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Journal of Microbiological Methods 32 (1998) 93–105

Journal of Microbiological Methods

In situ microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere and in biofilms

David C. White[a,b,*], Cecily A. Flemming[a], Kam T. Leung[a], Sarah J. Macnaughton[a,c]

[a]*Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37932-2575, USA*
[b]*Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6036, USA*
[c]*Microbial Insights, Inc., 2340 Stock Creek Boulevard, Rockford, TN 37953-3044, USA*

Abstract

Numerous studies have established a relationship between soil, sediment, surface biofilm and subsurface contaminant pollution and a marked impact on the in situ microbial community in both microcosms and in the field. The impact of pollution on the in situ microbial community can now be quantitatively measured by molecular 'fingerprinting' using 'signature' biomarkers. Such molecular fingerprinting methods can replace classical microbiological techniques that relied on isolation and subsequent growth of specific microbes from the in situ microbial community. Classical methods often revealed less than 1% of the extant microbial communities. Molecular fingerprinting provides a quantitative measure of the in situ viable microbial biomass, community composition, nutritional status, relative frequency of specific functional genes, nucleic acid polymers of specific microbes, and, in some cases, the community metabolic activity can be inferred. Current research is directed at establishing correlations between contaminant disappearance, diminution in toxicity, and the return of the viable biomass, community composition, nutritional status, gene patterns of the in situ microbial community towards that of the uncontaminated soil, sediment or subsurface material with the original uncontaminated microniche environments. Compared to the current reliance on disappearance of pollutants and associated potentially toxic products for detection of effective and quantitative bioremediation, assessment of the in situ microbial community will be an additional and possibly more convincing risk assessment tool. The living community tends to accumulate and replicate toxic insults through multiple interactions within the community, which may then effect viable biomass, community composition, nutritional status, community metabolic activities, and specific nucleic acid polymer patterns.

Keywords: Natural attenuation; Risk assessment; Microbial ecology; Signature biomarker analysis

1. Introduction

One of the major problems in remediation of contaminated environments is the determination of effectiveness and completeness to satisfactory status. Current practice requires the disappearance of the pollutants (and products) to regulatory levels with further toxicity testing, usually on a single organism species, to be sure there is no product or induced change resulting in residual toxicity. Technically, these tests are often impossible and so expensive that they are neither practical nor useful. Herein, we propose an ecologically based test of 'how clean is clean' using assessment of the microbiota as a comprehensive risk assessment tool to complement

*Corresponding author: Tel.: 423 974 8001; fax: 423 974 8027; e-mail: milipids@aol.Com

0167-7012/98/$19.00
PII S0167-7012(98)00017-7

contaminant disappearance. Where the environment is considered safe and clean, e.g. where a drinking water well could be sited, then there is a defined microbiota that can be argued legally as safe and clean with a risk considered acceptable by the body politic. The microbial community response may prove a much more comprehensive indicator of residual toxicity that is more sensitive than single organism species toxicity screens and will serve as a complement to the disappearance or sequestration of pollutants and products (Guckert et al., 1991a; Napolitano et al., 1994; Kohring, 1994; White, 1988a; White and Wilson, 1989).

Molecular fingerprinting by 'signature' lipid biomarker analysis/environmental nucleic acid probes (SLB/ENAP) assessment will provide a quantitative relatively inexpensive means to assess the environment that can be tied directly to reduction in toxicity in microcosm simulations. When the community ecology determined in a site that is undergoing natural attenuation or managed remediation significantly approaches that of a similar environment considered safe and uncontaminated than the risk approaches that of the unimpacted sites.

How do we know if manipulations or natural attenuation in bioremediation clean-up is sufficiently effective so the risks of personal and environmental impacts are predictable? This is a major question for which herein we propose an answer. It is well established that in many environments, the impacts of contamination or environmental manipulations have marked and potentially devastating effects on ecosystems. Classically, impacts have been monitored by single species tests such as the responses of *Ceriodaphnia* and *Pimephales pomelas* in water or a wide variety of invertebrates in soils. Also used are specific toxicological responses for carcinogenesis/mutagenesis with Ames tests and teratogenicity with egg to egg tests. Such tests provide simple one number assessment (that manufacturers relish and regulators worry about) of the potential impact risk by inference. There is a vast literature that discusses the problems with these reductionist approaches to toxicity assessment (Wells et al., 1997). What if it were possible to quantitatively assess impacts on indigenous microbial communities such that analyses were readily performed and were sufficiently comprehensive to provide reproducible insights that could be related to the impacts? This could go a long way to providing increased rigor and breadth, and making risk prediction much more reassuring. The specific microbial response should provide a quantitative measurement that indicates that natural attenuation processes are occurring and that a baseline community is being reestablished. The return of this baseline community or a community approaching the baseline community will be an indication that the biological component of the attenuation process has significantly decreased the risk associated with the perturbation. If pollution has disappeared and the community has changed significantly then the microniche environment influencing the in situ biota will strongly indicate that the risk has been decreased even if a return to the baseline community has not occurred.

