Microbial Population Changes during Bioremediation of an Experimental Oil Spill

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ABSTRACT

A field experiment was conducted in Delaware (USA) to evaluate three crude oil bioremediation techniques. Four treatments were studied: no oil control; oil alone; oil + nutrients; and oil + nutrients + an indigenous inoculum. The microbial populations were monitored by standard MPN techniques, PLFA profile analysis, and 16S rDNA DGGE analysis for species definition. Viable MPN estimates showed high but steadily declining microbial numbers and no significant differences among treatments during the 14-weeks. Based on the PLFA results, the communities shifted over the 14-week period from being composed primarily of eukaryotes to Gram-negative bacteria. The Gram-negative communities shifted from the exponential to the stationary phase of growth after week 0. All Gram-negative communities showed evidence of environmental stress. The 16S rDNA DGGE profile of all plots revealed eight prominent bands at time zero. The untreated control plots revealed a simple, dynamic dominant population structure throughout the experiment. The original banding pattern disappeared rapidly in all oiled plots, indicating that the dominant species diversity changed and increased substantially over 14 weeks. Nutrient addition and the addition of the indigenous inoculum altered the nature of this change.

Introduction

Analysis of the microbial communities that take part in *in situ* hydrocarbon biodegradation activities has been a challenge to microbiologists for years because most (~90-99%) of the species diversity making up competent degrading communities do not form colonies under current laboratory conditions [5, 6, 10]. Phospholipid fatty acid (PLFA) or signature lipid biomarker (SLB) measurement, together with nucleic acid-based molecular techniques for fingerprinting the 16S rDNA component of microbial cells, has become a powerful combination of techniques for elucidating the microbial ecology of actively bioremediating communities. The SLB methods measure the lipid profiles of microbes in the environment irrespective of culturability, thereby avoiding culture bias. These methods provide insight into several important characteristics of microbial communities such as viable biomass, community structure, and nutritional status [10].

Microbial communities within long-term contaminated systems tend to be dominated by those organisms capable of utilizing and/or surviving toxic contamination. As a result, these communities are typically less diverse than those in non-impacted systems. Using molecular markers such as DNA and signature lipids, the microbial community of such

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systems can be assessed *in situ*. However, when Gram-negative bacteria dominate the system, as is usually the case in hydrocarbon-contaminated environments, the insight gained from lipid analysis primarily concerns nutritional or physiological status with little differentiation among species. A complementary method by which such a shift in the microbial community structure can be monitored in detail is denaturing gradient gel electrophoresis (DGGE). This method makes use of the 16S rDNA molecule carried by all bacteria, the sequences of which provide molecular markers for species identification.

This study was undertaken to gain novel insight on the progress of natural and accelerated (by the addition of nutrients and/or an inoculum of hydrocarbon degrading bacteria) attenuation during a controlled oil spill field experiment in Delaware [8]. Frozen samples from the field study were extracted and analyzed for PLFA and 16S rDNA DGGE profiles. Results were used to determine how the degrading and non-degrading communities changed during the course of the 14 week experimental investigation, and whether the community structure of the site was returning to the background control structure by the end of the test period.

Materials and Methods

Experimental Design

The experiment was a randomized complete block design. Five replicate blocks of beach were marked off, each large enough to accommodate four experimental units or test plots. Treatments consisted of a non-oiled control, a natural attenuation control (oiled with no amendments), an oiled treatment receiving nutrients, and an oiled treatment receiving nutrients and an indigenous inoculum of hydrocarbon-degrading microorganisms derived from the site. The four treatments were randomized in each of the five blocks. The four plots in each block were separated from each other by a 10 m buffer zone to mitigate transfer of amendments from one plot to another. Each plot was divided into four equal horizontal sectors to evaluate the effect of position within the intertidal zone. Slickbar™ containment booms were placed around the plots to contain the oiled sand within the plots and minimize edge effects. Oil was applied on 1 July 1 1994, and the experiment began four days later (defined as day 0). Mineral nutrients (sodium nitrate and sodium tripolyphosphate) were dissolved in seawater and applied daily via a sprinkler system to maintain a threshold level of nitrogen (approximately 1.5 mg nitrate-N/L interstitial pore water) at all times. The nutrients added to each of the ten designated reservoirs consisted of 2-kg technical grade $NaNO_3$ (330 g nitrogen) and 128 g $Na_5P_3O_{10}$. Although several-fold higher levels were achieved than on the untreated plots, the natural levels of nitrate due to agricultural runoff on the Delaware Bay shoreline were high enough (average 0.82 mg/L) to sustain a significant intrinsic rate of biodegradation. For the no-nutrient control plots, only seawater was applied through the sprinkler system. Bonny light crude oil, previously weathered by aeration for two days, was applied at the rate of 136 L/plot, resulting in a calculated crude oil contamination level of approximately 5 g/kg sand.

