deserts of Antarctica in the pore spaces of sandstone (Friedmann 1982); deep subsurface aquifers (Fliermans and Balkwill 1989); salt-producing seawater-evaporation ponds (ca. 3–4 M NaCl; Kushner 1978); acid mine drainages at pHs of l–3 (Lundgren et al. 1972); and extremely alkaline soils at pHs of 10–12 (Langworthy 1978). More commonly studied microbial communities are found in or on soils, marine and fresh waters, sediments, air, and normal fauna and flora. A microbial community attached to decaying plant litter is shown in Figure 1.

Classical microbiologists attempted to determine the function of particular kinds of microbes (mostly bacteria and fungi) in natural microbial communities by selective enrichment culture (Brock 1975). These investigators devised chemically defined media with only one carbon source. Upon mixing the medium with a sample of soil or sediment, for example, only those microbes that could degrade the added carbon source would grow. Those microbes would thus be selected and enriched, producing measureable biomass. The microbes would be further subcultured to obtain pure isolates.

This method has been used to demonstrate the role of microbes in the cycling of matter in nature. For example, the metabolism of methane was demonstrated by a selective enrichment of populations from soil environments using methane as the sole carbon and energy source (Foster 1962). Selective enrichment of microbes is, however, artificial in that it removes the community from its natural habitat and allows only certain microbes with specific metabolic properties to grow under the conditions of incubation. Studies have shown (Perfilev and Gabe 1969) that subculturing microbes on artificial media often reveals only 0.01–10% of the total microbes present.

To study the community structure of a natural microbial community, investigators would take a sample (e.g., soil), plate it on selective enrichment media, and then isolate pure cultures. After collecting a few hundred or a few thousand cultures, the investigators would then try to determine the identity of each culture by the classical microbial taxonomic techniques of staining, morphological observation, and biochemical testing. All of the data generated (sometimes requiring approximately 100 tests on a single culture) would then be analyzed by computer to reveal the most appropriate taxonomic name for each culture (Sneath and Sokal 1973).

Clearly, this numerical taxonomic approach for determining community structure is cumbersome, time consuming, expensive, and only reveals the presence of microbes that can be cultured on the medium chosen. Culturing microbes from natural samples can indicate the microbes' presence,

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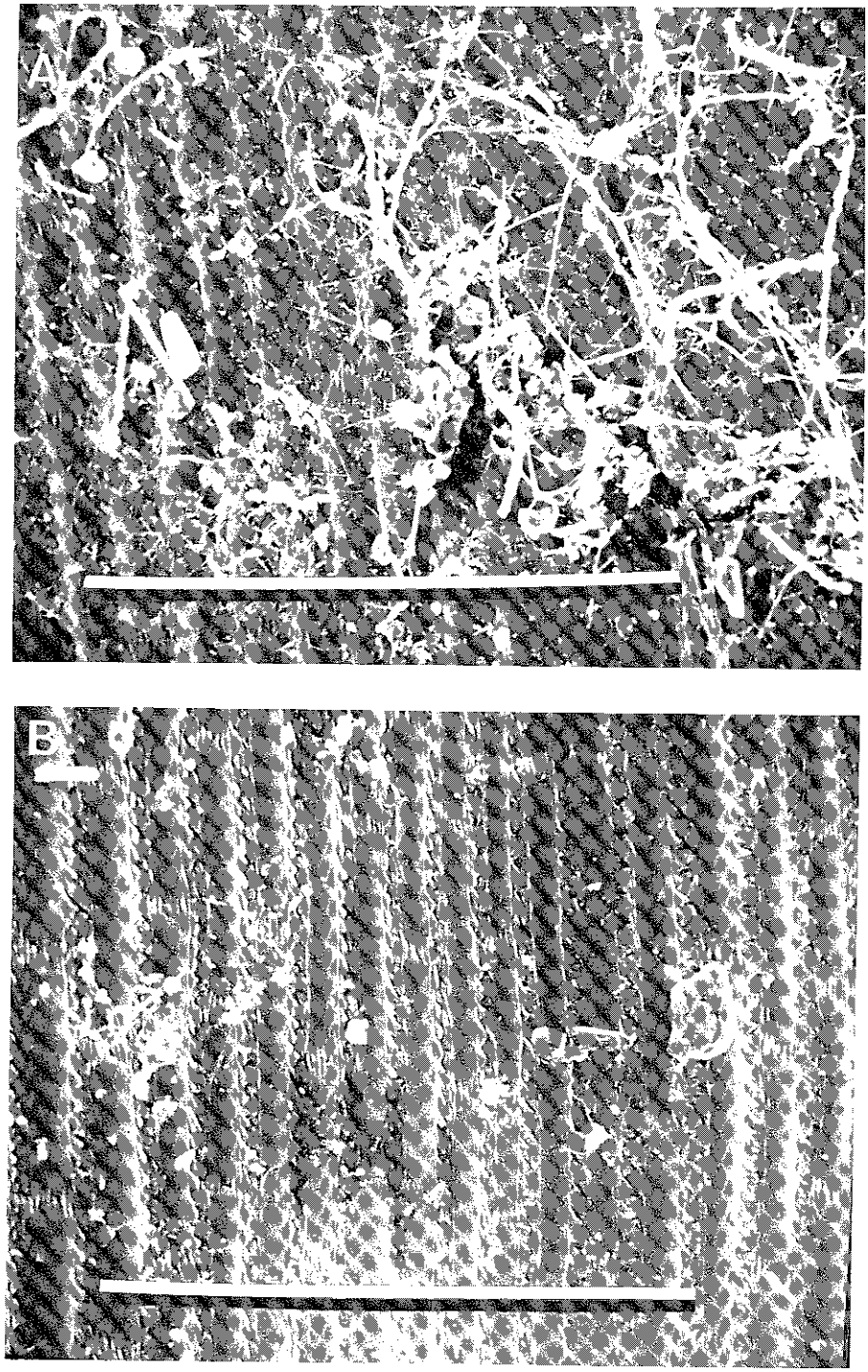

