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Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity

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Abstract

Chemical measures for the biomass, community structure, nutritional status, and metabolic activities of the microbial consortia based on analysis of components of cells and extracellular polymers represent a quantitative and sensitive method for determining the interactions between members of the consortia. The biomass of microbes can be determined by measuring the content of cellular components found universally in relatively constant amounts. If these components have a high natural turnover or are rapidly lost from viable cells, they can be utilized to measure the viable cell mass. The membrane phospholipids are a particularly useful biomass parameter. Phospholipids are found in all cellular membranes, they have a rapid turnover in living cells, are rapidly hydrolyzed on cell death, and are found in reasonably constant amounts in bacterial cells as they occur in nature. Estimates of the viable biomass by phospholipid content correspond to estimates from the content of muramic acid, ATP, several enzyme activities, direct cell counts, and in some cases viable counts of the subsurface sediments. The analysis of the ester-linked fatty acids of the phospholipids (PLFA) using capillary gas chromatography/mass spectrometry (GC/MS) provides sufficient information for the detection of specific subsets of the microbiota based on patterns of PLFA. With this technique shifts in community structure can be quantitatively assayed. Some of the microbiota form specific components such as poly beta-hydroxyalkakoate (PHA) under conditions of unbalanced growth. Others form polysaccharide glycocalyx when subjected to mechanical or chemical stress. The combination of analysis of phospholipids, PLFA, PHA, and glycocalyx provides a definition of the biomass, community structure, and metabolic status of complex microbial communities. These analyses do not destroy the vital interactions within microcolonies of mixed physiological types that characterize many environments. These assays have been validated by: 1) inducing shifts in communities by altering the microenvironment, 2) by isolating specific organisms or groups of organisms for "signature" biomarkers and then detecting the signatures 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.

The problem

Microbes found in environments present a complex problem for assays. Even in the water column the classical methods of microbiology that 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 (Jannasch & Jones 1959). With sediments and biofilms the problems with classical

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methods are more severe. In addition to the problems of providing a universal growth medium in the petri plate, the organisms must be quantitatively removed from the surfaces and from each other. Direct microscopic methods that require quantitative release of the bacteria from the biofilm can have the problem of inconsistent removal from some surfaces. High speed blending of sediments to remove the microbiota prior to staining and direct counting in epifluorescent illumination was neither quantitative nor reproducible when compared to chemical assay of the muramic acid of the prokaryotic cell wall in one study of marine sediments (Moriarty 1980). Direct microscopy can be performed on the sediment particles or thin biofilms by making estimations for organisms rendered invisible by sediment granules or overlapping organisms in biofilms. The application of computer-based image enhancing can allow calculations of microbial biomass in complex assemblies (Caldwell & Germida 1984). This methodology works best when the density of organisms in the sediments or biofilms is low and overlapping is minimal. Even with computer enhanced image processing in direct microscopy, we are left with the problem that the in situ methods often fail because the morphology of a microbe offers little insight into the metabolic function or activity of the cells. Methane-forming bacteria for example come in all sizes and shapes (Zeikus 1977). The problem is further complicated by the fact that in many environments only a tiny fraction of the organisms is active at any one time and aside from the observation of bacterial doubling (Hagstrom et al. 1979), the morphology gives little evidence of the activity of the cells. Microscopic changes induced by showing the fraction of active cells by tetrazolium reduction and formazan accumulation or nutrient stimulation in the presence of nalidixic acid with significant elongation of the cells appear to work in dilute planktonic environments (Maki & Remsen 1981). 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. All 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 artifically high levels of activity with the addition of the substrates. Application of the extremely sensitive chemical methods has shown the induction of activity in the sedimentary microbiota when subjected to minimal disturbances (Findlay et al. 1985).

The attachment and activity of microbes at surfaces is an extremely important feature of microbial ecology (Marshall 1976). Not only do microbes attach to surfaces, but there is abundant evidence that they exist in consortia of multiple metabolic types. The best studied consortium is probably the multiple species of anaerobes that ferment complex plant polymers to volatile fatty acids, carbon dioxide, and methane in the rumen of vertebrates (Wolin 1979). Microcolonies of mixed bacterial types bound together with extracellular polymers are readily detectable in marine sediments by transmission electron microscopy (Moriarty & Hayward 1982).

