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SUBSURFACE MICROBIOTA AS MONITORS OF CONTAMINENT MIGRATION AND MITIGATION

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The subsurface microbiota represent a diverse and living component of the subsurface that can have remarkable effects on the migration and possible mitigation of comtaminants. Methods for the recovery of subsurface materials free of contamination from the surface or drilling apparatus have made possible examination of this microbiota. Quantitative methods wherein "signature biomarkers" derived from the membranes of various physiological groups of bacteria can give indications of the viable biomass, community structure, nutritional status and in some cases the impacts of toxicants. The analysis is based on the extraction, fractionation, derivatization, and analysis of membrane components by gas chromatography/mass spectrometry of the neutral, glyco- and phospholipids. The microbiota rapidly respond to pollution with a change in activity and community sturcture. The nutritional status shifts from indications of unbalanced growth which induces the synthesis of poly beta-hydroxyalkanoate (PHA) and uronic acid containing polysaccharide glycocalyx to one of balanced growth with cell division with the concomitant formation of phospholipid ester-linked fatty acids (PLFA). Measurements in the field with a simple technique for the ratio of rates of incorporation of 14-C acetate into PHA/PLFA provide an excellent monitoring tool for nutritional status. The measurements of this ratio have proved an excellent monitor for the effectiveness of methane driven co-metabolism of volatile halogenated hydrocarbons. Presence PLFA indicates that specific types of microbes are present in viable bacteria as the polar groups of the membrane lipids are rapidly hydrolized on cell death. The presence of key signatue patterns can indicate that specific types of microbes are present. This can offers predicibility as to the biodegradative potential of the specific aquifer. The presence of specific groups of microbes can also indicate the potentiality of the aquifer permeability being modified as the hydrology of the system in impacted. The sensitivity of the microbiota to shifts in their microenvironment means that quantitiative analysis could be a developed into a instrument for rule making and regulatory action in remediation monitoring. The question of when is it clean could be defined. As the requirements for in situ treatments for ground water contamination become more acute, the detailed analysis of the response of the subsurface microbiota could be both the means for bioremediation and the test that it is complete.

INTRODUCTION

With the significant improvement in the sampling techniques that allowed recovery of subsurface aquifer material without contamination from surface microbiota pioneered by Dunlap et al. (1977), it became possible to demonstrate that there was an active and diverse subsurface microbial commuity. Direct microscopic enumeration and morphological examination revealed a sparse an unusual prokaryotic microbiota in uncontaminated aquifer materials (Ghiorse and Balkwill, 1983). The subsurface microbiota can be monitored by classical methods involving isolation from the substrata and cultivation in appropiate growth medium or by methods based on the detection of specific components of the cells which often do not require quantitative recovery of intact microbes from

the substrata. One such method involved the recovery of the polar lipids from the membranes of the microbes. The phospholipids of bacteria comprise by far the major portion of the extractible lipids of the cells. The content of phospholipids is an excellent measure of the cellular biomass in the subsurface when compared to direct microscopic counts with calculations of biomass, measures of the cell wall muramic acid (a unique bacterial wall component), and the cellular adenosine tri phosphate (Balkwill et al. 1988). By utilizing the analysis of phospholipid ester-linked fatty acids (PLFA) it was possible to show that uncontaminated subsurface aquifer sediments contained a unique microbiota, that was essentially prokaryotic, and distinct from surface or rhizosphere communities (Smith et al. 1985; 1986). This uniqueness of the subsurface microbiota extended from unconsolidated clays and sands to limestones in both the American South East and West in sediments 10-40 m from the surface to samples recovered from clays 410 m below the surface (White et al. 1983).

DEFINING THE SUBSURFACE MICROTIOTA

The GC/MS "signature biomarker" method involves the measurement of biochemical properties of the cells and their extracellular products. Those components generally distributed in all cellular life are utilized as measures of biomass. Components restricted to subsets of the microbial communities are 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 validated by using antibiotics and cultural conditions to manipulate the community structure. The resulting changes agreed both morphologically and biochemically with the expected results (White et al. 1980). Other validation experiments involved isolation and analysis of specific organisms and finding them in appropriate mixtures, utilization of specific inhibitors and noting the response, and changes in the local environment such as the light intensity are summarized in reviews (White, 1988).