The assessment of the microbes and their in situ interactions in various environments has proved to be a major problem, which requires the application of nontraditional methodology. Classical microbiological methods, that were so successful with infectious disease, have severe limitations for the analysis of environmental samples. Pure-culture isolation, biochemical testing, and/or enumeration by direct microscopic counting or most probable number (MPN) destroy most of the interactions between the various components within the environment. These disruptive methods, requiring isolation are not well suited for the estimation of total biomass or the assessment of community composition within environmental samples. Also, since several of these techniques are dependent on microbial growth, thus selecting against nonculturable microorganisms, such methods provide little insight into the in situ phenotypic activity of the extant microbiota. It has been repeatedly documented in the literature that viable counts or direct counts of bacteria attached to sediment grains are difficult to quantitate and may grossly underestimate the extent of the existing community (White, 1983, 1986, 1988b; Tunlid and White, 1991; Colwell et al., 1985). The traditional tests provide little indication of the in situ nutritional status or evidence of toxicity within the microbial community.

Over the past 15 years, the laboratories of D.C. White and colleagues in the Center for Environmental Biotechnology (CEB) have developed SLB analy-

ses and have introduced ENAP for microbial communities in various environmental matrices. Combined, SLB and ENAP provide a quantitative, comprehensive yet specific analyses of the extant in situ community for multi-species, multitrophic level toxicity assessment

2. Materials and methods

2.1. Signature lipid biomarker analysis

Environmental microbial lipid analysis is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography–mass spectrometry (GC–MS). Several unique classes of lipids, including steroids, diglycerides (DG), triglycerides (TG), respiratory quinones (RQ), poly β-hydroxyalkanoate (PHA), phospholipid lipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl ethers, sphingolipids and lipopolysaccharide hydroxy fatty acids (LPS-OHFA) can be used as signature lipid biomarkers (SLB) to characterize microorganisms or communities of microorganisms. The comprehensive analysis scheme proposed for SLB/ENAP analysis is diagrammed in Fig. 1.

One of the most important SLB classes phospholipids, are essential membrane components of living cells. Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers. Phospholipid fatty acid analysis can provide insight into the phylogenetic relationships between organisms similar to more specific phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (Guckert et al., 1991b; Kohring et al., 1994). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community as certain fatty acids, such as *trans* and cyclopropyl PLFA, provide indications of environmental stress. The PLFA and other biomarkers have been successfully extracted from environmental matrices such as soils and sediments providing a means for direct in situ measurements.

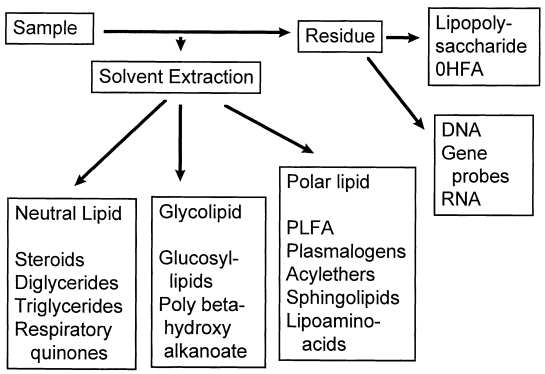

Fig. 1. Signature lipid biomarker/environmental nucleic acid probe analysis scheme.

Currently, lipids are extracted with liquid organic solvents and then separated into fractions using column chromatography. Fractions containing SLB may be further separated by thin layer or liquid chromatography with individual SLB determined using analytical instrumentation. The analysis was developed for quantitatively assessing microbial communities (bacteria, fungi, protozoa, and metazoa) in slimes, drilling muds, soils, filter retentates, bioreactors, deep subsurface sediments, and for the detection of specific bacteria in subsurface sediments (White, 1983, 1986, 1988b; Tunlid and White, 1991; Colwell et al., 1985; Federle et al., 1983; Ringelberg et al., 1997; White and Ringelberg, 1996; Lehman et al., 1995; Fredrickson et al., 1995a; Ringelberg et al., 1994; White and Ringelberg, 1995). Specifically, the SLB methodology provides a quantitative means to measure: (1) viable microbial biomass, (2) microbial community composition, and (3) community nutritional status.

