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MICROBIAL COMMUNITY STRUCTURE AND FUNCTION AS INDICATORS OF ENVIRONMENTAL HEALTH

David C. White
Institute for Applied Microbiology
University of Tennessee
10515 Research Drive, Suite 300
Knoxville, Tennessee 37932-2567

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David C. White, Institute for Applied Microbiology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2567

ABSTRACT

The microtiota represent the largest and most metabolically diverse biomass of the biota of soils and sediments. These microbes have the most rapid doubling times with favorable conditions and have the metabolic mechanisms for survival of compromising conditions in that environment. The microbiota which represent the base of the food web can respond rapidly to changes in the physical chemistry, nutrient availability, predation, or the pollutant impact, and thus can provide a means to define the conditions in their microniches if adequate methods are available to quantitatively define their biomass, community structure, nutritional status, and metabolic activity in sufficient detail. The traditional methods for microbial community analysis are inadequate because they require quantitative removal of the microorganisms from their habitat for cultural enumeration. Many organisms that can be removed and detected microscopically will not grow on the laboratory media. A biochemical methodology which utilizes analysis of cellular components found universally in all microbes can be utilized as a measure of cellular biomass. If the components of the cells utilized in these measurements are sufficiently unusual in their distribution amongst the various types of microbes then, they can be utilized as "signature" biomarkers for those specific types of microbes. If a sufficient catalogue of "signatures" can be developed, then changes in community structure can be defined. In this study it will be shown that the membrane phospholipids are sufficiently universal to be used as biomass indicators. The distribution of specific groups of phospholipid ester-linked fatty acids (PLFA) amongst specific groups of microorganisms is sufficiently asymmetric to provide "signatures" which can be used to define the community structure. Other lipids such as the endogenous storage polymer, poly beta-hydroxyalkanoate, together with the accumulation of specific PLFA, can be used to indicate the community nutritional status. Since the methods reported herein involve the chromatographic fractionation and mass spectral detection of the "signature" PLFA patterns, it is possible to define metabolic rates with mass labeled (13-C) components utilizing enrichments into specific microbes. These biochemical analyses have shown that microbial biofilms respond to changes in nutrient and electron donor/acceptor concentration and composition, predation, bioturbation (of sediments), and contamination of the sediments and soils. The richness and diversity of the microbiota, coupled with its potential for rapid response to changed conditions, make possible its use as a means to quantitatively access the impacts of toxicants on specific environmental sites. PLFA analysis can be utilized to quantitatively define the health of an environment.

INTRODUCTION

The microbes provide the largest biomass in nearly every environmental niche. The microbial component of a community is considerably greater in metabolic diversity than the metazoa. For example, the whole complex of anaerobic life is almost exclusively a microbial characteristic. Microbes have the greatest diversity in terms of physiological types of species. Nearly every possible chemical reaction with sufficient free energy has been exploited by a microbe. In addition microbes have the capacity for an extremely rapid response to selective conditions in the environment. With the great diversity of types and extraordinary metabolic versatility, shifts in microbial community structure should be the ideal way to study the impact of toxicants on an ecosystem, if a suitable assay system could be devised.

Classical microbiological methods that involve the detachment and subsequent culturing of organisms on petri plates can lead to gross underestimations of the numbers of organisms (White, 1983). The isolation and characterization of each specific group of microbe in a complex environment such as a sediment exposed to toxic runoff would be prohibitively expensive. If the extraordinary potential of the total microbiota are to be used in accessing the health of an ecosystem then new methods must be developed.

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). This paper will review these methods and demonstrate their effectiveness in monitoring shifts in the microbial community structure that could provide a quantitative assessment of the of the health of the ecosystem.

METHODS

Biomass estimation Phospholipids, intercellular adenosine nucleotides, and cell wall amino-sugars are biochemical components of cells that have been utilized to estimate microbial biomass (White, 1983). Of these, phospholipids have proven the most useful when examining predation effects on microbial biofilms. 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 intracellular adenosine nucleotides, and cell wall muramic acid (White et al. 1979b). Our laboratory has found it useful to measure the phosphate, the various polar head groups, and the ester-linked fatty acids that form the phospholipids. 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 and White 1983). As shown by Smith et al. (1986a), there appears to be a unique microbial community in uncontaminated subsurface sediments from below the root zone. The microbiota are sparse and have an identical coccobacillary morphology. In these subsurface sediments, the biomass and cell numbers estimated from direct cell count after acridine orange staining, agree with the numbers and biomass estimated from the extractible phospholipid phosphate and total fatty acids, the total adenosine triphosphate, the fatty acids from the lipopolysaccharide lipid A, and the cell wall muramic acid content (Balkwill et al. 1987).

