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ANALYTICAL MICROBIOLOGY METHODS

Chromatography and Mass Spectrometry

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CHAPTER 16

USE OF LIPID BIOMARKERS IN ENVIRONMENTAL SAMPLES

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INTRODUCTION

Even in a water column the classical methods of microbiology, which involve the isolation and subsequent culturing of organisms on petri plates, can lead to gross underestimations of the numbers of organisms detectable in direct counts of the same waters.[1] With sediments, soils, and biofilms, the problems with classical methods are more severe. In addition to the problems in providing a universal growth medium in the petri plate, the organisms must be quantitatively removed from the surfaces and from each other. Microscopic methods that require quantitative release of the microorganisms from the biofilm can have the problem of inconsistent removal from the surfaces.[2] Direct microscopy can sometimes be performed on thin biofilms by making estimations for organisms rendered invisible by particles or overlapping organisms in the biofilm.[3] This methodology works best when the density of organisms is low and overlapping is minimal. However, direct microscopic examinations offer a limited insight into the metabolic function or activity of the cells. Methane bacteria, for example, come in all sizes and shapes.[4] The problem is further complicated by the fact that in many environments only a tiny fraction of the organisms is active at any one level and aside from the observation of bacterial doubling time,[5] the morphology gives little evidence of the activity of the cells. The most direct method of determining the proportion of active cells in a given biofilm involves a combination of autoradiography and electron or epifluorescence microscopy. These methods require metabolic activity in the presence of the substrates and are subject to the limitations of density of organisms and thickness of the biofilm in the field of view. With the necessity for inducing metabolic activity there is a danger of inducing artificially high levels of activity with the addition of the substrates.[6]

The attachment and activity of microbes at surfaces is an extremely important feature of microbial ecology.[7] Not only do microbes attach to surfaces, but there is abundant evidence that they exist in consortia of multiple metabolic types.[8]

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Isolation of microbes from the environment for viable counting or direct microscopic examination may offer limited insight into the interactions taking place in microbial communities. Since these consortia have much more versatile metabolic propensities than simple species, it is important in ecological studies to preserve as much as possible of the structure and metabolic interactions of the microcolonies.

THE SOLUTION-BIOMARKERS

To improve the situation intensive efforts have been put into developing alternative methods that require neither growth, with its attendant problem of microbial selection, nor removal of cells from surfaces. With the alternative methods, the community as a whole can be examined with the microstructure of its multi-species consortia preserved. The methods involve the measurement of biochemical properties of the microbial cells and their extracellular products. Those components generally distributed in cells are utilized as markers for biomass. Components restricted to subsets of the microbial communities can be utilized to define the community structure. The concept of "signatures" for subsets of the microbial community, based on the limited distribution of specific components, has been shown for many monocultures.[9] A range of biomarkers exist, from those found in all organisms to those more or less specific to groups or species of microorganisms. Because of their comparable ease of extraction from even very complex environmental samples and their great taxonomic value,[10] lipid components have so far been shown to be the most useful biomarkers for microorganisms in environmental studies (Table 1).

Biomass estimations

Phospholipids are found in the membranes of all living cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids.[11] Phospholipids are not found in storage lipids and they have a relatively rapid turnover rate in sediments so the assay of these lipids gives a measure of the "viable" cellular biomass.[12] By using appropriate conversion factors, an estimate of biomass from the content of phospholipids corresponds well to estimates based on measurements of cell counts and cell volumes as well as analysis of cell wall muramic acid, lipopolysaccharide lipid A and adenosine triphosphate (ATP) in samples from subsurface sediments.[13]

Gram negative bacteria contain unique hydroxy acids in the lipid portion (lipid A) of the lipopolysaccharide (LPS) in the cell wall.[14] For example, β-hydroxy myristic acid has been used as a biomarker for Gram negative bacteria in sediments and body fluids (see Table 1).

Ergosterol is the predominant sterol in most fungi[15] and is absent or a minor constituent in plants.[16] It has been used as a marker for fungal biomass in soil and plant material (see Table 1).

Microbial community structure

The ester-linked fatty acids in the phospholipids (PLFA) are presently the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed.[17,18] The composition of fatty acids varies widely between different groups of organisms.[19] Bacteria unlike most other organisms do not usually synthesize polyunsaturated fatty acids. Furthermore, bacteria characteristically contain odd chain and branched fatty acids as well as cyclopropane and α- or β-hydroxy derivatives. Compositional patterns of fatty acids have been used for taxonomic and phylogenetic classification of bacteria.[10] By utilizing fatty acid patterns of bacterial

Table 1. Lipid biomarkers for biomass and community structure

Biomarker	Organism group	References
Phospholipids	All cells	87,45,13
Ergosterol	Fungi	88-91
LPS hydroxy-acids	Gram negative bacteria	43,51,53,55-56,92-94
Fatty acids in phospholipids	Sulfate reducing bacteria	57-62
	Methane-oxidizing bacteria	78,80
	Thiobacillus sp.	70,71
	Mycobacteria	95-97
	Francisella tularensis	74
	Dinoflagellates	54,98,99
Phytanyl and biphytanyl ether lipids	Archaebacteria	100-104 46,47
Plasmalogens	Anaerobic bacteria	12,24
Respiratory quinones	Aerobic/anaerobic bacteria	30
Sterols	Dinoflagellates	105-108

monocultures, Myron Sasser of the University of Delaware in cooperation with Hewlett Packard has been able to distinguish over 8000 strains of bacteria.[20] Isolation of bacteria from natural environments has shown that subsets of microbial communities contain specific patterns of fatty acids. For example, biomarker fatty acids have been identified in sulfate reducing, methane oxidizing, autotrophic mineral acid secreting, and pathogenic bacteria (see Table 1).

Although the analysis of PLFA cannot provide an exact description of each species or physiological type of microorganisms in a given environment, a quantitative description of the microbiota in the particular environment sampled is provided. With the techniques of statistical pattern recognition it is possible to provide a quantitative estimate of the differences between samples with PLFA analysis.

Potential problems with defining the community structure by analysis of PLFA come with the shift in fatty acid composition of some monocultures with changes in temperature and media composition, some of which were made in this laboratory.[21-23] There is as yet little published evidence for such shifts in PFLA in nature where the growth conditions that allow survival in highly competitive microbial consortia would be expected to severely restrict the survival of specific microbial strains to much narrower conditions of growth.