Isolation of the microbes in these consortia for viable counting or direct microscopic examination can provide little insight into the details of the interactions that take place in mixed microcolonies. Since these consortia have much more versatile metabolic propensities than single species, it is important in environmental effects testing to preserve as much as possible the anatomy and metabolic

interactions of these microcolonies. For that, a new type of analysis that does not involve quantitative removal of the microbes from surfaces or stimulation of new and possibly artifactual metabolic activities is necessary.

A solution-methods

Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of cultural selection of the classical plate count is eliminated. Since the total community is examined in these procedures without the necessity of removing the microbes from surfaces, the microstructure of multi-species consortia is preserved. The method involves the measurement of biochemical properties of the cells and their extracellular products. Those components generally distributed in cells are utilized as measures of 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 community based on the limited distribution of specific components has been shown for many monocultures (Lechevalier 1977, White 1983).

Biomass estimation

Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids (White et al. 1979c). Phospholipids are not found in storage lipids and have a relatively rapid turnover in some sediments so the assay of these lipids gives a measure of the "viable" cellular biomass when compared to enzyme activities, total intra-cellular adenosine nucleotides, cell wall muramic acid (White et al. 1979b). The phosphate of the phospholipids or the glycerol-phosphate and acid-labile glycerol from phosphatidyl glycerol-like lipids that are indicators of bacterial lipids can be assayed to increase the specificity and sensitivity of the phospholipid assay (Gehron & White 1983). As will be discussed below there appears to be a unique essentially prokaryotic microbial community in uncontaminated subsurface sediments from below the root zone (Smith et al. 1986a). These are sparse and have primarily a small cocco-bacillary morphology. In these sediments the biomass estimated from the extractible phospholipid agrees with estimates from total adenosine triphosphate, the fatty acids from the lipopolysaccharide lipid A, the muramic acid content as well as direct microscopic counts and viable counts on diluted medium incubated for a long time (Balkwill & White, unpublished observations).

Microbial community structure

The ester-linked fatty acids in the phospholipids (PLFA) are presently both the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed (Bobbie & White 1980, Guckert et al. 1985, White et al. 1984). The specification of fatty acids that are ester-linked in the phospholipid fraction of the total lipid extract greatly increases the selectivity of this assay as most of the enthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. By isolating the phospholipid fraction for fatty acid analysis it proved possible to show

bacteria in the studge of crude oil tanks. The specificity and sensitivity of this assay has been greatly increased by the determination of the configuration and position of double bonds in monoenoic fatty acids (Nichols et al. 1985, 1986a, Edlund et al. 1985) and by the formation of electron capturing derivatives which after separation by capillary GLC can be detected after chemical ionization mass spectrometry as negative ions at femtomolar sensitivities (Odham et al. 1985). This makes possible the detection of specific bacteria in the range of 10 to 100 organisms. Since many environments such as marine sediments often yield 150 ester-linked fatty acids derived from the phospholipids, a single assay provides a large amount of information. Combining a second derivatization of the fatty acid methyl esters to provide information on the configuration and localization of the double bonds in monounsaturated components provides even deeper insight. By utilizing fatty acid patterns of bacterial monocultures, Myron Sasser of the University of Delaware in collaboration with Hewlett Packard has been able to distinguish between over 8000 strains of bacteria (Sasser 1985). Thus analysis of the fatty acids can provide insight into the community structure of microbial consortia as well as an estimate of the biomass.

Despite the fact that the analysis of PLFA cannot provide an exact description of each species or physiologic type of microbes in a given environment, the analysis provides a quantitative description of the microbiota in the particular environment sampled. With the techniques of statistical pattern recognition analysis it is possible to provide a quantitative estimate of the differences between samples with PLFA analysis.

Potential problems with defining community structure by analysis of PLFA come with the shifts in fatty acid composition of some monocultures with changes in media composition or temperature (Lechevalier 1977) some of which were defined in this laboratory (Joyce et al. 1970, Frerman & White 1967, Ray et al. 1971). There is as yet little published evidence for such shifts in PLFA in nature where the growth conditions that allow survival in the highly competitive microbial consortia would be expected to severely restrict the survival of specific microbial strains to much narrower conditions of growth.

