Characterization of Bacterial Communities in Groundwater Samples From Mature Willows and Comparison to Bacterial Communities From Young Willows During Phytoremediation

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Executive Summary

Water samples were obtained from a phytoremediation site containing mature willows in the Guadalupe dunes (Guadalupe, CA). The mature willows could potentially be a part of a bioremediation project that aims at cleaning up a plume of dissolved petroleum in the ground water. The goal of this study was to first determine the taxonomic composition of the bacterial community in the water surrounding the mature tree roots. The composition of this bacterial community was compared to the bacterial community in groundwater samples previously obtained from a bioremediation site containing young willows. In the mature willow grove, samples were taken from five wells known to contain dissolved petroleum and five wells devoid of petroleum. A second sample from each of these ten wells was taken a week later. The taxonomic composition of the bacterial community in the samples was determined by using 16S rRNA Terminal Restriction Fragment (TRF) analysis. DNA was extracted from the water by filtering and then amplified by PCR using primers that are homologous to the 16S ribosomal gene of the bacteria. The primer is labeled with a fluorescent tag for detection. The resulting PCR amplicons were digested with *dpn*II, a restriction enzyme. The fluorescently labeled fragments were analyzed by TRF analysis to produce TRF patterns for each water sample. The TRF patterns from the mature willow water samples were compared to those of the young willow water samples. This comparison showed that there was a difference in the bacterial taxonomic composition in the mature and young willow samples containing petroleum. In contrast, there was no difference in the bacterial composition in the young and mature willow samples devoid of petroleum. It was also apparent that the bacterial community in groundwater around young willows was approaching that around the mature willows in petroleum affected conditions. In other words, when TPH is present there is a change in the bacterial composition in the young willows over time approaching that of the mature willows. If a future study could show that the bacteria in the groundwater around the mature willows is effective in reducing the TPH level, then the bioremediation site containing young willows might be successful in the future.

I. Introduction

Petroleum hydrocarbon (crude oil) contamination is one of the most significant forms of groundwater and soil contamination (1). "There have been more sites identified with petroleum hydrocarbon contamination than any other type of contamination" (1). Each year, huge amounts of crude oil are released into the environment due to human activities (3). The oil is released as a result of leaks from storage tanks, huge oil spills during transportation and distribution, as well as spills during refining (1). Some of the contaminated sites are relatively small and confined but others are wide spread due to large oil spills (1).

The nature of the contaminated sites became an issue when remediation techniques were first utilized. Soil excavation and groundwater pump and treat were the first techniques used but in most cases they both proved to be ineffective. Soil excavation was useful in sites that were small and defined but when the contamination was wide spread the technique was not successful. Due to the limited solubility of petroleum hydrocarbons and the variation in groundwater flow, groundwater pump and treat had very little impact on the contamination. Soil vapor extraction and bioventing developed as a result of the known volatile and biodegradable properties of petroleum hydrocarbons. These methods have proved to be more cost-effective than the previous ones mentioned but were still not optimal methods (1). Increasing awareness of hydrocarbon contamination led to the investigation of other remediation approaches including bioremediation.

Bioremediation is "the use of biological organisms such as plants or microbes to aid in removing hazardous substances from an area" (6). Bacteria use the hydrocarbons as a source of carbon and break them down into less harmful substances like water and carbon dioxide. Phytoremediation is a form of bioremediation in which plants are used to help remove

the contamination. Plants are able to heighten the bioremediation process by directly absorbing the hydrocarbons and by stimulating the activity of bacteria. In this study, the effects of trees on bacterial development and activity were analyzed. Plants stimulate bacterial activity and growth by leaking organic nutrients into the soil, which the bacteria use as carbon and energy sources. It is possible that the leaked organic nutrients may select for certain bacteria that have a tendency to degrade petroleum hydrocarbons.

In this study, groundwater samples were taken from the N13 site at the Guadalupe dunes oil field. This site contains naturally growing mature willows that could be potentially useful in cleaning up the petroleum hydrocarbons in the soil and water. A portion of the Guadalupe dunes was used by the Union Oil Company of California (Unocal) as an oil field from 1951 to 1994. (2) Diluent, a mid-cut petroleum distillate, was used to dilute the heavy crude oil for transportation and production purposes (2). During the time Unocal was utilizing the oil field, diluent leaked from the pipelines into the surrounding groundwater and soil. Unocal started various treatment programs including bioremediation to clean up the contaminated soil and groundwater. The Environmental Biotechnology Institute at Cal Poly has aided in some of these programs.

