

**Groundwater Sampling Methodology: A T-RFLP Comparison Between
Direct Groundwater Sampling and a Novel Biofilm Sampler.**

By
Chris D. Kornblatt

Biological Sciences Department
College of Science and Mathematics
California Polytechnic State University
San Luis Obispo
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AUTHOR: Christopher David Kornblatt

DATE SUBMITTED: October 2007

Dr. Chris Kitts
Senior Project Advisor

Signature

Dr. Michael A. Yoshimura
Department Chair

Signature

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Groundwater Sampling Methodology: A T-RFLP Comparison Between Direct Groundwater Sampling and a Novel Biofilm Sampler.

Abstract

The groundwater at Guadalupe Dunes, California, is affected with petroleum diluent and phytoremediation is being assessed as a viable remediation method. The C8 phytoremediation site is a restored coastal dune plant community situated over a dissolved petroleum plume. The plant roots have reached the groundwater as determined by unpublished observations. Dune Lupines (*Lupinus chamissonis*) are concentrated in the up gradient portion of the plume and the Arroyo Willows (*Salix lasiolepis*) concentrated down gradient. Plant roots are hypothesized to encourage the activity of petroleum degrading bacteria in the affected groundwater. Monitoring of bacteria in the plume has mostly been done with samples of groundwater taken from wells. However, the most active bacteria may in fact be associated with biofilms growing on soil in the saturated zone. This experiment was conducted to determine if there is a difference between the microbial communities collected with well water versus those that form biofilms on saturated soil. A secondary objective was to determine if Dune Lupines and Arroyo Willows had a similar effect on groundwater bacterial communities. Biofilm samplers were made of a 1 ft PVC pipe with a 6-inch screen of the same design as used in the end of ground water wells. Each biofilm sampler was filled with clean sand, capped, and lowered into the wells by rope until submerged. They remained in the wells for 90 days and removed at the same time as the phytoremediation groundwater sampling. Upon DNA extraction the biofilm sampler sand was separated into two portions, the inner core of the sand and the outer radius, to determine if there was a difference in colonization within the sampler. The sand was extracted using the same T-RFLP protocol employed for the groundwater samples, but used 5 replicates of 1 gram of sand per sample to

collect sample DNA. The samples yielded low amounts of microbial DNA, resulting in PCR primer-dimer artifacts. The inner core of each biofilm sampler had less DNA than the outer radius of sand. There was a difference between the communities sampled based on the method, though some common elements were observed. No conclusive results were found regarding the microbial community's association with each plant community. Both the groundwater samples and the biofilm samplers were similar in wells containing water with high concentration of iron and low concentration of sulfate, implying high levels of anaerobic respiration.

Keywords: microbiology, ecology, biofilm, sampling, groundwater, petroleum, biotrap, bioremediation, T-RFLP, Terminal Restriction Fragment Length Polymorphism

Introduction

In 1951 Unocal Corporation acquired the 3,000-acre Guadalupe Oil Field on the central coast of California and actively explored and extracted petroleum at this location until 1994. Unocal utilized steam injection and diluent-mixing methods to reduce the high viscosity crude oil and increase yield (Interactive Planning and Management, 2001). The diluent is a cut of petroleum similar to diesel and is known to contain many volatile compounds such as benzene, toluene, ethyl benzene and xylene (BTEX). The chemical characteristics of the diluent piped to the site varied because the diluent contents depended on the type of crude oil being distilled by the Conocco-Phillips petroleum distillation plant. An infrastructure of tanks and pipes were used to transport the diluent from the Conocco-Phillips to storage tanks then to the well sites. Corroded pipes resulted in almost 12 million gallons of diluent contamination in the dunes and ground water (Interactive Planning and Management, 2001). Bioremediation and phytoremediation was employed to remove the pollution from this sensitive site because it is a less invasive substitute to chemical and mechanical means of removing pollution. The C8 well site, the focus of this study, is in the slack of the dunes just inland of the pioneer dunes on the coast (Figure 1).