The community structure of the microbial consortia can be further defined by the analysis of plasmalogens and ether lipids (Table 1). The occurrence of plasmalogens in microbes is restricted to specific groups of anaerobic bacteria.[24,25] Archaebacteria (methanogens, halophiles, and thermophiles) are characterized by unique biphytanyl and di-biphytanyl ether lipids which are not found in other organisms.[26] These lipids have been utilized as biomarkers for archaebacteria in sediments, petroleum, hot-spring mats and fermenters (see Table 1).

The common bacterial respiratory quinones are ubiquinones, menaquinones, and desmethylmenaquinones. The type of quinones and length of the side chain varies with the type of bacteria, and their distribution has been utilized as taxonomic markers for bacteria.[27] The redox potential of the respiratory quinones suggests that the terminal electron acceptors of those bacteria containing ubiquinones should be of high potential as compared with those of bacteria containing naphthoquinones. Aerobes contain benzoquinones, some, but not all, anaerobes contain naphthoquinones.[28,29] Recently, Hedrick and White[30] showed that analyses of respiratory quinones can be utilized as sensitive biomarkers of aerobic versus anaerobic bacterial metabolism in environmental samples.

Nutritional status

Chemical methods can also be utilized to indicate the nutritional status of organisms. Many microorganisms accumulate lipid storage material under specific environmental conditions.[31] The nutritional status of microorganisms can be analyzed by measuring the proportions of these polymers relative to the cellular biomass. The nutritional status of microeukaryotes (algae, fungi and protozoa) has been monitored by measuring the ratio of triacyl glycerols to the cellular biomass.[32] Certain bacteria form the unique reserve polymer poly-β-hydroxybutyrate (PHB) under conditions in which the organisms can accumulate carbon, but have insufficient amounts of total nutrients to allow growth and cell division.[33,34]

Starvation induces the formation of minicells in some bacteria.[35] There is also a loss of cell components including membrane lipids but there is a marked increase in the portion of cyclopropane and monoenoic PLFA with the double bond in the trans configuration.[36] These changes can be utilized as biomarkers of the nutritional status of these bacteria.

Metabolic activity

The analyses described above all involve the isolation of components from microbial consortia. Since each of these components is isolated, the incorporation of labeled isotopes from precursors can be utilized to provide rates of synthesis or turnover in properly designed experiments. For example, incorporation of $H_3{}^{32}PO_4$ into phospholipids has been used to estimate the activity of the total microbiota, and incorporation of ^{35}S sulfate into sulpholipids has been used as a measure of the activity of microeukaryotes.[37,38] The ratio of the synthesis of PLFA (cellular growth) to that of PHB (carbon accumulation) has been shown to reflect the growth conditions in bacterial habitats.[6]

Mass spectrometry analyses allow the use of stable isotope-labelled precursors to study the rate of synthesis of biological markers. Stable isotope-labelled precursors are superior to radioactive isotopes in that the former have higher specific activities, in many cases approaching 100%. This enables the use of marker precursors at natural concentrations, which would help to avoid the distortions induced by the addition of high concentrations of substrates required for many radioactive precursors. A highly sensitive

and selective method has been developed to measure ^{13}C enrichment in bacterial fatty acids using mass spectrometry.[39]

THE METHOD

Generally, chemical analyses of lipid biomarkers involve the extraction of the sample with a suitable combination of organic solvents, followed by isolation and separation by various chromatographic techniques. In the past ten years this laboratory has developed a battery of methods for the analysis of lipid biomarkers in environmental samples.[9,40] These analyses are based on an efficient one-phase chloroform:methanol:water extraction of the sample (Figure 1).[12,41,42] The extracted lipids are then fractionated into three different classes on a silicic acid column: neutral lipids, containing triglycerides, sterols, and quinones; glycolipids with, e.g., PHB; and polar lipids that contain phospholipids, plasmalogens and ether lipids (Figure 1). Covalently bound lipids, such as lipid A in LPS, can be recovered from the residue of the Bligh and Dyer extraction.[43]

After extraction and isolation of the different lipid classes, the compounds are converted to suitable derivatives and separated by capillary gas chromatography (GC) (sterols, PHB-monomers, PLFA, aldehydes from plasmalogens and hydroxy acids from lipid A)[11,17,18,34,44,45] or high performance liquid chromatography (HPLC) (quinones and ether lipids).[30,46,47] Environmental samples contain a very complex mixture of lipids and care must be taken to correctly identify the biomarkers. For example, analysis of PLFA in marine sediments often yields 100-150 different fatty acids. The direct chemical verification of the lipids is, in most cases, performed with mass spectrometry (MS).[48] Special techniques are needed in some cases, for example, to determine the configuration and localization of double bonds in unsaturated fatty acids.[49]

The sensitivity for analyzing signatures with these techniques is at the picomolar level. For PLFA, this corresponds to the content in 5×10^6 bacterial cells like *Escherichia coli*.[9] However, a detailed examination of microbial communities can often require the analysis of components from a much smaller number of organisms. Introduction of more sensitive and selective detector systems such as selected ion monitoring (SIM) MS have made it possible to examine microbial constituents at sensitivities several orders of magnitude higher than more conventional GC techniques.[50] With this technique using chemical ionization (CI) and negative ion detection, microbial fatty acids can be determined at femtomolar levels, permitting detection of about 600 bacteria the size of *E. coli*.[51,52]

Nomenclature. Fatty acids are designated as total number of carbon atoms: number of double bond closest to the aliphatic (ω) end of the molecule indicated with the geometry "c" for *cis* and "t" for *trans*. The prefixes "*i*," "*a*," and "*br*" refer to *iso*-, *anteiso*-, and methyl-branching of unconfirmed position, respectively. Other methyl-branching is indicated as position from the carboxylic acid end, i.e., $C_{10\ Me\ 10:0}$. Cyclopropyl fatty acids are designated as "cy" with the ring position in parenthesis relative to the aliphatic end.

ENVIRONMENTAL STUDIES

The use of biomarkers for *in situ* studies of microbial communities has been validated in a number of studies by (1) inducing shifts in communities by altering the microenvironment, (2) by isolating specific organisms or groups of organisms for biomarkers and then detecting the markers after induction, (3) by detecting shifts in community nutritional status with

alteration in the environment, (4) by detecting specific organisms and their activity, and (5) by consequences of specific predation by grazers. These studies have been discussed in detail by White.[40] This section discusses the application of lipid biomarker analysis to a number of environmental studies.

