Organic Acids in Aquatic Ecosystems
eds. E.M. Perdue and E.T. Gjessing, pp. 25-42
John Wiley & Sons Ltd

The Application of Novel Approaches for Characterizing Organic Acids from Aqueous Matrices Focusing Biological Systems on Environmental Problems

D.C. White, D.E. Nivens, and M.W. Mittelman

Institute for Applied Microbiology
University of Tennessee
Knoxville, TN 37932-2567, U.S.A.

Abstract. This paper proposes that the spatial and temporal variability in the distribution of humic-derived organic acids in waters, sediments, and soils is primarily a function of the resident microbial biofilms which are able to process or generate these complex organics. These microbiota represent the largest biomass and have the metabolic versatility to control the humic-derived organic acid distribution. Mechanisms for the formation of humic organic acids include at least two major processes. First, microbes are involved in processing the products of primary photosynthesis such as soluble photosynthetate, cell bodies, litter, and other elaborations in the rhizosphere. Second, the microbes themselves liberate organic acids during microbial metabolism under conditions of growth-limiting concentrations of high potential electron acceptors. These microbially derived organic acids can be further processsed to form components of the humic organic acids. The microbial consortia responsible for the variability in the distribution of these humic organic acids are examined. In addition, the novel methods for characterization of both the biomass and community structure as well as the nutritional status and metabolic activities of the microbial biofilm consortia are described. New methods are required because the traditional methods for microbial community analysis are inadequate. Traditional methods 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 the 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 specific types of microbes. If a sufficient catalog of "signatures" can be developed, then

changes in community structure can be defined. This study will show 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 (PHA), together with the accumulation of specific PLFA, can be used to indicate the community nutritional status. The powerful methods of molecular biology have added a new dimension to microbial ecology. Nucleic acids can be quantitatively recovered from environmental samples and labeled oligonucleotide probes prepared against specific enzymes, antigens, etc. Particularly valuable probes can be prepared against the 16S RNA of the ribosomes. The differentially conserved portion of the sequence can be utilized for the preparation of oligonucleotide probes directed at the kingdom: either phylogenetically related groups or individual species. These labeled oligonucleotide probes readily permeate intact cells and are able to define individual cells in consortia or biofilms. However, the nucleic acids require that the organisms be isolated and the sequences be determined. Once determined, the probes can act in undisturbed consortia. Their primary difficulty is that the activity of the specific gene or cell line is not as yet readily determined. 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. New methodologies involving nondestructive electrochemical and/or spectroscopic techniques can be used to detect the formation of organic acids by living biofilms. These analyses have shown that microbial biofilms respond to changes in nutrient and electron donor/acceptor concentration and composition, predation, bioturbation (in sediments), and contamination of the sediments and soils. The richness (diversity of the microbiota coupled with its potential for rapid response to changing conditions make possible its use as a means to assess the impacts of toxicants on specific environmental sites quantitatively. Microbiota can also be utilized to begin to define the mechanisms involved in the temporal and spatial variability of humic organic acid distribution in the environment.

INTRODUCTION

The primary focus of this workshop was to identify the major processes that cause spatial and temporal variability in the properties of humic organic acids in the environment. This chapter deals primarily with one of the biological bases for the generation, modification, and fate of organic acids.

Hypothesis

The hypothesis of this presentation is that the critical factor in the spatial and temporal variability of humic organic acid distribution lies in the distribution and activities of the microbiota. The microbiota in the aquatic or terrestrial environments are primarily found attached to the surfaces in

the soils and sediments. 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. 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. These microbes have the most rapid biomass doubling times in the soils and sediments with favorable conditions yet have extraordinary metabolic mechanisms for survival in compromising conditions.

The microbes influence the spatial and temporal distribution and structure of organic acids in several ways:

