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Biochemical markers for measurement of predation effects on the biomass, community structure, nutritional status, and metabolic activity of microbial biofilms

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Abstract

Chemical measures for the biomass, community structure, nutritional status, and metabolic activities of microbes in biofilms attached to detrital or sediment surfaces based on analysis of components of cells and extracellular polymers represent a quantitative and sensitive method for the analysis of predation. These methods require neither the quantitative removal of the organisms from the surfaces nor the efficient culture of each group of microbes for analysis of predation effects on the biofilm. 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 have a naturally high turnover, are found in all cellular membranes, 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 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-hydroxyalkanoate (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 methods involve chromatographic separation and analysis so rates of incorporation or turnover into specific components can be utilized as measures of metabolic activities. With these methods it has proved possible to show that amphipod grazing can induce shifts in biofilm community structure, nutritional status, and metabolic activities. With this technology it proved possible to show resource partitioning amongst sympatric detrital feeding amphipods, prey specificity of feeding of benthic microvores, effects of sedimentary microtopology on predation, and shifts in the microbiota by exclusion of top epibenthic predators.

Introduction

The microbes that form biofilms on detrital surfaces or sediment granules present a complex problem for assay. Classical microbiological methods that involve the quantitative detachment and subsequent culturing of organisms on Petri plates can lead to gross underestimations of the numbers of organisms (White, 1983).

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 a series of experiments from our laboratory to elucidate the role of predation in the ecology of microbial biofilms.

Description of sites studied

Detrital microbiota developed on the surface of oak leaves (*Quercus virginiana* Mill) or Teflon sheets exposed in Apalachicola Bay, Florida (29°43.2'N; 84°57.3'W), sediments at that site or at one near the Florida State marine laboratory (29°54'N; 84°27.5'W) were utilized in these experiments. Complete descriptions of the study sites can be found in: (Morrison & White, 1980; Findlay & White, 1983a, b; Federle *et al.*, 1983a, b).

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 intra-cellular adenosine nucleotides; 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 asayed to increase the specificity and sensitivity of the phospholipid assay (Gehron & 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 a small cocco-bacillary 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 & 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 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 *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 physiological type of microbe 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 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 that result from starvation of some marine bacteria (Guckert *et al.*, 1986). This biomarker appears to indicate starvation with attachment to biofilms in the initial microfouling community.

From the residue of the lipid-extracted biofilm, muramic acid, a unique component of the bacterial cell wall can be recovered (Findlay *et al.*, 1983a). 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. Thus, 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 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 compo-

nents 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).

Assays for extracellular polysaccharide glycocalyx based on the specific content of uronic acids have been developed (Fazio *et al.*, 1982). These have 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 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 & 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 8 pg (90 femtomoles) of D-alanine from the bacterial cell wall (the equivalent of 1 000 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 induced shifts in the environment of the microniche that 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 that the communities were predominantly prokaryotic and that 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 & 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-potential 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 (summarized in Fig. 1) 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 Nucleopore filters with an extraction technique using hexane-isopropanol (Guckert, 1986). This solvent system does not affect the Nucleopore membrane.

Biomass

Cell Wall – Muramic Acid
Lipopolysaccharide Lipid A fatty acids
Membrane – Phospholipids (analysis of lipid phosphate, lipid glycerol phosphate, polar lipid total fatty acids)
Cellular Adenosine Triphosphate

Community Structure

'Signature Biomarkers'
Phospholipid Ester – Linked Fatty Acids (PLFA)
Lipopolysaccharide Lipid A hydroxy fatty acids
Phytopigments and Carotenoids
Steroids (microeukaryotes)

Nutritional Status

Ratio of PLFA to poly beta-hydroxy alkanoate (PHA)
Proportion of *trans* monoenoic PLFA
Respiratory Quinone ratio

Metabolic Activity

Ratio of rates of incorporation into PHA/PLFA. Excellent for determination of the 'disturbance artifact' in analysis of sediments
Incorporation of 13-C or 15-N labeled precursors into 'signature biomarkers' with gas chromatographic/mass spectral analysis
Rates of incorporation of 32-P or 35-S into lipids
Rates of 3-H thymidine incorporation into DNA
Rates of enzyme activity (FDA)
Cellular Adenosine Energy Charge

Fig. 1. Biochemical characterization of microbes in biofilms on Detritus or sediment granules. The same analysis has been applied to particulates recovered from the water column on membranes (Guckert, 1986).