Microbial communities in sediments

Groups of fatty acids have been identified as valid biomarkers for microorganisms in marine sediments. Perry et al.[53] proposed that *iso*- and *anteiso*- branched fatty acids, 10-methyl palmitic acid ($C_{10\ Me\ 16:0}$), cyclopropyl $C_{17:0}$ and $C_{19:0}$ acids, *cis* vaccenic acid ($C_{18:1\omega7c}$), the $C_{15:1}$, $C_{17:1\omega6}$ and $C_{17:1\omega8}$ isomers, and the branched monoenoic fatty acids $C_{i-15:1\omega8}$ and $C_{i-17:1\omega8}$ can be utilized as bacterial markers in sediment samples. Gillan and Hogg[54] defined nine subgroups (chemotypes) of bacteria in sediments based on fatty acid profiles. Gram negative bacteria in sediments can be detected using β-hydroxy acids as biomarkers.[53,55,56]

However, only in a few cases have detailed experiments been performed to study the relationship between specific bacterial biomarkers and the activity and dynamics of the corresponding microbiota. The sulfate reducing bacteria contain PLFA patterns which can be utilized to identify the lactate-utilizing *Desulfovibrio*, the acetate-utilizing *Desulfobacter*, and the propionate-utilizing *Desulfobulbos*.[57-62] These biomarkers allow the differentiation between sulfate reducing bacteria utilizing lactate, propionate, or those using acetate or higher fatty acids. Detailed analysis of sulfate reducing bacteria by N. Dowling of this laboratory strongly suggests that the majority of sulfate reducing bacteria found in marine sediments and in waters used in secondary recovery of oil are the acetate-utilizing strains. Sediments from a Scottish loch, when amended with lactate or propionate, show increases in the biomarkers for *Desulfovibrio* or *Desulfobulbos* respectively, that parallel increases in specific substrate induced sulfate reducing activity (Parkes, Dowling, and White, unpublished data). The biomarkers for sulfate reducing bacteria have also been utilized to study the interactions between consortia of glutamate fermenting and sulfate reducing bacteria in a continuous flow system inoculated with estuarine sediments.[63]

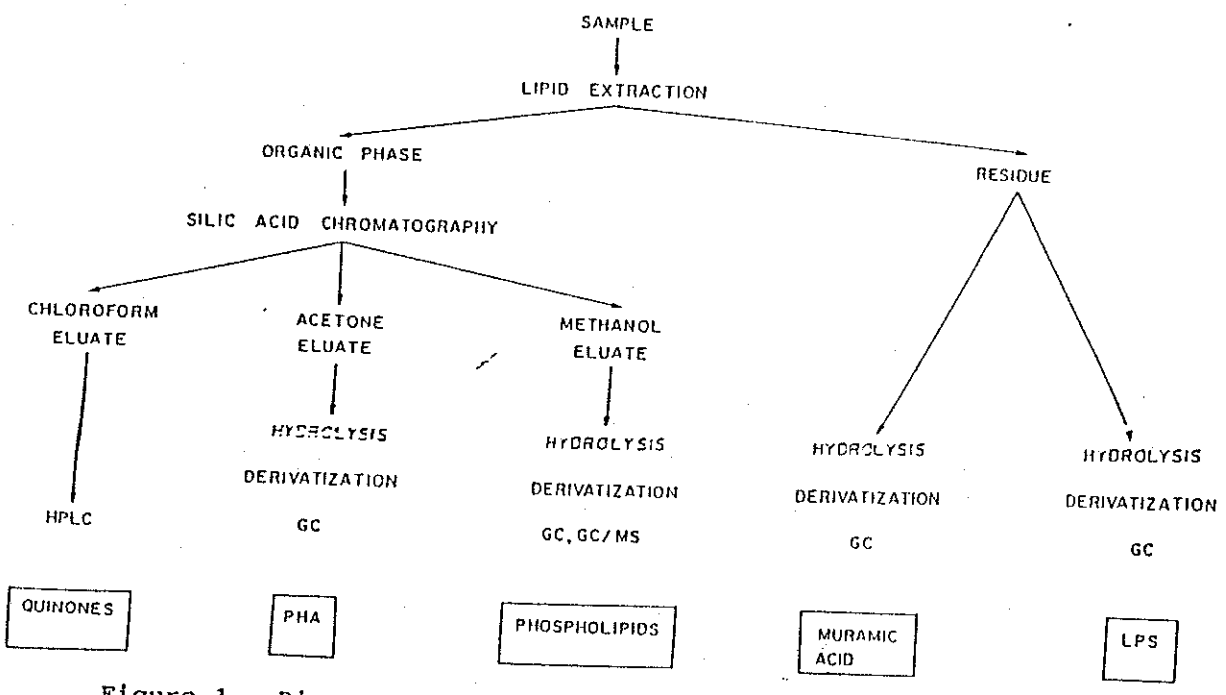

Figure 1. Diagram of the analytical scheme for the biochemical analysis of microorganisms in environmental samples.

Biofilms and fouling

The fouling of surfaces exposed to seawater generates enormous economic problems. The initial event in the fouling process involves an immediate coating of the surface with biopolymers that attract bacteria that binds to the surface.[7] The structure of the initial attached community of microorganisms was analyzed by SIM with chemical ionization and negative ion detection.[51] Examination of the PLFA profile indicated several properties of this community. The prominent fatty acids were characteristic of bacteria and the absence of polyenoic fatty acids indicated that microeukaryotes were lacking in the community.