Inoculum Preparation

The original culture consisted of a mixed consortium isolated from the same beach several months prior to the experiment and grown in the laboratory on the same Bonny Light crude oil. The indigenous inoculum was prepared by adding a portion of this culture into two 210 L stainless steel drums containing 170 L of seawater from Delaware Bay, the

weathered Bonny Light crude oil (600 mL), and the same nutrients as used on the beach. These enrichments were grown for two weeks. To allow weekly inoculation with fresh two-week cultures, each drum was offset in time from the other by one week. Once a week, 30 L of a suspended mixed population of hydrocarbon degrading bacteria was added to each of the inoculum plots.

Sampling

For hydrocarbon measurement, samples were collected from four separate sectors of each plot during each sampling event. Samples were collected in soil corers every two weeks for 14 weeks. For PLFA and DGGE analysis, the four samples from each plot were composited, frozen on dry ice, shipped to Cincinnati, and archived at -70 °C until the analyses were performed. Only samples from weeks 0, 8, and 14 were analyzed for 16S rDNA, with samples from 0, 2, 4, 8, 10 and 14 weeks analyzed for PLFA.

MPN Analysis

Sediment subsamples from each plot were placed in Whirlpak bags, brought back under ice to the on-site mobile laboratory trailer, and immediately processed for Most Probable Number (MPN) analysis of alkane and PAH degrading bacteria [11].

Petroleum Analyses

Sand samples from the field were collected every 14 days, frozen on dry ice, and shipped to Cincinnati for processing. The sand was mixed with an equal volume of anhydrous Na_2SO_4, the mixture extracted by sonicating three times for ten minutes with dichloromethane (DCM), and the final DCM extract was solvent-exchanged to hexane [8]. A Hewlett-Packard 5890 Series II gas chromatograph equipped with a HP 5971A Mass Selective Detector (MSD) was used for measuring the oil analytes. The MSD was operated in the selected ion monitoring (SIM) mode for quantifying specific saturated hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), and sulfur heterocyclic constituents. Operating conditions of the GC/MS instrument are described elsewhere [8]. Nitrate was analyzed by the cadmium reduction method [1] using an autoanalyzer (Technicon Instruments Corp., Tarrytown, NY).

PLFA Analysis

Lipids were analyzed using the modified Bligh and Dyer method [2, 7]. Briefly, samples were extracted in separatory funnels containing a single phase system (chloroform:methanol:phosphate buffer; 1:2:0.8 v/v/v) for a minimum of four hr before adding chloroform and deionized water (final solvent ratios, 1:1:0.9 v/v/v) to form two phases. The phases were allowed to separate for 24 hr, and the lipid phase dried by rotary evaporation. The lipids were fractionated into neutral-, glyco-, and polar-lipids on silicic acid columns as described [3]. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane [3]. PLFA was analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry (GC/MS). Fatty acids were identified by relative retention times and mass spectra.

DGGE Analysis

PCR amplification of 16S rDNA gene fragments was performed as described by Muyzer, et al. [4] with modifications. Thermocycling consisted of 35 cycles performed on a "Robocycler^{TM}" PCR block. DGGE used a D-Code 16/16 cm gel system maintained at a

constant temperature in TAE buffer. Gradients were formed between 20 and 55% denaturant with the gels stained in ethidium bromide. Gel images were captured by use of an Alpha Imager™ system. The central portion of each band of interest, which contain specific sequences, were excised and soaked in purified water. The products were re-amplified, purified by gel electrophoresis and isolated by use of a Gene-Clean™ kit. Purified DNA was sequenced with an ABI-Prism automatic sequencer model 373. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.htm).

