Characterization of Bacterial Communities in Capillary Fringe Soil Samples During Phytoremediation Using Terminal Restriction Fragment (TRF) Pattern Analysis

By
Kate Smith

Biological Sciences Department
College of Science and Mathematics
California Polytechnic State University
San Luis Obispo

Abstract

The goal of this study was to determine whether or not plant roots had an affect upon the bacterial communities in the soil. To determine this, samples were obtained from two phytoremediation sites at the Guadalupe dunes (Guadalupe, CA) where willows and cottonwoods were planted for bioremediation of dissolved petroleum in the ground water plume. The samples were taken from soil at the capillary fringe of the two phytoremediation sites. The samples were examined for the presence of roots, which were then removed from the samples and weighed to determine the percentage of roots in each sample. The number of aerobic heterotrophic bacteria from each sample was determined through plate counts. Counts were compared and statistically analyzed to show that plant material lead to increased numbers of aerobic heterotrophic bacteria. In addition the taxonomic composition of the bacterial communities in the samples, was analyzed using 16S rDNA Terminal Restriction Fragment (TRF) patterns. DNA was extracted from the soil and amplified by PCR using primers homologous to conserved regions in the eubacterial 16S ribosomal gene; the forward primer was fluorescently labeled. The PCR amplicons were digested with the restriction enzyme *Dpn*II. The resulting fluorescently labeled terminal restriction fragments (TRFs) were separated and detected by capillary electrophoresis to create TRF patterns. Comparison of TRF patterns from samples with and without plant roots, showed that the roots had a detectable affect on the taxonomic composition of the soil bacterial communities. TRF peaks representing bacteria with known petroleum degrading capacity, were present in higher proportion in the TRF patterns from soils with root material. This indicates that the trees used in the phytoremediation project are enhancing the degradation of petroleum in the groundwater by encouraging the proliferation of the appropriate bacteria.

I. Introduction

Hydrocarbons are one of the most common types of environmental pollutants found in the soil and groundwater. With the vast amount of oil exploration, transport and storage that has occurred over the last century, most of the hydrocarbon contamination that exists today can be blamed on the spills and leaks of oil companies. Due to these spills and leaks, there have been more sites identified with petroleum hydrocarbon contamination than any other type of contamination. These sites vary from large oil spills to the much more common leaking underground storage tank sites associated with many gas stations (1). With the large amount of pollution resulting from petroleum hydrocarbons, the cleanup of the contamination has become a topic of interest. Following realization of the enormous size of the hydrocarbon contamination problem, remediation approaches including soil excavation, and groundwater pump and treat, were put to use. However, these approaches were costly, environmentally detrimental and unsuccessful at recovering all of the contamination. Therefore other remediation approaches including bioremediation began to be investigated.

Bioremediation is a process through which hydrocarbons in soil and water can be naturally degraded by bacteria, which use the hydrocarbons as a source of carbon and other nutrients. Phytoremediation is a form of bioremediation, which incorporates trees and plants into the remediation process. Plants are able to enhance the removal of contaminants through direct uptake and stimulation of microbial activity (2). This study focused on the stimulation of microbial activity by the plants. It is believed that plants stimulate the bacteria due to the symbiotic relationship which they frequently form with each other; the bacteria benefit from the plants which leak organic nutrients and often result in an increased number of microorganisms near the plant roots (2). The release of organic nutrients by the plants may stimulate and select

for certain organisms and possibly lead to the proliferation of desirable phenotypes in the root zone which may be conducive to enhanced contaminant removal capabilities (2). Therefore, the effect of plant material on bacterial communities is an important topic to investigate in order to determine whether or not bacterial communities change in the presence of plant material.

The soil samples analyzed in this study were taken from the capillary fringe of the C8 and O13 phytoremediation sites at the Guadalupe oil field (Guadalupe, California). Willows and cottonwoods of varying diversities were planted in these phytoremediation sites for the bioremediation of dissolved petroleum in the groundwater plume. This area in Guadalupe, was used by the Union Oil Company of California (Unocal) as an oil field from 1946 to 1994. During this time period, diluent (a light petroleum distillate), was used to dilute the thick crude oil in order to ease pumping, leaked from the pipelines into the surrounding soil and groundwater. In an effort to clean up the petroleum hydrocarbons, various treatment programs including phytoremediation were started by Unocal with the aid of the Environmental Biotechnology Institute at Cal Poly State University in San Luis Obispo.

