Molecular Signatures of Petroleum Degrading Bacteria in Petroleum Affected Groundwater from the Guadalupe Dunes

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Abstract

The intent of this study was to identify microorganisms associated with the degradation of petroleum in groundwater samples from the leading edge of a dissolved petroleum plume at the Guadalupe Dunes Restoration site using molecular methods. The samples were collected from monitoring well I6-2, located down-gradient from the source of the petroleum plume. DNA was extracted and purified from the groundwater samples and a section of the genome was amplified by PCR using 16S rRNA gene primers. PCR products were digested with the enzymes *Hae*III and *Dpn*II and then analyzed by Terminal Restriction Fragment Length Polymorphism (TRFLP). Microorganisms were identified through statistical analysis of TRFLP data. The groundwater contained signatures of Proteobacteria species known for their role in bioremediation, showing that the process of petroleum degradation may be active in the leading edge of the plume, despite low petroleum concentrations.

Introduction

Monitored natural attenuation is the preferred method for reducing the toxicity level in contaminated environments such as soil and groundwater. It is a form of bioremediation that monitors the chemical changes of the site through bacteria that degrades contaminants as part of its metabolic process. Natural attenuation is a low-cost and effective process for restoring the environment [6].

In 1998, San Luis Obispo County approved the Guadalupe Dunes Restoration Project. The 2,700 acres Guadalupe oil field is a part of the Guadalupe Dunes, located on the central coast of California between San Luis Obispo County and Santa Barbara County [6]. Oil production began during the 1940s; in the 1950s, pipelines were built and diluent was pumped through the pipes to help the thick crude oil flow. A few years afterwards, the pipelines rusted and the diluent leaked into the dune sand aquifer. Due to the flow of the underground water, the contamination dispersed across the dunes, creating a plume originating from the diluent source [6].

Recently, Unocal has been working with the EBI to create research opportunities that will investigate new means of restoring the petroleum-contaminated sites [1]. Natural attenuation is being employed as a safe alternative to harsher methods of cleaning up the contamination at Guadalupe Dunes. The research developed at the EBI focuses on developing models to evaluate the effectiveness of this method. As part of this process, groundwater samples from a plume and the surrounding monitoring wells are analyzed for the relationship between the hydrogeochemical data and the microbial communities present.

