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MARINE BIODETERIORATION

Advanced Techniques Applicable to the Indian Ocean

Editors

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23. Assessment of Marine Biofilm Formation, Succession, and Metabolic Activity

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Marine biofilms often contain consortia of microbes of physiological diversity attached to surfaces. The evidence for succession, based on changes in the cellular morphology in the biofilm with time, has been quantitatively defined by changes in the microbial phospholipid fatty acid (PLFA) patterns. Shifts in PLFA patterns were induced by changes in light exposure, substratum composition, microtopology and biodegradability, essential nutrient limitation, predation, and biofouling. PLFA analysis indicated Gram-negative bacteria resembling those found in the particulate fraction of the water column formed the initial microfouling community. Examination of the biofilm by nondestructive Fourier transforming infrared spectroscopy revealed a correlation between corrosion and extracellular glycocalyx formation in biofilms, which was reversible with the removal of the biofilm.

INTRODUCTION

One of the most interesting new developments in microbial ecology is the importance of consortia that form biofilms attached to surfaces (Marshall 1976, White 1983). The attachment of these microbes greatly increases the metabolic activity and diversity when contrasted with specific monocultures in isolation. An example of this interaction is the activity of anaerobic bacteria such as sulfate-reducing bacteria within thin biofilms exposed to highly aerated waters. Sulfate-reducing bacteria exist in the anaerobic microniches created by the metabolic activities of heterotrophic aerobes in the biofilm consortia. Microbial communities bound together with extracellular polymers are readily detectable in numerous scanning electron micrographs of biofilms (Nickels et al. 1981a) and in marine sediments by transmission electron microscopy (Moriarty and Hayward 1982).

The study of these microbial consortia in surface biofilms to determine quantitatively biomass, community structure and metabolic activities has been limited by the availability of methods. Even in the water column, the

classical methods of microbiology, which involve the isolation and subsequent culturing of organisms on Petri plates, can lead to gross underestimations of the numbers of organisms detectable in direct counts of the same waters (Jannasch and Jones 1959). With sediments and biofilms, the problems with classical methods are more severe. In addition to the problem of providing a universal growth medium in the Petri plate, the organisms must be quantitatively removed from the surfaces and from each other. Direct microscopic methods, which require quantitative release of the bacteria from the biofilm, can have the problem of inconsistent removal from some surfaces. High speed blending of sediments to remove the microbiota prior to staining and direct counting in epifluorescent illumination were neither quantitative nor reproducible when compared with chemical assay of the muramic acid of the prokaryotic cell wall in one study of marine sediments (Moriarty 1980). Direct microscopy can be performed on sediment particles or thin biofilms by making estimations for organisms rendered invisible by sediment granules or overlapping organisms in biofilms. The application of computer-based image enhancing can allow calculations of microbial biomass in complex assemblies (Caldwell and Germida 1984). This method works best when the density of organisms in the sediments or biofilms is low, and overlapping is minimal. Even with computer-enhanced image processing in direct microscopy, we are left with the problem that the in-situ methods often fail because the morphology of a microbe offers little insight into the metabolic function or activity of the cells. Methane-forming bacteria, for example, come in all sizes and shapes (Zeikus 1977). The problem is further complicated by the fact that in many environments only a tiny fraction of the organisms is active at any one time, and aside from the observation of bacterial doubling (Hagstrom et al. 1979), the morphology gives little evidence of all activity. Isolation of microbes for viable counting or direct microscopic examination can provide little insight into the details of interactions that take place in mixed microcolonies. Since these consortia have much greater potential for metabolic diversity than single species, it is important in environmental effects testing to preserve, as much as possible, the anatomy and metabolic interactions of these microcolonies. To do this, a new type of analysis that does not require separation of bacterial cells from the substratum prior to analysis is needed to describe this community quantitatively.