From the residue of the lipid-extracted biofilm, muramic acid, a unique component of the bacterial cell wall can be recovered (Findlay et al. 1983). Muramic acid in the bacterial cell wall exists in a 1:1 molar ratio with glucosamine. Since the analysis gives both glucosamine and muramic acid and the chitin walls of many microeukaryotes yield glucosamine, the glucosamine to muramic acid ratio gives insight into the prokaryote to eukaryote ratio. This complements the information developed from the ester-linked PLFA.

Nutritional status

The nutritional status of biofilms or microbial consortia can be estimated by monitoring the proportions of specific endogenous storage compounds relative to the cellular biomass. The nutritional status of microeukaryotes (algae, fungi, or protozoa) in biofilms can be monitored by measuring the ratio of triglyceride glycerol to the cellular biomass (Gehron & White 1982).

Certain bacteria form the endogenous lipid PHA under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (Nickels et al. 1979). A more sensitive assay based on

GLC of the components of the PHA polymer showed the presence of a 3-OH acid longer than 4-carbons in these polymers (Findlay & White 1983b) which accounts for the changing of the name from poly beta-hydroxy butyrate (PHB) to PHA.

Assay for extracellular polysaccharide glycocalyx based on the specific content of uronic acids have been developed (Fazio et al. 1982). This assay has been utilized to show that poor growth conditions stimulate the formation of uronic acid containing exopolymers by a marine Pseudomonas (Uhlinger & White 1983).

Metabolic activity

The analyses described above all involve the isolation of components of microbial consortia. Since each of the components are isolated, the incorporation of labeled isotopes from precursors can be utilized to provide rates of synthesis or turnover in properly designed experiments. Measurements of the rates of synthesis of DNA with 3-H-thymidine provide an estimate of the rates of heterotrophic bacterial growth if short incubation times are utilized, isotope dilution is utilized to estimate precursor concentration, and DNA is purified (Moriarty & Pollard 1982). Incorporation of 35-S-sulfate into sulfolipid can be utilized to measure activity in the microeukaryotes (White et al. 1980, Moriarty et al. 1985). Incorporation of $H_3$32-PO_4 into phospholipids can be utilized as a measure of the activity of the total microbiota. The inhibition of phospholipid synthesis in the presence of cycloheximide represents the microeukaryote portion of the lipid synthesis (White et al. 1980, Moriarty et al. 1985). Measurement of rates of synthesis and turnover of both carbon and phosphate in individual phospholipids showed different turnover for the various lipids (King et al. 1977).

Analysis of signatures by GC/MS makes possible the utilization of mass labeled precursors that are non-radioactive, have specific activities approaching 100 %, include isotopic marker for nitrogen, and can be efficiently detected using the selective ion mode in mass spectroscopy. The high specific activity makes possible the assay of critical reactions using substrate concentrations in the biofilms that are just above the natural levels. This is not possible with radioactive precursors. Improvements in analytical techniques have increased the sensitivity of this analysis. Utilizing a chiral derivative and fused silica capillary GLC with chemical ionization and negative ion detection of selected ions, it proved possible to detect 8 pg (90 femtomoles) of D-alanine from the bacterial cell wall (the equivalent of 1000 bacteria the size of *E. coli* (Tunlid et al. 1985). In this analysis it proved possible to reproducibly detect a 1 % enrichment of 15-N in the 14-N-D-alanine.

Validation

The suite of methods for microbial biomass, community structure, nutritional status and metabolic activities described above have been validated in a series of experiments. These experiments can be classified as follows:

1) Induction of shifts in microbial community structure by altering the microenvironment result in changes that can often be predicted.