1. *Microbial catabolic production.* Under conditions of limited high potential electron acceptors, such as oxygen or nitrate, the metabolism of the obligate or facultative aerobes shifts so that organic acids rather than carbon dioxide and water become the primary metabolic end products. These anaerobic conditions are often the result of microbial metabolism itself. The anaerobic microbial communities routinely produce organic acids from biopolymers. A portion of these organic acids are further catabolized by the sulfate-reducing bacteria (in the presence of oxidized sulfur) or organic acids and/or hydrogen plus carbon dioxide with the production of methane. In more limited niches, nitrogen oxides and possibly even some oxidized metallic elements (such as Fe^{III}) can be reduced as electron acceptors.
2. *Microbial growth.* Viable microbes are surrounded by a lipid membrane consisting of hydrocarbon esters or ethers with a great structural diversity. The polar components of these membranes are enzymatically hydrolyzed following cell death. The hydrolysis results in the creation of neutral lipids that could form the raw materials for a portion of the organic carbon found in sediments.
3. *Microbial processing of photosynthetic products.* In the course of mineral recycling, the polymeric and simple products of photosynthesis are processed by the microbiota. These may represent detritus on the forest floor or stream bed, algal photosynthetate in the water column and sediments, or organic materials elaborated in the rhizosphere of higher plants. In many cases, organic material is depolymerized but not subsequently mineralized to carbon dioxide. This material may then be repolymerized or otherwise modified into products such as kerogen, which becomes increasingly resistant to biodegradation. The elegant work of W. Michaelis and his group has shown that selective depolymerization in the presence of mass-labeled isotopes allows the

recovery of labeled recognizable bacterial components such as bacterial hopanoids or archaebacterial ethers which are covalently bound to the polymers (Mycke et al. 1987).

Requisite Analysis

In addition to the analysis of the specific organic acids themselves, the determination of the heterogeneous distribution of the microbes, as well as their metabolic activities, is required. Analysis of the localization and activities of microbes in soil and sediments that could be responsible for organic acid distribution and structure requires special techniques. The classical microbiological techniques that proved so extraordinarily effective in the control of epidemic infectious diseases unfortunately are not as effective in helping to understand the distribution and activity of microbes in the environment. 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, 1988). Assays to define microbial consortia have been developed so that the bias of cultural selection associated with classical methods has been eliminated. Since the total community is examined in these procedures without the necessity of removing the microbes from surfaces, the microstructure of multispecies consortia is preserved. The method involves the measurement of biochemical properties of the cells and their extracellular products. The 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 (Ratledge and Wilkinson 1988). Phospholipids seem an ideal component for estimating active or potentially active microbial biomass (Fig. 1).

ANALYSES OF THE MICROBES IN SEDIMENTS AND SOILS

Biomass and Community Structure

Phospholipids, intracellular adenosine nucleotides, and cell wall amino-sugars are biochemical components of cells that have been utilized to estimate microbial biomass (Balkwill et al. 1988; White 1983, 1988). Of these, phospholipids have proven the most useful when examining predation effects on microbial biofilms (White and Findlay 1988). Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities, bacteria contain a relatively constant proportion of

CELL COMPONENTS	PERSISTENCE AFTER CELL DEATH
Cell Walls	
Muramic Acid	+
KDO	±
Lipid A	+
Nucleic Acids	
DNA (Genes)	+
16S RNA	?
mRNA	?
Exopolymers	
Uronic Acids	+
Nucleotides	
ATP	+
Enzymes	+
Lipids	
Neutral Lipids	+
Phospholipids	-

Fig. 1—Cellular components for estimation of potentially active microbial biomass.

their biomass as phospholipids (White, Bobbie et al. 1979). 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, Davis et al. 1979). As shown by Smith et al. (1986), 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 cocco-

bacillary morphology. In these subsurface sediments, the biomass and cell numbers estimated from direct cell counts after acridine orange staining agree with the numbers and biomass estimated from the extractable 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. 1988).

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 (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 many of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. For example, by isolating the phospholipid fraction for fatty acid analysis, it proved possible to show bacteria in the sludge of crude oil tanks. The extraction of the lipids provides both a purification and a concentration of the components. The transesterification of the PLFA to form the volatile esters necessary if the resolution of capillary gas-liquid chromatography (GLC) is to be utilized can be performed under conditions in which free fatty acids will not be derivatized. Utilizing the exquisite sensitivity of mass spectral detection with soft chemical ionization and selective ion monitoring, the sensitivity can be in the femtomolar range. At these sensitivities, contaminants result in the solvents; this determines the signal-to-chemical noise ratio. The specificity of the PLFA assay has been greatly increas by the determination of the configuration and position of double bonds in monoenoic fatty acids (Nichols et al. 1986) and also 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 allows the detection of specific bacteria with signature PLFA in the range of 100 to 1000 organisms. This sensitivity makes it possible to extract specific areas in biofilms possibly differentiated by microelectrodes to localize microbes. If the biofilms or consortia have been exposed to nonradioactive, mass-labeled (^{13}C) precursors, then the activity of specific microorganisms can be quantitated. 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. Figure 2 provides a diagram of the methods used in the signature biomarker analysis. These methods could be applied to the analysis of the humic organic acids as well.