2.1.1. Viable biomass

The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. The viable microbes have an intact membrane, which contains phospholipids (and PLFA). Cellular enzymes hydrolyze and release the phosphate group within minutes to hours following cell death (White et al., 1979). The lipid remaining is diglyceride (DG). The resulting diglyceride contains the same signature fatty acids as the original phospholipid, at least for some period of time. Consequently, a comparison of the ratio of phospholipid fatty acid

profiles to diglyceride fatty acid profiles provides a measure of the viable to nonviable microbial abundance and composition. A study of subsurface sediment showed that viable biomass as determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intercellular ATP, cell wall muramic acid, and very carefully conducted acridine orange direct counts (AODC) (Balkwill et al., 1988).

2.1.2. Community composition

The presence of certain groups of microorganisms can be inferred by the detection of unique lipids that originate from specific biosynthetic pathways (Edlund et al., 1985; Dowling et al., 1986; White et al., 1980; Parker et al., 1982; Bhat and Carlson, 1992; Fredrickson et al., 1995b; Hedrick et al., 1991a). Consequently, the analysis of SLB classes provides a quantitative definition of the microbial community. For example, specific PLFA are prominent in *Desulfovibrio* sulfate-reducing bacteria, whereas the *Desulfobacter* types of sulfate-reducing bacteria contain distinctly different PLFA (Edlund et al., 1985; Dowling et al., 1986). The analysis of other lipids such as the sterols (for the microeukaryotes–nematodes, algae, protozoa) (White et al., 1980), glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids from the lipid A component of lipopolysaccharide of gram-negative bacteria (Parker et al., 1982; Bhat and Carlson, 1992), sphinganines from sphingolipids (Fredrickson et al., 1995b), fatty dimethyl acetals derived from vinyl ether containing plasmalogens (Tunlid and White, 1991), and alkyl ether polar lipids derived from the Archae (Hedrick et al., 1991a) can provide a more detailed community composition analysis.

2.1.3. Nutritional status

Bacterial poly β-hydroxyalkanoic acid (PHA) (Findlay and White, 1983; Doi, 1990) and microeukaryotic triglyceride (Gehron and White, 1982) are endogenous storage lipids. The relative amounts of these compounds, as compared to the PLFA, provide a measure of the nutritional status. Many bacteria form PHA under conditions of unbalanced growth when a carbon source and terminal electron acceptor(s) is present but cell division is limited by the lack of some essential nutrient (Findlay and White, 1983; Doi, 1990). Specific patterns of PLFA can indicate physiological stress (Guckert et al., 1985, 1986; Heipieper et al., 1992). Exposure to toxic environments can lead to minicell formation and a relative increase in specific PLFA. Increased conversion from *cis* to *trans* PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol in the absence of bacterial growth (Heipieper et al., 1992). Prolonged exposure to conditions inducing stationary growth phase induces the formation of cyclopropane PLFA (Guckert et al., 1985, 1986). Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity (Hedrick and White, 1986). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce formation of benzoquinones in bacteria in contrast to microbes respiring on organic substrates that form naphthoquinones. Some specific but useful insights come from analysis of organisms like the *Pseudomonas* species which form acyl-ornithine lipids when growing with limited bioavailable phosphate (Minnikin and Abdolrahimzadeh, 1974) while some gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at suboptimal acid pH levels (Lennarz, 1970).

2.2. Recovery of DNA

Recently it has been shown that the solvent extraction utilized in the SLB lyses cells in the environmental matrix which facilitates the subsequent extraction of nucleic acids from the lipid-extracted residue (Kehrmeyer et al., 1996). These cellular nucleic acids can be used for enzymatic amplification and gene probing (Kehrmeyer et al., 1996). Over 50% of the gene *nahA* present in intact *Pseudomonas fluorescens* cells added to soil was recovered using the lipid extraction protocol as compared to recovery by the standard techniques (Ogram et al., 1987). The DNA recovered from the lipid extraction was of high quality, and is suitable for enzymatic amplification. The combined lipid extraction and recovery of nucleic acids can be very useful in biomass and community composition determinations. The DNA-probe analysis offers powerful insights because of the exquisite specificity in the detection of genes. The concomitant DNA/lipid

analysis readily provides quantitative recoveries independent of the ability to isolate or culture the microbes. While the lipid analyses give indications of the phenotypic properties of the community and the extant microbial activity by providing in situ indications of starvation, growth rate, exposure to toxicity, unbalanced growth, deficiencies of specific nutrients, and the aerobic/anaerobic metabolic balance (White, 1995), analysis using DNA probes can help define the physiologic potential of the microbial community. The combined DNA/lipid analysis overcomes some deficiencies in microbial ecology studies involving only nucleic acid analysis (White, 1994).

2.2.1. Nucleic acid analysis

Specific metabolic (degradative) gene probes can be used to quantify the response of the indigenous microbial community to the down-gradient movement and subsequent community metabolic response to a contaminant.