The microbiota that colonizes teflon strips suspended in a subtropical estuary when incubated in seawater containing antibiotics with the pH and nutrient com-

position altered to create a "fungus heaven" and "fungus hell" showed the presence of large branched mycelia by scanning electron microscopy (SCM) in "heaven" together with a significant increase of fungal biomarkers such as long chain polyenoic PLFA of both the alpha and gamma linolenic series, ergosterol type steroids, lipid inositol, serine, and sulfolipid synthesis. The samples in which prokaryotic growth was stimulated showed a morphology typical of bacteria by SCM, and increased levels of cell wall muramic acid, short branched PLFA, monounsaturated PLFA of the omega 7 from the anaerobic desaturase pathway, cyclopropane PLFA, lipid glycerol, ethanolamine and carbohydrates, and thymidine incorporation into DKA (White et al. 1980). The effect of light on marine biofilms resulted in the formation of sheets of diatoms by SCM and corresponding increases in biomarkers typical of these organisms such as chlorophyll a, lipid galactose, glycolipid glycerol, 16- and 18-carbon polyenoic PLFA, lipid inositol, and incorporation of sulfate into sulfolipids (Bobbie et al. 1981). The dark incubated controls showed predominantly prokaryotic morphology and biochemical "signatures".

With these techniques it has been possible to show succession in marine biofouling films (Morrison et al. 1977, Nickels et al. 1981a). The morphology by SCM shows an initial colonization by coccobacillary bacteria followed by bacteria with more complex morphology and microeukaryotes (predominately diatoms) that are then followed by other algae and microeukaryote larvae. The analysis of the initial microfouling film shows PLFA typical of gram-negative marine bacteria (Odham et al. 1985). This is followed by signature patterns typical of the more complex morphology.

The shifts in the terminal electron acceptors from high potential (oxygen or nitrate) to lower potential (sulfate, or carbon dioxide) induces changes in the microbial community structure. In experiments utilizing an inoculum from marine sediments it has proved possible to manipulate the community structure of the benthic microbiota by shifting from aerobic to anaerobic conditions (Guckert et al. 1985). The fatty acid profiles of independent flasks showed reproducible shifts when manipulated identically and significant differences when manipulated with different treatments. The absence of long chain polyenoic fatty acids indicated the communities were predominantly prokaryotic and the differences in the phospholipid ester-linked fatty acids were primarily in the proportions of cyclopropane fatty acids and the proportions and geometry of the monounsaturated fatty acids.

In similar experiments a subsurface sediment inoculum was grown through two cycles of aerobic growth and compared to organisms from the same inoculum grown through two cycles of anerobic growth with no supplement, or with sulfate or nitrate (Hedrick & White 1986). Again there were reproducible shifts in the microbial community structure as reflected in the profiles of ester-linked phospholipid fatty acids. Hedrick also developed a sensitive assay for the respiratory quinones from subsurface samples utilizing HPLC with electrochemical detection. Benzoquinone isoprenologues are formed by microbes grown with high potential terminal electron acceptors such as oxygen or nitrate (Hollander et al. 1977). Naphthoquinones are formed by bacteria with respiratory systems with various potential terminal electron acceptors. As might have been predicted, the aerobic culture formed the most benzoquinone relative to naphthoquinone, the nitrate supplemented anaerobic culture formed less benzoquinone, the sulfate supplemented culture formed still less benzoquinone, and the anaerobic fermentation formed the least.

It has been postulated for a long time that changes in rates of predation at the top of estuarine food chains would reverberate through the various trophic stages and finally affect the microbiota at its base. After developing methods for preserving sediment samples (Federle & White 1982) and sampling strategies for mud flats (Federle et al. 1983a) it was possible to show statistically significant differences in the community structure of the sedimentary microbiota by eliminating predation by the crabs and fish at the top of the food chain with properly designed caging experiments (Federle et al. 1983b). These experiments also showed significant differences in the benthic microbiota between continuous predation (crabs and fish caged inside) and the random predation of control areas. With the same type of technology it was possible to validate microcosms meant to mimic the estuarine waters nearby. The laboratory microcosms showed microbial biomass and community structures that were detectably different but the degree of difference was not large and did not increase with time when compared to the field in the system taken from a shallow, turbid, highly disturbed bay that is enriched by riverine runoff and is characterized by low macroscopic species diversity and high biomass. Microcosms prepared from a more stable, higher salinity, system with a much more diverse macroscopic community that is controlled by epibenthic predators showed a great difference from the field site. The differences between the microcosms in the laboratory and the field site increased drastically with time in this system (Federle et al. 1986).