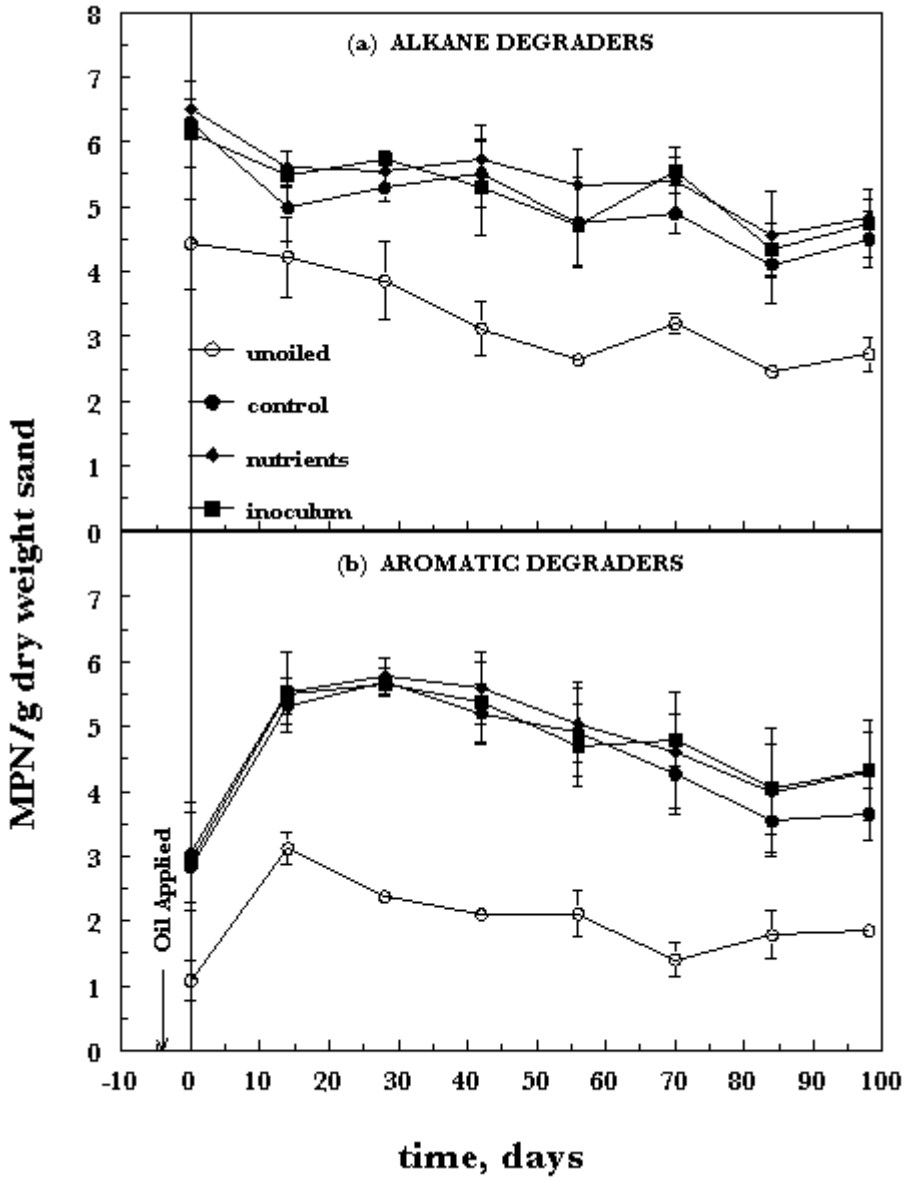

Fig. 1. Temporal changes in the MPN densities of the alkane and PAH degraders.

Results and Discussion

Biodegradation

The nitrogen concentrations naturally present along the coast of Delaware Bay were high enough to sustain rapid natural attenuation rates in the unamended plots. Despite the high intrinsic biodegradation rate in the controls, results from the biweekly samplings indicated that both the alkane and the aromatic biodegradation rates in the nutrient- and inoculum-treated plots were significantly greater than the unamended control (see [8] for a detailed discussion), but were not significantly different from each other.