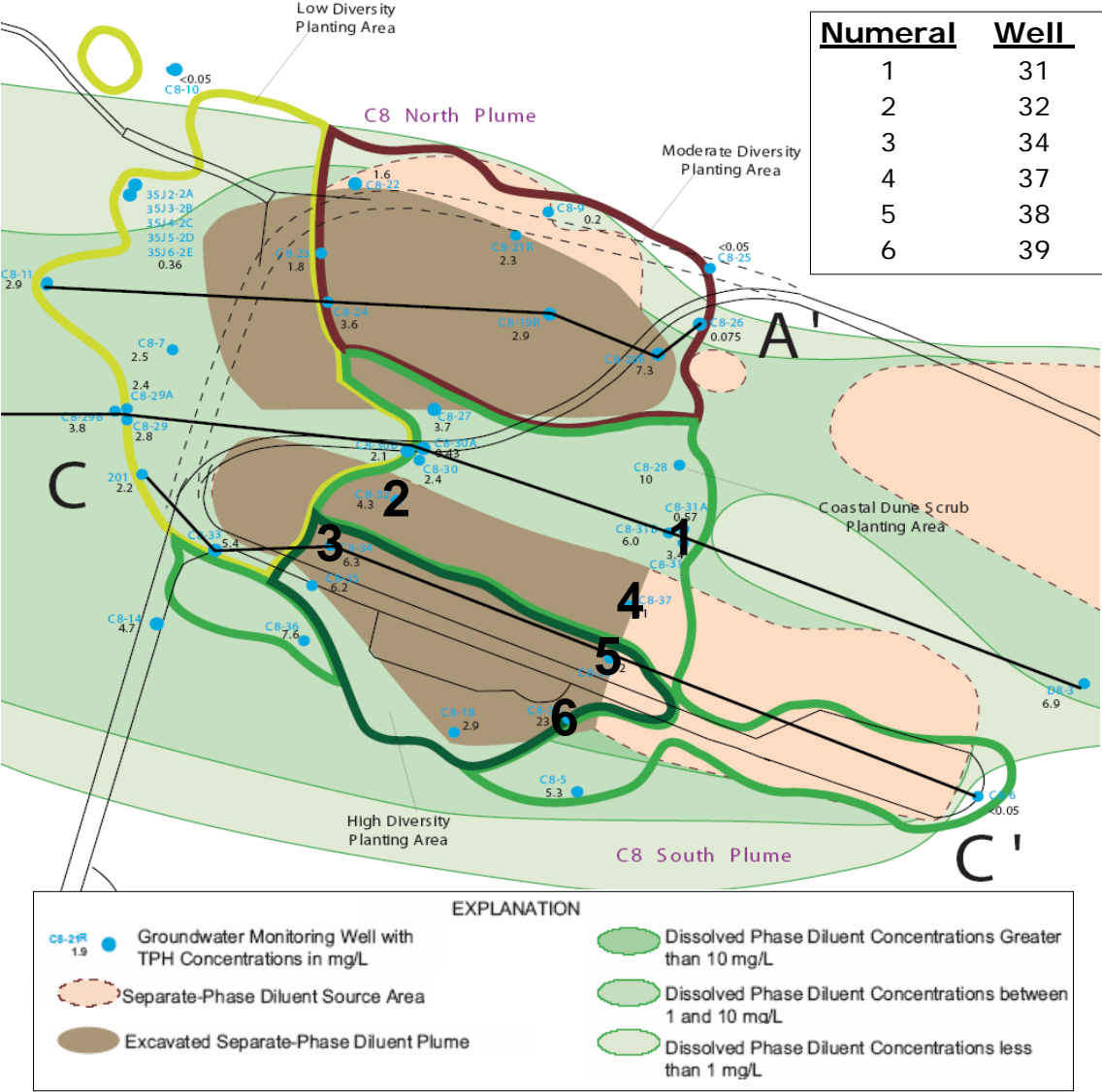


Figure 1: Guadalupe Oil Field C8 well site. The Wells 31, 37, 38, and 39 are up-gradient in the diluent plume compared to wells 2 and 3. For more well information on each well, see Table 1.

Phytoremediation is being used to remediate C8 Well site because it is less invasive and is half the cost to clean groundwater by the pump and clean method (Schnoor, 1997). In phytoremediation there are two important constituents in the clean up of the groundwater: the plants and the microbial communities. The plants utilized at this site, Arroyo Willow (*Salix*

lasiolepis) and Dune Lupine (*Lupinus chamissonis*), were chosen because they are indigenous to area and have a root system deep enough to reach the dune sand aquifer where the diluent has created an underground plume. Plant root systems work with the microorganisms because they can potentially slow the diluent the plume and can release 10 to 20% of its photosynthetic products into the groundwater as exudates thereby encouraging microbial growth in the groundwater (Schooner, 1997). Little is known about the microorganisms involved in in-situ phytoremediation due to challenges with laboratory isolation and the mechanical difficulty of sampling the subterranean microbial communities. At the Guadalupe Dunes in California, phytoremediation studies utilize groundwater to determine bacterial constituents of the community. The Phytoremediation project at the C8 well site requires 4 liters of water filtered through a 0.2 micron membrane to concentrate the sample before microbial community analysis is performed using Terminal-Restriction Fragment Length Polymorphism (T-RFLP). This method of sampling was used because alternative sampling methods for groundwater microbial communities were not feasible at the beginning of this study. The main purpose of this study was to determine if the Phytoremediation project was studying a representative sample of the microbial communities in the well. Groundwater sampling methods may produce a biased profile of the eubacterial community; the water sample may contain primarily planktonic bacteria and may under-represent the bacteria associated with biofilms. In a porous-flow column 99% of the aerobic heterotrophic bacteria were found to colonize the substrate not the water (Lehman, et. al, 2001). The biofilm samplers were designed to mimic the aquifer substrate thereby allowing for biofilm growth to provide a better representation of bacterial communities (Peacock, et. al, 2004). We anticipate a similar level of biodiversity between the communities sampled through the groundwater and biofilm sampler method. A close similarity between the biofilm samplers