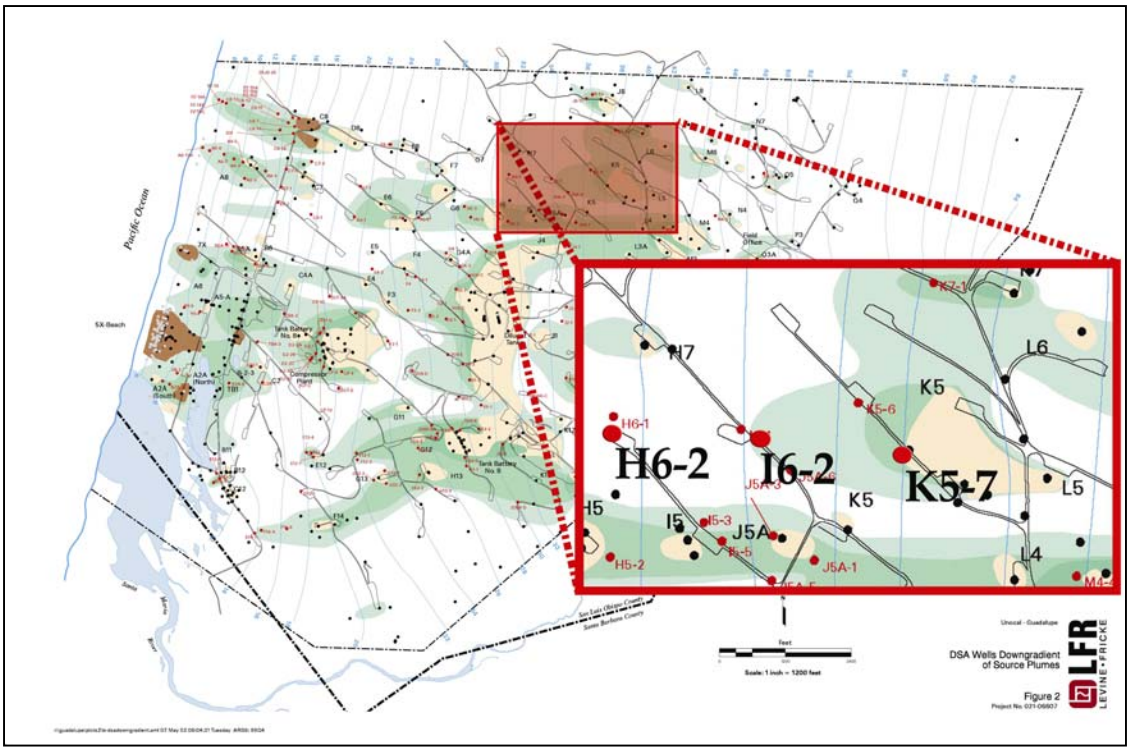
The following study used terminal restriction fragment length polymorphism (TRFLP) to classify petroleum degrading microorganisms from contaminated groundwater. TRF analysis is a quick and effective method for this study, and TRF patterns can be analyzed to examine the dynamics of microbial communities [5,6]. Groundwater samples were retrieved from one specific monitoring well, I6-2, and DNA extracted from the groundwater undergo PCR to amplify a sequence of the 16S ribosomal DNA. The PCR products (labeled with a fluorescent dye) were digested with two enzymes, *Dpn*II and *Hae*III, and the size of the labeled fragments was determined. The TPH (total petroleum hydrocarbon) degrading microorganisms were identified through further statistical analysis of the TRF peaks. Microbial communities were then assessed for correlation to physical data from the well.

Materials and Methods

Sampling

Samples used in this study were obtained from I6-2, a monitoring well positioned on the edge of the petroleum plume and farthest from the diluent source (Figure 1). The well is screened within the dune sand aquifer so groundwater samples may be extracted and studied. I6-2 is sectioned into smaller wells located vertically at increasing depth intervals; these are labeled I6-2A, I6-2B, I6-2C, and I6-2D, with I6-2D being nearest to the surface. Samples taken from each of the smaller wells were tested for physical and hydrogeochemical data by outside laboratories (BC labs, Bakersfield, CA; Inland Empire Labs, CA). The compounds of most interest in this study are listed in Table 1. This data was used in the study to identify petroleum-degrading microorganisms by correlating the TRF patterns with chemical data of the well groundwater.

Figure 1. Map of the Guadalupe Dunes contaminated site. Magnified region shows the position of I6-2 in relation to the diluent source. The green regions represent the petroleum plume. Darker green color shows areas where TPH levels are concentrated.



DNA was extracted from the samples by following the protocol for the MoBio Power Soil® DNA extraction kit (MoBio Inc, Carlsbad, CA). DNA samples were electrophoresed in a 1% agarose gel (100V for 20 minutes), then stained with ethidium bromide and photographed under UV light to verify the presence of genomic DNA.

PCR

PCR was performed to detect a section of the 16S rRNA gene; the primers 46-Ba2F (forward, 5'-GCYTAACACATGCAAGTCGA-3') and K2R (reverse, 5'-GTATTACCGCGGCTGCTGG-3') were used [6]. Each well sample requires three replicates (3 uL of DNA sample each) to ensure that an adequate amount of PCR product will be obtained. *E. coli* was used as a positive control and a closed tube was used as a negative control. Reagents and the amounts used for each PCR reaction are listed in Table 1.

The PCR reaction was performed under the following conditions: 94° C for 10 minutes; 30 cycles of 94° C for 1 minute, 46.5° C for 1 minute, 72° C for 2 minutes, then 72° C for 10 minutes. The presence of PCR product was verified by agarose-gel electrophoresis. The MoBio PCR Ultra-clean® kit (MoBio Inc, Carlsbad, CA) was used for PCR cleanup; the three replicates are combined during this process. The concentration of amplified DNA is determined with the Bio-Tek Fluorometer.