The goal of this study was to characterize the bacterial community from the groundwater at the N13 site and compare it to the bacterial community at the O13 site, which contains young willows. The groundwater samples were analyzed by using Terminal Restriction Fragment (TRF) patterns to determine the taxonomic composition of the bacterial communities. The TRF patterns were the final step in a process that included DNA extraction, PCR amplification, restriction enzyme digestion, and ethanol precipitation. Capillary gel electrophoresis was used to generate the TRF patterns. These patterns were then compared to patterns from the bacterial

community of the O13 site using Principal Components Analysis (PCA). It was determine that TPH (Total Petroleum Hydrocarbons) has an effect on the bacterial communities regardless of the age of the willow trees that the bacteria are living around. The trees also have an effect on the bacterial communities when TPH is present. There is a change in the bacterial composition in the young willows over time approaching that of the mature willows, with respect to TPH.

II. Materials and Methods

DNA Extraction

The bacteria were filtered from the water samples using filters with a pore size of 0.2 µm. DNA was extracted from the material caught in the filters by using the procedures that accompanied the UltraClean MoBio Soil DNA Kit. DNA concentration was measured using the SPECTRAmax UV Spectrophotometer (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA).

PCR Amplification

PCR was performed on the DNA samples using two primers that are homologous to highly conserved regions of eubacterial 16S rRNA genes. The forward primer was Ba2F and was fluorescently labeled with phosphamide dye (Applied Biosystems, Fremont, CA). The reverse primer used was K2R (Applied Biosystems). The PCR reactions were carried out using 1 µL of 1ng/µL of extraction product dilution, 30.7 µL DI water, 5 µL of 10X Buffer, 4 µL of 10mM dNTP, 2 µL of 20 µg/mL BSA, 5 µL of 25 mM MgCl2, 1 µL K2r, 1 µL Ba2F, and 0.3 µL of 5U/µL TaqGold enzyme. All reactions were placed in a thermocycler and heated to 94 for 10 min. followed by 35 cycles of 94 for 1 min., 46.5 for 1 min., 72 for 2 min., and ending with 72 for 10 min.

PCR Clean-up

Gel electrophoresis of the PCR products was performed in order to confirm successful PCR. 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 5 µL of PCR product was added with 1 µL of 5X loading buffer. The gel was run at 100 V for about 40 minutes. The gel was stained with ethidium bromide and visualized using the Bio-Rad Gel Doc system. After 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 digestion was performed on the cleaned-up PCR products using the New England Biolabs restriction endonuclease *dpn*II. Each reaction contained 0.4 µL of *dpn*II (10,000 U/mL), 4 µL of *dpn*II buffer, and 75 ng of DNA. The samples were placed in a thermocycler and were digested at 37 for 4 hours and then inactivated for 20 min. at 65.