Predation effects on microbial biofilms

Biofilm succession

In the absence of predation a natural succession of microbes during the maturation of marine biofilms has been detected. Shifts in morphology seen 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 microeukaryote larvae (Smith *et al.*, 1982).These studies form controls for analysis of predation effects.

Effects of predation by amphipods on the detrital microbiota

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 esturary 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 com-

munity 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 in 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 & 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 bacterial 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 by a factor of two over the ungrazed control that paralleled the formation of secondary bacterial biofilm. The unexposed leaves contained about 10% of the phospholipid and 0.04% of the ATP found in the bacterial biofilm. This indicated that the biomass indicators were 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 utilization 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 than twice that of 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 (Fig. 2).

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 glyceryl-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

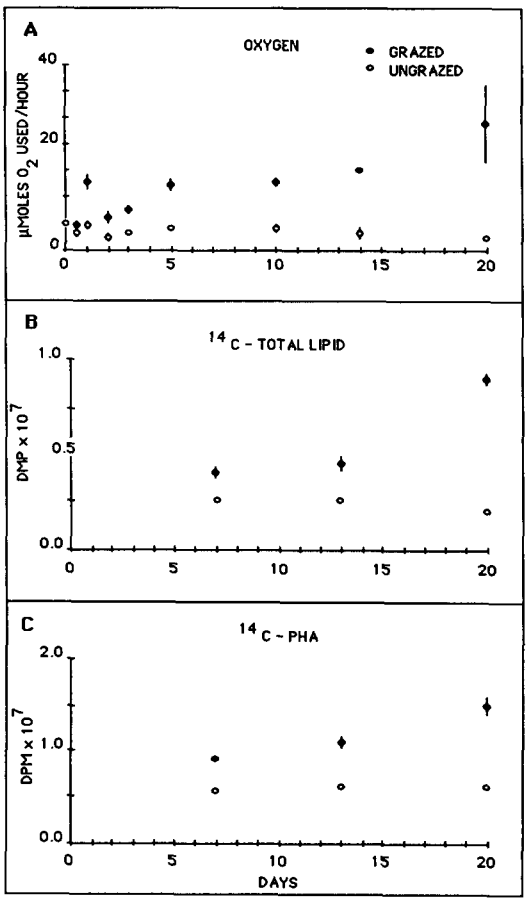

Fig. 2. Comparison of microbial activity in grazed and ungrazed microbial biofilms. Grazed biofilms are indicated with solid symbols, ungrazed with open symbols. Rates of oxygen utilization (A, top panel), 14-C released from prelabeled biofilms (B, middle panel), and 14-C PHA biosynthesis (C, lower panel) (data from Morrison & White, 1980).

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 at too high a density 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).

Determination of the nutritional status of the grazers

These methods also allow definition of the nutritional status of the grazing animal. The amphipods when allowed to feed freely on the detrital microbiota exhibit a ratio of triglyceride glycerol to membrane phospholipid of about 0.7. Starvation by isolation from the detritus for a week depressed the ratio to 0.13 (Gehron & White, 1982). Similar experiments showed the same findings for fungi, yeast, arthropods and the protozoa of the detritus. With this technology it was possible to establish that the feeding of the amphipods on the estuarine detrital microbiota resulted in the same nutritional status as the amphipods recovered from the field.

Resource partitioning between sympatric amphipod grazers

Using the PLFA analysis of the detrital biofilm it was possible to show that two sympatric amphipods *Gammarus mucronatus* (Say) and *Melita appendiculata* (Say) which have markedly different mouth part morphology partition the biofilm microbiota (Smith *et al.*, 1982). These experiments were performed much as those described above except that the substratum was teflon sheets rather than oak leaves. The morphology of the feeding apparatus of two sympatric estuarine detrital-feeding amphipods