CHEMICAL "SIGNATURES" IN THE ANALYSES OF BIOFILMS

Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of culture 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 consortia are preserved. The method involves the measurement of biochemical properties of the cells and their extracellular products. Those components, generally distributed in cells, are used as measures of biomass. Components restricted to subsets of the microbial communities can be used to define the community structure. The concept of "signatures" for subsets of the community, based on limited distribution of specific components, has been validated by using antibiotics and culture conditions to manipulate community structure. The resulting changes agreed morphologically and biochemically with the expected results (White et al. 1980). Other validation experiments that involved isolation and analysis of specific organisms and finding them in appropriate mixtures, use of specific inhibitors and noting responses, and changes in the local environment such as the light intensity are summarized in a review by White (1983).

Polar lipids are found in the membranes of all cells. Under the conditions expected in natural communities, bacteria contain a relatively constant proportion of their biomass as phospholipids (White et al. 1979b). Phospholipids are not found in storage lipids and have a relatively rapid turnover in some sediments. Thus, assay of these lipids gives a measure of the "viable" cellular biomass (White et al. 1979a).

The ester-linked fatty acids in the phospholipids (PLFA) are presently the most sensitive and most useful chemical measures of microbial biomass and community structure (Bobbie and White 1980, Guckert et al. 1985). The specification of fatty acids that are ester-linked in the phospholipid fraction of the total lipid extract greatly increases the selectivity of this assay as most of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. By using fatty-acid patterns of bacterial monocultures, Sasser (University of Delaware) in collaboration with the Hewlett Packard Company was able to distinguish between over 8,000 strains of bacteria. Thus, analysis of fatty acids can provide insight into the community structure of microbial consortia as well as an estimate of the biomass. Since biofilm PLFA analyses 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. The importance of the determination of double-bond configurations in monoenoic fatty acids improves the resolution of microbial "signatures" in complex biofilm microbial assemblies (Nichols et al. 1985, Edlund et al. 1985).

Analysis of PLFA cannot provide an exact definition of each species or

physiologic type of microbes in a given environment; it does provide a quantitative description of the microbiota in the particular environment sampled. With the techniques of pattern recognition, PLFA analysis allows distinction between communities.

Potential problems in 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 these were defined in this laboratory (Joyce et al. 1970, Freeman and White 1967, Ray et al. 1971). There is, as yet, little published evidence for such shifts in PLFA in nature.

The nutritional status of microbial consortia in biofilms can be estimated by monitoring the proportions of specific endogenous storage compounds relative to the cellular biomass. Certain bacteria form the endogenous storage lipid poly-beta-hydroxybutyrate (PHB) under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (Nickels et al. 1979).

Metabolic activity can also be determined. The analyses described all involve the isolation of components of microbial consortia. Since each of the components are isolated, the incorporation of labeled isotopes from precursors can be used to provide rates of synthesis or turnover in properly designed experiments. Measurements of synthesis rates and turnover of carbon and phosphate in individual phospholipids showed different turnovers for the various lipids. Glycerol phosphorylglycerol derived from phosphatidylglycerol showed rates of loss suggesting that the most rapid growth rates of biofilm microbiota were in the order of 100 hours in estuarine waters (King et al. 1977). Glycerol phosphorylcholine derived from phosphatidylcholine showed extremely slow turnover in biofilm microbiota after pulse chase exposure to ^{14}C-labeled precursors. Grazing by amphipods markedly increased the loss of ^{14}C-glycerol phosphorylcholine, providing a quantitative estimate of grazing pressure (Morrison and White 1980).

Analysis of signatures by gas chromatography-mass spectrometry (GC/MS) makes possible the use of mass labeled precursors that are nonradioactive, have specific activities approaching 100%, include an isotope marker for nitrogen, and can be efficiently detected using the selective ion mode in mass spectroscopy. The high specific activity makes possible 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. Using a chiral derivative and fused silica capillary gas liquid chromatography (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 10^3

bacteria the size of *Escherishia coli* (Tunlid et al. 1985). In this analysis, it proved possible to detect reproducibly a 1% enrichment of ^{15}N-D-alanine in the ^{14}N-D-alanine.