Pollution of the microenvironment induces changes in the microbial community structure. Exposure to xenobiotics in the ug/l range markedly influenced colonization of azoic marine sands in experiments designed to test the response to biocides in oil and gas well drilling muds (Smith et al. 1982b). These methods can be utilized with animals. The reef-building coral *Montastrea annularis* showed dose-response related shifts in amino acid pools, a drop in total phospholipid content, a shift from saturated to polyunsaturated fatty acids, a loss of triglycerides, and an increase in phospholipid fatty acids characteristic of bacterial infections on exposure to parts per million levels of oil and gas well-drilling muds (Parker et al. 1984).

The PLFA of the microbiota in subsurface sediments carefully protected from contamination by surface soil microbes during recovery and was not exposed to contaminants in situ shows a distinctive pattern that is remarkably consistent in subsurface clays and sands collected from several states in the USA and in limestones from England (Smith et al. 1986a). The shifts in biomass, community structure, and metabolic activity in the subsurface aquifer microbiota resulting from contamination by improper disposal of creosote wastes showed significant changes (Smith et al. 1985, 1986a). Contamination resulted in an increase in microbial metabolic activity, a decrease in the rate of formation of PHA, shifts in the patterns of PLFA, and a switch from predominantly gram-positive to gram-negative cells. These changes are consistent with the predicted shift to a gram-negative pseudononas-like microflora predicted from isolation experiments with the phenols as carbon sources. Gram-negative bacteria contain distinctive patterns of amide or ester linked aliphatic and hydroxy fatty acids in the lipid A of their lipopolysaccharide wall polymers (Parker et al. 1982). Gram-positive bacteria often contain teichoic acid polymers as the substituted poly-glycerol or ribitol phosphate esters. Teichoic acid glycerol and ribitol can be released specifically by hydrolysis with cold concentrated hydrofluoric acid on the lipid extracted sediment (Gehron et al. 1984). With this assay it proved possible to show that contamination of subsurface

aquifer sediments induces a shift from predominantly gram-positive to gram-negative based on the ratios of teichoic acid glycerol to phospholipid. The PLFA patterns isolated from the contaminated subsurface sediments were typical of pseudomonas like bacteria.

In an anaerobic environment in which nitrogen induced limitations of microbial growth, it proved possible to demonstrate that photosynthetic bacteria capable of nitrogen fixation in the presence of hydrogen were able to proliferate even in the dark (Mikell et al. 1986). Their detection was based on the appearance of bacteriochlorophyll in the pigments.

One would predict that the microbiota colonizing a substratum that was biodegradable would be greater than that found on a non-degradable substratum. Microbial biomass and metabolic activity was higher on pine needles than on polyvinyl chloride needles after incubation in an esturary for 14 weeks (Bobbie et al. 1978). Increasing the smoothness of silica grains of the same size and water pore space exposed to running seawater for 8 weeks fostered a decrease in the biomass of the prokaryotic biofilm with an increase in the microeukaryote grazer density of *Substratum microtopology* (Nickels et al. 1981b).

The microbial biomass of abyssal sediments subjected to abyssal storms showed increased spatial heterogeniety, biomass, and aerobic prokaryotes when compared with sediments from an undisturbed abyssal plain (Baird et al. 1985a, 1985b). The benthic macroinvertebrates of near-shore sediments from the McMurdo Sound in the Antarctic showed a gradient in diversity and biomass from east to west that is paralleled in the microbial biomass and microbial metabolic activity (Smith et al. 1986b).

2) The isolation of specific organisms or groups of organisms for signature biomarker analysis and the detection of these organisms in microbial consortia under conditions where their growth is induced is a second validation of the chemical methodology.

"Signatures" for some of the microbial groups involved in anaerobic fermentations have been developed. The rate limiting step in fermentations is the degradation of polymers (Wolin 1979). A second tier of microbes converts the carbohydrates and amino acids released from the biopolymers into organic acids, alcohols, hydrogen, and carbon dioxide. These are the anaerobic fermenters and some of these organisms contain plasmalogen phospholipids that are limited to this physiological class of anaerobes in the microbial world (Goldfine & Hagen 1972). Plasmalogens can be assayed by their resistance to alkaline methanolysis and extreme sensitivity to mild acid (White et al. 1979d). Anaerobic sediments have been shown to be enriched in plasmalogens (White et al. 1979d). Other groups of anaerobic fermenters contain phosphosphingolipids with unusual sphingosine bases. These were detected in Bacteroides (Rizza et al. 1970). Sphingosines are readily assayed in acid hydrolysates of the polar lipids by their amino groups or by GLC of the long chain bases (White et al. 1969). With these techniques in hand it proved possible to predict the "crash" of an anaerobic fermenter subjected to increased sulfate in the feedstock (Mikell et al. 1986).