was reflected in partitioning of the microbial resource as determined by biomarker analysis of the microbial biofilm (Smith *et al.*, 1982). The *Melita appendiculata* with its less setosed and more complexly articulated mandibles and maxillae apparently fed on non-photosynthetic microeukaryote grazers and had less effect in increasing the community microbial metabolic activity than the general feeding (bacteria and diatoms) *Gammarus mucronatus*. Grazing by *M. appendiculata* left a detrital biofilm greatly enriched in bacteria (increased muramic acid and higher proportions of the bacterial PLFA such as cis vaccenic acid, and the short iso and anteiso branched saturated fatty acids), a larger photosynthetic biomass (increased glycolipid galactose, increased rate of sulfolipid biosynthesis), and a higher total microbial biomass (greater extractible lipid phosphate and rate of phospholipid biosynthesis) than the biofilm remaining after grazing by *Gammarus mucronatus*. The biofilm exposed to *Melita* showed an enrichment in bacteria and diatoms by SEM. This possibly reflected the removal of the microfaunal grazers by the selective feeding of the *Melita*. The enrichment of the residual biofilm by diatoms was reflected in the increase in the twenty carbon pentaunsaturated polyenoic PLFA with alpha linolenic acid type of unsaturation characteristic of diatoms.

The surface grazed by the *Gammarus* with its highly setosed and less articulated mandibles and maxilla showed removal of the bacteria and cleaner detrital surface by SEM. More of the microeukaryotes remained, as reflected in the higher total lipid glycerol (primarily found in the triglyceride). The grazed surface showed intense metabolic activity as reflected in the higher total extractible adenosine nucleotide relative to the microbial biomass estimated by the extractible phospholipid. This was also reflected in the higher ATP, and the higher adenylate energy charge. The residual microbiota after grazing by *Gammarus* showed a ratio of phospholipid synthesis to phospholipid (essentially a measure of bacterial activity) and of sulfolipid synthesis to glycolipid galactose (a measure of sulfolipid synthesis) that is about half that of the *Melita* grazed detritus. The more generalized grazing of the *Gammarus* produced a smaller, more active residual microbiota that had a smaller specific synthetic activity. These two sympatric amphipods clearly partition in the detrital microbiota as evidenced by the chemical biomarker technique.

In these experiments the nutritional status of the amphipods in the laboratory exposures and in the field were essentially identical as measured by the adenylate energy charge and the ratio of triglyceride glycerol to phospholipid (Smith *et al.*, 1982).

Effects of predation in sediments

Another experiment shows that the effects of predation from the top of the food chain can affect the benthic microbiota. 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. A stepwise discriminant analysis showed distinct differences between the PLFA of the benthic microbiota between the control, the sediments subjected to continuous predation and those with random predation. The predator exclusion cages were rapidly overgrown by a dominant polychaete *Mediomastus ambiseta* and showed markedly decreased proportions of the polyenoic fatty acids characteristic of nematodes and algae and a markedly increased proportion of PLFA characteristic of bacteria. There followed a rapid increase in linoleic acid which is characteristic of microeukaryotes and could represent an increase in protozoa. The PLFA from the sediments suggested a shift in the bacterial community to a more anaerobic sulfate-reducing consortium.

With this technology it was possible to validate

microcosms set up to model estuarine sediments (Federle *et al.*, 1986). 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.

Effects of 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 (1986) described the consequences of disturbing sediment. The disturbance of sieving through 998 μm 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 a mud flat sediment. Subsequently there was a drop in the ratio to below predisturbance levels by 8 hours. The decrease continued for 3 days after which the ratio increased again to predisturbance levels by the fifth day. Total growth rates measured as phospholipid synthesis or incorporation of thymidine into DNA relative to the extractible phospholipid decreased initially in the first hour, then increased for a short time with a maximum in 2–4 hours and then decreased to a maintenance level at about half the predisturbance rate throughout the 5 day experiment. 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 growth rates. The long-chain polyunsaturated PLFA decreased. These PLFA are characteristic of 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. 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 colonized these newly aerobic sediments. There was a greater decrease in the proportions of PLFA associated with facultative and anaerobic bacteria in disturbed than in the control sediments.