Molecular probes of over 100 biodegradative or physiologically distinct systems for biotransformation of 40 different chemical contaminants are available for environmental assessment (Sayler et al., 1996). The application of those probes to quantify 'like' gene abundance in extracted nucleic acids from impacted and nonimpacted samples is the fundamental measurement basis for monitoring biological response in the natural or accelerated attenuation process. The dynamic response as measured by such probe technology does not measure the population or community structure changes of the microbial diversity during and after attenuation. Such changes in genus or species specific structure are best measured using 16S rRNA phylogenetic analysis. This challenging task is nearly impossible by conventional microbiological cultivation. Analysis is approachable using 16S rRNA hierarchical hybridization analysis with group or genus specific probe technology or by PCR/RFLP of amplified ribosomal RNA genes or their cDNA counterparts.

A more appropriate technique by which PCR amplification of rDNA and rRNA from the in situ microbial community can be utilized to monitor shifts in microbial diversity in soils is denaturating-gradient gel electrophoresis (DGGE) or temperature-gradient gel electrophoresis (TGGE). Gradient gel electrophoresis enables DNA fragments of the same length to be separated according to their melting properties (sequences). In DGGE, double stranded DNA is separated in a linearly increasing denaturing gradient of urea and formaldehyde at elevated temperatures (Muyzer et al., 1995). In TGGE, the double stranded DNA is separated using a linearly increasing temperature gradient in a uniform concentration of urea and formamide (Heuer and Smalla, 1997). With either technique, mixed amplified PCR products form a banding pattern. Utilizing rRNA genes, DGGE/TGGE analysis of PCR products has provided structural diversity of communities that has proved reproducible (Muyzer et al., 1995; Heuer and Smalla, 1997; Rolleke et al., 1996). Analysis by PCR with specific primers of selected bands in the gel has enabled identification of specific bands to organisms. This technique has recently been utilized to map differences in the *Nitrosomonas* and *Nitrospira* communities in environments with different pH and salinity (Kowalchuk et al., 1997).

2.3. Microcosm experiment

A fine sandy loam (pH 6.4;:1:1 soil: water), was obtained from the University of Tennessee Agricultural Experimental Station and stored at 4°C for 3 months prior to use. Half the microcosms were inoculated with a surrogate jet fuel mix containing naphthalene, 100; phenanthrene, 100; *n*-decane, 1000; *n*-octane, 1000; *o*-xylene, 50; and acetone, 1700 (final concentrations mg/gram dry weight soil) delivered to half the microcosms in 100 μl acetone with thorough mixing. Bacterial inocula of 3.3×10^7 cells per g dry wt soil each of *Pseudomonas putida* ATCC 33015; *Pseudomonas oleovorans* ATCC 29347, and *Sphingomonas* sp. ATCC 39723 (previously *Flavobacterium* sp.) was added to half the microcosms. Strains were grown for 24 h in media recommended by the ATCC at 28°C before inoculating the microcosms. Microcosms were covered loosely with foil and incubated at 22°C in the dark at 100% humidity. Water loss was minimal as determined by periodic mass measurements. Duplicate microcosms were sacrificed at 0, 7 and 14 days and stored at −20°C for DNA-probe and signature lipid biomarker analysis (SLB) analyses.

DNA primers for *alk*B, *xyl*E, and *pcp*C were synthesised to amplify by PCR 382bp-, 309bp- and

771bp-DNA fragments of alkane hydroxylase, catechol 2,3 dioxygenase, and tetrachloro-hydroquinone dehalogenase. The sensitivity of the analyses for gene probes was: *xyl*E-, *alk*A-, and *pcp*C-containing bacteria were about 60, 60, and 630 pg genomic DNA per g dry soil, respectively. Two ml of the purified soil-DNA sample was used in the PCR. Thirty-five cycles were performed in the reaction with 1 min of denaturation, annealing and extension. The PCR products were analysed by agarose gel electrophoresis. Digoxigenin (DIG)-labeled gene probes were synthesised by PCR using a DIG-labelling system (Boehringer Mannheim, Indianapolis, IN 46250). Genomic DNA extracted from *Pseudomonas putida* ATCC 33015; *Pseudomonas oleovorans* ATCC 29347, and *Sphingomonas* sp. ATCC 39723 served as templates in the PCR-labeling reactions for the *xyl*E, *alk*B, and *pcp*C gene probes, respectively. Dot-blot hybridization was performed according to the Boehringer Mannheim Genius System User's Guide (Boehringer Mannheim, Indianapolis, IN 46250). Chemiluminescence detection was performed by a Boehringer Mannheim DIG luminescent detection kit and chemiluminescence was recorded with a CCD camera.