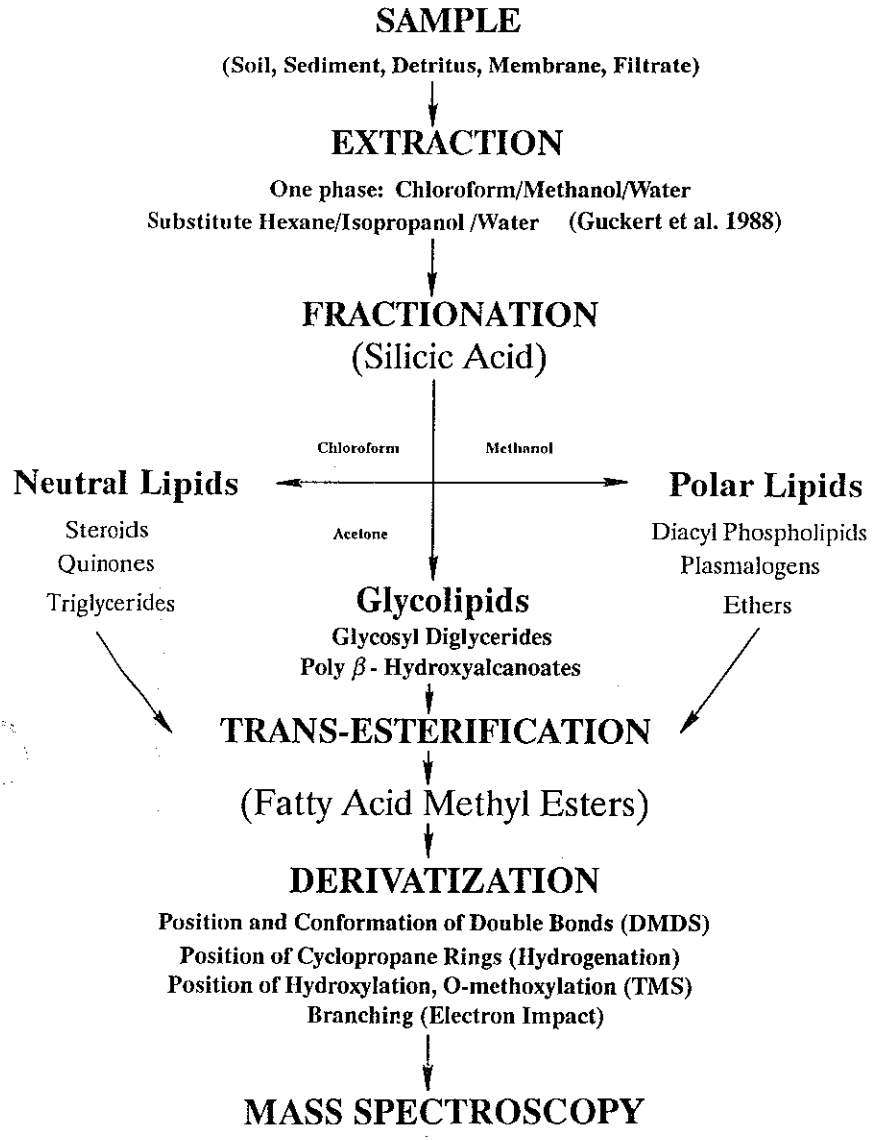

Fig. 2—Processing for lipid analysis.

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. 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 restrict severely 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.

The techniques of molecular biology have provided new tools to examine the distribution and community structure of microbial consortia in soils and sediments. Techniques have been developed which enable the quantitative recovery of DNA and ribosomal RNA from sediments. It is particularly difficult to recover nucleic acids from soils or sediments rich in clay, since the nucleic acids recovered are typically fragmented. This emerging technology is enormously powerful as oligonucleotides made with sequen of more than 10–20 bases provide a specificity that is virtually absolu Once the sequence is known and the appropriate fragment defined, the synthetic DNA probe is made automatically in substantial quantities. This is then tagged most often with ^{32}P by nick-translation. The environmental samples are treated with detergents to lyse the bacteria and nucleic acids recovered and purified. Once the nucleic acid is recovered from the environment, it is placed in appropriate ionic strength buffers and heated to denature (form single strands). The single strands are then allowed to anneal (hybridize) with the probe and the unhybridized single-stranded nucleic acid, and excess probe removed by various techniques. The degree of homology can be controlled by the hybridization conditions. Under proper conditions it is possible to detect single gene copies.