Table 1. Reagents and the amount used (in uL) for each PCR reaction

Reagents	**Amount (uL)**
10X GOLD buffer	5
dNTPs (10 mM)	3
BSA	2
$MgCl_2$ (25 mM)	7
46F (10 uM) labeled forward primer*	1
K2R (10uM) reverse primer	1
Taq GOLD (5 U/uL)	0.3
PCR H_2O	27.7
Total Reaction Volume	47

* 46F forward primer is labeled with a Cy5 fluorescent dye.

Enzyme Digests

PCR products were digested separately with the enzymes *Hae*III (10,000 U/mL) and *Dpn*II (10,000 U/mL). For each sample, 75 ng of DNA (from the PCR) was used in the digest. *Dpn*II digest - 0.6 uL *Dpn*II enzyme and 4 uL buffer were used for each reaction. DNA and PCR water were added to reach a volume of 40 uL. *Hae*III digest – 1 uL *Hae*III enzyme and 4 uL buffer were used per reaction. DNA and PCR water were added to reach a volume of 40 uL.

Precipitation and Fragment Analysis

After enzyme digestion was complete, excess salts were removed from DNA by ethanol precipitation. The samples were mixed with formamide and a base pair standard, and then ran through the CEQ™8000 Genetic Analysis System.

TRF pattern analysis

TRF patterns obtained after sequencing were aligned using the CEQ™8000 software and the data (TRF peak area and fragment size in nucleotides) transferred to Microsoft Excel for further analysis. Data from both the *Hae*III and *Dpn*II digestions were analyzed to give more accurate results when identifying the microorganisms.

Statistical analysis was performed by calculating the area under each TRF peak as a percentage of the total area of the TRF pattern. These new values were written as parts per million (ppm). The data was then truncated to remove any TRF peaks with an area that was less than 1.0% of the total sample pattern, as these peaks were not considered significant for analysis. [6]. TRF peaks of around 80 and 120 represent primer dimers and were excluded from analysis, as well as peaks in the 400-500 range which indicate uncut DNA.

Results and Discussion

Physical Data of the Wells

A contaminated site may harbor a variety of microorganisms that utilize different chemicals as energy sources for petroleum degradation. Hydrogeochemical data of the wells was collected and organized in Excel (Table 2). The relationship between TPH and other groundwater chemicals can be used to look for evidence of bioremediation and provide insight into the kind of microorganism present. TPH was plotted with CO_2 and oxygen, methane, ferrous iron, and sulfate. Figures 2-5 demonstrate how the values for each compound change with depth while also comparing this with changes in the levels of TPH. For easier visual comparison, TPH data was plotted on a secondary Y axis for all graphs.

Figure 2 illustrates the relationship between TPH, oxygen, and CO_2. Levels of petroleum should be higher near the bottom well (I6-2A) where the contamination is concentrated. The top well (I6-2D), located nearer to the surface, contains less TPH and the highest level of oxygen. With increasing depth, oxygen is being depleted due to TPH degrading bacteria that utilize oxygen as an electron acceptor. Oxygen and carbon dioxide should have an inverse relationship because as the microbes are using oxygen to degrade TPH, they are producing carbon dioxide as a waste product. It can be assumed from Figure 4 that the bacteria in well I6-2 are performing aerobic respiration.

In anaerobic conditions, methanogenic petroleum degradation occurs as a collective process thru the interaction of different microbes. Hydrogen producing bacteria first degrade petroleum and produce CO_2 and H_2, and then methanogens utilize the H_2 and CO_2 to produce methane [2]. High levels of TPH should result in more growth of

TPH degrading bacteria, more H_2 produced, and more methanogenic bacteria. Therefore, it would be expected that methane levels would increase as the level of TPH rises in the environment. Figure 3 shows a negative correlation between TPH and methane levels, which suggests that there are no methanogenic microorganisms in the well. Even though the data does not suggest the presence of methanogenic bacteria, a high level of methane still exists in well I6-2D. Perhaps the methane produced by bacteria up gradient (nearer to the source of the plume) trailed downstream to well I6-2. New methane is not being produced in well I6-2, which is indicated by the drop in the level of methane between wells I6-2D and I6-2C.