The initial attached bacterial community attracts a succession of microorganisms leading to the formation of a complex biofilm containing fungi, protozoa, algae as well as bacteria.[64] The properties of the surface influence the development and structure of the biofilm. The microbiota colonizing the surface of non-degradable polyvinylchloride needles contain a significantly lower biomass and activity than the community found on biodegradable pine needles.[65] The microtopography of the surface also markedly affects the biomass and structure of the biofilm.[66]

Corrosion and biodeterioration

There is increasing evidence that corrosion of metals exposed to seawater is facilitated by the presence of microbes and their products at the surface.[67] Microbes of different physiological types when acting in consortia appear to be more destructive than monocultures. The sulfate reducing bacteria are of particular importance in microbially facilitated corrosion and it has been proposed that these obligate anaerobic bacteria can function in an apparently aerobic environment by occupying anaerobic microniches in biofilms formed by the utilization of oxygen by facultative anaerobic consortia. Analysis of PLFA markers has shown that sulfate reducing bacteria can form biofilms with the heterotrophic bacteria *Vibrio natrigiens* on stainless steel coupons exposed to aerobic seawater that facilitates corrosion.[68,69]

The acid-forming *Thiobacillus* form a remarkable collection of unique PLFA: methoxy-, cyclopropyl-, hydroxy-monounsaturated, hydroxy-cyclopropyl, and monounsaturated components with the double bond in unusual positions.[70] The signature PLFA of these organisms were readily detected in a microcosm designed to measure the degradation of concrete exposed to biologically generated acid and in concrete samples from sewers that suffered structural failure.[71]

Microorganisms on plant roots

Plant roots are important sites for microbial activity in soil and the root surface represents an area where microbial biomass is often substantially increased when compared with the soil itself. The root microorganisms influence the growth and development of the plant by competing for nutrients and by taking place in symbiotic or pathogenic interactions.

The dynamics of bacteria associated with the rape plant *Brassica napus* have been examined by analyzing PLFA and PHB.[72] Bacteria isolated from soil surrounding rape plant roots showed profiles of fatty acids that were distinctively different from those of sterile roots. The prominent PLFA of the bacteria were short and branched saturated, cyclopropane and monoenoic fatty acids. The bacteria were added to sterile rape roots. After three weeks of growth bacteria associated with the roots showed active growth but no formation of PHB whereas organisms recovered from the soil away from the roots showed less growth and the accumulation of high amounts of PHB.

Fatty acid analysis has also been utilized to characterize bacteria that suppress *Rhizoctonia* damping-off on cucumber roots grown in bark compost media.[73] Cucumber roots grown in a naturally suppressive medium had higher proportion of $C_{18:1\omega7c}$ and $C_{1-17:1\omega8}$ but lower proportion of several methyl branched fatty acids compared to roots grown in a conducive medium. These results suggest major differences in bacterial community composition between suppressive and conducive systems. Fatty acid analysis also demonstrated that colonization of the cucumber roots by a biocontrol agent (*Flavobacterium balustinum*) induced major changes in the composition of the rhizosphere bacterial community.

Detection of pathogens

The pathogenic bacterium *Francisella tularensis* has been shown to contain unusual long-chained monoenoic PLFA that can be used as biomarkers.[74] Organisms with this pattern have been isolated from patients with serological evidence of infection. The biomarker PLFA pattern has been detected in human and animal tissues with serological, clinical and cultural evidence for the infection as well as in soils and vaccines that have been shown to contain the organism.

Detection of the covalently bound hydroxy acids of the lipid A of LPS in Gram negative bacteria has proved to be a valuable assay in the definition of Gram negative infection. With this assay it is possible to detect Gram negative bacterial infection in mammalian tissue or secretions.[51]

Degradation of organic pollutants

Groundwater is becoming increasingly important as a resource of clean fresh water for industrial and domestic use. At the same time, more of this vital resource is found to be contaminated with potentially toxic wastes. Analysis of lipid biomarkers in subsurface samples, collected with careful attention to avoid contamination from surface soil, revealed the presence of a specific microflora.[75,76] The microbiota was sparse compared with that present in surface soils. PLFA analysis showed absence of long chained polyenoic fatty acids typical of microeukaryotes and high proportions of fatty acids typical of bacteria. The bacteria of the uncontaminated subsurface aquifer sediments showed nutritional stress as evidenced by high levels of PHB and extracellular polymers. Contamination of the sediments increased the microbial biomass, decreased the rate of PHB formation and shifted the community to a more Gram negative bacterial consortium.

Trichloroethylene (TCE) is a major contaminant of the subsurface ground water aquifers and methane oxidizing bacteria have been shown to be associated with a consortium that can degrade it.[77] Analysis for PLFA biomarkers in type I and type II methane-oxidizing bacteria has shown the presence of unique monoenoic fatty acids.[78] Type I methane-oxidizers have predominantly C_{16} fatty acids with unusual double bond positions: $C_{16:1\omega8c}$, $C_{16:1\omega8t}$, $C_{16:1\omega5c}$ and $C_{16:1\omega5t}$. Type II methane-oxidizers have 18 carbon monoenoic fatty acids with similar double bond positions: $C_{18:1\omega8c}$, $C_{18:1\omega8t}$, $C_{18:1\omega7t}$ and $C_{18:1\omega6c}$. Type II methanogens also produce PHB.[79] Both the total microbial biomass and signature components typical of type II methane-oxidizing bacteria as well as the level of PHB increased dramatically in soil exposed to natural gas.[80] The soil columns enriched in microbial biomass and specific type II methane-oxidizing bacteria showed that methane disappearance was correlated with rapid biodegradation of TCE.[80] Preliminary evidence indicates similar increases in signature lipids in the subsurface sediments recovered from zones where the TCE concentration increases the microbial metabolic activity (Phelps, Davis, Fliermans, and White, unpublished data).

Monitoring of fermenters

The rate limiting step in fermentations is the degradation of polymers.[81] A second consortia of microorganisms converts the carbohydrates and amino acids released from biopolymers into organic acids, alcohols, hydrogen and carbon dioxide. These are the anaerobic fermenters and some of the organisms contain plasmalogen phospholipids that are limited to this physiological group of anaerobes in the microbial world.[24] Other groups of anaerobic fermenters contain phosphosphingolipids with unusual sphingosine bases. These were detected in *Bacteriodes*.[82]

The microbial biomass, community structure and nutritional status of microbes in thermophilic methane producing digesters have been analyzed with lipid biomarkers.[83] Analysis of PLFA showed a simple PLFA pattern of saturated and unsaturated normal fatty acids. The loss of polyenoic fatty acids was utilized to monitor degradation of the plant biomass feedstock. After inoculation and incubation the PLFA showed a markedly different pattern with increases in PLFA typical of microbes from anaerobic digesters. The continuous addition of volatile fatty acids such as propionate and butyrate, or the terminal electron acceptors nitrate or sulfate induced markedly shifts in the community structure as indicated in the PLFA analysis.[83]