Ethanol Precipitation of Digestion Products

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

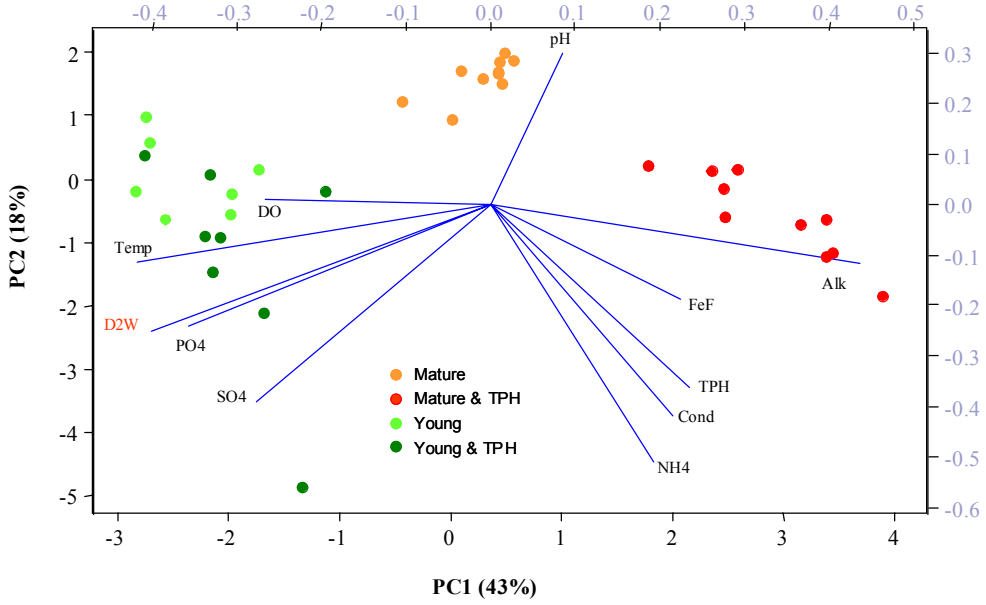
Capillary Electrophoresis

The DNA pellets were resuspended in 20 µL of formamide and 0.25 µL 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 in order to produce the TRF patterns.

III. Results and Discussion

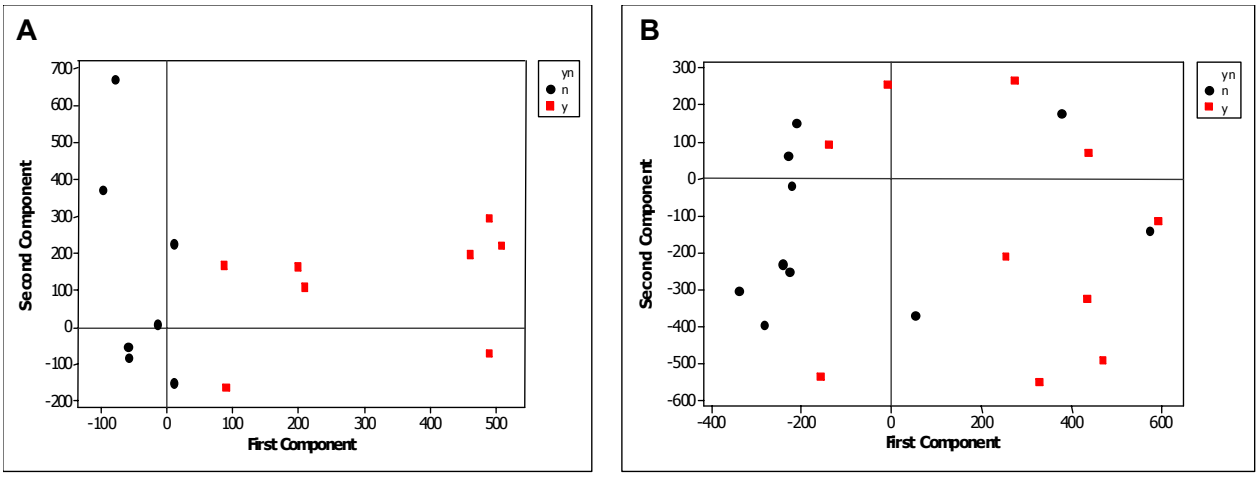
The chemical and physical parameters of the groundwater at the N13 (mature willow) and O13 (young willow) sites were compared statistically using Principal Components Analysis (PCA). "PCA involves a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called *principal components*. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible" (4). PCA indicated that there was a difference between the chemical and physical parameters of the groundwater at the N13 and O13 sites (Figure 1). There is a separation between the parameters along principal component 1. The groundwater in the young willows (O13) has higher values of depth to groundwater (d2w), dissolved oxygen, temperature, phosphate, and sulfate. The groundwater in the mature willows has a higher alkalinity, ferrous iron, pH, conductivity, and ammonia. Within the samples from the mature and young willows, there is a separation based on TPH.

Figure 1: Difference in chemical/physical parameters at N13 vs. O13.