and the groundwater samples will allow a greater confidence in a more inclusive sampling of microbial communities by groundwater collection, despite other studies.

Another benefit to this study will be a resolution to the theory that roots from Dune Lupines and Arroyo Willows at the studied wells may have an effect on the microbial communities. At this site, Arroyo Willows were planted primarily in the down-gradient portion of the plume; a Dune Lupine community as well as a mixed community of these two plants is located up gradient in the site. These plant communities have roots that have reached the ground as determined by observation during groundwater sampling. Plants roots have been shown to influence microbial communities due to the release of exudates and dissolved oxygen into the rhizosphere (Geyer, et. al, 2005). A comparison of bacterial communities sampled from the wells associated with each plant root will elucidate any significant difference between bacterial communities. The willow roots are anticipated to encourage the most petroleum degradation because willows were recognized for their ability to remediate groundwater and leachate of BTEX by phytotransformation (Schnoor, 1997).

T-RFLP is a common method of microbial community comparison. In this process 16S rDNA is extracted from a sample and is targeted for replication during Polymerase Chain Reaction (PCR) using specific primers with the forward primer tagged with a fluorescent label. A restriction endonuclease digest fragments the DNA and is analyzed by polyacrylamide gel capillary electrophoresis. Labeled amplicon fragments are compared to an internal standard to determine their nucleotide length and their relative abundance analyzed by the intensity of the fluorescent label. The data is portrayed by a graph of peaks with two axes: relative abundance based on dye signal and nucleotide length. Each peak is associated with a cluster or genus of bacteria allowing for a comparison by Euclidean distance of overall bacteria in each sample (Englebrektson, 2005).

Material and Methods

Experimental Design: Six wells were chosen at the C8 well site based on their association with the rhizosphere of the planted communities. Two wells were located in different communities with a dominant plant-type: Dune Lupine, Arroyo Willow, and mixture of Lupine and Willow. The biofilm samplers resided in the zone of saturation for 90 days until they were removed in August 2005 in concert with the groundwater removed for the Phytoremediation project. The samples had the bacterial DNA extracted, amplified by PCR, fragmented by restriction enzyme, and analyzed by T-RFLP.

Sampling: Biofilm samplers were made of a 1 ft PVC pipe with a 6-inch screen area with slits of 0.1 millimeter wide. These mimicked the design of the screen at the end of the studied ground water wells. Each sampler had a screw-on PVC cap at both ends, the top cap had a hole to attach a nylon cord for sampler removal from the well and the other with approximately eight 1 mm holes to allow for water to flow through. Each cap was lined with standard window screen made of fiberglass (Picture 1).



Picture 1: Biofilm sampler with one cap removed. Made by Bob Pease at the Unocal Central Coast Group. The sampler has a 1-inch diameter.

Clean sand from a dune in the same dune system was used as substrate for the biofilm growth. The fines were removed by filling a 5-gallon bucket $\frac{1}{4}$ full of the donor sand and the remaining

portion was filled with water. The sand was swirled by hand. The suspended fines were poured off with the water; this was continued until sand grains of equal size were left. The sand was sterilized in an autoclave and then used to fill the biofilm samplers the day sampling began. Each sampler was lowered into the well by nylon rope until submerged in the saturation zone. The samplings were placed in selected wells for 90 days and removed when the groundwater sampling occurred.

Sample Removal: Each biofilm sampler was removed from the well by the attached rope and sent to the laboratory in a plastic re-sealable partially filled with well water from the same well then placed in an additional re-sealable bag. The samples were transported in a cooler and refrigerated at 10°C at the laboratory until substrate removal within 3 days of arrival in the laboratory. Each end of the sampler was unscrewed and the center of the sampler substrate was cored with a ½ inch diameter sample corer. This portion of substrate was labeled as the inner portion of the sample, the remaining sand was removed by sterile spatula and labeled as the outer portion and stored separately. Sand was also collected from the opposite side and combined and homogenized with sand collected from the first side. Next, samplers were stored in a -10°C freezer until DNA extraction. DNA was extracted using a MO BIO PowerSoil™ DNA Extraction kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the supplied protocol using 5 replicates of 1 gram of sand per sample; inner and outer sand portions of each biofilm sampler treated as independent samples. The sand substrate was shaken at 5 meters per second for 45 seconds using a BIO 101 FastPrep® Bead Beater (Qbiogene, Irvine, CA) and stored at -4°C until further analysis also following the MO BIO PowerSoil™ DNA extraction kit protocols. Groundwater samples were collected using a mobile pump that was purged and used to collect 4 liters of well water into a carboy and transported to the laboratory refrigerator within 1 day.

