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LIPID/ DNA BIOMARKER ANALYSIS FOR ASSESSMENT OF *IN SITU* BIOREMEDIATION EFFECTIVENESS

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ABSTRACT: The combination of signature lipid biomarker and DNA gene probe analysis provides a powerful tool to quantitatively characterize bioremediation effectiveness throughout the entire microbial community without the necessity for isolation and culture. Lipid biomarker/ gene probe analysis enables assessment of viable biomass, community composition and nutritional/physiological status of the extant *in situ* subsurface microbial community. Herein we show the impact of the addition of petroleum hydrocarbons on the microbial ecology in a sandy loam with and without a bacterial inoculum. These community composition data can be used in the *in situ* assessment of the potential of indigenous microbiota for bioremediation.

INTRODUCTION

Shifts in the microbial community provide the ideal assessment of the effectiveness of bioremediation. The presence of specific genes indicate the potential for enzymatic activity while the viable biomass, community structure, and physiological status provide critical insight into the *in situ* conditions that allow effective expression of the metabolic bioremediation potential. Signature lipid biomarker (SLB)/gene probe polyphasic analysis provides this insight.

MATERIALS AND METHODS

Soil Microcosms. Soil (a fine sandy loam, pH 6.4) was obtained from the University of Tennessee Knoxville Agricultural Experiment Station, and stored at 4°C for 3 months prior to use. A hydrocarbon (HC) mixture was delivered to half the microcosms (125 mL Erlenmeyer flasks containing 20.0 g dry wt soil (20 % w/w moisture content) in 100 µL acetone with thorough mixing. Final concentrations of HC in the treated soils were: napthalene, 100; phenanthrene, 100; n-decane, 1000; n-octane, 1000; o-xylene, 50; and acetone, 1700 (µg/g dry wt soil). Bacterial strains (*Pseudomonas putida* (ATCC 33015); *Pseudomonas oleovorans* (ATCC 29347), and *Sphingomonas* sp. (ATCC 39723; previously *Flavobacterium* sp.)) were grown for 24 h at room temperature in media recommended by ATCC and each was added at 3 $x10^7$ cfu's/g dry wt to the inoculated microcosms. Microcosms contained inoculum but no HC, no inoculum and no HC, inoculum and HC and no inoculum and HC. Foil covered microcosms were incubated in the dark at 22°C. Duplicate

microcosms were sacrificed at 0, 7 and 14 days and stored at -20°C for DNA-probe and SLB analyses.

DNA Extraction and Purification. Soil DNA was extracted as described (Stephen *et al.*, 1996) with some modifications. Briefly, soil (0.5g) was mechanically extracted twice in 0.75 mL of 0.12M sodium phosphate buffer (pH 8), to which 0.5g of glass beads (0.17 mm dia.) and 0.5 mL of phenol/chloroform/isoamyl alcohol (25:24:1 ratio) was added. Crude DNA extracts were polyethylene glycol precipitated (1/10 vol. of 5M NaCl and 1 vol 30% PEG 8000) and glass milk purified (Leung *et al.*, 1995). Purified DNA was dissolved in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8), quantified by UV spectrophotometry at 260 nm, and stored at -20°C.

DNA Amplification/Probing. DNA primers for *alkB* (Kok et al. 1989), *xylE* (Nakai *et al.* 1983), and *pcpC* (Orser *et al.* 1993) were synthesised by PCR to amplify fragments of genes coding for alkane hydroxylase (382bp), catachol 2,3 dioxygenase, (309bp), and tetrachloro-hydroquinone dehalogenase (771bp), respectively. The PCR conditions were as follows; 2µL of sample DNA was used with 35 cycles each with 1 min denaturation (94°C), annealing (55°C), and extension (72°C). The PCR products were analyzed by agarose gel electrophoresis. Digoxigenin (DIG)-labeled gene probes were synthesised by PCR using a DIG labeling system (Boehringer Mannheim). Genomic DNA extracted from the inocula strains served as templates in the PCR labeling reactions for the *xylE, alkB*, and *pcpC* gene probes, respectively. Dot blot hybridization was performed according to the Boehringer Mannheim Genius System User's Guide. Chemiluminescence detection was performed by a Boehringer Mannheim DIG luminescent detection kit and recorded with a CCD camera.

Signature Lipid Biomarker Analysis. Samples were extracted using the modified Bligh and Dyer extraction (White *et al.*, 1979). Lipids were fractionated on silicic acid columns into neutral, glyco- and polar fractions (Guckert *et al.*, 1985). Neutral lipids were fractionated by thin layer chromatography and the diglyceride fraction recovered, transesterified, and the diglyceride fatty acid methyl esters (DGFA) analyzed by gas chromatography-mass spectrometry (GC/MS). The glycolipid lipid fraction was subjected to ethanolysis and the β-hydroxy acids from the poly β-hydroxyalkanoate (PHA) analyzed by GC/MS. The polar lipid fraction was subject to a mild alkaline transmethanolysis (Guckert *et al.*, 1985) prior to GC/MS analysis with the residue subjected to mild acid methanolysis for recovery of the plasmalogen derived dimethylacetals (DMA).