MPNs

Data showing the changes in the densities of alkane and PAH degraders are summarized in Fig. 1 [8, 9]. The alkane degraders were already at their maximum carrying capacity at T_0 (which is defined as four days after oil had been applied to the plots, when nutrients were first added). They slowly declined over the course of the next 14 weeks, and there were no significant differences among the three treatments. The PAH degraders increased about three orders of magnitude within two weeks after the experiment was started, but they also slowly declined with time thereafter.

Hierarchical Cluster Analysis of PLFA profiles

PLFA patterns indicated that the microbial communities were predominantly eukaryotic at T_0 but shifted rapidly to Gram-negative prokaryotic by week 2 and thereafter. This eukaryote PLFA was interpreted to have been caused by the predominance of eggs

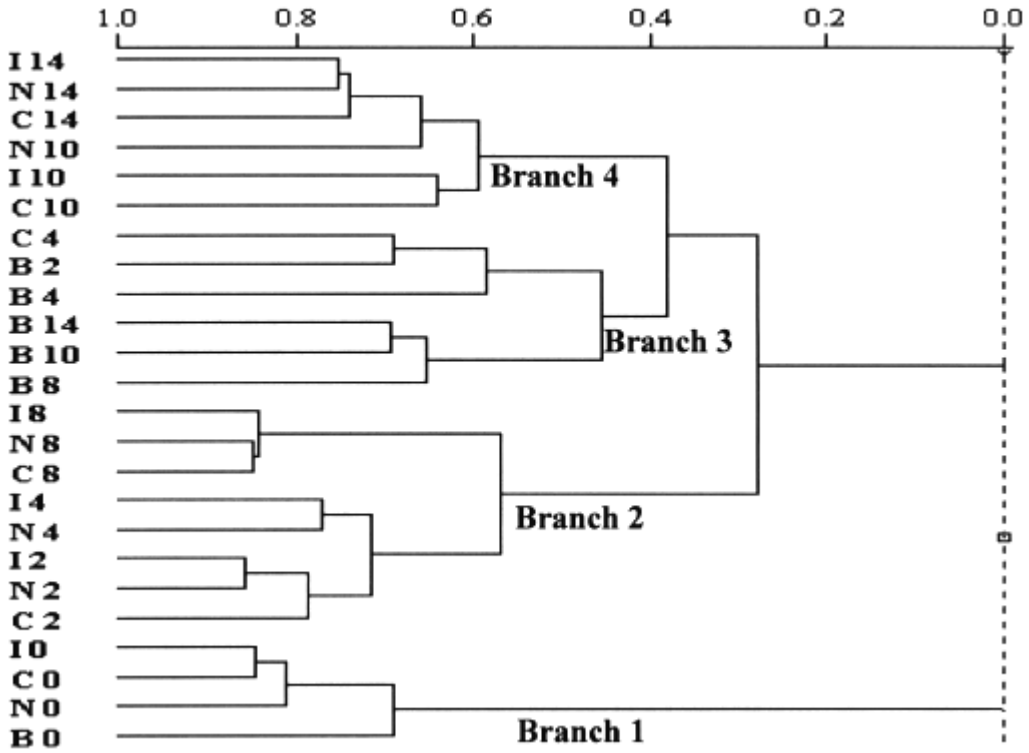

Fig. 2. A dendogram representation of a hierarchial cluster analysis of the PFLA profiles (B = background; C = oiled control; N = nutrient treatment; I = nutrient and inoculum treatment).

deposited by gravid horseshoe crabs in the early months of summer (May and June). Using Hierarchical Cluster Analysis (HCA), similarities between samples were calculated and compared. The primary purpose of HCA is to present data in a manner that emphasizes natural groupings. The results of the HCA analysis are given in the form of a dendrogram. The HCA showed distinct groupings throughout the project (Fig. 2). All week 0 samples clustered within Branch 1 indicating little or no difference between treatments at this time point. Natural attenuated, nutrient-amended, and inoculum-amended plots from weeks 2, 4 and 8 clustered together (Branch 2), with the remaining background samples clustering at Branch 3. Branch 4 shows clustering of the natural attenuation, nutrient-amended, and inoculum-amended plots for weeks 10 and 14.

These findings suggest first that the communities present at T_0 were essentially the same regardless of their exposure to crude oil. This implies that at T_0 the hydrocarbon degraders detected by MPN analysis (Fig. 1) formed only a minor fraction of the total biomass, otherwise the HCA analysis would not have given similar clustering patterns at that time.