The proper sequence must be selected for the probe, and the enzyme (or gene) must have been sequenced to provide the sequence. With access to gene libraries it is possible to select for a wide variety of genes. Genes that are involved in functional groups of bacteria can be selected if the distribution of the enzyme is known. Of all the naphthalene degrading aerobes isolated from several contaminated soils, less than 25% hybridized with the DNA oligonucleotide probe in the best-studied pathways. Clearly, there are other

enzymes involved in naphthalene degradation. As more of the sequences of genetic determinants of specific processes become known, the probes can be modified or mixed probes utilized. The probe defines the presence of the specific gene, not the enzyme of the metabolic activity. G.S. Sayler has established that certain genes can be detected outside of bacteria adsorbed to the sediment (Ogram and Sayler 1988). Even with these provisos, the technique is powerful and can give insight into the enzyme distribution in a community. Problems may exist in referring the presence of the gene to the presence of a specific bacteria. Some genes are widely distributed; for example the APS-reductase enzyme, which activates sulfate and is essential to all the sulfate-reducing bacteria, was also found to occur in sulfur-oxidizing bacteria as well. These DNA probes are particularly useful in the detection of genetically engineered microbes in the environment where the specificity of the probe can be controlled. The use of DNA gene probes has been reviewed (Jain et al. 1988).

An alternative or complementary method that allows detection of the ribosomal RNA (rRNA) with probes has some additional properties. The rRNA are remarkably conserved molecules that are involved in protein synthetic systems common to all life. Initial work concentrated on the 5S rRNA which consists of about 120 nucleotides. This short sequence was sufficiently invariate in that it showed a paucity of independently varying nucleotide positions. The 16S rRNA (~1600 nucleotides) was ideal for phylogenetic comparisons and, with the advent of DNA cloning and sequencing methods, provides exciting possibilities. There are invariate sequences universal to all life and sequences common to the major kingdoms—eukaryotes (plants, animals, and microeukaryotes) and prokaryotes (eubacteria and archaebacteria). There are sequences which can be used to define closely phylogenetically related groups (which may not be functionally obvious, such as the plant mitochondrial rRNA, the methane-oxidizing bacteria, and the plant tumor-inducing agrobacterium) and individual species or strains. The fact that there are 10^4 copies of the rRNA in each cell, that the analytical systems can detect 50 or so molecules, and that the probe nucleotides can penetrate the intact cells of bacteria in environmental samples provides a system in which appropriately labeled probes can be used to identify specific bacteria or groups of bacteria in biofilms. When fluorescent probes are used on biofilms the localization of specific cells can be readily determined. This exciting new technology has been reviewed (Olsen et al. 1986). An elegant use of this technology in an examination of the effects of a commonly used antibiotic on the community structure in the bovine rumen has been reported (Stahl et al. 1988).

The disadvantage of the rRNA probe technology is that for maximum effectiveness the sequence of the 16S RNA must be known. This means the organism must be culturable and the sequencing performed. Determination

of the sequence is a procedure involving several difficulties, not the least of which comes in defining the best positioning (placement of "skipped" bases) to use in phylogenetic matching. There is also a constant problem of almost ubiquitous RNAase contamination which can easily ruin the experiments. Nonetheless, the application of nucleic acid probe technology to environmental systems is a powerful means for defining the distribution of microbes in samples and their community structure.

Nutritional Status and Metabolic Activity

A major problem with the nucleic acid probes is in the detection of metabolic activities and nutritional status. DNA probes provide an elegant definition of the presence of the gene but do not indicate the activity of the enzyme in question. G.S. Sayler is in the process of using molecular manipulations to insert the LUX operon (the genes from a phosphorescent bacteria for its light generating system) into the operon for specific biodegradative enzymes (pers. comm.). When this is done and the bacteria are induced to form the enzymes, they phosphoresce, thus indicating their activity. Individual metabolically active cells can be detected. This requires considerable metabolic insight into the specific microbes and the ability to generate stable specific genetic constructs. Techniques are developing to allow the definition of the ratio of rRNA to DNA. Generally, the faster the bacteria grow, the higher the ratio between rRNA and DNA. However, this indicates only potential for rapid growth.

The advantages of PLFA technology lie in the ability to detect the incorporation of label from precursors into specific molecules. This is particularly powerful when used with nonradioactive ^{13}C and detection by capillary gas chromatography/mass spectrometry (GC/MS).

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 poly beta-hydroxyalkanoate (PHA) under conditions in which the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division. Conditions in which PHA is accumulating and PLFA are not being formed represent unbalanced growth. In unbalanced growth the organisms accumulate PHA but are unable to form new membranes or divide. PLFA formation represents new membrane formation with cell division. The ratio of incorporation of labeled acetate into PHA/PLFA is an exquisitely sensitive indicator of

nutritional status. Some metabolites, including some organic acids, are metabolized by bacteria only during conditions of unbalanced growth.