Each groundwater sample was filtered through 0.2 micron synthetic membranes by pump until either entirely filtered or filtered for a maximum of 8 hours. The membranes were aseptically cut in half, one half was stored in sterile aluminum foil at -10°C, the other half was pulverized using sterile mortar and pestle and liquid nitrogen. The powder was transferred to 3 MO BIO PowerSoil™ bead tubes to serve as triplicates and were physically shaken at 4 m/s for 45 seconds using a Bio 101 FastPrep instrument. DNA extraction success was determined by a 1.5% agarose gel electrophoresis in 1x TBE buffer was stained with ethidium bromide and visualized by using a Gel-Doc 2000 system (Bio-Rad, Hercules, CA). DNA was quantified by absorbance using a UV spectrophotometer 96-well plate at 260 nm.

Polymerase Chain Reaction: A reaction volume was prepared for a 50 µL volume PCR reaction for each sample including an *E. coli* control. Each PCR reaction was prepared for each sample in triplicate by creating a master mix of 5.0 µL of 10x Buffer, 3.0 µL 10mM (2.5 mM of each nucleotide) dNTP, 2.0 µL 20 µg/mL BSA, 7.0 µL 25mM MgCl₂, 1.0 µL Ba2F primer labeled with a Cy5 fluorescent tag (New England Biolabs, Ipswich, MA), 1.0 µL K2R primer (New England Biolabs), 20.7 µL of PCR-grade H₂O, 0.3 µL 5 U/mL *AmpliTaq* Gold® polymerase (Applied BioSystems, Fremont, CA) for each replicate. The 96-well plate was loaded with 40 µL of PCR master mix and capped until 10.0 µL of DNA extract and 1.0 µL *E. coli* DNA template control was added. The sample plate was placed in a thermocycler set to 94°C for 10 minutes then cycled 35 times between 94°C for 1 minute, 46.5°C for 1 minute, 72.0°C for 2 minutes, and ended with 72.0°C for 10 minutes. Once the thermocycler was finished, it remained at 4°C until the sample plate was retrieved and stored at -10°C. Gel electrophoresis using 1.5% agarose gel and stained by ethidium bromide submersing solution was used to qualitatively assess the PCR amplicon. A MoBio Ultraclean™ PCR Cleanup kit (MO BIO

Laboratories Inc.) was used to remove the byproducts and residual reagents of the PCR by following the manufacturer's protocol. The PCR clean-up product was stored at -10°C. PCR amplicon was quantified using a Flx8000 Bio-Tek fluorometer (Bio-tek Instruments Inc., Winooski, VT) set to determine the amplicon concentration using the fluorescent forward primer.

Enzyme Digest: Restriction endonuclease enzyme *DpnII* was used to cut 75 ng/mL of amplicon, originally obtained from the PCR cleanup, was aliquoted in a 96 well plate. 4.6 µL of master mix, created by combining 0.06 µL 10,000 U/mL *DpnII* enzyme and 4.0 µL of *DpnII* buffer per reaction (New England Biolabs), was added to each well containing PCR amplicon. A control well during each enzyme digest containing was created by adding 5.0 ng of *E. coli* amplicon generated by the same primers used during PCR as well as 4.6 µL of master mix. PCR-grade H₂O was added to each reaction to bring the final volume to 40.0 µL. The sample plate was placed in a thermocycler initially set to 37°C for 4 hours then to 65°C for 20 minutes then ultimately to 4°C until sample plate was stored at -10°C. An ethanol precipitation was performed to remove residual salts from the enzyme digest. A master mix was created with 250 µL of cold 95% ethanol, 2 µL 3M sodium acetate, and 1 µL glycogen per sample. 103 µL of master mix was added to each enzyme digest sample then the 96-well plate was capped and inverted 5 times. The sample plate was incubated for 30 minutes at -4°C then centrifuged for 15 minutes at 5,300 rpm at 4°C using a U20-434 rotor arm in a Avanti® J-E BeckmanCoulter instrument (Beckman Coulter Inc, Fullerton, CA). The sample plate was inverted onto a paper towel to drain the ethanol supernatant. A solution of 100 µL of cold 70% ethanol was added and centrifuged for 5 minutes at 4°C. Ethanol was removed by inverting the sample plate onto paper towels and sample plate was centrifuged upside-down on paper towels for 1 minute at 700 rpm at

4°C to dry pellet. Ethanol was further removed by evaporation in a fume hood with negative pressure for 15 to 20 minutes. The plate was recapped and stored at -10°C.