The goal of this project was to determine whether or not plants could stimulate and diversify the bacterial community structure and thereby aid in the increased rate of diluent degradation. In order to determine whether or not plant material affected the bacterial populations, the samples were examined for the number and type of bacteria present. The number of aerobic heterotrophic bacteria present was determined through plate counts. The taxonomic composition of the bacterial communities in the samples was analyzed using 16S rDNA Terminal Restriction Fragment (TRF) patterns. TRF patterns can be used to track the dynamics of specific populations of bacteria in complex communities (3). The development of a TRF pattern involves the initial extraction of DNA from a sample, PCR amplification using a

fluorescently labeled forward primer homologous to the eubacterial 16S rDNA, restriction digestion with a restriction endonuclease, ethanol precipitation to remove excess reagent, the separation of TRF fragments by capillary gel electrophoresis, and laser detection. The pattern which results is referred to as an electropherogram and is analyzed and compared to a database of known endonuclease cut sites, to determine the relative abundance and diversity of the bacteria in each sample.

II. Materials and Methods

Analysis of plant material in the soil

To determine the percentage of plant material in the samples, roots and other plant materials were separated from the soil. The soil samples were dried and weighed and then sifted to remove plant material. Any plant material that passed through the sifting apparatus was recovered by placing the sifted soil into beaker of water and collecting any plant material that floated to the top. The plant material was removed and then dried and weighed and the percentage of plant material in each soil sample was determined.

Aerobic Heterotrophic Bacteria Quantification

To determine the number of aerobic heterotrophic bacteria present, ten fold dilutions of each soil sample were made in Bushnell-Haas media and the 10^{-2} through 10^{-5} dilutions were plated on R_2A media. The colonies were counted following both one and two weeks of incubation. The CFU/gram of soil was calculated from these plate counts.

DNA Extraction

DNA was extracted from the soil samples according to the procedure accompanying the UltraClean® MoBio Soil DNA Kit (MoBio Laboratories Inc., Solano Beach CA.)

DNA Quantification

To measure the concentration of DNA obtained from the samples, 1:20 dilutions of the samples were made. The DNA in the diluted samples was quantified using a SPECTRAmax UV spectrophotometer® (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA.).

PCR Amplification

PCR was performed with the use of two primers homologous to the highly conserved region of the eubacterial 16S rDNA. The forward primer Ba2F (5'-GCY TAA CAC ATG CAA GTC GA-3'), was fluorescently labeled with phosphamide dye. The reverse primer used was K2R (5'-GTA TTA CCG CGG CTG CTG G-3'). The reactions were carried out using 1uL of 1 ng/uL dilutions of extraction product, 5 uL of 10X Buffer, 3 uL of 10 mM DNTP, 2 uL 20 ug/mL BSA, 7 uL 25 mM $MgCl_2$, 1 uL K2R, 1 uL Ba2F, 29.7 uL water, and 0.3 uL 5 U/ul TaqGold®. All reactions were performed in triplicate and placed in a thermocycler and heated to 94 °C for 10 min followed by 35 cycles of 94 °C for 1 min., 46.5 °C for 1 min., and 72 °C for 2 min., ending with 72 °C for 10 min.

Clean-up of PCR Products

To confirm successful PCR a gel electrophoresis of the PCR products was performed. The PCR products were run on a 1.5% agarose gel in TBE buffer (89.2 mM Tris, 88.9 mM boric acid, 2.47 mM disodium EDTA). To each well 3 uL of the PCR product/DNA was added with 1 uL of 5X loading buffer. The gel was stained using ethidium bromide and visualized using the Bio-Rad gel Doc system. Following confirmation of PCR product, the PCR reactions were cleaned up using the MoBio UltraClean® PCR Cleanup Kit following the manufacturer's protocol. The DNA in each sample was again quantified using the Spectramax spectrophotometer.

Enzyme Digestion

An enzyme digest was performed on the cleaned up PCR products using the New England Biolabs restriction endonuclease *Dpn*II. Each digestion reaction included 6 units of *Dpn*II, 4 uL of *Dpn*II buffer and 75 ng of DNA. The samples were digested at 37 °C for 4 hours and inactivated for 20 min. at 65 °C.

Ethanol Precipitation of Digest

The digestion reactions were precipitated to remove the buffer and restriction enzymes from the samples. Each reaction was mixed with 100 uL of cold 95% ethanol, 2 uL of 3 M sodium acetate (pH 4.6) and 1 uL of glycerol and incubated at 4 °C for 30 minutes. Following incubation the sample was centrifuged for 30 min. at 3490 RPM to pellet the DNA. The pellet was then washed in 100 uL of cold 70% ethanol followed by centrifugation for 15 minutes at 3490 RPM. The ethanol was removed by inverting and the sample was centrifuged inverted, for 1 min. at 700 RPM to dry the pellet.