During petroleum degradation, ferric iron (Fe^{3+}) can serve as an electron acceptor for certain microorganisms when oxygen levels are low, thus reducing it to ferrous iron (Fe^{2+}) [2]. With increasing depth, there is more TPH available for metabolic use, so it is expected that ferrous iron (the waste product) would be produced at higher levels. Figure 4 shows a positive correlation with TPH and ferrous iron. The graph suggests that there exists a TPH degrading microbial community in the well that uses ferric iron as an electron acceptor.

Some sulfate reducing bacteria are known to be TPH degraders; sulfate can be consumed during metabolism as an electron acceptor while TPH acts as the electron donor [3]. As TPH levels increase, the bacteria should be reducing the level of sulfate in order to degrade petroleum. The plots in Figure 5 show an apparent difference from the relationship expected between TPH and sulfate. This implies there was no sulfate reducing, TPH degrading bacteria in well I6-2. The sulfate rich mud that lies underneath the dune sand aquifer may explain the correlation seen in Figure 5. It's likely that sulfate

from the mud dissolved into the aquifer, resulting in a gradient from high concentration to lower concentration as the sulfate moves up toward the surface. Therefore, the positive correlation between TPH and sulfate is not due to bacteria, but rather the diffusion of sulfate from the mud into the aquifer.

Table 2. Physical and hydrogeochemical data for Well I6-2A to I6-2D

Well Number	Depth Below Groundwater (feet)	Screen Interval (feet bgs)	Ferrous Iron (mg/L)	O_2 (mg/L)	CH_4 (mg/L)	CO_2 (mg/L)	SO_4 (mg/L)	Average TPH* (mg/L)
I6-2A	9.71	76.55-77.75	1.9	5.16	0.003	4.43	140	0.60
I6-2B	6.174	73.25-74.25	1.89	5.00	0.003	5.16	130	1.083
I6-2C	2.747	69.75-70.75	0.47	5.2	0.007	5.33	64	0.57
I6-2D	-0.742	66.25-67.25	0.39	6.96	0.029	2.72	34	0.407

* TPH = Total Petroleum Hydrocarbons

Figure 2. Line Plot Comparing the levels of TPH, Oxygen, and Carbon Dioxide in Wells I6-2D to I6-2A.