T-RFLP: A master mix was created using 20 µL of formamide and 0.25 µL 600 base pair standard per sample. 20 µL of master mix was added to each well with digested PCR amplicon. A drop of mineral oil was added to each sample to prevent evaporation. T-RFLP was promptly performed on a Beckman Coulter CEQ 8000 Genetic Analysis system (Beckman Coulter Inc.) or the sample was stored at -4°C.

Analysis: The T-RFLP data was output into a Microsoft Excel (Microsoft, Seattle, WA) sheet where the fragment lengths, or peaks on the T-RFLP pattern, were aligned into integer length bins and bins lacking information were removed (Alignment program by Dr. Chris Kitts, California Polytechnic University San Luis Obispo). The data was truncated by removing peaks with less than 1% of the total peak area to eliminate background signal. The low concentration of DNA extracted from the Biofilm Samplers resulted in the creation of primer-dimers during the annealing stage of the PCR. Primer-dimers formed peaks at 80, 120, and 160 nucleotides (Fig. 2A). The primer-dimers were manually removed from the T-RFLP patterns in the ranges of 0-90 and 115-130 nucleotides. Residual primer-dimer artifacts around 160 nt in length were not removed because organisms in unpublished data were found to produce peaks at this fragment size. If the primer-dimer peaks were not removed as much as possible they would have falsely increased the similarity between the biofilm samplers and would have decreased similarity to the groundwater samples. The square root of each T-RFLP peak's area was taken to de-emphasize large peaks while maintaining peak abundance for Bray-Curtis similarity analysis. These data were analyzed by PRIMER5 software (Primer-E Ltd., Plymouth, England). The Bray-Curtis similarity was calculated as described in Englebrektson, 2005.

Results & Discussion

Table 1: Environmental parameters of each well at the C8 wells site were determined at the time of the sampling. Total petroleum hydrocarbons (TPH), dissolved oxygen (DO), iron, and sulfate data were collected to give us a picture of the metabolic status of the microbial community. TPH data from Well 34 is anomalous as determined by TPH concentration trends for this well as observed in unpublished data and is deduced to be at most 2.5 mg/L.

Well	Plant Type	TPH (mg/L)	DO (mg/L)	Iron (mg/L)	Sulfate (mg/L)
31	Lupine	1.3	0.37	0.005	57
32	Willow	2.4	1.38	0.01	60
34	Willow	6.7	0.27	0.19	37
37	Lupine	6.3	0.69	6.18	28
38	Willow/Lupine	4.3	0.56	7.52	27
39	Willow/Lupine	6.7	2.18	3.87	22

The concentration of TPH, which at this site was introduced through human activities, can serve as a potential energy source for microbial communities. Some chemoheterotrophic organisms are able to utilize these hydrocarbons for their metabolism in the absence of light and DO. The bacterial communities live in an anoxic subterranean environment; sulfate is used as an electron donor in anaerobic metabolism with iron taking the place of oxygen as an electron acceptor. Wells 37, 38, and 39 have the greatest iron and the lowest concentrations of sulfate. Wells 31, 32, and 34 have at least 1 log-lower concentration of iron but have a higher concentration of sulfate. From this data it appears that Wells 37, 38, and 39 have a greater amount of anaerobic metabolism than Wells 31, 32, and 34. It is also important to note the relationship between the locations of the wells because this is the cause for the variance in TPH concentrations between the wells. Wells 37, 38, and 39 are the closest of the wells to the source of the diluent plume and within the diluent in the previously excavated C8 Phytoremediation area (Figure 1). Well 31 is also up gradient of Wells 32 and 34 but is not directly in line with the

direct flow of the diluent plume from the source and therefore contains lower concentrations of TPH through diffusion. Wells 32 and 34 are directly down gradient of the diluent source in the excavated sand, but are further from the source than Wells 37, 38 and 39. The lower concentration of TPH in Well 31 may be the main cause for its lower level of anaerobic metabolism comparable to those of the microbial communities in Wells 32 and 34.