In experiments in cooperation with P. D. Brooks, C. A. Mancuso (of this laboratory) and D. P. Chynoweth (University of Florida) at the Disney World water hyacinth digester, the eubacterial community structure was markedly different in samples recovered from different parts of the digester. Comparing the feedstock chopped hyacinth material, the stored feedstock, the bottom material, the top of the bottom material, the bottom of the floating mat, and the top of the floating mat in the digester, there was a progressive decrease in the proportions of polyenoic and monoenoic PLFA. This decrease correlated with an increase in the proportion of branched and cyclic as well as saturated PLFA. The proportion of methanogenic archaebacteria measured as the di- and tetraphytanylglycerol ether phospholipids increased from the feedstock, to traces in the stored and partially fermented feedstock with more in the material at the bottom of the digester to the highest levels at the top of the digester.[84]

Changes in microbial pigments during digestion in the Disney World fermenter has been shown by J. J. Olie of this laboratory. Chlorophyll a and b, phaephytin a, phaephorbide as well as several bacterial carotenoids increased between the feedstock hyacinths and the digester samples. Possibly the most interesting finding of these analyses was the detection of large amounts of bacteriochlorophyll in the digester samples. None was detected in the feedstock. The role of these photosynthetic organisms that are known to utilize hydrogen in the dark to fix nitrogen is currently under investigation.

CONCLUSION AND PROSPECTS

Chemical measures for the biomass, community structure and nutritional status of microbial consortia based on analysis of lipid biomarkers provide a quantitative definition useful for studies of interactions between microorganisms and their environment. Areas that will benefit greatly from the application of these methods are the role of microbes in the facilitation of corrosion, studies of interaction between plant roots and microorganisms, detection of pathogens in the environment, the role of microorganisms in the degradation of pollutants and monitoring of fermenting processes.

Recently developed chemical analytical techniques will make it possible to determine new classes of biomarkers, to improve the detection limit and to

assay complex biological microenvironments. One approach to increase the sensitivity and speed of the analysis is to utilize the extraordinary separation efficiency of tandem mass spectrometry (MS-MS).[85] In combination with extended mass range, soft ionization techniques and new inlet systems, it is now possible to analyze large and complex lipid molecules.[86] Techniques for desorption of ions directly from a surface also have great potential in studies of microbial consortia. In combination with microprobes, desorption techniques can be used to analyze the distribution of specific molecules on micrometer scale. Such methods can be of great significance in studies of the critical initial phases of biodeterioration processes, such as biofouling and corrosion and in studies of interactions between pathogenic microorganisms and plants or animals.

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REFERENCES

1. H. W. Jannasch and G. E. Jones, Bacterial populations in seawater as determined by different methods of enumeration, Limnol. Oceanogr. 4:128 (1959).
2. D. J. W. Moriarty, Biogeochemistry of ancient and modern sediments, in: "Problems in the Measurement of Bacterial Biomass in Sandy Sediments," Australian Academy of Science, Canberra (1980).
3. D. E. Caldwell and J. J. Germida, Evaluation of difference imagery for visualizing and quantitating microbial growth, Canad. J. Microbiol. 31:35 (1984).
4. J. G. Zeikus, The biology of methanogenic bacteria, Bacteriol. Rev. 41:514 (1977).
5. A. Hagstrom, U. Larsson, P. Horstedt, and S. Normark, Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments, Appl. Environ. Microbiol. 37:805 (1979).
6. R. H. Findlay, P. C. Pollard, D. J. W. Moriarty, and D. C. White, Quantitative determination of microbial activity and community nutritional status in estuarine sediments: evidence for a disturbance artifact, Canad. J. Microbiol. 31:493 (1985).
7. K. C. Marshall, "Interfaces in Microbial Ecology," Harvard University Press, Cambridge (1976).
8. J. W. Costerton, T. J. Marrie, and K. -J. Cheng, Phenomena of bacterial adhesion, in: "Bacterial Adhesion Mechanisms and Physiological Significance," C. D. Savage and M. Fletcher, eds., Plenum Press, New York (1985).
9. D. C. White, Analysis of microorganisms in terms of quantity and activity in natural environments, in: "Microbes in Their Natural Environments," Society for General Microbiology Symposium, 34:37 (1983).

10. M. P. Lechevalier, Lipids in bacterial taxonomy-- a taxonomist's view, Crit. Rev. Microbiol. 7:109 (1977).
11. D. C. White, R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison, Biochemical measurements of microbial mass and activity from environmental samples, in: "Native Aquatic Bacteria: Enumeration, Activity and Ecology," ASTM STP 695, American Soc. for Testing and Materials (1979).
12. D. C. White, W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie, Determination of the sedimentary microbial biomass by extractible lipid phosphate, Oecologia 40:51 (1979).
13. D. L. Balkwill, F. R. Leach, F. T. Wilson, J. F. McNabb, and D. C. White, Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments, Microb. Ecol. 16:73 (1988).
14. O. Luderitz, M. A. Freudenberg, C. Galanos, V. Lehmann, E. Th. Rietschel, and D. H. Shaw, Lipopolysaccharides of Gram negative bacteria, in: "Current Topics in Membranes and Transport," vol. 17, S. Razin and S. Rottem, eds., Academic Press, New York (1982).
15. J. D. Weete, Sterols of the fungi: distribution and biosynthesis, Phytochemistry 12:1843 (1973).
16. W. R. Nes, The biochemistry of plant sterols, Adv. Lipid Res. 15:233 (1977).
17. R. J. Bobbie and D. C. White, Characterization of benthic microbial community structure by high resolution gas chromatography of fatty acid methyl esters, Appl. Environ. Microbiol. 39:1212 (1980).
18. J. B. Guckert, C. B. Antworth, P. D. Nichols, and D. C. White, Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments, FEMS Microbiol. Ecology 31:147 (1985).
19. J. L. Harwood and N. J. Russell, "Lipids in Plants and Microbes," George Allen and Unwin, London (1984).
20. M. Sasser, Identification of bacteria by fatty acid composition, Am. Soc. Microbiol. Meet. March 3-7, 1985.
21. G. H. Joyce, R. K. Hammond, and D. C. White, Changes in membrane lipid composition in exponentially growing *Staphylococcus aureus* during shift from 37 to 25°C, J. Bacteriol. 104:323 (1970).
22. F. F. Frerman and D. C. White, Membrane lipid changes during formation of a functional electron transport system in *Staphylococcus aureus*, J. Bacteriol. 94:1854 (1967).
23. P. H. Ray, D. C. White, and T. D. Brock, Effect of growth temperatures on the lipid composition of *Thermus aquaticus*, J. Bacteriol. 108:227 (1971).
24. H. Goldfine and P. O. Hagen, Bacterial plasmalogens, in: "Ether Lipids: Chemistry and Biology," E. Snyder, ed., Academic Press, New York (1972).
25. Y. Kamio, S. Kanegasaki, and H. Takanshi, Occurrence of plasmalogens in anaerobic bacteria, J. Gen. Appl. Microbiol. 15:439 (1969).
26. M. DeRosa, A. Gambacorta, and A. Gliozzi, Structure, biosynthesis, and physical properties of archaebacterial lipids, Microbiol. Rev. 50:70 (1986).
27. M. D. Collins and D. Jones, Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications, Microbiol. Rev. 45:316 (1981).
28. C. R. Whistance, J. F. Dillon, and D. R. Threlfall, The nature, intergeneric distribution and biosynthesis of isoprenoid quinones and phenols in gram-negative bacteria, Biochem. J. 111:461 (1969).
29. R. Hollander, G. Wolf, and W. Mannheim, Lipoquinones of some bacteria and mycoplasmas, with considerations on their functional significance, Antonie van Leeuwenhoek J. Microbiol. 43:177 (1977).
30. D. B. Hedrick and D. C. White, Microbial respiratory quinones in the