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 and 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 many of the anthropogenic 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 sludge 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 by 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 et al. 1984; Hewlett-Packard, 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 from the total PLFA.

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 and 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.

The shifts in microbial PLFA patterns, with changes in physiological conditions, can be utilized to gain insight into the nutritional status of the organisms in a particular biofilm so long as the behavior of the specific groups of microbes under consideration is carefully validated by studies of cultures under defined conditions. A potentially very useful finding is the detection of increased proportions of trans monoenoic fatty acids in the minicells which result from the starvation of some marine bacteria (Guckert et al., 1986). This biomarker appears to indicate starvation with attachment to biofilms in the initial microfouling community.

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 and White 1982).

Certain bacteria form endogenous lipid PHA under conditions where 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-ØH acid longer than 4-carbons in these polymers (Findlay and White 1983b) which accounts for the changing of the name from poly beta-hydroxy butyrate (PHB) to PHA.

Assays 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 and White 1983).

Metabolic activity The analyses described above all involve the isolation of components of microbial consortia. Since each of the components is 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 and 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 ^{32}P phosphoric acid 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 8pg (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.

Reproducibility The changes in biofilm or sedimentary microbiota, which induced shifting the environment of the microniche, are reproducible. 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 PLFA 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 PLFA 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 anaerobic growth with no supplement, or with sulfate or nitrate (Hedrick and White 1986). Again there were reproducible shifts in the microbial community structure as reflected in the profiles of PLFA. Not only were the PLFA patterns reproducible, but the ratio of respiratory quinones reflected the redox environment in the bacterial biofilms. 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 utilizing low-potenital electron acceptors such as sulfate or organic substrates. Aerobic consortia 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.

These methods can be applied to biofilms attached to substrata (detrital surfaces, sediments, biofouled surfaces) or from suspended particulate matter in the water column after collection through methanol-washed Nuclepore filters with an extraction technique using hexane-isopropanol (Guckert, 1986). This solvent system does not affect the Nucleopore membrane.

RESULTS AND DISCUSSION

Validation of the "signature" biomarker technique

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 provides a validation of the signature biomarker 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 and 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 (HPLC) 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 is possible to show a relationship between the sediment methanogenic biomass, the methane released into the air (Martz et al. 1983) and 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 and Taylor 1983; Taylor and Parkes 1983, Parkes and Calder 1985; Dowling et al. 1985) 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. 1985). Sediments from a Scottish Loche when ammended 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, and White unpublished data). PLFA biomarkers for specific 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).

Response of the microbial community to shifts in the microenvironment

Induction of shifts in microbial community structure by altering the microenvironment result in changes that can be readily demonstrated using the biomarker techniques defined above. The microbiota that colonizes teflon strips

suspended in a subtropical estuary, when incubated in seawater containing antibiotics with the pH and nutrient composition 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 significantly increased fungal biomarkers such as long chain polyenoic PLFA of both the alpha and gamma linolenic series, ergosterol type steroids, lipid inositol and 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 DNA (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, 16ω and 18ωcarbon 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 an anaerobic environment which has 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. Supplementation of the system with nitrogen resulted in depression of the nitrogen fixing photosynthetic bacteria.

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 estuary for 14 weeks (Bobbie et al. 1978).

The microbiota also shift biomarker components when the nutritional conditions change. Eukaryotic microbes form triglycerides when exposed to a rich medium and lose the triglyceride under conditions of starvation (Gehron and 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).

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 ^{14}C-acetate. This ratio has been shown to be an extraordinarily sensitive measure of the nutrient environment in the bacterial habitat (Findlay and White 1984; Findlay et al. 1985). Using this measure, while carefully infecting the isotope into cores of sediment, it proved possible to detect the effects of raking intertidal sediments with a garden rake prior to measuring the rates of incorporation of ^{14}C-acetate into PLFA and PHA. 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 seagrass 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 disappears 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 rizosphere 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).