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. 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 (White et al. 1979).

The ester-linked fatty acids in the phospholipids (PLFA) are both the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed (Bobbie and White, 1980, Guckert et al. 1985). 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.

PLFA analysis has provided a quantitative means to document that a specific subsurface microbiota clearly different from the surface soil exists in samples of sandy clays recovered from several states in the southeastern United States and England (Smith et al. 1986).

Examination of the lipid content of the subsurface microbiota also provides insight into the nutritional status of the microbial community. The nutritional status of microbial consortia can be estimated by monitoring the proportions of specific endogenous storage compounds relative to the cellular biomass. Certain bacteria form the endogenous lipid poly beta- hydroxyalkanoate (PHA) under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (Nickels et al. 1979). The ratio of the rate of 14-C acetate incorporation into PHA relative to PLFA provides a very sensitive measure of the microbial community nutritional status. The relative rates synthesis of these two lipid components are readily determined by comparing the PHA (recovered in the glycolipid fraction of a silicic acid fractionation of the total lipid) from the polar lipid (PLFA) fraction (Findlay and White, 1988). A second biomarker for nutritional status can also be detected in the PLFA patterns. Starvation induces the formation of minicells in some bacteria. There is a loss of cell components including the membrane lipids but there is a marked increase in the proportion of monoenoic PLFA with the double bond in the trans configuration (Guckert et al. 1986). The accumulation of this trans monoenoic PLFA synthesized from 14-C acetate has been shown in natural communities of pelagic marine organisms (Guckert et al, 1988). Preliminary investigations with D. Balkwill have shown that monocultures isolated from the deep subsurface environments also accumulate the trans monoenoic PLFA when starved.

A third measure of community nutritional status is increased formation of extracellular polysaccharide glycocalyx by the microbiota with stress. Uronic acid-containing glycocalyx forms maximally in the marine Pseudomonas atlantica under conditions of nutritional stress (Uhlinger and White, 1983).

Uncontaminated subsurface aquifer sediments contain microbiota with high levels of PHA relative to the PLFA, high proportions of trans monoenoic PLFA, and high levels of extracellular polysaccharides indicating poor nutrient conditions (White et al., 1983; Smith et al., 1986).

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

Are these measurements reproducible? The changes in biofilm or sedimentary microbiota, which induced shifting the environment of the microniche, are reproducible. The shifts in the terminal electron acceptors from high potential (oxygen or nitrate) to lower potential (sulfate, or carbon dioxide) induces changes in the microbial community structure. In experiments utilizing an

inoculum from marine sediments it has proved possible to manipulate the community structure of the benthic microbiota by shifting from aerobic to anaerobic conditions (Guckert et al. 1985). The PLFA of independent flasks showed reproducible shifts when manipulated identically and significant differences when manipulated with different treatments. The absence of long chain polyenoic fatty acids indicated the communities were predominantly prokaryotic and the differences in the PLFA were primarily in the proportions of cyclopropane fatty acids and the proportions and geometry of the monounsaturated fatty acids.

In similar experiments a subsurface sediment inoculum was grown through two cycles of aerobic growth and compared to organisms from the same inoculum grown through two cycles of anaerobic growth with no supplement, or with sulfate or nitrate (Hedrick and White 1986). Again there were reproducible shifts in the microbial community structure as reflected in the profiles of PLFA. Not only were the PLFA patterns reproducible, but the ratio of respiratory quinones reflected the redox environment in the bacterial biofilms. Benzoquinone isoprenologues are formed by microbes grown with high potential terminal electron acceptors such as oxygen or nitrate (Hollander et al., 1977). Naphthoquinones are formed by bacteria utilizing low-potenital electron acceptors such as sulfate or organic substrates. Aerobic consortia formed the most benzoquinone relative to naphthoquinone, the nitrate supplemented anaerobic culture formed less benzoquinone, the sulfate supplemented culture formed still less benzoquinone, and the anaerobic fermentation formed the least.