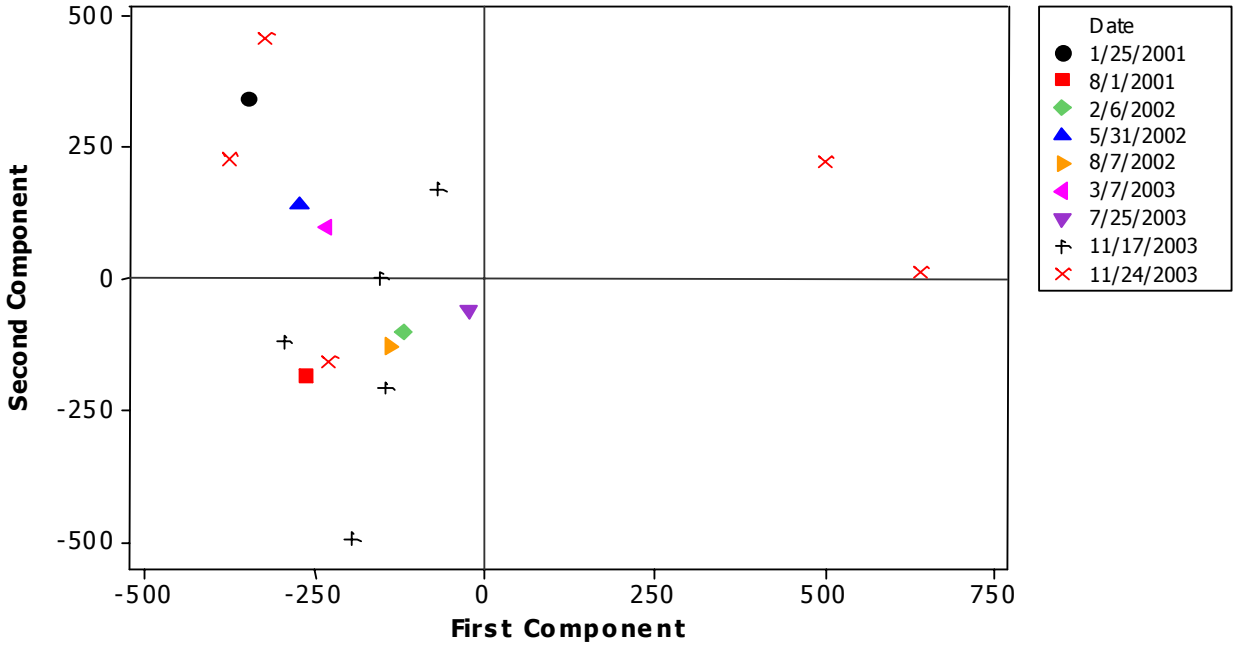
TRF patterns from the groundwater samples were compared using PCA to illustrate the effect of TPH on the bacterial communities. PCA showed that TPH makes a difference in the bacterial communities at both the N13 and O13 sites (Figure 2). In other words, TPH changes the composition of the bacterial communities whether the samples are from the mature willows or the young willows.

Figure 2: Principal components analysis of TRF Patterns based on presence of TPH. Red symbols are samples containing TPH and black symbols are samples without TPH. A. Analysis of samples from young willows. B. Analysis of samples from mature willows.