The SLB were analysed from lyophilized and weighed 10-g samples of sediments prior to extraction with the one-phase chloroform–methanol–buffer extractant. The lipids were recovered, dissolved in chloroform and fractionated on disposable silicic acid columns into neutral-, glyco- and polar-lipid fractions. The neutral lipids were fractionated by thin-layer chromatography and the diglyceride fraction recovered, transesterified, and the diglyceride fatty acid methyl esters (DGFA) analyzed by GC–MS. The glycolipid lipid fraction was subjected to ethanolysis and the β-hydroxy acids from the PHA analyzed by GC–MS. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane, the residue was subjected to mild acid methanolysis for recovery of the plasmalogen derived dimethylacetals and the lipid residue subjected to strong acid hydrolysis to recover the amide linked fatty acids. The PLFA were separated by capillary gas–liquid chromatography with detection and identification by electron impact mass spectrometry (GC–MS). The position of double bonds in monoenoic PLFA was determined by GC–MS after forming adducts with dimethyl disulfide. The lipid-extracted residue was subjected to strong acid hydrolysis and the hydroxy fatty acids of the lipopolysaccharide (LPS) analyzed by GC–MS. Detection limits for the lipids was about 0.1 pmole PLFA which corresponds to between 10^4–10^5 organisms the size of *Arthrobacter*.

3. Results and discussion

3.1. Community responses in anaerobic microcosms

The impacts of environmental stress can be readily demonstrated on the microbial communities of high-solids fermentation systems designed to produce methane. Examination of the SLB of a thermophilic, high-solids, high output biomass reactor showed remarkable correlations between methane production and the viable biomass, community structure and nutritional status (Hedrick et al., 1991b,c,d, 1992). In batch-fed reactors, differences between a starved community, a disturbed community, and one that is overfed are clear. The differences are clear with respect to methane production, carbon dioxide production, and acetate turnover. Disturbed and starved communities rapidly respond to feeding. Disturbance from the addition of solid substrate has little effect on the community structure or the activity. Starvation stimulates methanogen growth without much effect on the eubacterial biomass. Starved communities readily recover and produce methane. The resilience in the overfed community is much decreased. In the overfed community, the evidence of toxicity is reflected in a decrease in pH, and increases in ammonia and acetate. This evidence for toxicity is paralleled by a marked decrease in ether lipid synthesis (ether lipids are characteristic of methanogenic bacteria). Overfeeding results in a marked decrease in the methanogenic bacteria and if prolonged sufficiently, results in a reactor crash. Signs of toxicity in overfed reactors in the eubacterial community are reflected in increases in *trans*/*cis* monoenoic PLFA and accumulation of cyclopropane PLFA that accompany the marked decrease in methanogens.

The SLB analysis of the perturbations induced by

adding 26 μmol chloroform/gram or 300 μmol oxygen/gram had clearly different effects on the bioreactor community (Hedrick et al., 1991d). Both resulted in a marked decrease in methane production. Total CO_2 production increased with oxygen and decreased with chloroform. Chloroform was directly toxic to the methanogens; oxygen induced shifts in the eubacterial fermentative and acetogen communities with increased competition for reducing equivalents with the methanogens again showing how shifts in community structure relate to bioprocess effectiveness.

It proved possible to separate the hydrolytic/acetogenic and methanogenic components of a biomass-to-methane process. In a phase-separated methanogenic reactor (Hedrick et al., 1991b), the first phase had a volatile fatty acid production representing 50% of the applied chemical oxygen demand (COD) and methane production of only 9% of the biodegradable COD. The methanogenic phase reactor had a 73% efficiency for conversion of applied COD to methane and only 2% of the COD appeared as volatile fatty acids. The SLB analysis showed the two reactors had markedly different microbial communities with a 3-fold greater biomass of eubacteria and an 18-fold greater biomass of methanogenic bacteria in the methanogenic reactor. The eubacterial community in the hydrolytic/acetogenic phase showed a community dominated by short, branched, saturated PLFA compared with a monoenoic PLFA-containing community in the methanogenic reactor. The hydrolytic/acetogenic community showed signs of metabolic stress. In these experiments the community ecology indicated by SLB analysis were clearly correlated with shifts in the metabolic activity. Insights into the community ecology allow predictions of activity and potential of environmental risks.