EXPLORING THE SUBSURFACE MICROENVIRONMENT VIA THE MICROBIOTA

Knowing the physiological responses of specific bacteria to changes in their environment and the limits of the physical-chemical milieu that permits survival, it is possible to define the microniche in which these bacteria can function (White, 1986). This definition of the microenvironment based on the microbial community structure can be further refined if consequences of suboptimal conditions can be reflected in measurable refections of the bacterial nutritional status. The addition of activity and biomass measures should provide a microbiological means to explore the subsurface microenvionment.

Can changes in the subsurface microenvironment be detected in changes in the microbiota? When the microbiota from contaminated subsurface aquifer material were examined marked changes in the microbiota were detected. The rates of biodegradation of alkylbenzenes and chlorobenzene by the subsurface microbiota recovered from a creosote contaminated site in Conroe, TX was roughly related to microbial biomass (Wilson et al., 1986). Examination of the subsurface microbiota from this site showed an increase in the total microbial biomass, a progressive decrease in the content of glycerol teichoic acid, an the synthesis of PLFA that roughly paralled the degree of contamination (Smith et al., 1985; 1986). The decrease in glycerol teichoic acid paralleled the decrease in gram positive bacteria (Gehron et al., 1984) and an increase in gram-negative bacteria.

The response of the subsurface microbiota to contamination has been demonstrated by the examination of a series of cores recovered from a drilling that penetrated a lense of contamination by trichloroethylene (TCE). Cores recovered between 30 and 48 m below the surface showed greater than 500 mg

TCE/liter. At these high concentrations there was a depressed recovery of "viable" bacteria indicated both by the lack of incorporation of 3-H labeled thymidine into DNA, 14-C acetate into lipids, extractible PLFA or viable bacteria measured by plate count. At areas just above (25-28 m below the surface) or below (52-54 m) the lens of maximal TCE concentration where the TCE concentration was less than 100 mg/l, the microbial biomass measured at PLFA, the rates of 3-H thymidine incorporation into DNA and the rate of 14-C acetate synthesis into lipid showed activities two to four orders of magnitude greater than the uncontaminated subsurface aquifer material above and below the contaminated zone (Phelps et al., 1989a). Microbial consortia able to degrade TCE when provided a variety of electron donors, were recovered with much higher frequency from this zone of increased activity than from surrounding core material above and below these sites. These microbial consortia have been shown to degrade TCE when exposed to concentrations up to 100mg/l (Fliermans et al., 1988).

Exposure of subsurface soils to methane and air induces a shift in microbial community structure with an increase in microbial biomass by about 5-fold with at least an 2000-fold increase in the type II methane-oxidizing bacteria. Authentic monocultures of methane-utilizing bacteria of both type I and type II show distinctive "signature" fatty acid biomarker profiles (Nichols et al., 1985). High proportions of the unique 18:1w8c PLFA characteristic of the type II methane-oxidizing bacteria have appeared on at least 5 occasions when subsurface sediments from different locations were exposed to methane and air. Similarly when subsurface sediments are exposed to propane a microbial community enriched in putative actinomycetes with high proportions of 10 methyl 18:0 and the unique 16:1w6c PLFA routinely appear. These PLFA patterns are reproduced in propane-degrading isolates recovered from these sediments (Ringleberg et al., 1988).