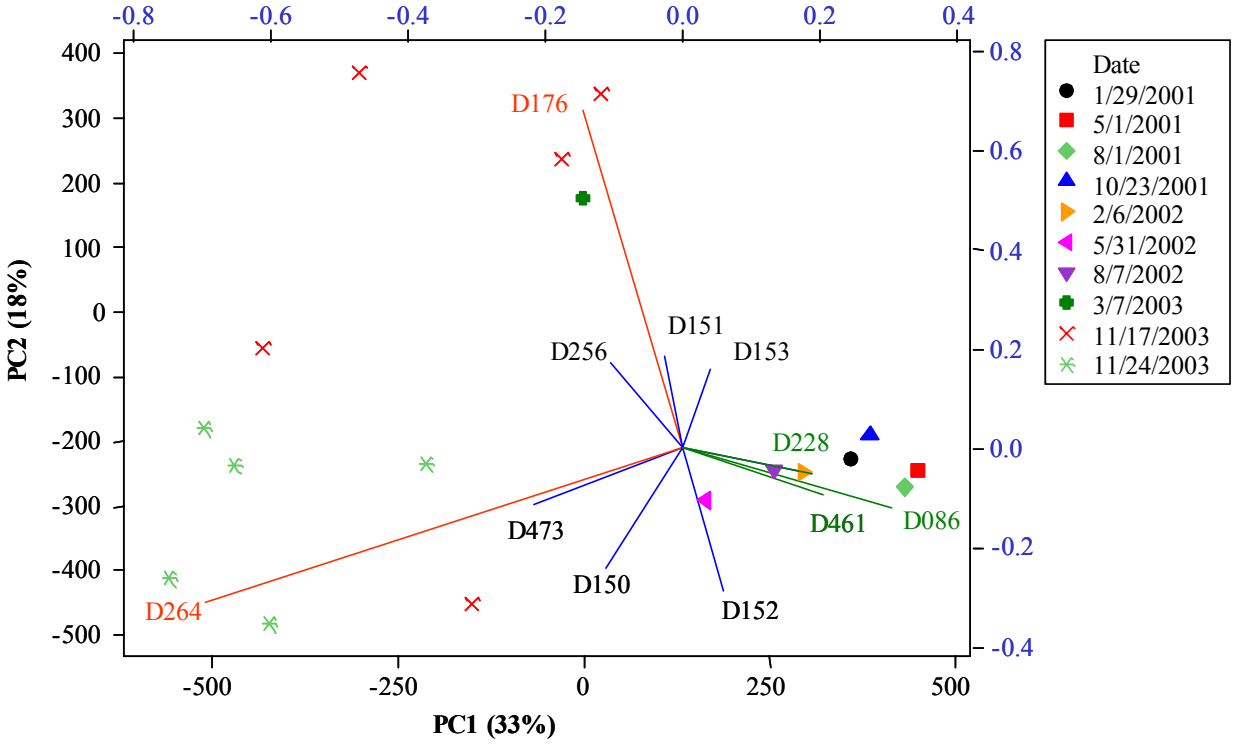
TRF patterns from the N13 and O13 samples that did not contain TPH were analyzed using PCA. The analysis showed that when TPH is not present, there is no significant difference in the bacterial communities between the young and mature willows (Figure 3). In other words, in the absence of TPH the age of the trees did not have an effect on the bacterial communities. The dates for the young and mature willows are spread over the graph indicating that there is no separation between the bacterial communities of the N13 and O13 sites.

Figure 3: PCA of TRF Patterns from N13 and O13 sites in the absence of TPH indicating no significant separation between the bacterial communities. The samples from the mature willows are dated 11/17/2003 and 11/24/2003 and the samples from the young willows are from the remaining dates.



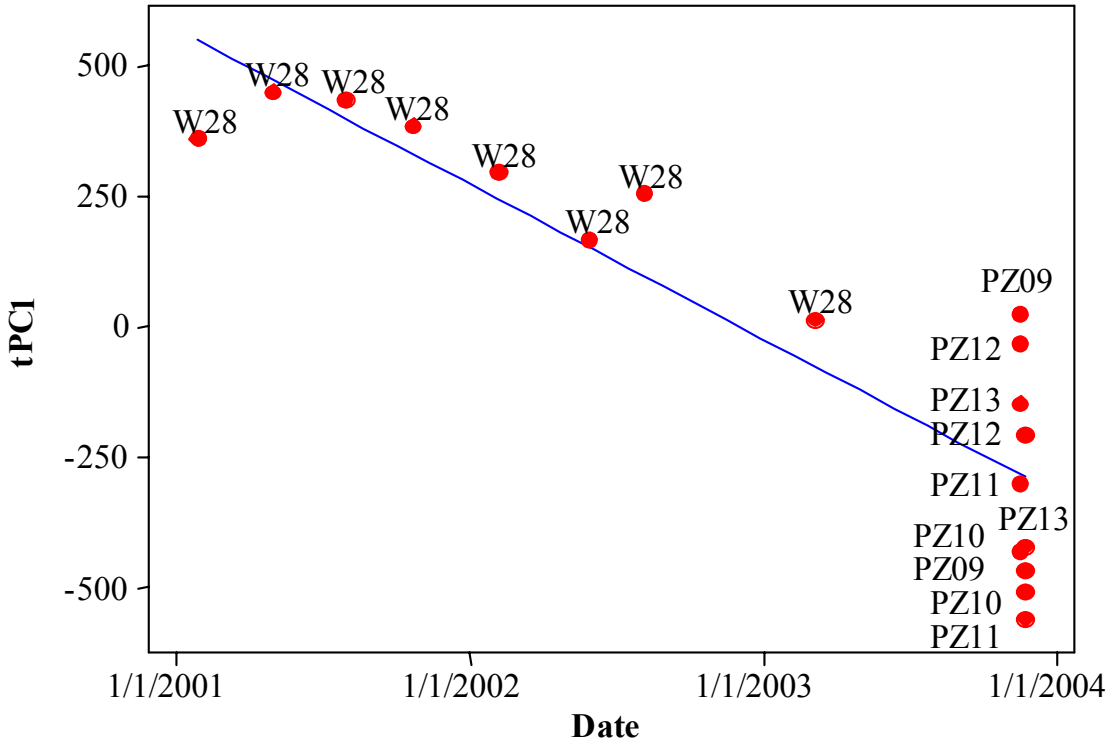
Samples from the young and mature willows that contained TPH were analyzed by PCA to highlight the effect of the trees on the bacterial communities in the presence of TPH. PCA illustrated that the age of the trees did have an effect on the bacterial communities when TPH was present (Figure 4). There is a clear separation between the samples from the mature willows and the samples from the young willows. Figure 4 also begins to illustrate that the bacterial communities from the young willows are moving towards those of the mature willows over time. In addition, the PCA loadings indicate that a certain set of TRF peaks increased while others decreased over time in the young willows.