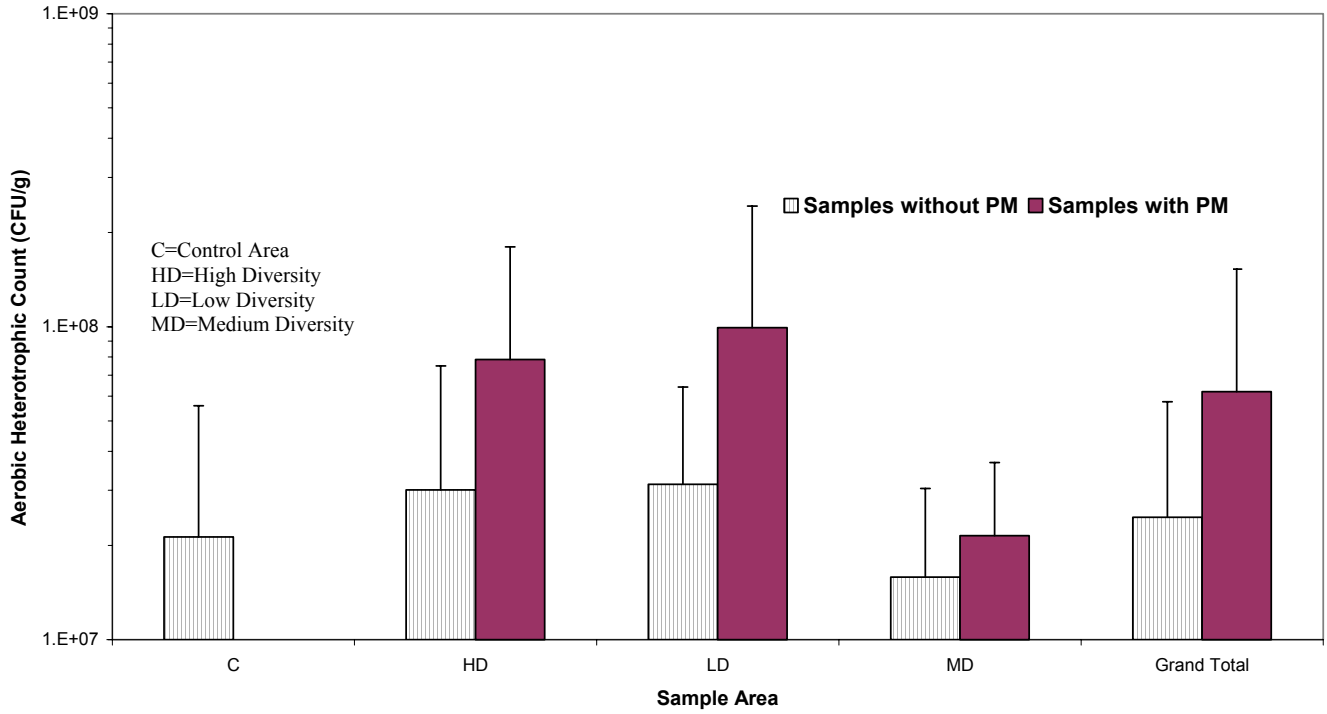
Capillary Electrophoresis

The DNA pellets were resuspended in 20 ul of formamide and 0.25 ul of CEQ 600 bp standard, one drop of mineral oil was added to each sample to prevent evaporation. The samples were then run in the Beckman Coulter CEQ 8000 Genetic Analysis System.