Starvation in microbes stimulates the secretion of exopolymers by microbiota. These may be critical to attachment to substrata. Generally, bacteria possess negatively charged exopolymers containing uronic acids: methods for their detection based on GC/MS with reduction with sodium borodeuteride of activated esters prior to hydrolysis have been utilized (Fazio et al. 1982). A much easier assay of exopolymer formation utilizes the ratios between the carbon–oxygen stretch (~1150 cm^{-1} of carbohydrates) and the amide II (~1550 cm^{-1} of the proteins in the bacteria) determined by diffuse reflectance with the Fourier transforming infrared spectrometer (DRIFT). With this system it is also possible to detect high concentrations of PHA by the carbonyl absorbance (~1730 cm^{-1} [Nichols et al. 1985]). With the DRIFT microscope these ratios can be determined in areas as small as 20 μm in diameter.

The microbial component analysis utilizing capillary GC/MS involves the isolation and characterization of specific molecules by MS. If the soils and sediments in which the distribution and activities of the microbes are to be examined are exposed to ^{13}C-labeled precursor such as acetate prior to the analysis, then the enrichment of ^{13}C in specific biomarkers can be used to establish the activity of specific bacteria. The mass-labeled precursors are nonradioactive, have specific activities approaching 100%, and can be efficiently detected using the selective ion mode in mass spectroscopy. Their 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. It is also possible to utilize ^{15}N to follow nitrogen metabolism by specific microbes. 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 about 1000 bacteria the size of *E. coli*) (Tunlid et al. 1985). In this analysis it proved possible to detect reproducibly a 1% enrichment of ^{15}N in the ^{14}N–D-alanine.

The phospholipid signature technique based on MS offers a further tantalizing possibility. When lyophilized bacteria or biofilms are subjected to bombardment by ions or atoms of modest intensity, the naturally charged phospholipids are among the first molecules to be desorbed from the microbial films. Because of their charge, they are readily analyzed by MS. The technology is rapidly being developed to allow focused beams to sputter phospholipids from the biofilms directly. The sputtered lipids can be readily identified by MS. Phospholipids readily lose specific polar head groups (such as M^- and M-141 loss of ethanolamine representing phosphatidylethano-

lamine). If the sensitivity can be increased, it should be possible to image, at least crudely, the lipid distribution and to detect the areas of activity in biofilms previously exposed to ^{13}C (prior to lyophilization).

Validation

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, provide a validation of the signature biomarker methodology. This and other validation criteria for the biomarker methodology have been reviewed (White 1988).

Microbial Acid Formation

The actual formation of organic acids by living biofilm bacteria can be detected. Both direct and indirect nondestructive methods can be employed. Direct methods involve microelectrodes that respond to pH and have now been made with active surfaces of between 20 and 200 μm in diameter, which have sufficient stability to be useful. These electrodes can be placed into the biofilm and the pH monitored at specific places in the biofilm. The electrodes can be combined with oxygen electrodes (reported with diameters of 5 μm) (Revsbech and Jorgensen 1986). Microbial biofilms have been shown to be able to generate highly localized pHs as low as 5.2 in some areas and as high as 9.2 at the metal–biofilm surface using glass microelectrodes in growth systems with neutral pH in the bulk phase (Little et al. 1989). This area of research is rapidly developing. Other chemically modified ion-selective microelectrodes that respond directly to pH have been tried but stability problems have been rumored. Optrodes as terminal components of fiberoptic cables in which dyes respond to pH changes are currently under development.

A second direct method of acid formation detection involves the use of "reporter" molecules whose infrared spectrum shifts with changes in pH, which are detected in the evanescent wave on crystals exposed in the attenuated total reflectance (ATR) cells of the FT/IR. In this system, a biofilm is formed on the crystal in a flow cell connected to a continuous culture system. The microbes in the biofilm are then fed with an appropriate substrate to allow acid generation, which can be detected in the shifts of the reporter molecules.

Indirect methods are based on the changes in the ionized double layer of metal working electrodes on which microbial biofilms are grown. These systems also use a continuous culture system with flow rates so that the major microbial community in the apparatus is attached to surfaces. The electrochemical properties of the metal are affected by the propensity of

the biofilm to elaborate organic acids. In these systems, the bacteria lower the pH by secreting acids, primarily acetate, with traces of propionate and butyrate. Other common interactions involve oxygen utilization, chelation of metals, and concentration of anions at the metal surface. These changes result in the local breakdown of the metal passivation layers with subsequent changes in the polarization resistance.