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 14C acetate and has been shown in natural communities of pelagic marine organisms (Guckert, Ph. D. thesis, Florida State University).

Response of the microbiota to changes in predation

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 and White 1982) and strategies for gathering mud flats samples (Federle et al. 1983a), it was possible with properly designed caging experiments 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, high saline system with a 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).

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).

In the absence of predation, a natural succession of microbes during the maturation of marine biofilms has been detected. Shifts in morphology, observed by using scanning electron microscopy (SEM), can be measured quantitatively with PLFA analysis (Morrison et al. 1977; Nickels et al. 1981a). The morphology by SEM 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 filamentous bacteria, the diatoms, the algae and the microeukaryote larvae (Smith et al., 1982). These studies form controls for analysis of predation effects.

Amphipod predation of the detrital microbiota can be detected using the biomarker technique. Brown fallen oak leaves from a live oak (Quercus virginiana Mill) provide a surface on which a succession of microbiota attach (Morrison et al., 1977). When these leaves are exposed to seawater in a subtropical estuary such as Apalachicola Bay, Florida a biofilm forms on the detritus. The biofilm is initially comprised of bacterial microcolonies that are quickly overlain by a more diverse community characterized by filamentous bacteria, fungal hyphae, cyanobacteria, diatoms and microalgae as detected by scanning electron microscopy (SEM). The initial microfouling community is characterized by a high ratio of extractible adenosine triphosphate (ATP) to muramic acid. This is followed by a community with a lowered ratio of ATP to muramic acid. Muramic acid is a unique component of the bacterial cell wall.

After a two week exposure in the bay in litter baskets, the leaves were frozen and thawed three times to decrease the macrofaunal populations and exposed to filtered estuarine seawater for an additional day. The dominant amphipod Gammarus mucronatus Say 1818 is a surface biofilm feeder. These animals were recovered, counted, and starved for 24 hours by incubation and the absence of leaves with a biofilm. These starved amphipods rapidly grazed the surface of the leaves when exposed to the mature biofilm. They appear to graze the biofilm exclusively as delicate stellate trichome structures, on the ventral sides of the leaves, were never damaged in these experiments. The experiments consisted of exposing the mature detrital biofilm generated by a two week exposure in the bay to grazing amphipods at a natural density (one amphipod/ 8.5 cm^2 leaf surface area) and recovering leaves over a period of 20 days. The exposures were maintained at ambient conditions and there was no amphipod mortality. Leaves were sampled by removing 6.5 mm diameter disks with a cork borer and utilizing the disks for the determinations described below (Morrison and White, 1980).

Amphipod grazing resulted in a progressive decrease in the total colonizing microbiota with an increase in the exposed leaf surface. In the first day there was a rapid loss of biomass followed by a rapid recovery of the biofilm. There was essentially no change in the biofilms not exposed to the grazing amphipods throughout the experiment. In the grazed biofilms high resolution SEM showed a shift in morphology with grazing. The mature biofilm with the fungal hyphae, complex baterial colonies containing filaments, diatoms, cyanobacteria, and microalgae were replaced by a bacterial biofilm containing much more extracellular polymer glycocalyx. The shift in biofilm community structure defined by SEM was confirmed by the changes in the patterns of PLFA. There was a shift in the proportions of monoenoic PLFA from those characteristic of both the bacteria and microeukaryotes to those formed by bacterial anaerobic pathways, a marked decrease in the polyenoic PLFA characteristic of the microeukaryotes, and an increase in the short and branched saturated PLFA characteristic of gram-negative bacteria (Smith et al., 1982). The biofilm biomass estimated by the extractible ATP and the lipid phosphate both showed increases that paralleled the formation of the secondary bacterial biofilm of a factor of two over the ungrazed control. The unexposed leaves contained about 10% of the phosphohlipid and 0.04% of the ATP found in the bacterial biofilm. This indicates that the biomass indicators are primarily related to the biofilm microbes. These experiments were repeated with teflon coupons so that changes observed with SEM could be confirmed by analysis of PLFA without interference from oak leaf lipids.