Phytanyl glycerol diethers found in the Archaebacteria can be assayed by high pressure liquid chromatography (HPLX) after appropriate derivatization (Martz et al. 1983). C. Mancuso in this laboratory has improved the sensitivity and

resolution of the analysis of the diphytanylglycerol and for the first time the bi-diphytanylglycerol ether lipids of the methanogenic bacteria by HPLC (Mancuso et al. 1986). In the course of this work she has also been able to show the presence of isoprenologues of the aliphatic side chains of the diether lipids using highly sensitive GC/MS techniques (Mancuso et al. 1985). With this analytical system it has proved possible to show a relationship between the sediment methanogenic biomass and the methane released into the air (Martz et al. 1983) and in fractions isolated from an anaerobic digester (Mikell et al. 1986).

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 *Desulfobulbus* (Edlund et al. 1985, Parkes & Taylor 1983, Taylor & Parkes 1983, Parkes & Calder 1985, Dowling et al. 1986) that allows differentiation between those utilizing lactate, propionate, or those using acetate and 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 the secondary recovery of oil are the acetate-utilizing strains (Dowling et al. 1986). Sediments from a Scottish Loche when amended with lactate or propionate show increases in the signature biomarkers of the *Desulfovibrio* or *Desulfobulbus* respectively, that parallel increases in specific substrate induced sulfate-reducing activity (Parks, Dowling & White, unpublished data). PLFA biomarkers for sulfate-reducing bacteria and indications of sulfate-reducing activity were readily detected in anaerobic fermenters supplemented with sulfate (Mikell et al. 1986).

The acid-forming *Thiobacillus* sp. form a remarkable collection of unique PLFA – methoxy, cyclopropyl- or hydroxy-monounsaturated, hydroxy-cyclopropyl, and monounsaturated components with the double bond in unusual positions (Kerger et al. 1986a). The signature PLFA of these organisms were readily detected in a microcosm designed to measure the degradation of concrete exposed to biologically generated acid (Sand et al. 1986) and in concrete samples from sewers that suffered structural failure (Kerger et al. 1986b).

Analysis of the PLFA of type I and type II methane-oxidizing bacteria have shown that they contain components that are sufficiently unusual that they can serve as signatures (Nichols et al. 1985b). Both the total microbial biomass and the signature components typical of type II methane-oxidizing bacteria increase dramatically in soil columns made with subsurface soil exposed to natural gas (Nichols et al. 1986c). Trichlorethylene (TCE) is the 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 (Wilson & Wilson 1985). The soil columns enriched in microbial biomass and specific type II methane-oxidizing bacteria showed methane disappearance was correlated with rapid biodegradation of TCE (Nichols et al. 1986c). Preliminary evidence indicates similar increases in the signature lipids in the subsurface sediments recovered from zones where the TCE concentration increases the microbial metabolic activity (Phelps, Davis, Fliermans & White, unpublished data).

3) Induction of shifts in the microbial community nutritional status as a result of alterations in the environment is a third test of the validity of the chemical methods.

Eukaryotic microbes form triglycerides when exposed in a rich medium and lose the triglyceride under conditions of starvation (Gehron & White 1982). Antarctic ice diatoms showed marked increases in formation of triglyceride paralleling the austral summer blooms with a decrease in triglyceride formation and increase in glycolipid formation with the decline in the blooms (Palmisano et al. 1986). With this assay it was possible to determine that amphipods existing in the estuary at the Florida State University marine laboratory have a triglyceride glycerol to phospholipid ratio typical of starvation. Toxicity testing with these amphipods should be done with organisms in the starving nutritional state if the exposure is to be in the estuary.