Figure 1. Scanning electron micrographs of the microbial community on sedge (*Carex aquatilis*) leaf litter (**a**) from the surface, a rich microbial community, or (**b**) at 4-meter depth, a sparse microbial community, in Toolik Lake, Alaska. Bar markers = 200 μm. Photos: Thomas W. Federle.

but it does not reveal whether the microbes biodegrade the particular compound under environmental conditions. In addition, most microbial communities are tightly attached to a substratum and cannot be quantitatively removed, so every microbe is not counted (White 1983).

To overcome these problems, microbial ecologists have recently sought to develop ways to measure microbial community biomass, structure, metabolic status, and activity under in situ conditions in an attempt to reveal more closely the functional role of the community in nature. So far, no perfect method for analyzing the role of microbes under natural conditions has been developed, although ribosomal RNA isolation and sequencing shows great promise for determining natural community structure (Olsen et al. 1986). The investigator must determine which available methods will best answer the experimental question at hand. Numerous methods using biochemical analyses to measure the biomass, presence, and activity of microbes under natural conditions have been discovered, and their uses and shortcomings have been reviewed (Parkes 1988, White 1983, 1986, 1988).

This article introduces the use of lipid analysis to study the biomass, community structure, metabolic status, and activity of natural microbial communities (Figure 2). This method is relatively sensitive and quantitative, and it accounts for most, if not all, of the natural microbes present. Using this methodology, an investigator can study microbial population changes through time brought about by physical or chemical environmental perturbations. Changes in indigenous microbial communities can indicate the effects of environmental contamination.

Extraction of lipids

For the study of microbial communities, lipid analysis basically entails the extraction of lipids from a sample with organic solvents followed by analysis of certain fractions of the extracted material. The extraction and analysis are straightforward. In the field or laboratory, the sample is exposed to a single-phase mixture of chloroform, methanol, and water in an initial ratio of 1:2:0.8, as originally described by Bligh and Dyer (1959). When these solvents are added, the lipids dissolve almost instantly (White and Frerman 1967) and further lipid metabolism stops. This technique provides a snapshot of the lipids at the time of extraction. After a short period of extraction (approximately 2 hours) in this monophasic system, water and chloroform are added to separate the phases by changing the polarity of the mixture. The total lipid fraction will

be found in the lower chloroform phase, whereas the more polar proteins, nucleic acids, cell walls, and other components remain in the upper methanol-water phase or at the chloroform-water interphase. As diagrammed in Figure 2, the organic (lipid-containing) phase can be further fractionated into phospholipids for analysis of community structure and biomass; the residue at the interphase can be used to measure Gram negative, Gram positive, and total eubacterial biomass.