Figure 4: PCA biplot of TRF data from both sites in the presence of TPH. Filled symbols are from the O13 site and line symbols are from the N13 site. Only loading vectors > 0.1 are presented. Red loading vectors represent TRF peaks that dominate samples from N13 willows. Green vectors represent dominant TRF peaks in O13 willows.



A plot of PC1 against sampling date was investigated due to the observation that most of the variation in TRF patterns over time appeared to be associated with PC1 (33%). The groundwater samples from the young willows were taken over a two and a half year period while the mature willow samples were taken over the period of a week. This analysis makes it clear that bacterial communities from the young willows are becoming more similar to the bacterial communities from the mature willows (Figure 5). It seems that as the young willows are maturing, the bacterial communities in the groundwater are maturing along with the trees.

Figure 5: PCA of TRF patterns over time for O13 bacterial communities (samples marked "W28") as compared to the N13 willows (samples marked PZ09-PZ13).



In order to determine how the bacterial communities in the young willows were changing over time, TRF peaks that had a high correlation to sampling time or a high loading for PC1 were plotted against the sampling time. The relative abundance of TRF peaks 154, 176, 258, 264, and 271 increased over time in the young willows (Figure 6). These bacteria were abundant in the mature willow samples and this is partly what made the bacterial communities in the young willows become similar to those in the mature willows. In contrast, there was another group of bacteria in the groundwater from the young willows that was decreasing in abundance over time. The bacteria with the TRF peaks 86, 130, 228, 461, and 204 decreased in relative abundance over the two and a half year period (Figure 7). Since these bacterial types were less abundant in the mature willows, their decrease also contributed to the increasing similarity between the bacterial communities of the mature willows and the maturing young willows.

Figure 6: Relative Abundance of O13 TRF peaks that are increasing over time according to the loadings from the PCA. The regression line is in a log function. TRF peaks in red are those noted in Figure 4.