3.2. Periphyton assessment as a surface run-off predictor

At Oak Ridge National Laboratory, a multitrophic level, multi-species assay system based on periphyton (the slime covering rocks in streams) was developed to monitor pollution abatement in streams (Guckert et al., 1991a; Napolitano et al., 1994). Unglazed tiles or rocks were incubated for a month in an unpolluted stream and then transferred to three sites in East Fork Poplar Creek. Each site had different levels of toxicity as estimated by the responses to *Ceriodaphnia* and *Pimephales pomelas* larval assays. After a month the tiles were recovered and a portion incubated in ^{14}C-acetate for an hour and the rest of the tiles were subjected to SLB analysis. The ratio of rates of PLFA (membrane) synthesis to PHA (storage lipid) synthesis showed an increased ratio as the system was more highly impacted. Toxicity increased the formation of membrane lipids without much effect on the storage lipids. Principal components analysis of the PLFA showed three distinct clusters in which signature PLFA of diatoms were associated with the least-impacted cluster and signature PLFA of green algae were associated with the most impacted cluster. The intermediate site was intermediate. The experiment was repeated three times at different seasons with identical results. Additionally, the periphyton analysis of wastewater abatement was repeated in a different stream with an identical result (Napolitano et al., 1994). Outdoor artificial streams made from plastic rain gutters, seeded with tiles and over which water was pumped from ponds and into which the toxicant was added showed a similar response (Kohring, 1994). A major detergent-making company set up a multimillion dollar artificial stream facility and now uses periphyton analysis by SLB as a toxicity assessment for effects of detergents in effluents on streams. Regulatory bodies in North America and Europe have very favourably received the results based on the periphyton as a toxicity assessment assay. The periphyton analysis data resulted in a safety multiplier factor for allowable discharge levels for detergents of 1.0. The regulators were willing to use the responses directly from this data without a safety factor (multiplier) for uncertainty that would protect for unknown interactions in the toxicity of these discharges.

3.3. Application of SLB to risk assessment in subsurface sediments

The application of SLB/ENAP for monitoring the environmental health in the subsurface has been proposed and discussed (White, 1988a; White and Wilson, 1989). Shifts were detected in the viable

biomass and community composition following pollution in the subsurface (Smith et al., 1986). In soil columns gassed with methane and air there were increases in viable biomass, proportions of PLFA characteristic of Gram-negative heterotrophs, and the type II methane-oxidizing bacteria (Nichols et al., 1987). Addition of different fatty acid substrates to anaerobic sediment cores induced marked and expected changes in the bacterial community structure reinforcing the contention that the subsurface microbial community exhibits a dynamic response to perturbation (Parkes et al., 1992). Subsurface sediments perfused with methane, propane, and air, show shifts in community structure, and metabolic capabilities that correlate with trichloroethylene (TCE) biodegradation (Ringelberg et al., 1988; Cox et al., 1994). Active biodegradation of petroleum hydrocarbons in subsurface sediments resulted in increases in viable biomass, shifts to aerobic heterotroph PLFA, decrease in biomarkers indicative of stationary phase growth, decrease in PHA/PLFA ratio, and increases in the proportion of benzoquinone respiratory quinones indicative of aerobic electron transport activity (Ringelberg and White, 1992). The nutritional status of microbial consortia actively degrading petroleum differs markedly from the organisms fortuitously degrading TCE. Effective fortuitous degradation of TCE correlated with high levels of reducing power indicated by a high PHA/PLFA ratios (Ringelberg and White, 1992; Nichols and White, 1989; Lackey et al., 1993). Maintenance of high levels of reducing power correlated with continuing TCE reduction. Clearly the subsurface microbiota responds to shifts in the environment. Unpublished experiments from the Savannah River in situ TCE biodegradation demonstration showed that the changes detected in the recovered sediments were reflected in the ground water microbes collected as membrane filter retentates. Also, the recovery of specific gene probes for methane monooxygenase and the signature PLFA of the methane-oxidizing bacteria correlated well. What are currently unknown are the factors that affect the rates of change of the subsurface microbiota to markedly different communities characteristic of uncontaminated conditions as the pollutants are removed. Experiments where an accurately dated pollution event occurred in areas of homogeneous sediments with well characterized hydrology and plume geometry offer ideal test sites for testing the utility of the shifts in microbial community ecology correlating with the decreasing risk from the pollution. SLB/ENAP analysis from sediments and ground water membrane filter retentates from a uniform glaciofluvial aquifer with well-characterized hydrology that was the site of a tragic 1988 crash site with a JP-4 fuel spill (including monoaromatic and aliphatic hydrocarbons) at Wurtsmith Air Force Base, MI are currently being examined as a nearly ideal site to validate the relationship between in situ community ecology shifts and decreases in pollutants and products. Shifts in in situ microbial community ecology in various portions of the plume will be compared with those of the uncontaminated sediments.

3.4. Microcosm demonstration of impact and initial stages of recovery by SLB/ENAP

In a recently reported microcosm experiment (White et al., 1997), the SLB/ENAP analysis established the impacts of amendments of the surrogate jet fuel and to a much smaller extent the effects of the bacterial inoculum. This soil contained between 13 and 23 nmoles of phospholipid ester-linked fatty acids (PLFA) and between 0.8 to 8 μg DNA per gram between the start and end of the experiment. The PLFA corresponded to between $4–16\times10^8$ cells per gram and the DNA to approximately 10^7 to 10^8 extracted genomes per g in the unamended soil.