FIGURE 2: T-RFLP results had the potential of being artificially similar because of primer-dimer artifacts in each biofilm sample stemming from the low DNA template during PCR. T-RFLP graphical outputs were compared between the clean undigested PCR amplicon and undigested PCR amplicon of each sample. The peak area data was outputted as a dendrogram and statistical data to visually display relative similarity and to reveal any trends between the samples. A). Biofilm Sampler well 38 (inner core of sand substrate) T-RFLP of the uncut PCR amplicon. The peaks at 80, 120, and 160 nucleotides (nt) were primarily caused by primer-dimers. The arrows point to the primer-dimer peaks manually removed from the statistical calculations. B). A T-RFLP output of the same sample as in part A but fragmented by a *DpnII* restriction enzyme digest. The primer-dimer peaks were not digested and are still present at the same nucleotide lengths. The arrows point to the primer-dimer peaks manually removed from the statistical calculations.

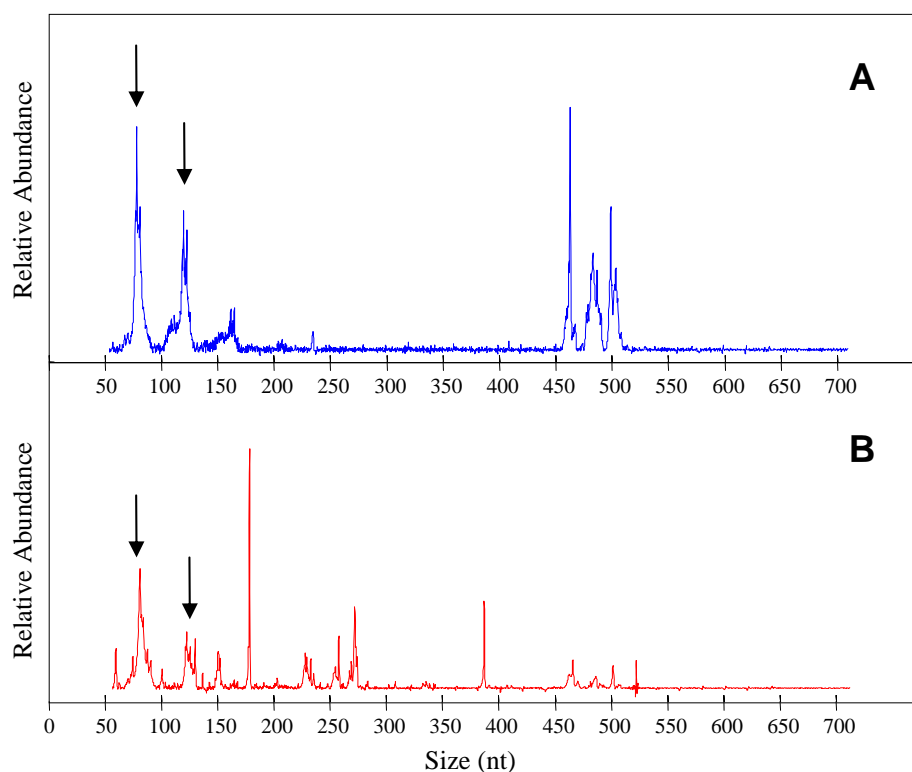
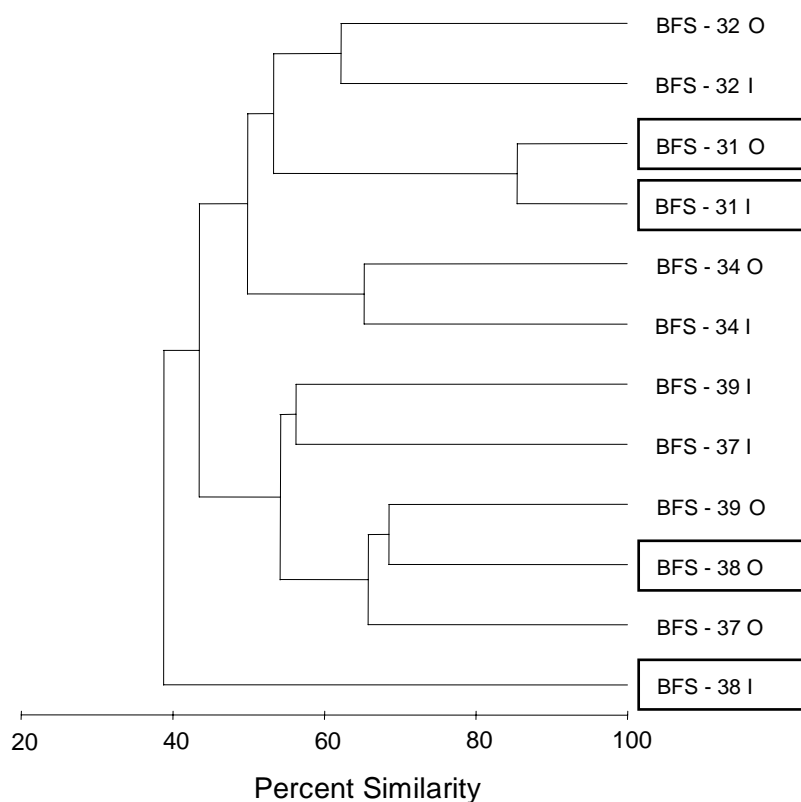
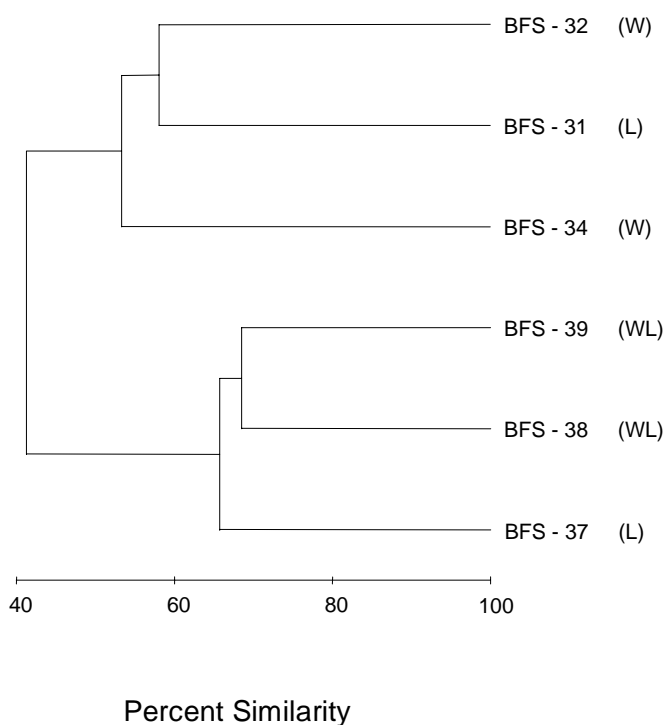