Many bacteria form PHA under conditions of unbalanced growth when organisms can accumulate carbon precursors but are unable to divide because of some limiting nutrient (Herron et al. 1978, Nickels et al. 1979). The rates of formation or degradation of PHA has proved a useful means of defining the nutritional status of microbes in various environmental habitats. PHA synthesis is formed under conditions that compromise growth with cellular division whereas PLFA formation accompanies the growth (and cell division) of bacteria. A useful measure thus is the ratio of the rate of formation of PLFA to PHA from 14C-acetate. This ratio has been shown to be an extraordinarily sensitive measure of the nutrient environment in the bacterial habitat (Findlay & White 1984, Findlay et al. 1985). With this measure it proved possible to detect the effects of raking intertidal sediments with a garden rake prior to measuring the rates of incorporation of 14C-acetate into PLFA and PHA if the isotope was carefully injected into cores of sediment. Other commonly utilized methods of measuring activity by forming slurries or filtering the isotopically labeled precursor through the core completely obscured the effects of the raking. Measuring the ratio of incorporation rates greatly increases sensitivity and allows measurement of the "disturbance artifact" involved in the application of labeled precursors to highly stratified environments such as sediments (Findlay et al. 1985). In his Ph. D. studies R H. Findlay showed a hierarchy of disturbance in a tidal sand bar. The undisturbed sediment showed the smallest values for the ratio of incorporation into PLFA/PHA. Increasing ratios based on the greater synthesis of PLFA (cellular growth) and lesser formation of PHA (carbon accumulation) for sands in the course of sand dollar feeding and bioturbation, bioturbation in sting ray elasmobranch feeding pits, areas subjected to wind and tide disturbance were detected. The highest level was for the sediments slurried in a common method of measuring microbial activities. Similar findings of increased rates of PLFA synthesis have been detected with disturbance of stratified sediments by measuring the incorporation of $H_3$32-PO_4 into phospholipids (Moriarty et al. 1985).

With this sensitive measure of the microbial community nutritional status microniches known to be limiting for bacterial growth could be examined as a validation of the method. The leaf surface of some living seagrasses has been shown to contain a microflora with specific community structure. The leaf secretions apparently control the epiphytic community. The epiphytic microbiota on sea grass blades has a very high PHA/phospholipid ratio indicating that the leaf surface is an environment where the nutrients induce unbalanced growth (Herron et al. 1978). The chelating activity of the tannin-rich brown runoff water from the pine plantations of north Florida induces rapid accumulation of PHA in the estuarine detrital microbiota attached to oak leaves (Nickels et al. 1979). The accumulated PHA dis-

appears with the addition of appropiate nutrients to the seawater. Recently it has been possible to study the rhizosphere of the rape plant *Brassica napus* (L.). Bacteria from the rhizosphere when added to sterile plants show differences in community nutritional status. Organisms that are attached to the roots show active formation of signature PLFA but no formation of PHA whereas organisms recovered from rhizosphere away from the roots show less growth and the accumulation of large amounts of PHA (Tunlid et al. 1986).

Uncontaminated subsurface aquifer sediments show a microbiota with high levels of PHA relative to the phospholipids (White et al. 1983) as well as a specific PLFA pattern that is similar in clays, sands, and limestones from various locations and distinctly different from surface soils (Smith et al. 1986a). Contamination of the subsurface sediments with aromatic phenols induces bacterial growth in the vadose zone with a decrease in the rate of PHA biosynthesis (Smith et al. 1985).

A second measure of community nutritional status is the formation of extracellular polysaccharide glycocalyx. Uronic acid-containing glycolalyx forms maximally in the marine *Pseudomonas atlantica* under conditions of nutritional stress (Uhlinger & White 1983). Uncontaminated subsurface aquifer sediments contain microbiota with very high levels of extracellular polysaccharides indicating poor nutrient conditions (White et al. 1983). Excessive amounts of these exopolymers are not formed when nutrients are added to subsurface sediment microcosms (Balkwell & White, unpublished data). These polymers form on the surfaces of metals exposed to rapidly flowing seawater and may be responsible for inducing reversible acceleration of corrosion (Nivens et al. 1986).

Starvation induces the formation of minicells in some marine bacteria. There is a loss of cell components including the membrane lipids but there is a marked increase in the proportion of monoenoic PLFA with the double bond in the trans configuration (Guckert et al. 1986, Hood et al. 1986). The accumulation of this trans monoenoic PLFA represents a synthesis from 14-C acetate and has been shown in natural communities of pelagic marine organisms (Guckert, Ph. D. thesis, Florida State University).