The original sample can also be incubated for a short time with ^{14}C isotopes and the lipids extracted to measure the incorporation of ^{14}C as an indication of microbial metabolic activity. Or the sample can be extracted with boiling chloroform and the intracellular storage molecules measured as an indication of metabolic or nutritional stress.

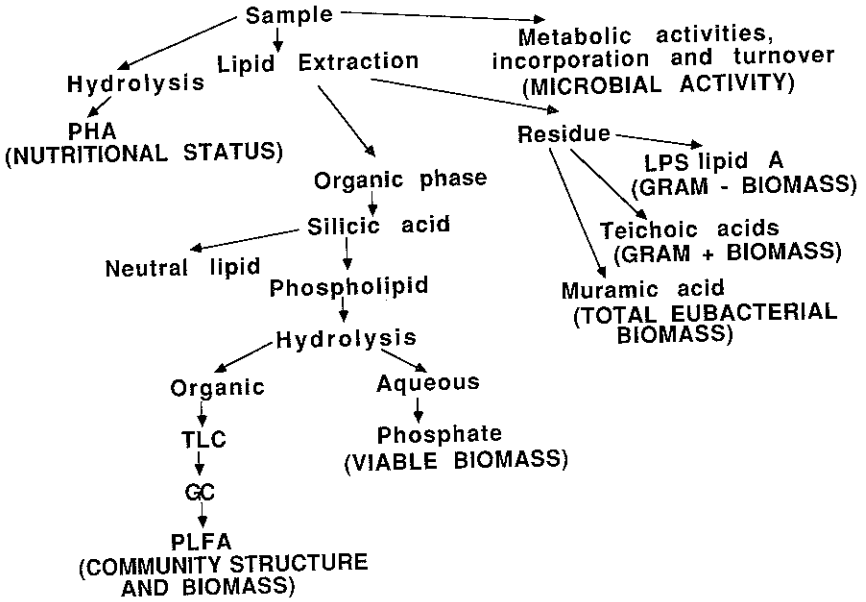

Figure 2. Flow diagram for the biochemical analysis of natural microbial communities using lipids (after White 1983).

Microbial biomass

Determining the viable biomass of a microbial community provides an estimate of the amount of active microorganisms in a particular environment and, therefore, the capability for metabolic transformations in that environment. The viable biomass of a microbial community is determined by measuring a cellular component that is common to all cells of the microbiota and quickly degraded on cell death. The ATP content of a microbiota, for example, has been successfully measured (Karl and LaRock 1975). There are, however, numerous problems with the most common procedures of ATP extraction and analysis (Karl 1986).

The extraction and analysis of the phospholipid components of a community is another way to measure biomass (White et al. 1979). In their membranes, all cells contain phospholipids, which are not stored but are turned over relatively rapidly during metabolism (White et al. 1979). Consequently, they indicate the viable biomass. The total lipids are extracted (as described above) and digested with acid, and the remaining phosphate is measured colorimetrically (Figure 3). The procedure is quantitative, fairly sensitive (to approximately 1 nanomole phosphate), and can detect about 10^8 bacteria the size of *Escherichia coli* (White 1983).

Determining the total phospholipid fatty acid (PLFA) composition can provide an estimate of microbial community biomass. A typical phospholipid contains 2 moles of fatty acids per mole of phospholipid; thus, by dividing the total fatty acid composition by 2, it is possible to determine the phospholipid biomass. This procedure is more sensitive than measuring the lipid phosphate, because capillary gas chromatography is used to detect the amount of fatty acids. Using this procedure, Federle (1986) was able to show a dramatic decrease in microbial biomass as a function of depth in three soils (Figure 3).

Another biomass measure using lipid components of microbial cells involves the analysis of lipid glycerol (Gehron and White 1982). This procedure is somewhat more complicated, requiring the hydrolysis of the phosphate from the lipid glycerol backbone using hydrofluoric acid, with subsequent analysis of the derivitized glycerol by gas chromatography. The procedure can detect approximately 10^6 bacteria the size of *E. coli*, an increase in sensitivity of approximately two orders of magnitude over lipid phosphate analysis.