There are two electrochemical methods of detection that can be utilized to detect changes in the passivation that result in corrosion. The first method involves passively monitoring electrochemical events. Measuring the open-cell corrosion potential (OCP) between working stainless steel and reference electrodes has established that breakdown of passivation in local areas (pitting) is associated with a 200 to 300 mV negative shift in OCP relative to the calomel electrode. This shift is associated with the activity of microbial biofilms actively secreting acid. Preliminary experiments utilizing ultrafast multimeters which monitor electrochemical noise (ECN) between working and reference electrodes show that microbial activity on surfaces produces marked shifts in amplitude when the frequency spectrum of the noise is factored. When factored, spontaneous pulses of about 1 mV show a decrease in noise at higher frequencies compared to lower frequencies. These shifts between "pink" and "white" noise occurring at about 90 Hz appear to correlate with microbial activity and may be associated with local breaches in passivation films on the metal.

The second type of electrochemical monitoring involves the current responses when a small potential is applied to the working electrode. It is important to know that the currents or potentials applied to the biofilms are of short duration and low in magnitude, so no detectable damage to the electrode surface or to the cell metabolism is induced. The standard linear polarization analysis that employed linear potential sweeps of hundreds of millivolts from the open-cell potential irreversibly damaged the biofilms. Two methods, small amplitude cyclic voltametry (SACV) and electrochemical impedance spectroscopy (EIS), have shown utility in on-line nondestructive monitoring of biofilm microbial activity (Dowling et al. 1988, 1989).

SACV involves the application of a sawtooth (triangular) signal that is sufficiently small so that the change of the electrode potential remains in the linear range. This technique allows the accurate determination of the polarization resistance and provides as well some indication of the capacitance of the system. The polarization resistance is related to the reciprocal of corrosion rate. Generally, sweeps involve a maximum of 1 μA over a period of 64 s. This is slow enough to provide a pseudo-equilibrium that overcomes problems of steady state. The capacitive component of the system is indicated by the hysteresis. Both the capacitive contribution to the hysteresis and the problems of nonsteady state decrease at slower sweep rates. The limits on

the slowness of the sweep rates are usually related to the stability of the potentiostat. In such a system the activity of acid-generating bacteria induced an increase in SACV of 3.7 Kohm cm^2 compared to 18 Kohm cm^2 for the uninoculated control.

The second transient response method involves EIS. EIS differs from SACV in that a small sinusoidal potential is applied to a working electrode over 5 decades of frequencies and the phase shift of the resultant sinusoidal current is measured. The results are usually plotted using a Nyquist diagram of Z″ (imaginary impedance) versus Z′ (real impedance). This analysis gives more information about the system than the SACV measurement. However, the analysis can take several hours and the equipment tends to be more expensive. EIS subjects the microbially influenced corrosion (MIC) biofilm to greater perturbations than SACV and could lead to artifacts originating in the electrochemical measurement itself. Evidence that EIS does not significantly affect a living microbial biofilm comes from the fact that sequential EIS analyses show no differences. The small current resulting from the impression of a potential equivalent to the corrosion potential does not change during the analysis. Both of these observations indicate that the corrosion system including the biofilm is unchanged during the EIS analysis. Laboratory experiments can be used to demonstrate readily the effectiveness of EIS in the detection of microbial activity. EIS provides an additional advantage as it not only gives nondestructive estimates of the corrosion potential but also provides indications of local inhomogeneities in the corrosion process that become more pronounced as the sweep frequency decreases. The complication results from the influence of the local processes on the average corrosion rates which in turn are related to microbial biofilm activities. The use of these devices in monitoring microbially influenced corrosion has been reviewed (Dowling et al. 1988, 1989).

These electrochemical devices have the advantage of being nondestructive and can be utilized on-line to monitor changes in biofilm activities. The problem is that any process affecting the Helmholtz double layer can induce a response, and microbial organic acid production or bacterial growth are major but not exclusive processes monitored by these systems.

Independent on-line monitoring of the rate of bacterial colonization may be possible utilizing a piezoelectric Quartz Crystal Microbalance. In this device a transverse AT-cut quartz crystal is expected to detect changes in the viscoelastic properties of the surface exposed to the solution as the bacteria attach and colonize the surface. These changes are detected nondestructively as shifts in the resonant frequency of the crystal. This device can be combined with the electrochemical monitors described above to increase the specificity.