4) The detection of specific organisms in environments with the expected consequences in a fourth type of validation of the chemical methods of community analysis.

The pathogenic bacterium *Francisella tularensis* has been shown to contain a signature PLFA pattern (Nichols et al. 1985a). Organisms with this PLFA pattern have been isolated from patients with serologic evidence of infection. This unique PLFA pattern has been detected in human and animal tissue with serologic, 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 fatty acids of the lipopolysaccharide lipid A of gram-negative bacteria has proved to be an extremely 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 (Odham et al. 1985).

In section 2 we have described the detection of signature lipids biomarkers for methanogeic bacteria, methane-oxidizing bacteria, acid producing *Thiobacillus* sp., anaerobic fermenters, and sulfate-reducing bacteria in consortia in which the

specific activity has been demonstrated. Preliminary investigations suggest strongly that soil columns enriched with propane or butane stimulate the growth of mycobacteria with the capacity to degrade these gases and that contain the characteristic 10-methyl octadecanoic acid in the PLFA (Nickels, Pfiffner, Trexler, Hensen, Wilson & White, unpublished data).

5) The fifth evidence for the validation of chemical methods for biomass, community structure, nutritional status and metabolic activity rests on the changes in microbial community structure after grazing by predators with known specificity.

The sand dollar *Mellita quinquiesperforata* bioturbates sandy sediments and grazes specific components of the benthic biota. Using PLFA analysis the grazing of the sands by this organism was shown to selectively remove the non-photosynthetic microeukaryotes from the sediment (Findlay & White 1983a). This is in agreement with the studies of the morphology of the organisms found in the feeding apparatus. As described above the bioturbation associated with sand dollar predation increases the ratio of 14-C incorporation into PLFA and decreases incorporation into PHA.

Amphipod grazing of the detrital microbiota affects the community structure and metabolic activity of the biofilm. Grazing by the relatively nonspecific *Gammarus mucronatus* results in decreases of PLFA characteristic of algae and fungi and replacement by a largely bacterial community (Morrison et al. 1977, Morrison & White 1980). The chemical measures agree with the morphology shown with SCM. Grazing increases the metabolic activity and biomass of the detrital microbiota reflected higher rates of oxygen utilization, lipid synthesis, and release of 14-C carbon dioxide from prelabeled microbiota. Grazing stimulates the turnover of PHA and synthesis of PLFA again showing increased metabolic activity. Analysis of specific polar lipids showed that glycerol phosphorylglycerol derived from phosphatidyl glycerol showed rates of loss that parallel the most rapid rates of growth of the detrital microbiota (King et al. 1977). Glycerol phosphorylcholine derived from phosphatidyl choline showed extremely slow turnover in the detrital microbiota after pulse chase exposure to 14-C-labeled precursors. Grazing by amphipods markedly increased the loss of 14-C-glycerol phosphorylcholine providing a quantitative estimate of grazing pressure (Morrison & White 1980, Smith et al. 1982).

The morphology of the feeding apparatus of two sympatric estuarine detrital-feeding amphipods was reflected in partitioning of the microbial resource as determined by PLFA analysis of the microbial biofilm (Smith et al. 1982). The complex apparatus of *Melita appendiculata* fed on non-photosynthetic microeukaryotes and had less effect in increasing the community microbial metabolic activity than the general feeding (bacteria and diatoms) *Gammarus mucronatus*.

Conclusions

The methods described above provide insight into the biomass and community structure of microbial consortia at the time of the analysis. This is in some respects like the anatomy of a higher organism and it defines the potential of activities possible for this community. Phospholipids, adenosine nucleotides,

muramic acid, and the lipopolysaccharide of dead bacteria are rapidly lost from marine sediments (Davis & White 1980, White et al. 1979b, 1979d, King et al. 1977, Moriarty 1977, Saddler & Wardlaw 1980). This indicates that the chemical markers provide good estimates for the standing viable or potentially viable microbiota. Rates of formation or loss of endogenous storage lipids or exocellular polysaccharide polymers of synthesis or turnover of specific membrane signature biomarkers provide insight into the nutritional status and actual metabolic activities of the biofilms. Application of this suite of methods to diverse environments has provided both insight and validation of these measures.

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