The biomass of certain organisms of a microbial community can be estimated by extracting compounds unique to those microbes (Fazio et al. 1979). Eubacteria in a sample can be estimated by measuring the amount of muramic acid. It can be extracted from the residual interphase of a typical lipid extraction (Figure 2), then purified and analyzed using gas chromatography to a sensitivity of approximately 10^{-13} moles, or approximately 2×10^6 *Pseudomonas fluorescens* equivalents (Findlay et al. 1982). A drawback to this method is that the quantity of muramic acid varies dramatically among Gram-positive and Gram-negative, as well as cyanobacterial, cells. Another confounding factor is muramic acid's relative persistance (low turnover rate), so the estimate includes dead bacteria.

Lipopolysaccharide (LPS) is a molecule characteristic of Gram-negative bacteria and cyanobacteria. It contains unique moieties that can be exploited for use as biomass markers. LPS, in its lipid moiety, contains β-OH fatty acids. These can be found in the residual interphase of a lipid extraction (Figure 2). After mild acid hydrolysis, the β-OH fatty acids from the purified LPS can be methylated and analyzed by gas chromatography. The method detects picomolar quantities, approximately 10^6 *E. coli* equivalents (Parker et al. 1982).

Gram-positive bacteria usually contain in their cell walls unique polyhydric alcohols, the teichoic ac-

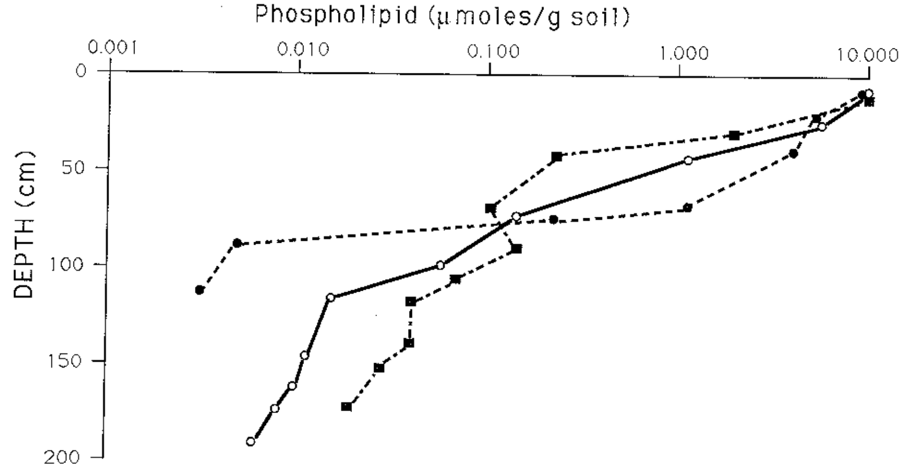

Figure 3. Phospholipid concentration of the soil microbiota from various soil types as a function of depth. ○, Loam; ●, sand loam; ■, clay loam (after Federle 1986).

ids. These polyols, made of erythritol or ribitol, can be extracted from the residue of a lipid extraction (Figure 2), derivitized, and analyzed by gas chromatography to picomolar concentrations, approximately 5 × 10^6 *Staphylococcus aureus* equivalents (Gehron et al. 1984).

Community structure

The types of microbes present in an environment, or the microbial community structure, determine whether a certain microbial transformation could take place. An investigator may wish to know, for example, if the microbes present in a soil sample (i.e., eubacteria, fungi, and actinomycetes) can degrade a particular xenobiotic chemical.

For the study of microbial community structure, it is important to discriminate among different types of microbes. Analysis of patterns of PLFA content provides one such means. In their membranes, most cells have phospholipids that contain ester-linked fatty acids. (But archaebacteria contain ether-linked mono- or diphytanyl lipids and thus cannot be measured by these methods.) The fatty acids of these phospholipids maintain membrane fluidity so the transport of nutrients can occur. Because the total lipids of a cell can be quantitatively extracted almost instantly (Figure 2), a quantitative and qualitative analysis of the phospholipid fraction for fatty acids can reveal the presence and abundance of certain types of microbes under natural conditions, giving a picture of the microbes present at a particular moment. Representative fatty acid structures and the nomenclature used to describe them are shown in Table 1. The presence of certain phospholipid fatty acids can serve as markers for certain microbial types (Table 2).