The reproducible shift in subsurface microbial community structure when exposed to either methane + air or propane, butane + air is particularly important as it validates the signature biomarker technology for the methane-utilizing and propane-utilizing bacteria in the subsurface. Furthermore the newly induced subsurface microbiota show greatly increased ability to degrade halogenated hydrocarbon subsurface contaminants such as perchloroethane (PCE), trichloroethane (TCE), various dichloroethenes, chloroform, methylene chloride, and ethylene dibromide (Nichols et al. 1987). Similar experiments with propane, butane + air amended soil columns have shown similar abilities. The growth of the methanotrophic bacteria stimulated the growth of a microbial consortium as evidenced by the phospholipid fatty acid patterns (Ringelberg et al. 1988). From studies of isolates some of the organisms whose growth was stimulated by the elaborations from the methanotrophic bacteria were responsible for the halogenated hydrocarbon degradations. The consortium induced with the growth of the methanotrophic bacteria was stable and did not significantly change the flow rates. The methanotrophs are microaerophilic (require oxygen) and no trace of vinyl chloride has been detected as a product of the degradation.

USEFULNESS OF THE SUBSURFACE MICROBIAL COMMUNITY IN RULE MAKING AND REGULATORY ACTION

The subsurface microbes provide the largest biomass in aquifer sedimentary

materials. These microbes provide a staggering metabolic diversity when compared to other communities. For example, the whole complex of anaerobic life is almost exclusively a microbial characteristic. Microbes have the greatest diversity in terms of physiological types of species. Nearly every possible chemical reaction with sufficient free energy has been exploited by a microbe. In addition microbes have the capacity for an extremely rapid response to selective conditions in the environment. With the great diversity of types and extraordinary metabolic versatility, shifts in microbial community structure and nutritional status should be the ideal way to study the impact of toxicants on the subsurface ecosystem, if a suitable assay system could be devised.

Examination of the subsurface microbiota utilizing the signature lipid technology provides a quantitative description of the biomass, community structure, nutritional status and with preincubation with mass labeled precursors measures of specific metabolic activities. These methods have established that pollution induces changes in the subsurface microbiota.

Monitoring the living subsurface microbiota offers advantages over measures of contaminant concentrations in determining pollution. The microbiota as with all living systems integrate metabolic insults over time, so assessments based on the biological responses provide increased reliability when compared to ephemeral pollution plumes in the subsurface.

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

The methods described in this paper provide quantitative insight into the biomass and community structure, nutritional status, and metabolic activities of microbial consortia in sediments that do not require quantitative recovery of the organisms from the biofilm or that they all be cultured successfully. These methods are not complicated by fossil components from non-viable cells remaining in the microbial consortia. Phospholipids, adenosine nucleotides, muramic acid, and the lipopolysaccharide of dead bacteria are rapidly lost from sediments (Davis and White, 1980; White et al., 1979; King and White, 1977; Saddler and Wardlaw, 1980). This indicates that these chemical markers provide good estimates for the standing viable or potentially viable microbiota. Rates of formation or loss of endogenous storage lipids or exocellular polysaccharide polymers or synthesis or turnover of specific membrane signature biomarkers provide insight into the nutritional status and actual metabolic activities of these microbial consortia.

The methodology described herein can be validated by showing that specific microorganism types can be induced in soils and sediments which show the same patterns of signature biomarkers as isolates from the specific environment. The microbiota has been shown to be rapidly responsive to shifts in the

microenvironment as well as to predation. The microbiota in sediments with redox gradients rapidly respond to artificial distrubance and to the disturbance induced by bioturbation. It is then not surprising that the microbiota respond to pollution. The response to pollution has possibly been best demonstrated in the subsurface sediments where primarily prokaryotic subset of the soil microbiota show a clear shift from predominantly gram positive microbiota with the chemical markers of starvation are changed into a predominately gram negative community showing growth and increased biomass with contamination by a biodegradable mix of toxicants. Preliminary use of the PLFA methodology to assess the impact of heavy metal contamination of esturine sediments in a carbonate rich semitropical system (Biscayne bay, Florida) and North Florida bay containing terrigenous aluminosilicate sediments show a linear relationship using cannonical correlation between shifts in microbial community structure estimated by subsets of the PLFA and the pollutant metal concentrations (Schropp et al. 1988). This analysis required a large weighting factor based on sediment grain size and shows that the accessment of the microbiota provides a quantittative tool to monitor the effects of estuarine pollution.