Findlay (1986) then examined the effects of distur-

bance 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 indicated non-bacterial lipids, possibly from the worm. These lipids disappeared rapidly and 2 hours after extrusion

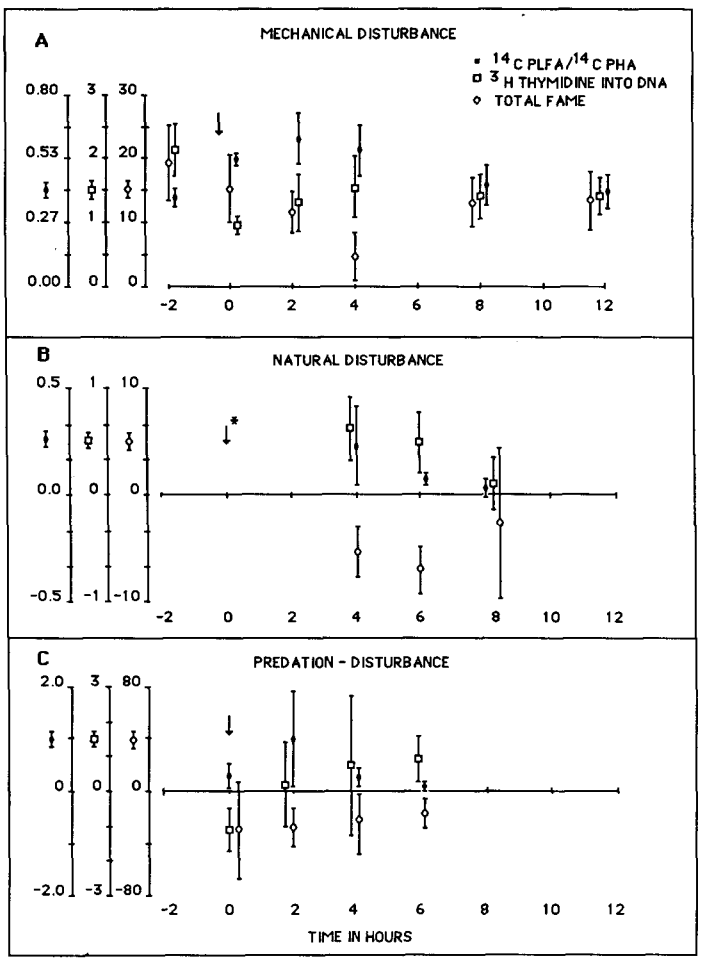

Fig. 3. Comparison of the effects of mechanical disturbance (A, top panel), natural disturbance (B, middle panel), and predation-disturbance (C, lower panel) on microbial nutritional status, metabolic activity, and biomass in shallow marine sediments. Natural disturbance was by the sting ray *Dasyatis sabina* creating pits in its search for infauna. Disturbance-predation was examined in the fecal castings of the Enteropneust *Ptchyodera bahaminasis*. Nutritional status is assayed as the ratio of the relative rate of 14-C acetate incorporation into PLFA versus PHA (solid squares). Microbial metabolic activity is given as the rate of 3-H-thymidine incorporation into DNA (open squares) in units of pmoles thymidine/min/μmole lipid phosphate. Microbial biomas was assayed as PLFA (open diamonds) in nmoles PLFA/g dry weight of sediment. The arrow indicates the point at which the sediments were disturbed (* with the arrow is where the time of disturbance is estimated). In the mechanical disturbance experiment (A, top panel) the t = -2 hr samples were taken before the sediment was disturbed. Data for the natural disturbance (B, middle panel) and predation-disturbance (C, lower panel) is presented as average differences (treatment − control). Values greater than zero indicate biomass or activity was greater in the disturbed sediments vs the control sediments whereas values less than zero indicate biomass or activity was greater than the control sediments. Error bars represent plus and minus one standard deviation. Experimental details are presented in Findlay (1986).

the muramic acid, phospholipid fatty acids and phosphate indicated a bacterial biomass less than the control sediments. The PLFA patterns suggested the loss of microeukaryotes, algae, aerobic and facultative bacteria, and anaerobic bacteria. The PLFA patterns indicated that 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 accumulate preferentially in the mature fecal casts. The microbiota of the fecal casts indicate the enteropneust fed rather nonselectively on the total microbiota and although recolonization occurred somewhat more slowly, the pattern of recovery was similar to that of sediments subjected to simple physical disturbances. The effects of disturbance and disturbance-predation are illustrated in Fig. 3.