FIGURE 3: A comparison of Biofilm Samplers (BFS) inner core (I) and outer radius (O) of substrate after 90-day incubation time in the wells. The horizontal axis shows similarity between samples using Bray-Curtis similarity. This comparison is to determine the similarity of the BFS's within each sampler and to the other wells. The sample names boxed in this figure were to draw your attention to the wide degree of variance between individual samples from the same Biofilm sampler.



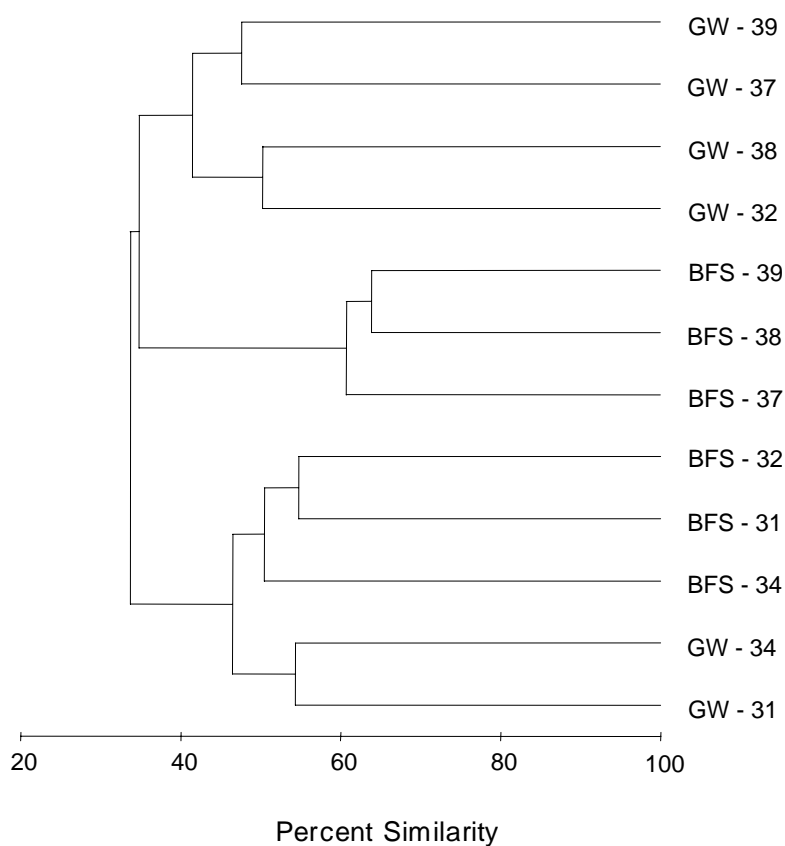
The Bray-Curtis similarity of the inner and outer sample from BFS – 31 was determined to have 85% similarity. The least similar inner and outer sample was extracted from BFS – 38 and had a similarity of 40%. Because of the high variance between the samples, it is difficult to draw conclusions regarding the colonization and uniformity of the colonies in each Biofilm Sampler. The two clusters of wells seen in Table 1 can be seen in this figure with the first cluster comprised of Wells 31, 32, and 34 while the second cluster includes Wells 37, 38, and 39.