The biofilm microbial activity in the grazed microbiota showed increases. The oxygen utlization expressed per gram dry weight of leaf increased 4-fold over the ungrazed detritus. The rate of the loss of 14-C carbon dioxide from pre-labeled detrital biofilms was more that twice the ungrazed control. The synthesis of PHA and total microbial lipid was likewise higher in the grazed biota. The alkaline phosphatase however was roughly equal in the first few days of the experiment but then was consistently lower in the grazed bacterial biofilms. The rate of loss of 14-C labeled PHA was significantly greater in the grazed microbiota.

The turnover of specific components labeled by a short exposure to precursors and then incubated with a higher concentration of unlabeled precursor gives an indication of the minimal rate of metabolism of that component. If the "pulse chase" experiment is done at the same time as the exposure to grazing, then the loss of label from a specific component becomes the combination of the rates of metabolism by the bacteria plus the rate of removal from the biofilm by the grazer. Examination of lipid classes in this experiment showed that the deacylated phospholipid glycerol esters gave insight into the rate of predation. In the ungrazed biota the glycerol ester glyceroyl-phosphorylcholine (GPC) derived from phosphatidyl choline showed essentially no turnover. When the biofilm was exposed to the amphipods the rate of disappearance of GPC paralleled the rate of loss of muramic acid. Glyceroyl-phosphorylglycerol derived from phosphatidyl glycerol has an active metabolism in bacteria. Its rate of turnover increased in the grazed microbiota indicating both the loss due to removal of the cells plus the greatly stimulated metabolic activity induced in the bacterial biofilm that is grazed.

In preliminary experiments it was possible to show that there is an optimum density of grazing amphipods for stimulating the biofilm microbial activity. Densities too low or too high result in either a mature biofilm with the complex morphology of the ungrazed control or a grazing rate that does not allow the secondary bacterial biofilm to develop. With exposures of detrital microbiota to high enough grazing pressure the capacity for recovery by the biofilm is exceeded and the activity and biomass of the detrital microbiota are depressed (Morrison, 1980).

These experiments clearly indicate that grazing shifts the detrital microflora from a metabolically stable community with complex morphology to one of metabolically active fast growing bacteria and diatoms. The loss of alkaline phosphatase activity in the grazed community suggests an increase in the availability of phosphate released by the grazers. These are the conditions of perpetual "youth" imposed by grazing that were predicted by Johannes (1965).

Response of the sedimentary microbiota to bioturbation

In sediments in which a redox gradient exists, the analysis of the effects of predation is complicated by the effects of disturbance. The addition of oxygen to reduced sediments can markedly affect the benthic microbiota. Findlay developed a sensitive measure of disturbance based on the ratio of the rate of 14-C acetate incorporation into PHA and PLFA (Findlay et al., 1985). PHA is formed by bacteria under conditions when cell division is compromised (unbalanced growth, Nickels et al., 1979). PLFA synthesis accompanies cell growth and cell division; conditions during which PHA formation stops and utilization accelerates. Findlay, in his Ph. D. thesis, described the consequences of disturbing sediment. The disturbance of sieving through 998 um

sieve resulted in a rapid initial increase in the ratio of 14-C incorporation into PLFA/PHA. This burst of growth indicated bacterial growth on the reduced carbon components in the anaerobic sediment induced in the presence of the added oxygen. This response was maximal in 2 hours after sieving mud flat sediment. Subsequently there was a drop in the ratio to below predisturbance levels by 8 hours. The decrease continued for three days after which the ratio increased again by the fifth day to predisturbance levels. Throughout the five day experiment, total growth rates measured as phospholipid synthesis or incorporation of thymidine into DNA relative to the extractible phospholipid decreased initially in the first hour and then increased for a short time with a maximum in 2-4 hours and then decreased to a maintainence level of about half the predisturbance rate. The microbial biomass showed an initial fall in the first 2-4 hours then a slow recovery to predisturbance levels measured as muramic acid or total extractible phospholipid phosphate. The PLFA patterns showed shifts during the initial fall and rebound of the growth rates. The long-chain polyunsaturated PLFA decreased. These PLFA are characteristic of the the microeukaryotes. PLFA characteristic of anaerobic bacteria initially decreased and then showed a disproportionate increase that paralleled the increased rate of PLFA synthesis. PHA initially disappeared then did not accumulate for the 2-4 hour period of recovery. Increased proportions of trans monoenoic PLFA shown to be characteristic of starved bacteria by Guckert et al., (1986) were detected in the 2-4 hour period. The microbiota apparently underwent an initial shock from which a few microbes recovered and exploited the increased nutrients and high potential electron donor. This secondary growth spurt continued until growth depleted the environment of an essential micronutrient and unbalanced growth ensued (PHA and trans monoenoic PLFA increased). This community was eventually replaced by a microbially dominated assembly greatly enriched in anaerobes and facultative heterotrophs.