Branch 2 was composed of PLFA profiles from all oiled plots at weeks 2, 4, and 8. After week 8, however, a major change took place in microbial community composition giving rise to a different community structure on the oiled plots as shown by the single branch (Branch 4) containing the week 10 and 14 oiled samples. During this time period climatic changes had occurred (temperature declines and storms) that might have influenced this population shift.

DGGE

Fig. 3 summarizes the DGGE gel patterns of the various treatments on the Delaware shoreline. At T_0, all the sites demonstrated simple eubacterial community structures (eight prominent bands), in agreement with the PLFA data that suggested the communities did not change during the first 4 days of exposure to oil. Sequence analysis of the excised bands suggested that they were derived from species related to cultured pseudomonads, planococci, and cytophagae. The unoiled background site maintained a structure of a few dominant bacteria through week 8. At week 14 three further species were detectable,

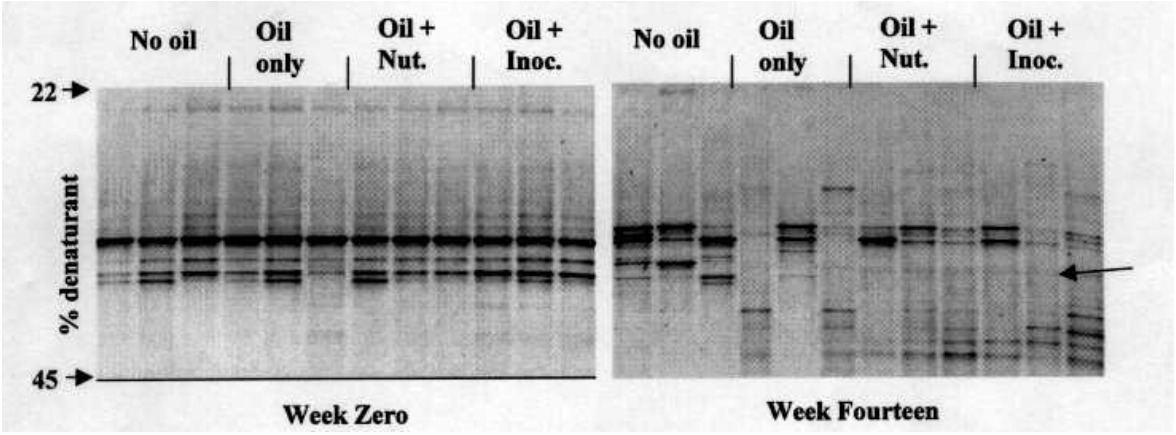

Fig. 3. DGGE Gel patterns in the four Delaware Treatments at weeks 0 and 14 (Nut. = nutrient treatment; Inoc. = nutrient and inoculum treatment). The three lanes under each treatment signify the three independent replicate samples.

related to the planococci, cytophagae, and the low-GC Gram-positive phylum. The communities of all the oiled sites regardless of treatment changed by week 8 to being much more diverse in nature such that most of the original species were undectectable (data not shown). By week 14, the microbial community was highly diverse and few of the original species were abundant enough to be detected. There were no obvious differences between the nutrient-amended and inoculum-amended plots, but a single band (indicated by an arrow), which was absent from all unamended oiled plots, appeared consistently in both.

Conclusions

The GC/MS analyses of the oil analytes were able to detect significant differences in rates of biodegradation between the amended and unamended natural attenuation plots but not between the nutrient and inoculum plots [8]. The PLFA data indicated that the communities shifted from being eukaryote biomass dominated at week 0, to being dominated by Gram-negative bacteria at week 14. Despite this large shift in biomass, the HCA indicated that the microbial communities from the unoiled plots at weeks 2-14 had significantly different PLFA "fingerprints" to those obtained from any of the amended and unamended oiled plots. Based on the DGGE results, all sites at T_0 were dominated by a few eubacterial community members. The community structures of the unoiled plots were unchanged through week 8, but three additional species of bacteria were detectable by week 14. Species diversity in all oiled treatments increased substantially by week 8 and continued to change until week 14. Community changes over the 14 weeks studied varied considerably within each of the four treatments, but some changes were specific to treatment. No effect of the inoculum was detected by any of the analytical methods used in this study.

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