Specificity of the feeding of benthic grazer

The sand dollar *Mellita quinquiesperforata* bioturbates sandy sediments and grazes specific components of the benthic biota (Findlay & White, 1983b). Sediments through which the animal has passed show an increase in the average depth of the oxidized zone of the sediments from 0.2 cm in the control to 0.8 cm. The total microbial biomass estimated as the extractible lipid phosphate or the phospholipid fatty acids was unchanged and the muramic acid and several PLFA characteristics of bacteria were slightly decreased after passage of the sand dollar through the sediment. The microeukaryotic biomass estimated as triglyceride glycerol, and total PLFA polyenoic fatty acids decreased, but the photosynthetic biomass measured as the chlorophyll was not significantly affected. Examination of the sediments for vitally staining harpacticoids, nemadodes, and foraminifera showed almost complete removal of the foraminifera without effect on the other two groups of non-photosynthetic meiofauna. Confining the sand dollars to microcosms in the laboratory showed that multiple feeding passes through the sediment resulted in significant decreases in the bacterial biomass measured as their specific PLFA. PLFA analysis of the grazing of 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 increased the ratio of 14-C incorporation into PLFA and decreased incorporation into PHA.

Conclusions

The methods described above provide quantitative insight into the biomass and community structure, nutritional status, and metabolic activities of microbial consortia in biofilms that do not require quantitative recovery of the organisms from the biofilm or that they all be cultured successfully. The examples given in this review establish that the effects of grazing can be measured easily in biofilms and the complications in the analysis of grazing in sediments by bioturbation can be resolved using properly designed experiments and these methods. 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 & White, 1980; White *et al.*, 1979b; 1979d; King *et al.*, 1977; Moriarty, 1977; Saddler & Wardlaw, 1980). This indicates that these chemical markers provide good estimates for the standing stock of viable microbiota. Rates of formation or loss of endogenous storage lipids or exocellular polysacchride 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 effects of predation on the microbial community in biofilms on marine detritus or sediment sub-

Grazing of Biofilms

Increase in metabolic activity (oxygen uptake, carbon dioxide release, PHA formation, PHA turnover, lipid synthesis)
Increased biomass (ATP, total lipid, phospholipid)
Increased bacteria (increase in muramic acid/ATP or phospholipid, bacterial PLFA)
Decreased alkaline phosphatase (increase in available phosphate)
Shift in biofilm morphology (from complex climax in succession to bacterial and diatom dominance)
Specificity-sympatric amphipod grazers partition the biofilm

Sediments

Bioturbation of sediments with a redox gradient
Initial anaerobic die-off (PLFA patterns, DNA and lipid synthesis)
Rapid bacterial proliferation (PHA disappearance, PLFA synthesis)
Recovery and unbalanced growth (PHA accumulation, *trans* monoenoic PLFA accumulation)
Shift in community structure (loss of microeukaryotes, increased anaerobic bacteria – PLFA pattern shifts)

Predation of sediments
Direct effects
Enteropneust fecal casts (loss of microbial biomass and activity with rapid recolonization by bacteria – PLFA patterns)
Specific predation – Sand Dollar (minimal bioturbation + specific decrease in biomarkers for non-photosynthetic microeukaryotes)

Indirect effects
Sting Ray feeding pits – bioturbation effects + recovery with increased aerobic bacteria (PLFA patterns)
Polychaete overgrowth – decreased mieofaunal PLFA, increase in anaerobic bacterial PLFA followed by increase in possible protozoal PLFA (PLFA patterns)

[Refer to text for details]

Fig. 4. Summary of effects of grazing of detrital biofilms and bioturbation and/or predation in sediments. (refer to the text for details, PLFA and PHA defined in Fig. 1).

stratum are complex and far reaching (Fig. 4). Predation by its very nature, initially decreases microbial biomass. In most instances, unless grazing pressure is severe, the secondary microbial community that develops will have increased rates of metabolic activity and growth. Total microbial biomass will be greater and the turnover rates of both the substrates and microorganisms will also increase. The complexity of the community will decrease with the community structure shifted towards faster growing organisms. Many predators appear selective and only remove a subset of the microbial community further altering community structure. In sediments, predation is usually accompanied by bioturbation. The effects of these two phenomena are similar except that predation makes detrital nutrients and carbon available to higher trophic levels whereas nutrients liberated by death or injury of microorganisms during physical disturbance are likely to be available only to the microbial components of the benthic food web.

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