III. Results and Discussion

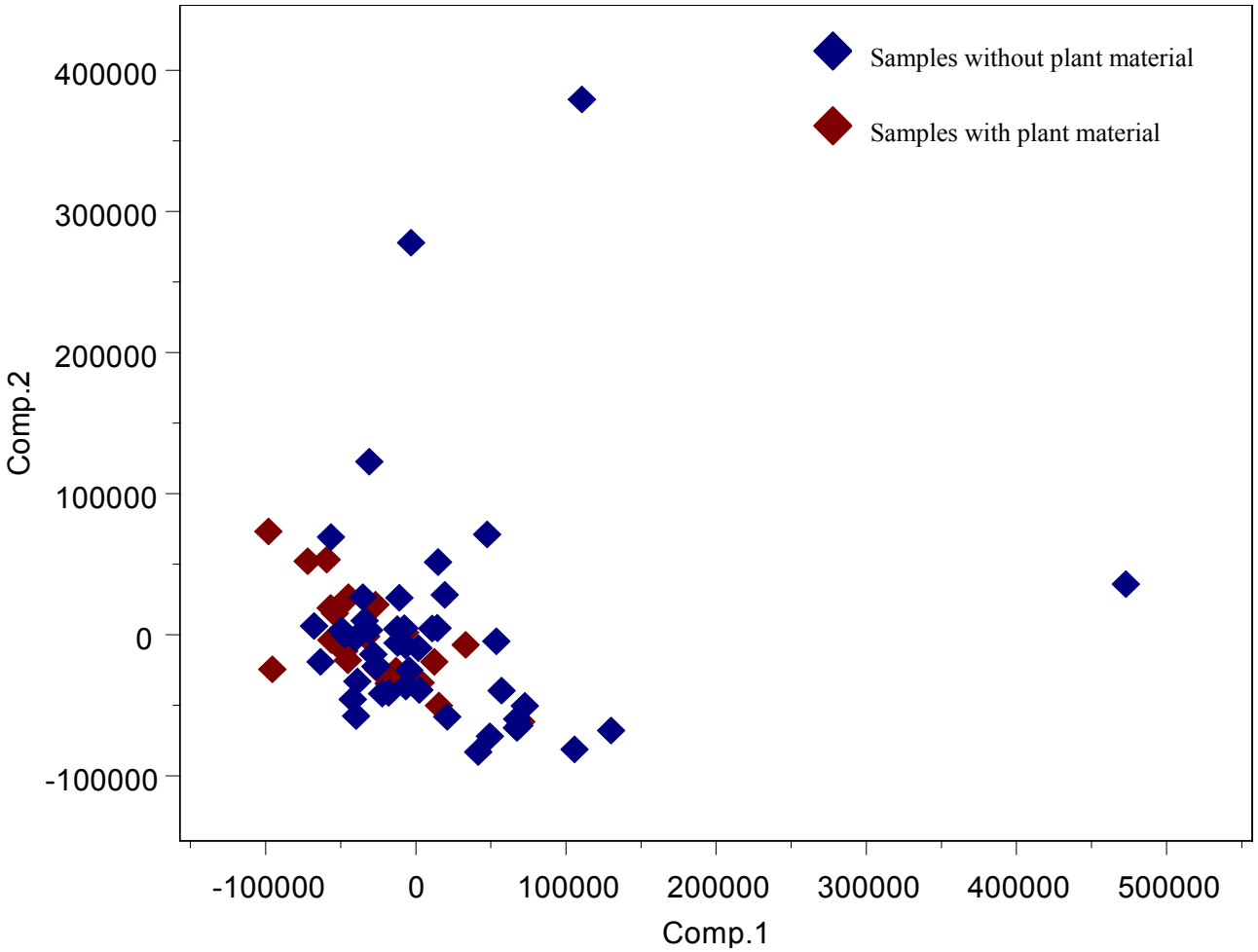
Aerobic heterotrophic bacterial counts were generated from the R_2A plate counts. The bacterial counts were compared between all samples as well as between samples from the same area. It was concluded with statistical significance (p-value=0.04), that for the comparison of all samples, the samples with plant material had higher numbers of aerobic heterotrophic bacteria than those samples without plant material (Figure 1). Therefore it can be said that plant material lead to increased numbers of aerobic heterotrophic bacteria.

Figure 1: Effect of Plant Material (PM) on Bacterial Counts



TRF patterns generated from the soil samples were compared and statistically analyzed to illustrate the differences in the taxonomic composition of samples with and without plant material. Principal components analysis was performed on the TRF patterns in order to compare the taxonomic composition of the samples. "Principal Components Analysis (PCA) is a multivariate statistical method that determines the primary sources of variation in a dataset and reduces the dimensionality by creating linear combinations of variables that best preserves the overall variation in the dataset, referred to as Principal Components (PC)"(4). Principal components analysis indicated a separation between the bacterial communities of the two sample types along component 1 (p-value=0.004) (Figure 2).

Figure 2: Principal Components Analysis of TRF Patterns



From the Principal Components Analysis, it was concluded that there was a difference in the overall taxonomic composition of the two sample types. In order to examine the specific differences and to determine whether or not plant material had an effect upon the abundance of individual peaks, a T-test was performed for each individual TRF peak. Following statistical analysis it was concluded that there was a significant difference between samples, with and without plant material, for the peaks located at 201bp (p-value= 0.006), 205bp (p-value=0.000), 214bp (p-value=0.017), 230bp (p-value=0.013), and 264bp (p-value=0.019)(Figure 3). These peaks were much more abundant in those samples with plant material than those without (Figures 4&5). Therefore, it can be concluded that plant material enhances bacteria associated with those peaks.