31. E. A. Dawes and P. J. Senior, The role and regulation of energy reserve polymers in micro-organisms, Adv. Microb. Physiol. 20:135 (1973).
32. M. J. Gehron and D. C. White, Quantitative determination of the nutritional status of detrital microbiota and grazing fauna by triglyceride glycerol analysis, J. Exp. Mar. Biol. Ecol. 64:145 (1982).
33. J. S. Nickels, J. D. King, and D. C. White, Poly-β-hydroxybutyrate metabolism as a measure of unbalanced growth of the estuarine detrital microbiota, Appl. Environ. Microbiol. 37:459 (1979).
34. R. H. Findlay and D. C. White, Polymeric β-hydroxy alkanoates from environmental samples and *Bacillus megaterium*, Appl. Environ. Microbiol. 45:71 (1983).
35. R. Y. Morita, Starvation-survival of heterotrophs in the marine environment, Adv. Microb. Ecol. 6:171 (1982).
36. J. B. Guckert, M. A. Hood, and D. C. White, Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans-cis* ratio and proportions of cyclopropyl fatty acids, Appl. Environ. Microbiol. 52:794 (1986).
37. D. C. White, R. J. Bobbie, S. J. Morrison, D. K. Oosterhof, C. W. Taylor, and D. A. Meeter, Determination of microbial activity of estuarine detritus by relative rates of lipid biosynthesis, Limnol. Oceanogr. 22:1089 (1977).
38. D. J. W. Moriarty, D. C. White, and T. J. Wassenberg, A convenient method for measuring rates of phospholipid synthesis in seawater and sediments: its relevance to the determination of bacterial productivity and the disturbance artifacts introduced by measurements, J. Microbiol. Methods 3:321 (1985).
39. A. Tunlid, H. Ek, G. Westerdahl, and G. Odham, Determination of ^{13}C-enrichment in bacterial fatty acids using chemical ionization mass spectrometry with negative ion detection, J. Microbiol. Methods 7:77 (1987).
40. D. C. White, Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity, Arch. Hydrobiol. Beih. Ergeben Limnol. 31:1 (1988).
41. E. G. Bligh and W. J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37:911 (1959).
42. J. D. King, D. C. White, and C. W. Taylor, Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora, Appl. Environ. Microbiol. 33:1177 (1977).
43. J. H. Parker, G. A. Smith, H. L. Fredrickson, J. R. Vestal, and D. C. White, Sensitive assay, based on hydroxy-fatty acids from lipopolysaccharide lipid A for gram negative bacteria in sediments, Appl. Environ. Microbiol. 44:1170 (1982).
44. D. C. White, R. J. Bobbie, J. S. Nickels, S. D. Fazio, and W. M. Davis, Nonselective biochemical methods for the determination of fungal mass and community structure in estuarine detrital microflora, Botanica Marina 23:239 (1980).
45. M. J. Gehron and D. C. White, Sensitive assay of phospholipid glycerol in environmental samples, J. Microbiol. Methods 1:23 (1983).
46. R. F. Martz, D. L. Sebacher, and D. C. White, Biomass measurement of methane-forming bacteria in environmental samples, J. Microbiol. Methods 1:53 (1983).
47. C. A. Mancuso, P. D. Nichols, and D. C. White, A method for the separation and characterization of Archaebacterial signature ether lipids, J. Lipid Res. 27:49 (1986).
48. C. Asselineau and J. Asselineau, Fatty acids and complex lipids, in: "Gas Chromatography/Mass Spectrometry Applications in Microbiology," G. Odham, L. Larsson, and P. -A. Mardh, eds., Plenum Press, New York (1984).