FIGURE 4: Comparison of biofilm samplers (BFS) at each of the studied well sites. The horizontal axis denotes the percent similarity between biofilm samplers based on Bray-Curtis similarity. BFS – 31, 37, 38, and 39 are up-gradient in the plume. BFS – 32 and 34 are down gradient. A symbol of (W) was used for wells associated with willow root systems, (L) for lupine and (WL) for both willow and lupines. The outer radius of each BFS was used over the inner core because of its closer proximity to the groundwater outside the BFS.



The similarity is visually demonstrated in the dendrogram of the biofilm samplers at each well. The wells associated with willows, BFS – 32 and 34, have a 55% similarity and wells associated with lupine, BFS – 31 and 37, have a similarity of 40%. Wells with willow and lupine have similarity of 68%. The large range reveals a great variance in the similarities between the samples and does not show any correlation between the presence or absence of plants. In light of the previous figures, the dendrogram clusters do not support clustering based on plant type but rather further supports the clustering seen in Table 1 and Figure 3.

FIGURE 5: The percent similarity based on the Bray-Curtis similarity of corresponding groundwater samples (GW) and Biofilm Samplers (BFS). The sampling of the GW occurred at the same date as the removal of the BFS. The outer radius of each BFS was used to represent each well's BFS because of its closer proximity to the groundwater outside the BFS than the BFS samples using the inner core.



This dendrogram also appears to support clustering of bacterial communities around the environmental conditions in Table 1. Wells 37, 38, and 39 have a greater amount of oxidized iron, a waste produce of anaerobic bacteria, compared to Wells 31, 32, and 34. These two clusters also show the same split between the differing concentrations of sulfate in the well water. Wells 37, 38, and 39 have lower concentrations of sulfate than Wells 31, 32, and 34. This clustering is not as clear as in the other figures because GW samples and BFS samples

clustered separately implying that sampling method was more important than well sampled in bacterial community structure. BFS – 37, 38, and 39 are almost as similar to the GW – 37, 38, and 39 as they are to the samples that we expect to be very different based on the well environmental conditions and from what was seen in the Figures 3 and 4. The samples from Well 32 did not conform to the clustering seen in the previous figures or from Table 1; GW – 32 and BFS – 32 did not cluster together and had similarity of 34%.

Conclusion

There is not a great similarity between the T-RFLP patterns of the groundwater samples and the BFS samples, but the same general trend of anaerobic metabolism was apparent. In each dendrogram created by Bray-Curtis similarity, well clusters matched the well water characteristics associated with anaerobic metabolism. Wells 31, 32, and 34 had lower concentrations of iron and TPH and greater concentrations of sulfate in the well water and therefore low relative levels of anaerobic metabolism. The other cluster of wells had a greater rate of anaerobic metabolism, Wells 37, 38, and 39, had greater concentrations of iron and TPH but lower concentrations of sulfate. Therefore the Groundwater Sampling method successfully assessed the degradation of petroleum diluent in groundwater as confirmed by the Biofilm Sampler method. There were no significant trends observed between the microbial communities and the associated plant rhizosphere. Plant type was not a primary cause of differentiation in bacteria community structure between wells. There was a clear difference between the sampling methods, groundwater sampling and biofilm samplers, and within each biofilm sampler substrate, the inner core and outer radius. The low levels of similarity between the inner core and outer radius of substrate did not affect the overall clustering of wells based on anaerobic metabolism as seen in Table 1 and Figures 3, 4 and 5. But within the two clusters of wells there was a lower level of similarity than expected. Because of the great degree of variance between the inner core and outer radius of the substrate in the samplers only general trends for studies with a large enough scope for success at this time. Both methods do not collect a large enough sample size to provide a complete analysis of the microbial community otherwise the samples would cluster by well and not by method.

Future Studies

A study utilizing more biofilm samplers with longer residence times within the wells would result in a greater level of bacterial colonization. In this study we have not determined the residence time required for the most representative sample. Therefore a long-term study is required. Longer Biofilm Sampler incubation period within the wells is advised because very low 16S rDNA was extracted from these samples, which led to large concentrations of primer-dimer and allowed any DNA contamination to have a greater impact on each T-RFLP. More samplers concentrated in single site will allow for a better comparison of groundwater characteristics to the microbial community. A BFS study employing a range of BFS diameters would be beneficial to determine microbial colonization rates and variances in the population.

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