With the effects of mechanical disturbance carefully documented, Findlay (1986) examined natural disturbances. The sting ray Dasyatis sabina digs extensive pits in the sediment in search of infaunal prey. The disturbed sediments represent resuspended deep sediments brought to the surface in the feeding activity. The disturbed sediments, sampled after the nocturnal feeding, showed initially lower biomass and growth rates than control sediments with time dependent increases much like the sieving experiment. The PLFA patterns indicated shifts to more aerobic bacteria and microeukaryotes containing linoleic acid as these organisms colonize the newly aerobic sediments. There was a concomitant decrease in the proportions of PLFA associated with facultative and anaerobic bacteria than in the control sediments.

Findlay (1986) then examined the effects of disturbance in the field combined with predation. The Enteropneust Ptchyodera bahaminasis feeds by ingesting sediment from near the surface and generates mounds of fecal castings from the other end of a U shaped feeding tube. The fecal castings initially contained a low bacterial biomass measured by muramic acid and low rates of phospholipid synthesis and thymidine incorporation into DNA when compared to control sediments. Initial high levels of phospholipid fatty acids and phosphate indicate non-bacterial lipids possibly from the worm. These lipids disappeared rapidly and 2 hours after extrusion the muramic acid, phospholipid fatty acids, and phosphate indicated a bacterial biomass less than the control sediments. The PLFA patterns indicated the loss of microeukaryotes, the algae, the aerobic and facultative bacteria, and the anaerobic bacteria. The PLFA patterns indicated the facultative anaerobes appeared unchanged from the control sediments. Six hours after extrusion, microbial growth rates were significantly

greater in the fecal castings than in control sediments. The fecal casts degenerated after 8 hours so long term effects could not be studied. The branched saturated fatty acids characteristic of the phospholipids of the sulfate-reducing bacteria Desulfovibrio and Desulfobacter seemed to preferentially accumulate in the mature fecal casts. The microbiota of the fecal casts indicate the enteropneust feeds rather nonselectively on the total microbiota and although recolonization occurs somewhat more slowly, the pattern of recovery is similar to that of sediments subjected to simple physical disturbances.

Responses of the microbiota to pollution

Pollution of the microenvironment induces changes in the microbial community structure. Exposure to xenobiotics in the ug/l range markedly influenced the 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 not exposed to contaminants in situ, show 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, predicted from isolation experiments with the phenols as carbon sources, are consitent with the predicted shift to a gram-negative pseudononas-like microflora. 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 induced 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.

Analysis of the PLFA of type I and type II methane-oxidizing bacteria have shown that they contain components that are sufficiently unusual and 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 groundwater aquifers and methane oxidizing

bacteria have been shown to be associated with a consortium that can degrade it (Wilson and Wilson, 1985). The soil columns enriched in microbial biomass and specific type II methane-oxidizing bacteria showed methane disappearence 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 (Fliermans et al. 1988).

Addition of propane to subsurface soils induces the differential growth of bacteria with PLFA patterns of actinomycetes when isolated form typical colonies with branched mycelia (Ringelberg et al. 1988). The propane-oxidixing actinomycetes when isolated from these soils also form large amounts of the unusual monoenoic PLFA 16:1w5c, and 16:1w8c (Ringelberg et al. 1988). The patterns found in the isolates can be demonstrated in soils exposed to propane and air even for a very short time. These consortia also degrade TCE and other halogenated hydrocarbons.

Uncontaminated subsurface aquifer sediments from various locations show a microbiota which contains high levels of PHA relative to the phospholipids (White et al. 1983) as well as a specific PLFA pattern which is similar in clays, sands, and limestones but distinctly different from suface 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 and 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 and 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).