49. P. D. Nichols, J. B. Guckert, and D. C. White, Determination of monunsaturated double bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts, J. Microbiol. Methods 5:49 (1986).
50. A. Tunlid and G. Odham, Ultrasensitive analysis of bacterial signatures by gas chromatography/mass spectrometry, in: "Perspectives in Microbial Ecology," Proc. of the Fourth International Symposium on Microbial Ecology, Ljubljana, Yugoslavia, (1986).
51. G. Odham, A. Tunlid, G. Westerdahl, L. Larsson, J. B. Guckert, and D. C. White, Determination of microbial fatty acid profiles at femtomolar levels in human urine and the initial marine microfouling community by capillary gas chromatography-chemical ionization mass spectrometry with negative ion detection, J. Microbiol. Methods 3:331 (1985).
52. G. Odham, A. Tunlid, G. Westerdahl, P. Marden, Combined determination of poly-β-hydroxyalkanoic and cellular fatty acids in starved marine bacteria and sewage sludge using gas chromatography with flame ionization or mass spectrometry detection, Appl. Environ. Microbiol. 52:905 (1986).
53. G. J. Perry, J. K. Volkman, and R. B. Johns, Fatty acids of bacterial origin in contemporary marine sediments, Geochem. Cosmochim. Acta 43:1715 (1979).
54. F. T. Gillan and R. W. Hogg, A method for the estimation of bacterial biomass and community structure in mangrove-associated sediments, J. Microbiol. Methods 2:275 (1984).
55. P. A. Cranwell, The stereochemistry of 2- and 3-hydroxy acids in a recent lacustrine sediment, Geochem. Cosmochim. Acta 45:547 (1981).
56. H. Gossens, W. I. C. Rijpstra, R. R. Duren, J. W. deLeeuw, and P. A. Schenck, Bacterial contribution to sedimentary organic matter; a comparative study of lipid moieties in bacteria and recent sediments, Org. Geochem. 10:683 (1986).
57. J. J. Boon, J. W. deLeeuw, G. J. v.d. Hoek, and J. H. Vosjan, Significance and taxonomic value of *iso* and *anteiso* monoenoic fatty acids and branched β-hydroxy acids in *Desulfovibrio desulfuricans*, J. Bacteriol. 129:1183 (1977).
58. R. J. Parkes and J. Taylor, The relationship between fatty acid distributions and bacterial respiratory types in contemporary mariane sediments, Estuarine Coastal Mar. Sci. 16:173 (1983).
59. J. Taylor and R. J. Parkes, The cellular fatty acids of the sulfate-reducing bacteria, *Desulfobacter sp.*, *Desulfobulbus sp.*, and *Desulfovibrio desulfuricans*, J. Gen. Microbiol. 129:3303 (1983).
60. A. Edlund, P. D. Nichols, R. Roffey, and D. C. White, Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species, J. Lipid Res. 26:982 (1985).
61. R. J. Parkes and A. G. Calder, The cellular fatty acids of three strains of *Desulfobulbus*, a propionate-utilizing sulfate-reducing bacterium, FEMS Microbiol. Ecol. 31:361 (1985).
62. N. J. E. Dowling, F. Widdel, and D. C. White, Analysis of phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria, J. Gen. Microbiol. 132:1815 (1986).
63. J. E. Dowling, P. D. Nichols, and D. C. White, Phospholipid fatty acid and infra-red spectroscopic analysis of a sulphate-reducing consortium, FEMS Microbiol. Ecol. 53:325 (1989).
64. J. S. Nickels, R. J. Bobbie, D. F. Lott, R. F. Martz, P. H. Benson, and D. C. White, Effect of manual brush cleaning on the biomass and community structure of the microfouling film formed on aluminum and titanium surfaces exposed to rapidly flowing seawater, Appl. Environ. Microbiol. 41:1442 (1981).
65. R. J. Bobbie, S. J. Morrison, and D. C. White, Effects of substrate biodegradability on the mass and activity of the associated estuarine microbiota, Appl. Environ. Microbiol. 35:179 (1978).

66. J. S. Nickels, R. J. Bobbie, R. F. Martz, G. A. Smith, D. C. White, and N. L. Richards, Effect of silicate grain shape, structure and location on the biomass and community structure of colonizing marine microbiota, Appl. Environ. Microbiol. 41:1262-1268 (1981).

67. N. J. E. Dowling, J. Guezennec, and D. C. White, Methods for insight into mechanisms of microbially influenced metal corrosion, in: "Biodeterioration," D. R. Hougton, R. N. Smith, and H. O. U. Eggins, eds., Elsevier Applied Science Publ., London (1988).

68. N. J. E. Dowling, J. Guezennec, and D. C. White, Facilitation of corrosion of stainless steel exposed to aerobic seawater by microbial biofilms containing both facultative and absolute anaerobes, in: "Microbial Problems in the Offshore Oil Industry," E. C. Hill, J. L. Sherman, and R. J. Watkinson, eds., John Wiley, Chichester (1986).

69. N. J. E. Dowling, J. Guezennec, M. L. Limoine, A. Tunlid, and D. C. White, Corrosion analysis of carbon steels affected by aerobic and anaerobic bacteria in mono and coculture using AC impedance and DC techniques, Corrosion 44:869 (1988).

70. B. D. Kerger, P. D. Nichols, C. A. Antworth, W. Sand, E. Bock, J. C. Cox, T. A. Langworthy, and D. C. White, Signature fatty acids in the polar lipids of acid producing *Thiobacilli*: methoxy, cyclopropyl, α-hydroxy-cyclopropyl and branched and normal monoenoic fatty acids, FEMS Microbiol. Ecology 38:67 (1986).

71. B. D. Kerger, P. D. Nichols, W. Sand, E. Bock, and D. C. White, Association of acid producing *Thiobacilli* with degradation of concrete: analysis by "signature" fatty acids from the polar lipids and lipopolysaccharide, J. Industrial Microbiol. 2:63 (1986).

72. A. Tunlid, B. H. Baird, M. B. Trexler, S. Olsson, R. H. Findlay, G. Odham, and D. C. White, Determination of phospholipid ester-linked fatty acids and poly-β-hydroxybutyrate for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* (L), Canad. J. Microbiol. 31:1113 (1986).

73. A. Tunlid, H. A. J. Hoitink, C. Low, and D. C. White, Characterization of bacteria that suppress *Rhizoctonia* damping-off in bark compost media by analysis of fatty acid biomarkers, Appl. Environ. Microbiol. 55:1368 (1989).

74. P. D. Nichols, W. R. Mayberry, C. P. Antworth, and D. C. White, Determination of monounsaturated double bond position and geometry in the cellular fatty acids of the pathogenic bacterium *Francisella tularensis*, J. Clin. Microbiol. 21:738 (1984).

75. D. C. White, G. S. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz, and H. L. Fredrickson, The ground water aquifer microbiota: biomass, community structure and nutritional status, Developments in Industrial Microbiol. 24:201 (1983).

76. G. A. Smith, J. S. Nickels, B. D. Kerger, J. D. Davis, S. P. Collins, J. T. Wilson, J. F. McNabb, and D. C. White, Quantitative characterization of microbial biomass and community structure in subsurface material: A prokaryotic consortium responsive to organic contamination, Canad. J. Microbiol. 32:104 (1986).

77. J. T. Wilson and B. H. Wilson, Biotransformation of trichlorethylene in soil, Appl. Environ. Microbiol. 49:242 (1985).