The SLB/ENAP polyphasic analysis clearly showed the effects of hydrocarbon exposure on microbial community composition of both the endogenous microbiota and the inoculum (Fig. 2). The viable biomass increased as indicated by the increase in PLFA and DNA, with a clear increase occurring by day 7, the first sampling after the amendments. Principal-components analysis showed that the PLFA that contributed most to the hydrocarbon-influenced increase were PLFA typical of the Gram-negative heterotrophs (16:1ω7c, 18:1ω7c), as well as 10 Me 18:0, a biomarker associated with aerobic actinomycetes. Conversely, there was a decrease in the proportion of i15:0 (a biomarker which, where the iso/anteiso 15:0 is <1, is usually associated with Gram-positive aerobes). There was a decrease in environmental stress indicated by the fall in the *trans*/*cis* monoenoic PLFA ratio and a small increase in stationary phase growth in the Gram-nega-

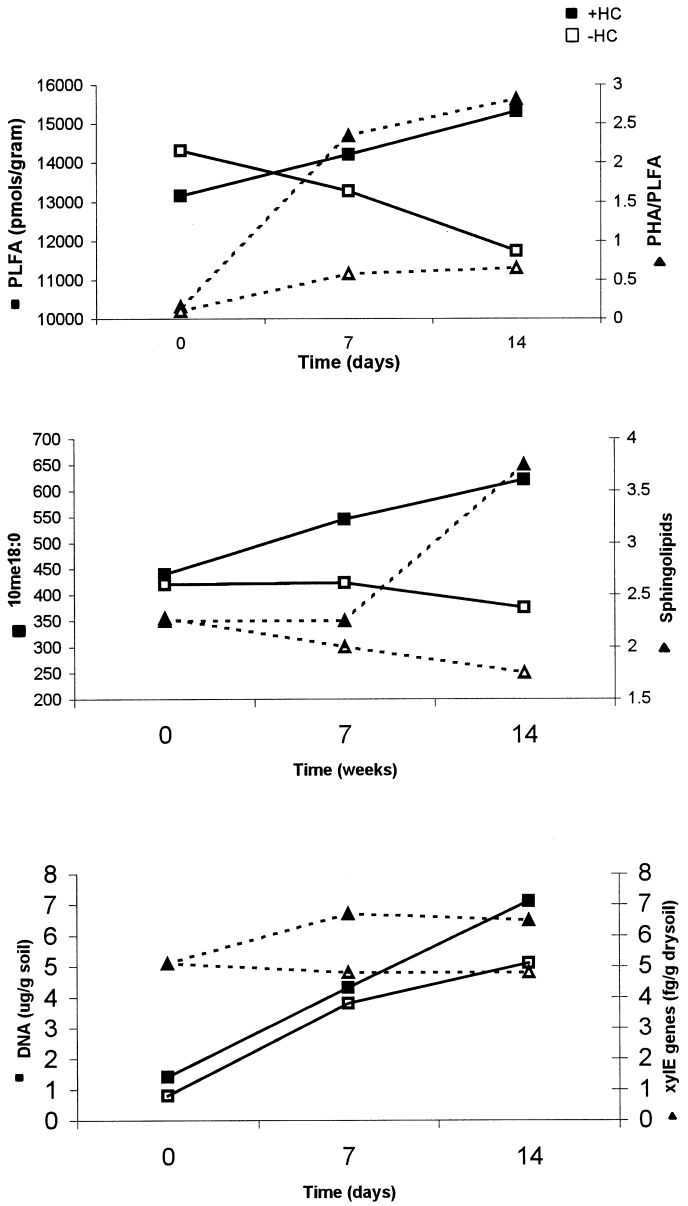

Fig. 2. Effect of the addition of hydrocarbons (HC) and their metabolism at initiation, after 7 and 14 days on the in situ microbial community biomass, community composition, nutritional/physiological status, and catabolic gene concentration. Each point is an average of two experiments.

tive heterotrophs as indicated by the proportion of cyclopropane 17:0.

In hydrocarbon-treated microcosms, the toxic stress reaction was observed in Gram-negative bacteria as evidenced by the increase in the *trans*/*cis* PLFA ratio. Aliphatic hydrocarbon-degrading potential was maintained as indicated by the *alk*B level as was the aromatic hydrocarbon-degrading potential (*xyl*E) and the chlorinated hydrocarbon-degrading potential indicated by *pcp*C. Following amendment with hydrocarbons there was a decrease in the ratio of diglyceride fatty acids (DGFA)/PLFA which is indicative of a decrease in cell death. Bacteria with abundant carbon source and terminal electron accep-

tor but lacking an essential nutrient undergo unbalanced growth with an accumulation of the endogenous storage polyester polymer PHA. The PHA/PLFA ratio increased much more markedly in the presence of biodegradable hydrocarbons in the uninoculated soil than in the inoculated hydrocarbon-exposed soil. This suggests that one or more limiting nutrients were supplied with the inoculum and supported more cell divisions when hydrocarbons were also added.