The total lipid fraction can be further fractionated using silica gel column chromatography (White 1983, 1988). This procedure separates the phospholipids from the neutral lipids, glycolipids, sterols, and other lipids. The phospholipid fraction can either be further purified by thin-layer chromatography or hydrolyzed using acid (Bobbie and White 1980) or alkaline (White et al. 1979) conditions. The resulting fatty acids can be esterified with methanol to form fatty acid methyl esters (FAMEs).

These FAMEs can then be analyzed quantitatively and qualitatively by high-resolution fused-silica capillary

Table 1. Representative fatty acids often found in microbial communities.

Fatty acids	Example	Shorthand*	Common name
Saturated fatty acid	$CH_3(CH_2)_{14}COOH$	16:0	Palmitate
Monounsaturated fatty acid	$CH_3(CH_2)_4CH{=}CH(CH_2)_8COOH$	16:1ω6	Palmitoleate
Polyunsaturated fatty acid	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	18:2ω6	Linoleate
Iso-fatty acid	$CH_3CH(CH_2)_{11}COOH$ with CH_3 on the CH	i16:0	*Iso*-palmitate
Anteiso-fatty acid	$CH_3CH_2CH(CH_2)_{11}COOH$ with CH_3 on the CH	a16:0	*Anteiso*-palmitate
Cyclopropane fatty acid	$CH_3(CH_2)_2CH{-}CH(CH_2)_{11}COOH$ with CH_2 bridging the two CH	cy18:0	Cyclopropylsterate

*The shorthand nomenclature used by lipidologists to describe the fatty acids is as follows (see also Table 2). The number before the colon indicates the number of carbons in the fatty acid. The number after the colon indicates the number of carbon-carbon double bonds in the fatty acid chain. The position of the initial unsaturation is indicated by the number of carbon units from the methyl, or ω, end of the molecule. The geometry of the double bond is shown by **c** for *cis* and **t** for *trans*. Because almost all unsaturations are *cis*, **c** is often omitted. Methyl branching can be indicated as iso (i; the second carbon from the methyl end) or anteiso (**a**; the third carbon) or just **br** (branch) if the position is unknown. When a branch is known but not in the **i** or **a** position, it is indicated by the position from the carboxyl end followed by **Me** before the carbon chain length (e.g., 10Me18:1ω3). Cyclopropane fatty acids are indicated as **cy**.

gas chromotography, and their chemical structures can be verified by capillary gas chromatography–mass spectrometry. These FAME profiles reveal the relative abundance of certain microbe biotypes in the natural sample. Although it is impossible to characterize the microbes as to genus (which may not be relevant anyway), it is possible to determine quantitatively and qualitatively the groups of microbes present when the sample was extracted (Table 2). These fatty acids represent general as well as specific markers for different types of microbial cells.

In an experimental manipulation designed to show shifts in the PLFA community structure of marine sediments incubated under aerobic, facultative, and anaerobic conditions, Guckert et al. (1985) found that the FAME profiles were quite different among the three microcosms (Table 3). The dramatic increase in cy17:0 and cy19:0 under facultative and anaerobic conditions indicate that these FAMEs would be good markers for anaerobic microbes. The presence of large quantities of 16:1w7, 16:1w7t, and 18:1w7 in the aerobic microcosms show that these fatty acids would be good markers of aerobic eubacteria. The lack of polyunsaturated fatty acids in these profiles indicates the absence of microeukaryotes. These FAME profiles also show that one can successfully follow changes in eubacterial community structure after environmental manipulation (e.g., altering oxygen availability) in natural microbial communities.

Metabolic status

Microbial communities may be living under conditions of metabolic stress, or unbalanced growth, because of such factors as inadequate moisture, pH, light, inorganic nutrients, available organic matter, or temperature. Because many bacterial and eukaryotic cells store intracellular molecules during periods of metabolic stress, analysis of these storage compounds can be used as indicators of the metabolic health of the community. Changes in these compounds can be followed during environmental manipulations to study the effects of the changes on community metabolism.

Many bacteria synthesize the unique molecules polybetahydroxyalkanoates (PHAs) during nutritional starvation. PHAs were previously thought to be polymers of betahydroxybutyrate (Findlay and White 1983a), but careful gas chromatographic analysis revealed that they consist of a variety of short-chain betahydroxy acids in addition to betahydroxybutyrate (Findlay and White 1983a). PHAs can be analyzed by gas chromatography after hot chloroform extraction, separation, and methylation. The sensitivity is in the picomolar range, and one can follow dramatic changes in PHA content during environmental manipulation (Findlay and White 1983a).