RESULTS AND DISCUSSION

The sensitivity of the analyses for gene probes was ~ 60, 60, and 630 pg genomic DNA/g dry soil for the *xylE*-, *alkA*-, and *pcpC* containing bacteria, respectively. The detection limit for the lipids was ~ 0.1 pmole PLFA (between 10^4-10^5 organisms the size of *Arthrobacter*). This soil contained between 13 and 23 nmoles of PLFA/g (4-16 x 10^8 cells/g; Figure 1) and 0.8 and 8.0 µg DNA/g (10^7 to 10^8 cells/g; Table 2).

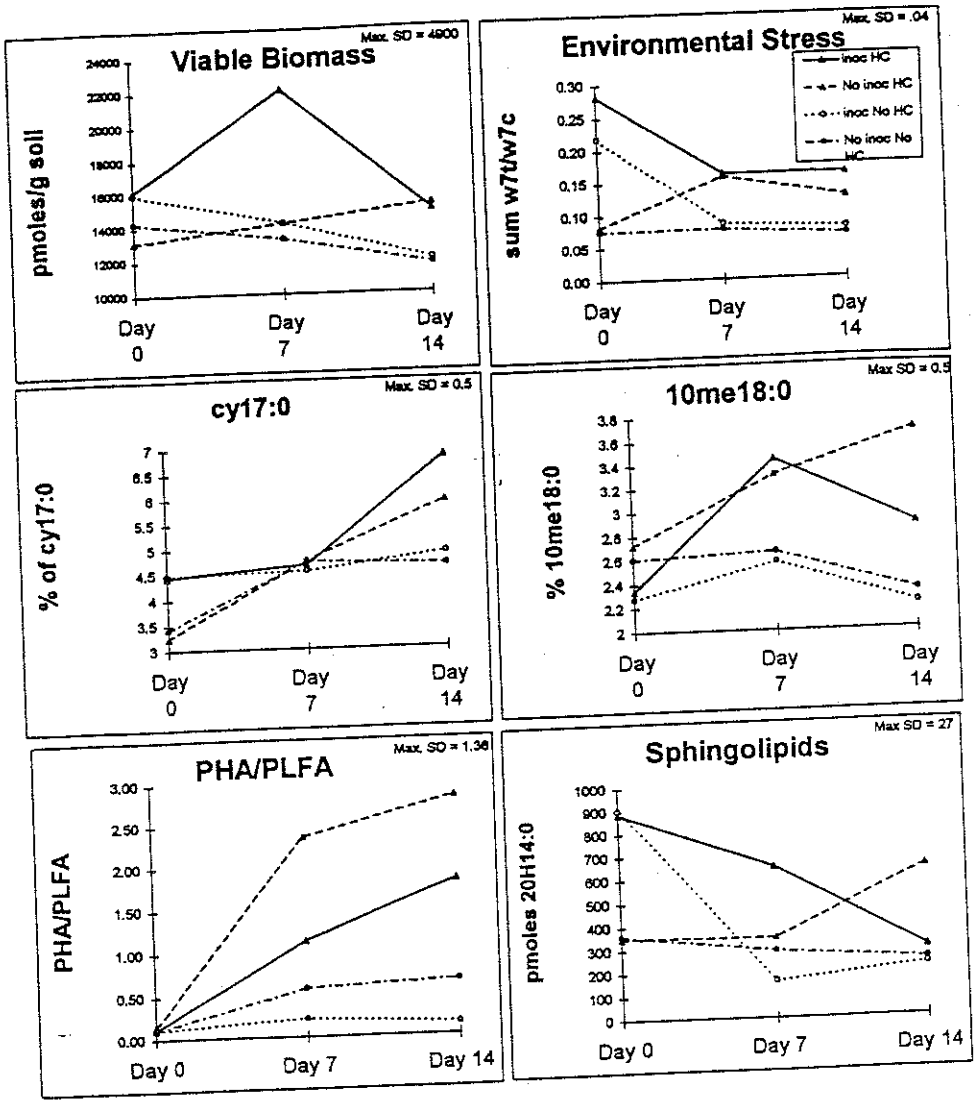

FIGURE 1. Comparision of lipid biomarkers over time

The SLB/gene probe polyphasic analysis shows the effect of HC exposure on both the indigenous microbiota and the inoculated soil microbial composition. Principal components analysis of the PLFA showed the specific PLFA that contributed most to the total variance between the communities after a 7 day incubation and indicates the distinctiveness of the communities resulting from the manipulations (data not shown). In all but the inoculated +HC soil, i15:0, (usually associated with Gram-positive aerobes), was prominent, whereas, in the inoculated +HC soil the community shifted to a PLFA pattern more typical of the Gram-negative heterotrophs.