3.5. Community change with time after hydrocarbon exposure

In a continuation of the experiments described in Section 3.4, the response of the in situ microbial community to the hydrocarbon amendment shown in the first 7 days was continued in the second 7 days (Fig. 2). The viable biomass as indicated by the total PLFA showed a rapid decrease in the absence of hydrocarbons even though the recoverable DNA increased but at a lower rate. The toxic exposure stress indicated by the ratio of *trans*/*cis* monoenoic PLFA ratio showed a significant decrease in both microcosms with and without hydrocarbons (data not shown) (White et al., 1997). The proportions of actinomycetes continued to increase (10 Me 18:0 PLFA) and the proportion of the community with sphingolipids (Sphingomonas) increased strikingly in microcosms exposed to hydrocarbons (Fig. 2). The proportions of *xyl*E remained constant in the hydrocarbon-amended soils and the control in the second week. *Xyl*E codes for a gene for a much more 'central' metabolic reaction many reactions down the pathway for aromatic contaminants in contrast to the more 'peripheral' metabolic activities coded by the *alk*B or *pcp*C genes. Thus *xyl*E would not necessarily be expected to change.

Within the period of 7 to 14 days after the amendment of the surrogate jet fuel there was a shift in the community. Hierarchical cluster analysis of the patterns of the PLFA show the community profiles after 14 days after hydrocarbon amendment were a distinct community different from the community at initiation of the experiment and from the community 7 days after hydrocarbon exposure (Fig. 3). These shifts in community ecology have been comprehensively documented with artificial neural net analysis (Almeida et al., 1998). To detect a change in the in situ microbial community more towards that of the uninoculated soil would take considerably longer and probably require the system

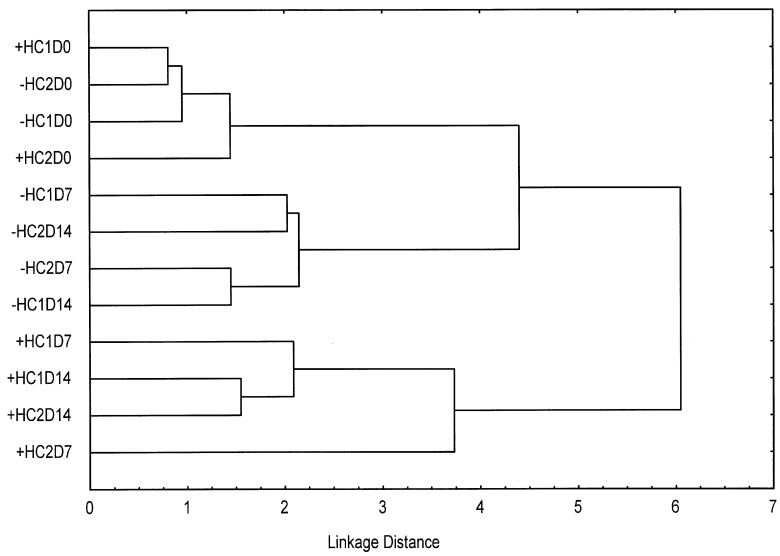

Fig. 3. Hierarchical cluster analysis of phospholipid ester-linked fatty acid patterns of the microbiota in sandy loam microcosms at the start and 7 and 14 days after amending with hydrocarbons (HC) contrasted with unamended control (-HC). HC1D7 indicates hydrocarbon-amended experiment replicate 1 extracted on day 7.

be incubated in a flow-through column to maintain the activity so community maturation could follow pollutant disappearance. Even in this very preliminary experiment there were significant changes in the community ecology in the second week.

These experiments with anaerobic digesters, periphyton exposed in surface waters, and subsurface soils and sediments establish the rapidity of shifts in the in situ microbial community in its response to changes in the microniche environments so pollutant disappearance should induce major shifts in community ecology. The SLB/ENAP analysis provides quantitative assessment of the viable biomass, community composition, nutritional/physiological status, dynamics of specific gene frequencies, and shifts in specific microbial components, which can be correlated, to natural and accelerated bioremediation. SLB/ENAP analysis can provide a powerful integrating and comprehensive determination of 'activity end-points' which with the disappearance of pollutants provide much more quantitative risk assessments.

Acknowledgements

This research was supported in part by grants DOE DE-FG02-96ER62163, from the Subsurface Science program, administered by F.J. Wobber and DE-FC02-96ER62278, from the Assessment Program Science Team Leadership in the Natural and Accelerated Bioremediation (NABIR) Program, Office of Health and Environmental Research, United States Department of Energy, administered by John Houghton and grant SY887-11 from the Strategic Environmental Research and Development Program, administered by Oak Ridge National Laboratory.

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