In a study of microbial communities in natural marine sediments,

Table 2. Marker phospholipid fatty acids (PLFA) in various cell types (Bobbie and White 1980, Delong and Yayanos 1986, Federle 1986, Gillian and Hogg 1984, Guckert et al. 1985, 1986, Parkes 1988, Sargent et al. 1987).

Eubacteria
Common markers: 15:0, i15:0, a15:0, 16:1ω9, 16:1ω5, i17:0, a17:0, 17:0, 18:1ω7t, 18:1ω5, i19:0, a19:0
Aerobes: 16:1ω7, 16:1ω7t, 18:1ω7
Anaerobes: cy17:0, cy19:0
Sulfate-reducing bacteria: i17:1ω7, 10Me16:0, 17:1ω6
Clostridia: cy15:1
Barophilic, psychrophilic bacteria: 20:5, 22:6
Cyanobacteria
Same as eubacteria plus 18:2ω6
Actinomycetes
Same as eubacteria plus 10Me18:0
Fungi
16:0, 18:1ω9, 18:2ω6, 18:3ω6, 18:3ω3
Protozoa
20:3ω6, 20:4ω6
Plants and green algae
Diatoms: 16:1ω3t, 20:5ω5, 20:5ω3
Green algae: 16:1ω13t, 18:3ω3, 18:1ω9
Microalgae: 16:3ω6
Higher plants: 18:1ω9, 18:1ω11, 18:3ω3, 20:5ω3, 26:0

Table 3. Fatty acid profiles for aerobic, facultative, and anaerobic microcosms of marine sediments expressed as average mol% (± 1 SD) (Guckert et al. 1985).

Fatty acid	Aerobic	Facultative	Anaerobic
Saturated			
13:0			0.2 (0.3)
14:0	1.8 (1.1)	2.3 (2.9)	3.3 (0.4)
15:0	0.1 (0.3)	0.1 (0.1)	1.9 (1.7)
16:0	22.6 (0.9)	34.7 (10.3)	33.5 (5.3)
17:0			0.5 (0.6)
18:0	0.2 (0.4)	2.5 (2.5)	0.4 (0.5)
Unsaturated			
15:1ω6		0.5 (1.0)	0.7 (0.5)
16:1ω7	42.8 (3.3)	26.6 (19.7)	2.2 (1.2)
16:1ω7t	13.5 (3.1)	0.4 (0.4)	0.4 (0.5)
16:1ω5	0.1 (0.1)	0.3 (0.4)	0.1 (0.1)
17:1ω8		0.1 (0.2)	0.6 (0.2)
17:1ω6		0.1 (0.1)	
18:2ω6	0.1 (0.2)		
18:1ω9	0.3 (0.7)	0.5 (0.4)	0.2 (0.3)
18:1ω7	17.3 (2.0)	15.4 (11.0)	2.9 (0.9)
Branched and cyclopropane			
a15:0			0.5 (0.5)
i16:0	0.3 (0.3)	0.4 (0.5)	
10Me16:0		0.2 (0.5)	0.1 (0.2)
i17:0		0.2 (0.4)	0.1 (0.1)
cy17:0		10.2 (8.5)	26.9 (5.1)
cy18:0	0.8 (0.5)		2.8 (2.7)
cy19:0		5.4 (8.5)	22.5 (6.9)
N	6	4	4

Table 4. Change in metabolic components of microbial communities in estuarine sediments from perturbation by sand dollars, *Mellita quinquiesperforata* (Findlay and White 1983b).

Component*	Control sand†	Grazed sand†
Total lipid / PL	2190 ± 975	2960 ± 456
PLFA / PL	711 ± 157	872 ± 172
PHA / PL	2230 ± 494	1700 ± 193
PLFA / PHA	0.33 ± 0.11	0.51 ± 0.1‡

*PL = phospholipids; PLFA = phospholipid fatty acids; PHA = polybetahydroxyalkanoates.
†pmoles/μmole lipid phosphate; n = 5.
‡Significant difference between means, $p < 0.05$.

Findlay and White (1983b) showed that in communities perturbed by grazing sand dollars (*Mellita quinquiesperforata*), the amount of PHA synthesized decreased compared with ungrazed controls (Table 4). The investigators concluded that the natural grazing disturbance caused the microbes to mobilize (catabolize) the stored endogenous PHA reserves, thereby demonstrating a change in the community's metabolic status.