The specificity of electrochemical devices is being enhanced by the application of polymer coatings that contain enzymes or reactants which

react only with specific analytes in the biofilm or solution. The membrane components in turn react with the electrochemical devices. If the electrodes themselves can be made sufficiently small (on the scale of microns), then many of the problems of inhomogeneity and imperfect mixing that plague electrochemistry will disappear. Microelectrodes also have the advantage of providing much greater spatial resolution.

Role of Microbes in Organic Acid Composition and Distribution

This paper has addressed the techniques by which microbial consortia in biofilms on soil, sediment, or suspended particles can be monitored. The biomass and community structure determinations can show who is there. If coupled to measures of labeled precursors incorporated, these techniques can also show the other critical consideration of not only who is there but who is metabolically active. The nutritional status can also be monitored with these techniques and can be important in processes where the organisms derive no direct benefit from specific metabolism yet in the presence of suboptimal growth conditions are able to modify chemical structures in the environment. This so-called co-metabolism has been demonstrated for microbial consortia in which aerobic methane or propane oxidation at suboptimal rates can degrade halogenated hydrocarbons (Ringelberg et al. 1988).

The role of specific microbial consortia in modifying the composition of nic organic acids could be examined in some direct experiments. Humic organic acid materials labeled with ^{13}C could be created by growth if plants were incubated with ^{13}C-carbon dioxide, such as cypress seedlings with $^{13}CO_2$. This material could be characterized as described in the preceding sections. If the labeled humic organic acids were placed in a flow-through microcosm where they were exposed to a microbial biofilm, the effects of shifts in microbial activity and community structure could be correlated with time to changes in the structures of the humic organic acids. Once specific microbial activities were associated with specific changes in the amount and composition of humic organic acids in the microcosm experiments, then insight into how the microbial metabolism affects the spatial and temporal distribution of these complex organics could be developed.

CONCLUSIONS

Other papers in this volume address novel methods by which the acids themselves can be detected, and these must be utilized coordinately with the techniques for monitoring the distribution, community structure, nutritional status, and metabolic activities of the microbes. The role of

microbes in the temporal and spatial distribution of both biodegradation and possibly biosynthesis of the humic organic acids can be better defined.

Acknowledgements. This work would not have been possible without the dedicated work of the colleagues who formed this laboratory and continue to generate the insight and hypotheses. Particularly we wish to acknowledge the essential functions of Norah Rogers, who for 13 years has managed the laboratory at Florida State University with exemplary care and responsibility. This work has been supported by grants N00014-87-K00012, N00014-83-K-0275, and N00014-88-K-0489 from the Office of Naval Research; OCE-80-19757 and DPP-86-12348 from the National Science Foundation; and DE-FG05-88ER60643 from the Office of Health and Environmental Research, Department of Energy. The FT/IR was purchased with grant N00014-83-G-01066 from the Department of Defense, University Research Instrumentation Program, Office of Naval Research, and the VG Trio-3 GC/MS/MS was acquired with funds from the University of Tennessee, Grant ARO 24187-LS-RI, Department of Defense, University Instrumentation Program, Army Research Office, 2-4-01018 DEG-Lab Equipment from the Department of Education (to G.S. Sayler), and DE-F605-8ER75375 from the Department of Energy, University Research Instrumentation Program. The generous gift of the Hewlett-Packard HP-1000 RTE-6/VM data system for the HP5996A GC/MS system greatly facilitated the research reported here.

REFERENCES

Balkwill, D.L., F.R. Leach, J.T. Wilson, J.F. McNabb, and D.C. White. 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct cell counts in subsurface sediments. *Microb. Ecol.* 16:73–84.

Dowling, N.J.E., J. Guezennec, M.L. Lemoine, A. Tunlid, and D.C. White. 1988. Corrosion analysis of carbon steels affected by aerobic and anaerobic bacteria in mono- and coculture using AC impedance and DC techniques. *Corrosion* 44:869–874.

Dowling, N.J.E., E.E. Stansbury, D.C. White, S.W. Borenstein, and J.C. Danko. 1989. On-line electrochemical monitoring of microbially induced corrosion. In: Microbial Corrosion: 1988 Workshop Proceedings, ed. G. Licena, pp. 1, 5–19. Research Project 8000-26, Electric Power Research Institute, Palo Alto, CA.

Fazio, S.A., J. Uhlinger, J.H. Parker, and D.C. White. 1982. Estimations of uronic acids as quantitative measures of extracellular polysaccharide and cell wall polymers from environmental samples. *Appl. Env. Microbiol.* 43:1151–1159.