CONCLUSIONS

The methods described above provide quantitative insight into the biomass and community structure, nutritional status, and metabolic activities of microbial consortia in soils and sediments and do not require quantitative recovery of the organisms from the biofilm or that they be cultured successfully. These methods are not complicated by fossil components from non-viable cells remaining in the microbial consortia. Phospholipids, adenosine nucleotides, muramic acid, and the lipopolysaccharide of dead bacteria are rapidly lost from marine sediments (Davis and White 1980; White et al. 1979b; 1979d; King et al. 1977; Moriarty 1977; Saddler and 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 or synthesis or turnover of specific membrane signature biomarkers provide insight into the nutritional status and actual metabolic activities of these microbial consortia as they are grazed. Selectivity in the grazing of biofilms and sediments by

specific grazers can also be determined based on the analysis of signature biomarkers.

The methodology described herein can be validated by showing that specific microorganism types can be induced in soils and sediments which show the same patterns of signature biomarkers as isolates from the specific environment. The microbiota has been shown to be rapidly responsive to shifts in the microenvironment as well as to predation. The microbiota in sediments with redox gradients rapidly respond to artificial distrubance and to the disturbance induced by bioturbation. It is then not surprising that the microbiota respond to pollution. This response to pollution has possibly been best demonstrated in the subsurface sediments where primarily prokaryotic subsets of soil microbiota show a clear shift in the chemical markers of starvtion, from predominantly gram-positive to predominantly gram-negative microbiota. Preliminary use of the PLFA methodology, using subsets of the PLFA and pollutant metal contamination of esturine sediments in a carbonate rich semitropical system (Biscayne bay, Florida and north Florida bay) containing terrigenous aluminosilicate sediments, shows an estimated linear relationship using cannonical correlation between shifts in microbial community structure (Schropp et al. 1988). This analysis required a large weighting factor based on sediment grain size and shows that the accessment of the microbiota provides a quantitative tool to monitor the effects of estuarine pollution.

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David Cleaveland White
Social Security # 344-24-4416
Born Moline, Illinois, May 18, 1929.

Education: Dartmouth College, A.B., Magna cum laude, Phi Beta Kappa, 1951.

Tufts University School of Medicine, M.D., 1955; Rockefeller University, Ph.D., 1962. Internship Hospital University of Pennsylvania, 1955-1956.

Military Service: Lt. M.C U.S.N.R., NAS Johnsville, Pennsylvania, 1956-1958, Aviation Medical Acceleration Laboratory, Naval Air Development Center, research in aviation medicine.

Academic Service: Assistant Professor to Professor of Biochemistry, University of Kentucky Medical Center, 1962-1972.

Professor, Department of Biological Sciences, Florida State University, 1973-1985; Associate Director Program in Medical Sciences, FSU, 1973-1984.

University of Tennessee/Oak Ridge National Laboratory Distinguished Scientist 1986-.

Professor of Microbiology/Ecology, University of Tennessee, Knoxville 1986-, Research Scientist, Oak Ridge National Laboratory, 1986-.

Director, Institute of Applied Microbiology, University of Tennessee, Knoxville, 1986-.

Adjunct Professorships: Professor of Community Medicine and Family Medicine, University of Florida Medical Center, 1975-1984; Professor +of Biological Sciences 1986-, Professor of Oceanography, Professor of Chemistry, FSU, 1981-; Professor Institute of Food and Agricultural Sciences, University of Florida, 1981-; Professor Interdepartmental Toxicology Program, University of Arkansas Medical Center, and the National Center for Toxicological Research, 1981-;

Co-director Center for Biomedical and Toxicological Research, Florida State University, 1982-1985.

Editorships: Founding Editor-in-chief, Journal of Microbiological Methods, Elsevier Biomedical Press, Amsterdam, 1982-; Editor, Applied and Environmental Microbiology, American Society for Microbiology, 1980-1985; Associate Editor, Journal of Contaminant Hydrology, 1985-; Annual Review of Microbiology 1987-.

Honors: P. R.Edwards Award,Outstanding Microbiologist, South-eastern Branch, American Society for Microbiology, 1981; Wellcome Visiting Professor- American Society for Microbiology and the Burroughs Wellcome Fund for 1984-1985, to the University of Oklahoma; Fellow American Academy Microbiology, 1978-1984; Foundation Speaker, American Society for Microbiology, 1971, 1978, 1979, 1985; Advisory Committee, Center for Theological Inquiry, Princeton University 1984-; Antarctic Service Medal, 1984.

Reviewed Publications: 210 as of January 1988.