Guckert et al. (1986) found other metabolic changes during microbial nutrient starvation. When *Vibrio cholerae* were starved, the bacteria decreased their content of PLFA 16:1w7c from 39% to 11% and increased their content of 16:1w7t from 1% to 17%. There was also a decrease from 19% to 13% in 18:1w7c and an increase from 0.2% to 5% in i8:1w7t during the 30-day experiment. The study indicated that a shift in the fatty acid trans:cis ratio is associated with starved or stressed bacteria in natural environments.

Many eukaryotic microbes, such as fungi and crustaceans, synthesize endogenous triglycerides when nutrients are depleted. A good indicator, therefore, of microeukaryotic metabolic status is the ratio of triglyceride glycerol to phospholipid content. Changes in this ratio indicate a change in the metabolic status of microeukaryotes in a microbial community (Gehron and White 1982).

Table 5. Effects of environmental toxicants on sediment microbial community activity (Barnhart and Vestal 1983).

Toxicant*	EC_{50}† (mg / l)
Cd	>100
Hg	23
Cr	91
Cu	6
Zn	60
Ni	68
SDOC	>1000
SDS	>1000
PCP	2
Phenol	245‡

*SDOC = sodium deoxycholate; SDS = sodium dodecyl sulfate; PCP = sodium pentachlorophenate.
†The EC_{50} is the effect concentration that inhibits microbial activity by 50%.
‡Concentration is in μl / l.

Metabolic activity

The metabolic activity of a microbial community is important in assessing the role of microbes in nature. Activity can be determined by measuring a metabolic property common to almost all of the cells in a community: ATP energy charge (Davis and White 1980); extracellular enzymes (Morrison et al. 1977); community respiration by oxygen uptake (Morrison et al. 1977); and incorporation of radiolabeled substrates into certain cellular components such as DNA (Moriarty and Pollard 1981), sulfolipids (White et al. 1980), phospholipids (White et al. 1979), and cellular lipids (White et al. 1977).

A simple measure of microbial activity is the incorporation of [^{14}C]acetate into cellular lipids (White et al. 1977). Acetate can be readily used by almost all eubacterial and fungal cells. Because cells synthesize and turn over lipids rapidly during metabolism, acetate is quickly incorporated into lipids (White et al. 1977) and provides a good measure of microbial community activity. Because of the relatively high specific activity of [^{14}C]acetate, nanomolar quantities can be added to natural samples and incubated for a short time. After incubation, the total lipids can be extracted and the incorporation measured (White et al. 1977).

This procedure has been miniaturized (McKinley et al. 1982) so that the incubation and extraction can be completed in a 20-milliliter glass scintillation vial. This method allows numerous samples to be analyzed rapidly, thus providing statistical accuracy to the measurements. The method has been used to study the microbial community activity in composting sewage sludge (McKinley and Vestal 1984), oligotrophic freshwaters (Hullar et al. 1986), coastal marine mangrove detritus (Findlay et al. 1986), marine sediments (White et al. 1977), antarctic rock microbiota (Vestal 1988), oligotrophic freshwater detritus (Federle and Vestal 1982), and soils (White et al. 1979).

The procedure has a drawback when used with a sample that is not normally aqueous. The mixing of sediment or soil with the [^{14}C]acetate usually creates a disturbance artifact, which can give a higher activity than occurs naturally. But the disturbance in slurries can be minimized by carefully inoculating the sample in a core tub with [^{14}C]acetate (Findlay et al. 1985).

The incorporation of [^{14}C]acetate into microbial lipids has application in environmental studies. The method has been used to measure the effects of environmental toxicants on the activity of aquatic sediment microbiota (Table 5; Barnhart and Vestal 1983). As can be seen from dose-response experiments, various toxicants can have a dramatic effect on the natural microbial activity of the sediment communities.

Conclusions

Sensitive analytical procedures have been developed for the quantitative study of natural microbial communities in situ. Although these methods cannot answer all experimental questions regarding natural microbial communities, they do provide a good set of tools for studying the microbes. With current interest in the effects of environmental pollutants on natural environments and the applied use of indigenous microbial communities to biodegrade environmental contaminants, these methods can provide quantitative data on the status and role of these most important ecosystem components.

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