Figure 3: Relative Abundance of *Dpn*II TRF Peaks

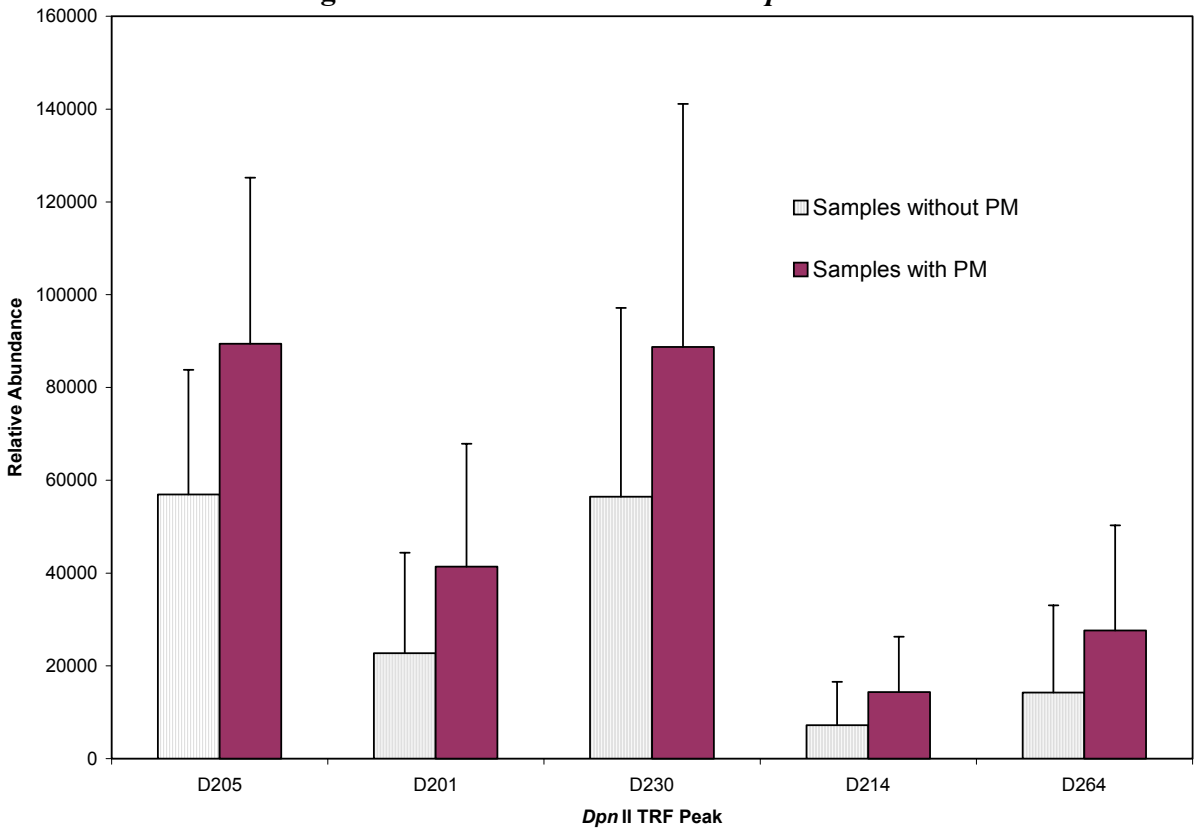
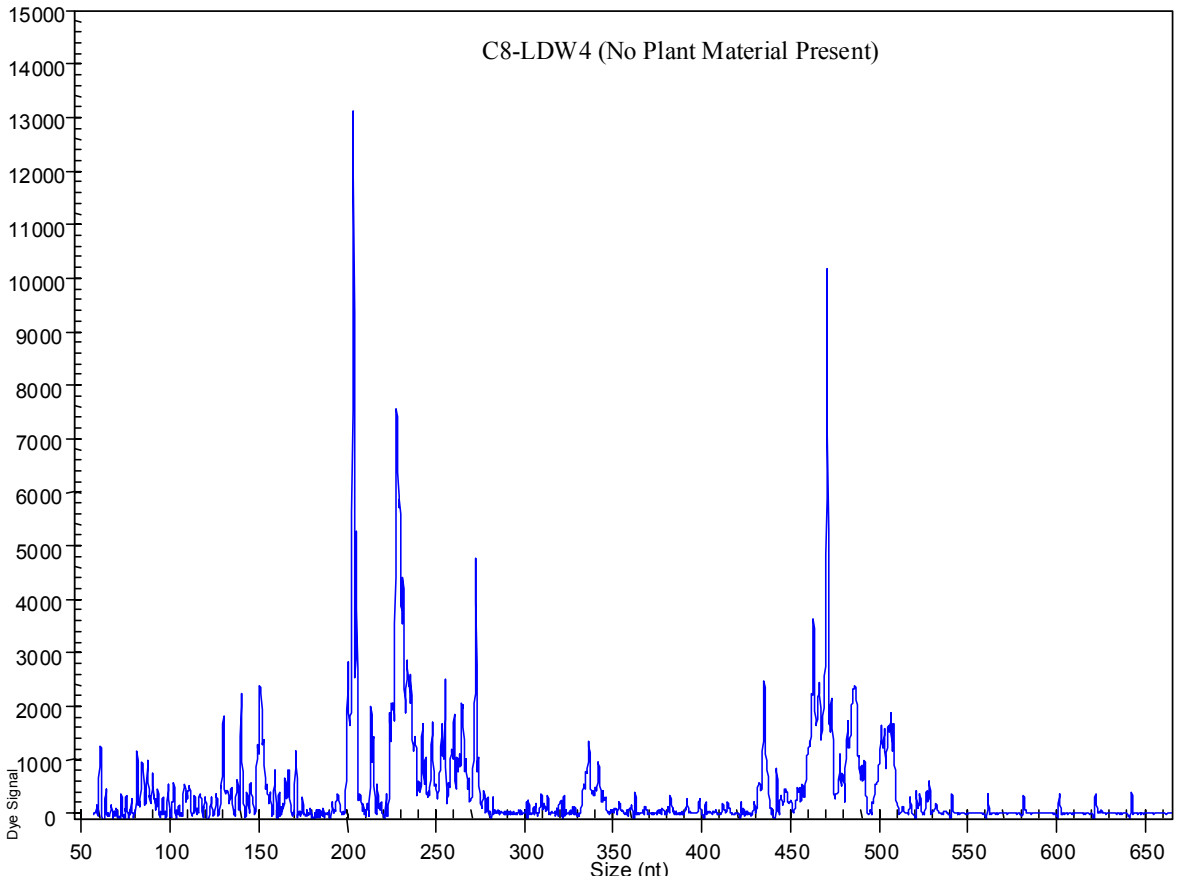