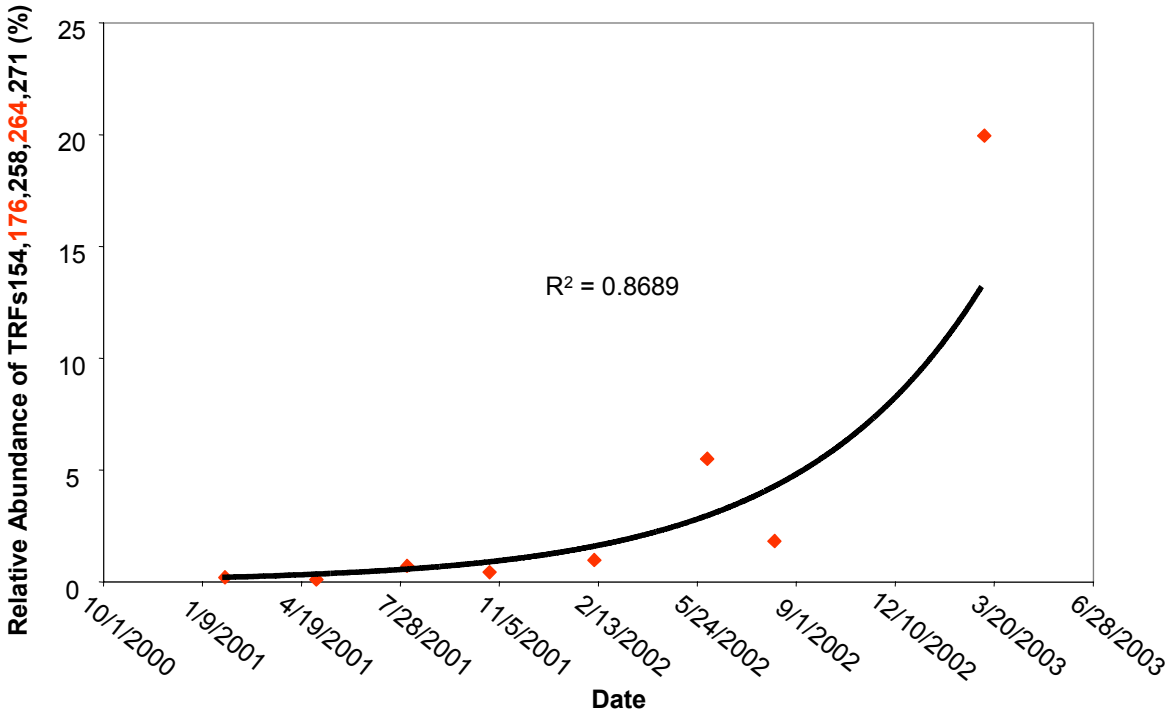
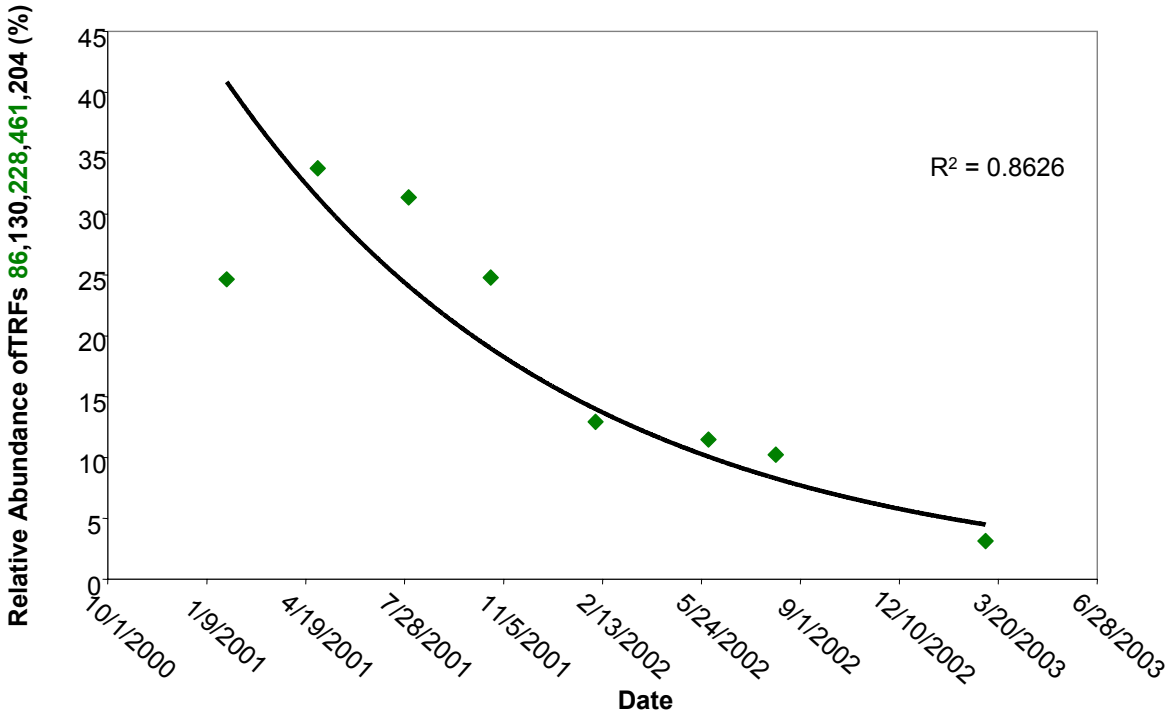