78. P. D. Nichols and D. C. White, Accumulation of poly-β-hydroxybutyrate in a methane-enriched halogenated hydrocarbon-degrading soil column, Proceed. Fourth Intl. Symp. Interaction between Sediments and Water, Melbourne, Australia (1987).

79. P. D. Nichols, G. A. Smith, C. P. Antworth, R. S. Hanson, and D. C. White, Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for the methane-oxidizing bacteria, FEMS Microbiol. Ecology 31:327 (1985).

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98.

80. P. D. Nichols, J. M. Henson, C. P. Antworth, J. Parsons, J. T. Wilson, and D. C. White, Detection of a microbial consortium including type II methanotrophs by use of phospholipid fatty acids in an aerobic halogenated hydrocarbon-degrading soil column enriched with natural gas, Environ. Toxicol. Chem. 6:8997 (1987).
81. N. R. Wolin, The rumen fermentation: a model for microbial interactions in anaerobic ecosystems, Adv. Microbiol. Ecol. 3:49 (1979).
82. B. Rizza, A. N. Tucker, and D. C. White, Lipids of *Bacteroides melanogenicus*, J. Bacteriol. 101:84 (1970).
83. J. M. Henson, G. A. Smith, and D. C. White, Examination of thermophilic methane-producing digesters by analysis of bacterial lipids, Appl. Environ. Microbiol. 50:1428 (1985).
84. A. T. Mikell, T. J. Phelps, and D. C. White, Phospholipids to monitor microbial ecology in anaerobic digesters, in: "Methane from Biomass. A Systems Approach," W. Smith, ed., Elsevier, New York (1986).
85. J. V. Johnson and R. A. Yost, Tandem mass spectrometry for trace analysis, Anal. Chem. 57:758A (1985).
86. K. L. Busch and R. G. Cooks, Mass spectrometry of large, fragile, and involatile molecules, Science 218:247 (1982).
87. D. C. White, R. J. Bobbie, J. D. King, J. S. Nickels, and P. Amoe, Lipid analysis of sediments for microbial biomass and community structure, in: "Methodology for Biomass Determinations and Microbial Activities in Sediments," ASTM STP 673, American Society for Testing and Materials, pp. 87 (1979).
88. M. Seitz, D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard, Ergosterol as a measure of fungal growth, Phytopathology 69:1202 (1979).
89. S. E. Matcham, B. R. Jordan, D. A. Wood, Estimation of fungal biomass in a solid substrate by three independent methods, Appl. Microbiol. Biotechnol. 21:108 (1985).
90. W. D. Grant and A. W. West, Measurements of ergosterol, diaminopimelic acid and glucosamine in soil: evaluation as indicators of microbial biomass, J. Microbiol. Methods 6:47 (1986).
91. W. F. Osswald, W. Holl, and E. F. Elstner, Ergosterol as a biochemical indicator of fungal infection in spruce and fir needles from different sources, Z. Naturforsch. 41c:542 (1986).
92. S. K. Maitra, M. C. Schotz, T. T. Yoshikawa, and L. B. Guze, Determination of lipid A and endotoxin in serum by mass spectroscopy, Proc. Natl. Acad. Sci. USA 75:3993 (1978).
93. N. Saddler and A. C. Wardlaw, Extraction, distribution and biodegradation of bacterial lipopolysaccharides in estuarine sediments, Antonie van Leeuwenhoek J. Microbiol. 46:27 (1980).
94. K. Kawamura and R. Ishiwatari, Tightly bound β-hydroxy acids in recent sediment, Nature 297:144 (1982).
95. L. Larsson, P. -A. Mardh, G. Odham, and G. Westerdahl, Detection of tuberculostearic acid in biological specimens by means of glass capillary gas chromatography--electron and chemical ionization mass spectrometry, utilizing selected ion monitoring, J. Chromatogr. 182:402 (1980).
96. G. Odham, A. Tunlid, L. Larsson, and P. -A. Mardh, Mass spectrometric determination of selected microbial constituents using fused silica and chiral glass capillary gas chromatography, Chromatographia 16:83 (1982).
97. L. Larsson, G. Odham, G. Westerdahl, and B. Olsson, Diagnosis of pulmonary tuberculosis by selected ion monitoring: improved analysis of tuberculostearate in sputum using negative-ion mass spectrometry, J. Clin. Microbiol. 25:893 (1987).
98. J. K. Volkman and R. B. Johns, The geochemical significance of positioned isomers of unsaturated acids from an intertidal zone sediment, Nature 267:693 (1977).

99. H. L. Frederickson, T. E. Cappenberg, and J. W. De Leeuu, Polar lipid ester-linked fatty acid composition of Lake Vechten seston: an ecological application of lipid analysis, FEMS Microbiology Ecology 38:381 (1986).
100. W. Michaelis and P. Albrecht, Molecular fossils of archaebacteria in kerogen, Naturwiss. 66:420 (1979).
101. S. C. Brassell, A. M. K. Wardroper, I. D. Thomson, J. R. Maxwell, and G. Eglinton, Specific acyclic isoprenoids as biological markers of methanogenic bacteria in marine sediments, Nature 290:693 (1981).
102. B. Chappe, W. Michaelis, P. Albrecht, and G. Ourisson, Fossil evidence for a novel series of archaebacterial lipids, Naturwiss. 66:522 (1982).
103. D. M. Ward, S. C. Brassell, and G. Eglinton, Archaebacterial lipids in hot-spring microbial mats, Nature 318:656 (1985).
104. G. G. Pauly and E. S. Van Vleet, Acyclic archaebacterial ether lipids in swamp sediments, Geochim. Cosmochim. Acta 50:1117 (1986).
105. J. J. Boon, W. I. C. Rijpstra, F. de Lange, and J. W. De Leeuu, Black sea sterol-- a molecular fossil for dinoflagellate blooms, Nature 277:125 (1979).
106. S. C. Brassell and G. Eglinton, Biogeochemical significance of a novel sedimentary C_{27}-stanol, Nature 290:579 (1981).
107. J. W. De Leeuu, W. I. C. Rijpstra, and P. A. Schenk, Free, esterified and residual bound sterols in Black Sea Unit I sediments, Geochim. Cosmochim. Acta 47:455 (1983).
108. N. Robinson, G. Eglinton, S. C. Brassel, and P. A. Cranwell, Dinoflagellate origin for sedimentary 4α-methyl steroids and 5α(H)-stanols, Nature 308:439 (1984).