Figure 4: C8 TRF Patterns



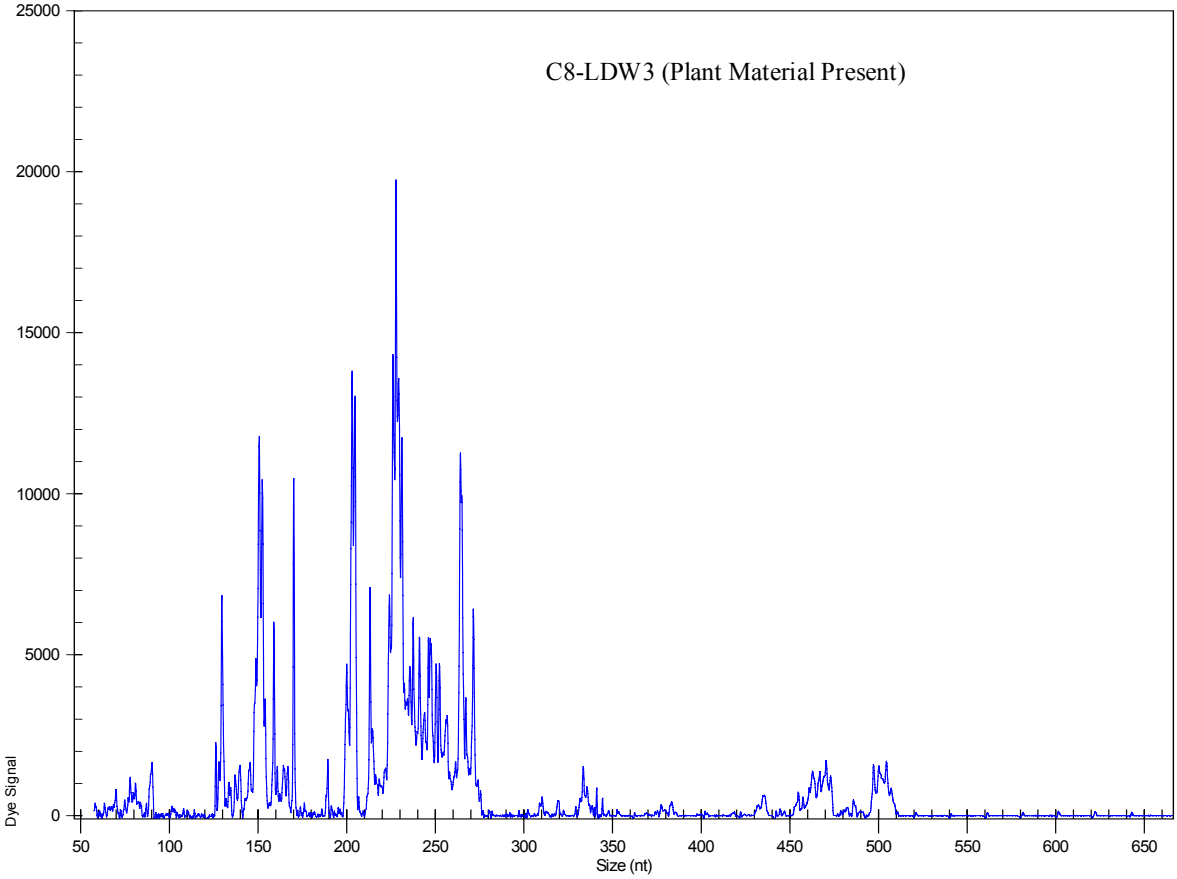
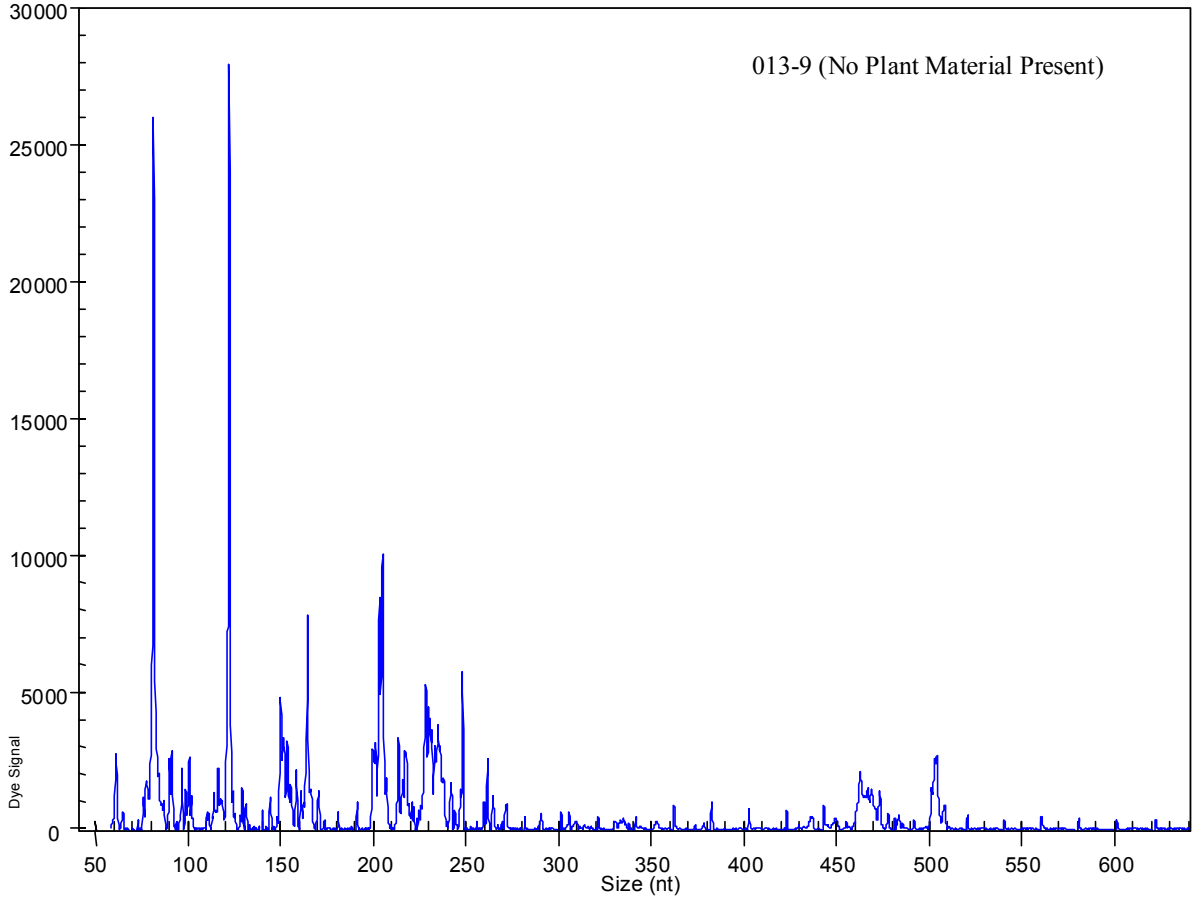
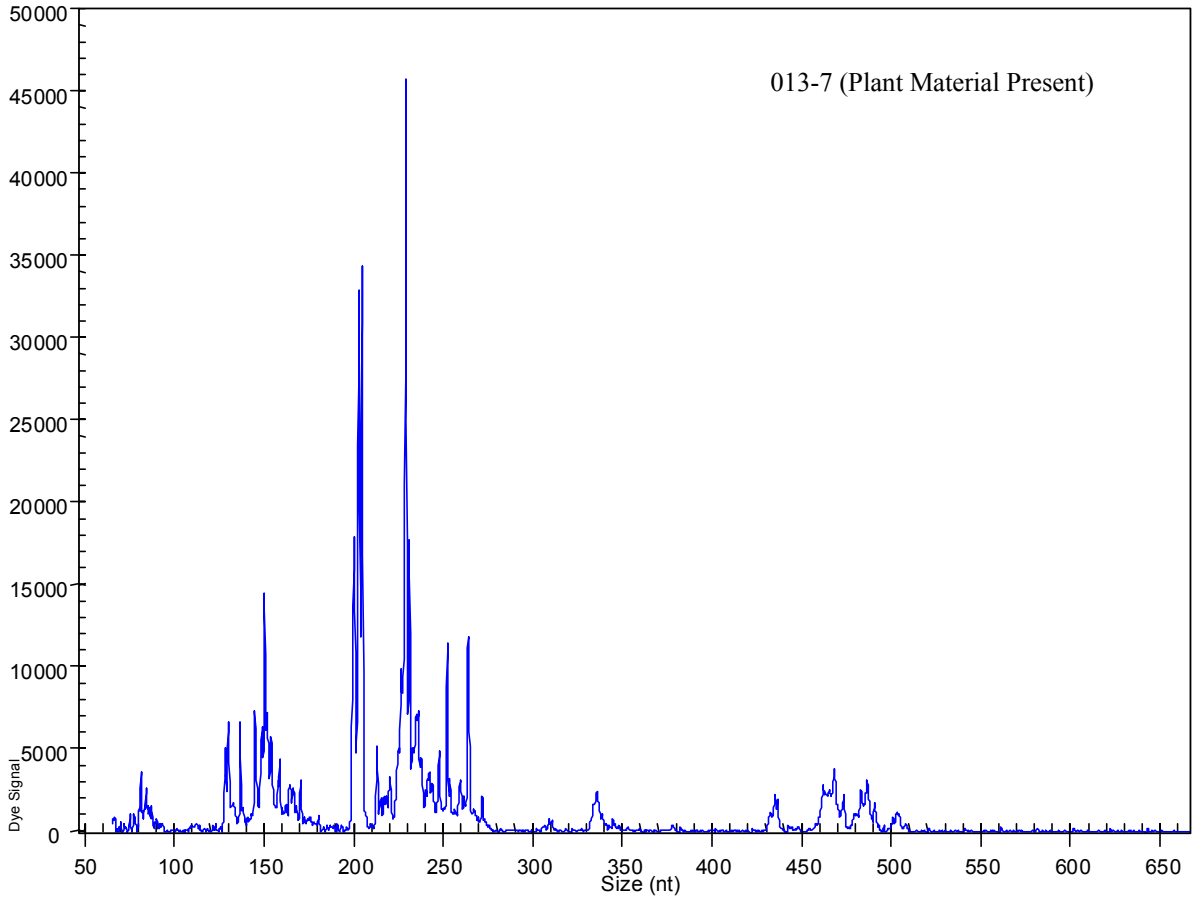


Figure 5: 013 TRF Patterns





Those TRF peaks, which proved to be significantly different (p-value<0.05) between samples with plant material and without, were compared to GenBank's database to identify the bacteria represented by the peaks. The peaks located at 201 bp, 205 bp, and 214 bp represent alpha-proteobacteria, which are known to be associated with plants. The peak at 264 is representative of *Streptococcus* spp. and *Streptomyces* spp.. *Pseudomonas* spp. are found to be associated with peaks at 230 bp. Bacteria in the genera *Pseudomonas* are known petroleum degraders and "have an ability to utilize a diverse range of substrates including those found in petroleum"(Greene et al., 2000, Esteve-Nunez et al., 2001, as cited by Kaplan)(4). The fact that petroleum degrading bacteria are more prevalent in samples with plant material is very important. This implies that the trees are involved in escalating the rate of petroleum degradation in the groundwater by promoting an increase in the number of petroleum degrading bacteria.

References:

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4) Kaplan, C.W.. "Bacterial Community Dynamics." Thesis. California Polytechnic State University, 2002.