Figure 7: Relative abundance of O13 TRF peaks that are decreasing over time according to the loadings from the PCA. The regression line is in a log function. TRF peaks in green are those noted in Figure 4.



The possible taxonomy of the changing bacterial populations in the young willows was determined by using a database of TRF fragment sizes generated from analysis of 16S rRNA gene sequences in GenBank. Of the bacteria that are decreasing over time, TRF 461 may represent several types of actinobacteria. This TRF peak was important in a related study of groundwater that had very high concentrations of dissolved TPH. This study was done at a site (TB8) in Guadalupe investigated for natural attenuation without phytoremediation. TRF 86 may represent anaerobic iron respiring organisms (5). TRF 228 may represent methane-oxidizing bacteria in the genera *Methylomonas* and *Methylobacter*.

The possible taxonomy of the bacteria associated with TRF 154, which are increasing over time, are *Pseudomonas* and *Azoarcus*. These bacteria are known to be petroleum degraders

so their possible presence in the groundwater may be lowering the level of TPH. TRF 271 was previously seen as associated with planted areas in another phytoremediation site (C8) also at Guadalupe.

Table 1: Possible bacterial taxonomy of the increasing and decreasing TRF peaks from the young willow samples.

Increasing over time		**Decreasing over time**	
TRF Peak	**Bacteria**	**TRF Peak**	**Bacteria**
154	*Pseudomonas*, *Azoarcus*	86	*Shewanella*, *Aeromonas*
176	actinobacteria	130	beta-proteobacteria
258	*Clostridium*, *Cytophaga*	204	alpha-proteobacteria
264	*Clostridium*, *Bacillus*, actinobacteria	228	gamma-proteobacteria
271	*Bacillus*, delta-proteobacteria	461	actinobacteria

IV. Conclusions

Based on TRF analysis and PCA, TPH has an effect on the bacterial communities in the young and mature willows. This is shown in the separation of the TRF patterns with and without TPH from both the N13 and O13 sites (Figure 2). On the other hand, there is no difference in the bacterial communities between the two sites when TPH is not present (Figure 3). If TPH is absent, then the age of the willows does not have a detectable effect on the bacterial communities. In contrast, willow age has an effect on the bacterial communities when TPH is present in the groundwater (Figure 4). In addition, certain groups of bacteria found in the young willow samples are decreasing as the willows mature while others are increasing over time (Figures 6 and 7). A TRF peak representing aerobic TPH degrading bacteria increased over time in the young willows and TRF peaks representing some anaerobic iron reducing bacteria decreased over time (Table 1). This illustrates how the O13 (young willow) bacterial community is approaching that of the N13 (mature willow) community (Figure 5). The bacterial community from the young willows should be mature soon given their trend toward the mature willow community.

The results from this study give rise to many questions that could be answered by future studies. To determine whether the phytoremediation project is working, a study could be done to find out if the mature willows and their associated bacteria are reducing the level of TPH in the groundwater. This could be carried out by taking groundwater samples from the Santa Maria River bed down-gradient of the mature willows and testing for the remaining TPH. These TPH levels could then be compared to those up gradient from the mature willows. This would help determine whether there is a decrease in the TPH level as the groundwater flows through the mature willows and is degraded by the associated bacteria. The bacterial community in the

young willows should be analyzed again by TRF analysis and PCA to determine whether they have matured to the bacterial communities of the N13 willows. Some 16S rRNA genes could also be cloned and sequenced from these samples to more accurately identify the bacteria involved in the changes we observed.

V. Acknowledgements

I would like to thank Dr. Kitts for his generous aid and support in working on this project. I would also like to thank all of the employees at the EBI for their assistance in working through each step of the process needed to complete my study. Thank you to Unocal for providing me with this project and for all of their support.

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