Gehron, M.J., and D.C. White. 1982. Quantitative determination of the nutritional status of detrital microbiota and the grazing fauna by triglyceride glycerol analysis. *J. Exp. Mar. Biol. Ecol.* 64:145–158.

Guckert, J.B., C.B. Antworth, P.D. Nichols, and D.C. White. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol. Ecol.* 31:147–158.

Guckert, J.B., M.A. Hood, and D.C. White. 1986. Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl. Env. Microbiol.* 52:794–801.

Jain, R.K., R.S. Burlage, and G.S. Sayler. 1988. Methods for detecting recombinant DNA in the environment. *CRC Crit. Rev. Biotech.* 8:33–84.

Little, B., R. Ray, P. Wagner, Z. Lewandowski, W.G. Characklis, J. Jacobus, and F. Mansfeld. 1989. Electrochemical reactions on stainless steels covered with biofilms. In: Corrosion Research Symposium 1989, ed. T.P. Ford and T.M. Devine, pp. 30–32. Houston, TX: National Association of Corrosion Engineers.

Mycke, B., F. Narjes, and W. Michaelis. 1987. Bacteriohopanetetrol from chemical degradation of an oil shale kerogen. *Nature* 326:179–181.

Nichols, P.D., J.B. Guckert, and D.C. White. 1986. Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. *J. Microbiol. Meth* 5:49–55.

Nichols, P.D., J.M. Henson, J.B. Guckert, D.E. Nivens, and D.C. White. 1985. Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria-polymer mixtures and biofilms. *J. Microbiol. Meth.* 4:79–94.

Odham, G., A. Tunlid, G. Westerdahl, G. Larsson, J.B. Guckert, and D.C. White. 1985. Determination of microbial fatty acid profiles at femtomolar levels in human urine and the initial marine microfouling community by capillary gas chromatography-chemical ionization mass spectrometry with negative ion detection. *J. Microbiol. Meth.* 3:331–344.

Ogram, A.V., and G.S. Sayler. 1988. The use of gene probes in the rapid analysis of natural microbial communities. *J. Ind. Microbiol.* 3:281–292.

Olsen, G.J., D.J. Lane, S.J. Giovanni, N.R. Pace, and D.A. Stahl. 1986. Microbial ecology and evolution: a ribosomal approach. *Ann. Rev. Microbiol.* 40:337–365.

edge, C., and S.G. Wilkinson. 1988. Microbial Lipids, vol. 1. New York: Academic.

Revsbech, N.P., and B.B. Jorgensen. 1986. Microelectrodes: their use in microbial ecology. *Adv. Microb. Ecol.* 9:293–352.

Ringelberg, D.B., J.D. Davis, G.A. Smith, P.D. Nichols, J.B. Nickels, J.M. Hensen, J.T. Wilson, M. Yates, D.H. Kampbell, H.W. Reed, T.T. Stockdale, and D.C. White. 1988. Validation of signature polar lipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials. *FEMS Microbiol. Ecol.* 62:39–50.

Smith, G.A., J.S. Nickels, B.D. Kerger, J.D. Davis, S.P. Collins, J.T. Wilson, J.F. McNabb, and D.C. White. 1986. Quantitative characterization of microbial biomass and community structure in subsurface material: prokaryotic consortium responsive to organic contamination. *Can. J. Microbiol.* 32:104–111.

Stahl, D.A., B. Flesher, H.R. Mansfeld, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Env. Microbiol.* 54:1079–1084.

Tunlid, A., G. Odham, R.H. Findlay, and D.C. White. 1985. Precision and sensitivity in the measurement of ^{15}N enrichment in D-alanine from bacterial cell walls using positive/negative ion mass spectrometry. *J. Microbiol. Meth.* 3:237–245.

White, D.C. 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. *Symp. Soc. Gen. Microbiol.* 34:37–66.

White, D.C. 1988. Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity. *Adv. Limnol.* 31:1–18.

White, D.C., R.J. Bobbie, J.S. Herron, J.D. King, and S.J. Morrison. 1979. Biochemical measurements of microbial mass and activity from environmental samples. In: Native Aquatic Bacteria: Enumeration, Activity and Ecology. *Soc. Test. Mater. Sp. Publ.* 695:69–81.

White, D.C., J.D. Davis, J.S. Nickels, J.D. King, and R.J Bobbie. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* 40:51–62.

White, D.C., and R.H. Findlay. 1988. Biochemical markers of predation effects on the biomass, community structure, nutritional status and metabolic activity. *Hydrobiol.* 159:119–132.