APPLICATION TO THE INITIAL MICROFOULING COMMUNITY

Morphological studies have indicated that the microfouling biofilm is initiated by attachment of rod-shaped bacteria to surfaces (Marshall et al. 1971). These organisms are first reversibly bound to the surface and then may form an extracellular polymer that irreversibly binds them to the surface. The initial colonizers are followed by bacteria with more complex shapes (such as prosthecate oganisms) and then by microeukaryotes such as diatoms and other algae. This morphological description of microfouling succession was documented by PLFA analyses of the microbiota (Morrison et al. 1977, Nickels et al. 1981a). The effects of substratum composition (Berk et al. 1981), biodegradability (Bobbie et al. 1978), influence of microtopology (Nickels et al. 1981b), mechanical disturbance (Nickels et al. 1981c), amphipod grazing and resource partitioning (Morrison and White 1980, Smith et al. 1982), essential elemental chelation (Nickels et al. 1979), and the effects of light (Bobbie et al. 1981) on the microbial consortia of biofilms have been followed with these analyses.

The quantitative description of the initial microfouling community by analysis of the signature lipids required the development of a measurement of sufficient sensitivity to detect a few microbes on a coupon. By creating electron withdrawing derivatives of the PLFA with pentafluorobenzyl bromide at room temperature, it was possible to create negative molecular ions by chemical ionization mass spectrometry at a very high efficiency (Odham et al. 1985). These derivatives gave excellent chromatographic separation, and with detection of negative ions after chemical ionization mass spectrometry in the Laboratory of Ecological Chemistry at the University of Lund, Sweden, we were able to detect routinely PLFA patterns at femtomolar sensitivity. This is equivalent to approximately 10 to 100 microbes the size of *E. coli*. With this technology, we examined the initial microfouling community attached to teflon strips exposed to running seawater for periods of one to five days. The samples showed PLFA patterns typical of marine Gram-negative bacteria. There was little change in community structure during this period. Washing the biofilms with a stream of seawater removed a specific subset of the community. The lack of long-chain polyenoic PLFA was interpreted to mean that there were no microeukaryotes in the initial microfouling community.

Recent studies by Guckert of this laboratory focused on water-column microorganisms that are the sources of the biofouling community. In the absence of a mass spectral analysis system with chemical ionization and

negative ion detection, GLC with flame ionization detection could give p molar sensitivity required to examine small samples. However, GLC analysis does not give information on the position and geometry of double bonds or substituents so necessary in the definition of "signatures" (Nichols et al. 1984). A compromise involved the development of a GLC system involving analyses of specific derivatives of PLFA, simultaneously, on polar and nonpolar GLC columns. Nuclepore-size fractionation of estuarine water showed differences in PLFA patterns for different size fractions. Preliminary results suggest that the initial microfouling community in the estuarine system we have been examining most closely approximates the microbiota of the particulate fraction. In recent experiments, Guckert was able to show that starvation of *Vibrio cholerae* results in the formation of dwarf cells containing a markedly elevated ration of *trans/cis* monoenoic and proportions of cyclopropane PLFA. It thus becomes possible to correlate the formation of components of the initial microfouling community with the nutritional status of water-column microbiota. Several organisms isolated from the initial microfouling film and cultured in unamended seawater contained high proportions of these *trans* monoenoic PLFA. As a part of this study, Guckert was able to show that manipulation of a marine microbial consortia between aerobic, anaerobic, logarithmic, and stationary growth conditions showed reproducible shifts in the PLFA profiles of independent flasks when identically manipulated and significant differences when manipulated with different treatments (Guckert et al. 1985). The differences in PLFA were in the proportions of cyclopropane fatty acids and the proportions and geometry of the monounsaturated fatty acids. We feel the analysis of PLFA will provide a means of defining the effects of shear environment, seasonality, input water-column composition, and specific countermeasures on the initial microfouling community.