In the inoculated +HC soil, viable biomass (PLFA) increased in the first 7 days, then decreased in contrast to the DNA which increased slightly between the 7 and 14 day exposure. There was an increase in the biomarker 10 Me 18:0 (actinomycetes), a decrease in environmental stress of Gram negative heterotrophs (decrease in the *trans/cis* monoenoic PLFA ratio), and an increase in the proportion of cyclopropane 17:0 (indicative of stationary phase growth in the Gram-negative heterotrophs). Aliphatic, aromatic and chlorinated HC degrading potential was maintained as indicated by the *alkB, xylE* and *pcpC* levels (Table1). Between days 7 and 14, the community in the inoculated +HC soil contained less actinomycetes, increased stationary phase cells, and less evidence of stress. No significant change was detected in the genetic potential as reflected in the proportions of *alkB, xylE,* and *pepC* or in the total DNA.

Microbes in the non inoculated +HC soil showed a lesser increase in viable biomass over days 2-7, but did not show decrease in viable bacteria over the 7 to 14 day period. The Gram negative bacteria showed increased environmental stress in the first week before levelling off in the second week. Gram negative bacteria showing stationary phase growth increased throughout the study as did the proportion of actinomycetes. Bacteria lacking an essential nutrient undergo unbalanced growth resulting in an accumulation of PHA (White et al., 1997). PHA accumulated more in the presence of HCs in the uninoculated soil than in the inoculated soil suggesting that the neceessary nutrients were added with the inoculum. In contrast to *xylE,* genes for which were detected in the uninoculated soil, the genes for alkane degradation (*alkB*) and chlorinated HC degradation (*pepC*) were not sufficiently prominent in the indigenous microbial community to appear following HC treatment.

Increased DNA was extracted from the inoculated soil lacking HCs although viable biomass decreased over the 14 days, however, there was an increase in the proportion of DGFA to PLFA (data not shown) indicating increased cell death. In the first week there was a decrease in environmental stress and no change in the proportion of cells in stationary phase, actinomycetes or in the PHA/PLFA ratio. The potential for chlorohydrocarbon degradation (*pcpC* genes) decreased slightly, while that for aromatic degradation (*xylE*) remained constant, and that for aliphatic degradation (*alkB*) decreased sharply suggesting selection against these bacteria in the absence of HCs.

The uninoculated untreated soil showed little change in community composition. The viable biomass decreased slightly whereas the total extractable

DNA increased 5-fold again reflected in an increased DGFA/PLFA ratio (data not shown).

Sphingomonas contain important biodegredation potential for refractory pollutants and heavy metal immobilising exopolymers (White et al 1996c) and can be detected by the presence of amide linked 2-0H fatty acids. Indigenous *Sphingomonas* increased in the HC treated uninoculated soil (Figure 1). When inoculum was present (with/without HC), there was a rapid decrease in the proportion of *Sphingomonas* signature amide linked 2-0H 14:0 reflected in the loss of *pcpC* in the inoculated but untreated soil. Unless HCs were added without the competition from the inoculum, *Sphingomonas* was out competed by other indigenous heterotrophs.

CONCLUSIONS

The polyphasic SLB/Gene probe analysis provides a comprehensive, quantitative means to monitor bioremediation effectiveness by asking the customers (i.e. the microbes) if the conditions are proper for their effective activity.

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TABLE 1. Effect of HC (HC) contaminants on degradative bacteria in natural (-I) and inoculated (+I) soil[a].

Treatment	Degradative bacterial population, log (fg DNA /g dry soil)		
	Day 0	Day 7	Day 14
	alkB-containing bacteria		
-I, -HC	<4.8	<4.8	<4.8
-I, +HC	<4.8	<4.8	<4.8
+I, -HC	9.1±1.1[b]	5.2±0.3	<4.8
+I, +HC	8.5±2.0	6.0±0.0	6.6±0.3
	xylE-containing bacteria		
-I, -HC	5.1±0.2	<4.8	<4.8
-I, +HC	5.0±0.3	6.7±0.8	6.5±0.6
+I, -HC	7.4±0.5	6.6±1.0	7.0±0.5
+I, +HC	7.3±0.9	6.8±0.7	6.6±0.1
	pcpC-containing bacteria		
-I, -HC	<5.8	<5.8	<5.8
-I, +HC	<5.8	<5.8	<5.8
+I, -HC	9.0±1.0	8.3±0.2	5.7 ±0.2
+I, +HC	8.8±0.5	8.6±0.2	7.3±0.2

[a] Cell densities of the degradative bacteria were determined by quantifying the amount of PCR-amplified DNA fragments using specific DIG-labeled chemiluminlescent gene probes; [b] Standard derivation at 95% confident level.

TABLE 2. Effect of HC contaminants on total soil DNA.

Treatment[a]	Total soil DNA (μg/g dry soil)		
	Day 0	Day 7	Day 14
-I, -HC	0.8±0.8	3.8±1.7	5.1±0.4
-I, +HC	1.4±1.0	4.3±3.5	7.1±1.7
+I, -HC	3.6±1.7	6.0±1.7	6.7±0.3
+I, +HC	4.2±0.8	7.9±1.4	8.3±1.0

[a] Indicated in Table 1.