THE BAD NEWS

With the establishment of PLFA analysis as a means to define the biomass and community structure of subsurface microbial communities in uncontaminated sediments and to show reproducible shifts in community structure with exposure to contamination or gaseous electron donors it became possible to establish some bad news. Ground water recovery is obviously the most expeditious way to sample the microbiota of subsurface aquifer materials.

The bad news is that the microbiota recovered from the ground water bear little relationship to the microbiota that exist in the subsurface aquifer materials. Elegant studies by T. Hasen and his students at the Savanah River Laboratory have shown that the microbiota recovered from ground water in series of wells screened at depths from which subsurface aquifer material had been previously recovered and analyzed, show wide differences. The ground water microbiota show several orders of magnitude less cells/unit volume, a considerably different genetic pool based on DNA hybridization, a much more straved nutritional status, and different metabolic activities than the microbiota found in the subsurface aquifers (unpublished observations). This is the same experience reported from Germany. Kolbel-Boelke et al. (1988) showed that ground water showed several orders of magnitude less total bacteria that were predominantly gram-negative whereas the sedimentary bacteria from the aquifer were predominately gram-positive with remarkably different physiologic properties. Hirsch and Rades-Rohkohl (1988) showed differences in the viability and morphologic properties of the ground water and aquifer sedimentary organisms. Great care needs to be taken in the recovery of the ground water organisms to achieve reproducability. The well water standing in the wells must be removed and the biofilms on the casing surfaces not disturbed before the samples are reproducible (Hirsch and Rades-Rohkohl, 1988).

THE HOPEFUL COMPROMISE

There is a possible compromise between the sampling problems of recovering uncontaminated subsurface aquifer materials and pumping seven well volumes of water through a tangential flow filter. Where it is possible to force water into

the aquifer system under sufficient pressure that there is a backflow it is possible to significantly increase the likelyhood of sampling a microbial community more like that in the aquifer material than the ground water. McKinley et al., (1988) showed that the recovery of microbes from a backflow treatment was maximal in the initial backflood water in two experiments and after two well volumes in two additional experiments. The biomass at the maximal recovery was between one to two orders of magnitude greater than the lowest levels (after eight well volumes had been pumped. More than 90% of microbes in the back flood were attached to particles and easily recovered in glass fiber filters. The PLFA patterns showed significant differences between the early and late recovery. The highest biomasses were associated with the highest proportions of cyclopropane 19, and monounsaturated 16 (both cis and trans) whereas the microbes after eight well volumes showed maximal 18:1w7t and branced PLFA. Maximal metabolic activity measured as lipid formation from 14-C acetate in the microbes recovered in the initial back flood. The backflood technology may offer the route to sampling the aquifer microbiota.

CONCLUSIONS

The sensitivity of the subsurface microbiota to perturbations in the aquifer material microenvironment coupled with the microbiota's pervasiveness, metabolic diversity, rapidity of response, and resilience, makes them ideal biomonitors. With the development of quantitative and reproducible assays based on signature biomarkers for biomass, community structure, nutritional status and with exposure to labeled precursors specific metabolic activities, the means to utilize these biomonitors is at hand. The major problem then is recovering the subsurface microbiota uncontaminated with surface or drilling mud organisms. This currently requires special precautions with expensive drilling technology. The backflow methods of recovering particulate bound subsurface aquifer microbiota utilizing existing monitoring wells could provide the means to recovering the microbial biomonitors in a cost effective way. Then the subsurface microbiota could be developed into a quantitative rule making instrument and a quantitative analysis developed that would be based on chemical analysis to define the state of subsurface microenvironments.
This definition of the subsurface microenvironments would then provide a reproducible means to monitor remediation and help define what is clean in a less arbitrary way. Ground water contamination is an increasingly serious threat but the subsurface microbiota provide both the means for <u>in situ</u> bioremediation and the mechanism for testing its effectiveness.

DISCLAIMER

The research described in this report has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the agency and no official endorsement should be inferred.

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