CONSEQUENCES OF BIOFILM MICROBIAL METABOLIC ACTIVITY

Rapid formation of extracellular polysaccharide glycocalyx is a major response to mechanical or chemical counter-measures in the control of biofouling on metal surfaces exposed to running seawater (Nickels et al. 1981a, c; White and Benson 1984). This glycocalyx appears to have higher specific heat-transfer resistance than cellular biofilm. We have developed a chemical assay for this glycocalyx based on the specific content of uronic acids (Fazio et al. 1982). This assay was then used to show that poor growth conditions stimulate the formation of uronic acid containing exopolymers by a marine *Pseudomonas* similar to those found in the initial microfouling community (Uhlinger and White 1983). The analysis of biofilms based on the isolation of chemical signatures is a destructive

analysis and cannot be readily automated or used to give real time monitoring of biofilms. The possibility of using a nondestructive technique to monitor the chemistry of living biofilms is now possible with the Fourier transforming/infrared spectrometer (FT/IR).

The infrared portion of the spectrum is extraordinarily rich in information regarding the vibration and rotation motions of atoms in molecules. Not only can specific infrared absorption be assigned to particular types of covalent bonds, but the modifications of these bonds by the local electronic environment can also be detected in the details of the spectra (Bellamy 1958, Parker 1971). One of the problems restricting the application of infrared spectroscopy has been that the atomic interactions sensed in the infrared portion of the spectrum are at relatively low energies and the detection is relatively inefficient. This has precluded the full usage of the power of the analysis using complex materials isolated from the environment.

FT/IR examination by diffuse reflectance (DRIFT) of freeze-dried, powdered bacterial monocultures showed significant differences between specific microorganisms (Nichols et al. 1985). These findings, together with the powerful technique of subtraction of one spectrum from another, suggest that DRIFT could be used to recognize differences in community structure. Preliminary experiments indicate that examination of planktonic microbiota on pre-extracted filters by DRIFT can be compared with a detailed examination of the lipid content.

The formation of the uronic acid containing exopolysaccharide glycocalyx can be detected with FT IR. This analysis replaces a three-week chemical tour-de-force involving GC/MS in the analysis of bacterial glycocalyx. The DRIFT spectrum of *E. coli* plus gum arabic and of *Pseudomonas atlantica* induced to form polysaccharide glycocalyx are similar in appearance.

With the DRIFT analysis it proved possible to demonstrate the reversible facilitation of corrosion of 304 stainless steel by the extracellular polysaccharide of *Vibrio natriegens*. This organism does not produce hydrogen sulfide in its metabolism. There was a 15-fold increase in the corrosion current density measured electrochemically from the Tafel constants and polarization resistance that correlated with the colonization of the stainless-steel disks by microcolonies of the bacteria. The colonization of the metal surface was detected by direct microscopy after staining and epifluorescent illumination, scanning electron microscopy, and by an increase in the DRIFT absorbance at the amide I area centered at 1660 cm^{-1} corresponding to the bacterial protein. Maximum rates of corrosion were associated with the appearance of extracellular material with a spectral maximum centered at 1440 cm^{-1}, similar to calcium hydroxide. Removing the biofilm, particularly calcium hydroxide with its absorption at

1440 cm^{-1}, decreased the corrosion current density ten-fold. In this instance, both the presence of a non-sulfate reducing bacterium and its extracellular products reversibly facilitated corrosion of stainless steel in seawater. Similar experiments showed that the obligate aerobe *P. atlantica* significantly increased the corrosion current density when it secreted its extracellular carbohydrate glycocalyx, which also contained the infrared signature of calcium hydroxide.

Development of FT/IR spectroscopy offers a potentially rapid and nondestructive method to examine marine biofilms on the scale of microbial consortia. The continued development of destructive analytical methods can be the essential validation for infrared signatures. Both techniques offer the opportunity to gain deeper insights into the microbial ecology of the microfouling biofilm.

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