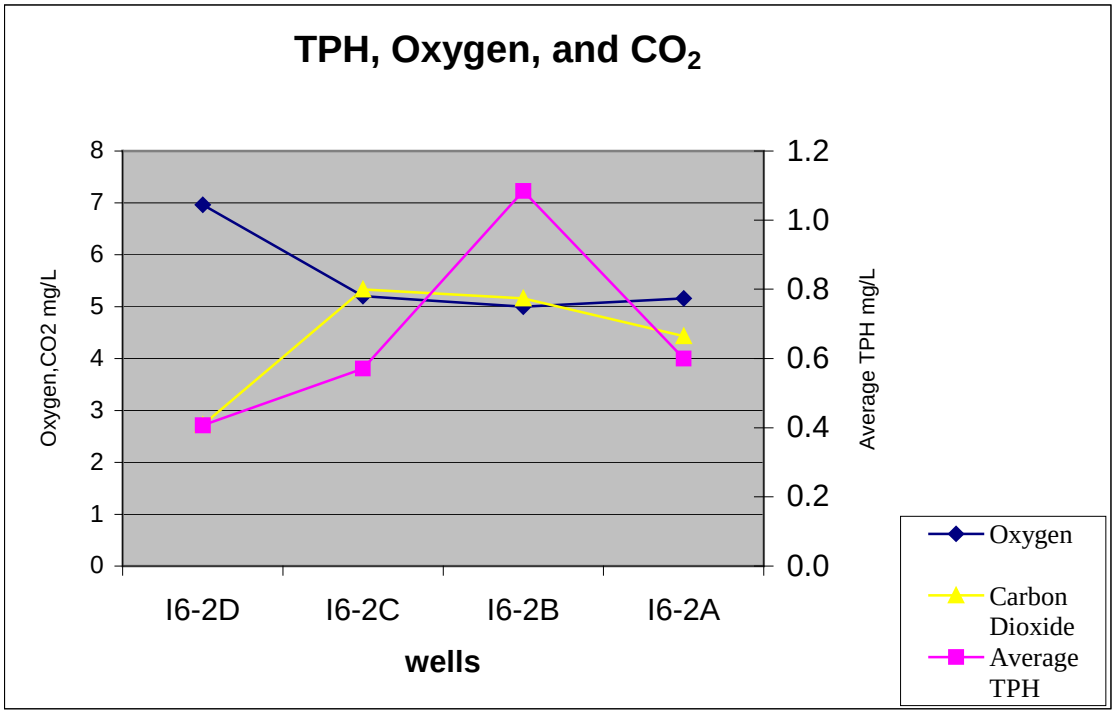


Figure 3. Line Plot Comparing the levels of TPH and Methane in Wells I6-2D to I6-2A.

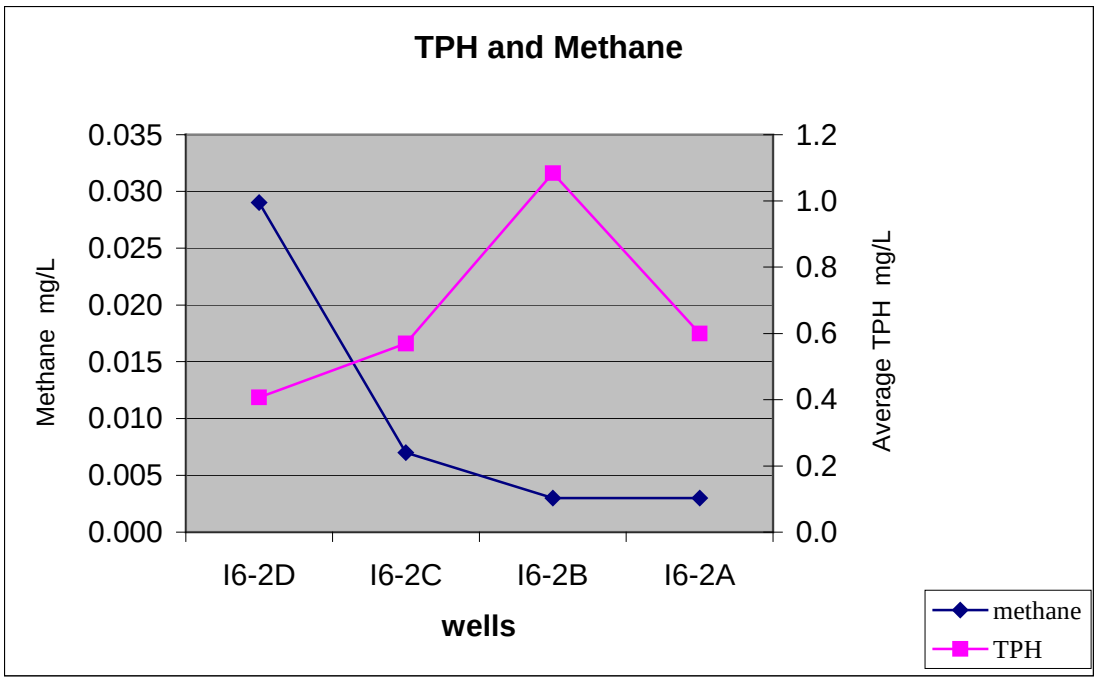


Figure 4. Line Plot Comparing the levels of TPH and Fe^{2+} in Wells I6-2D to I6-2A.

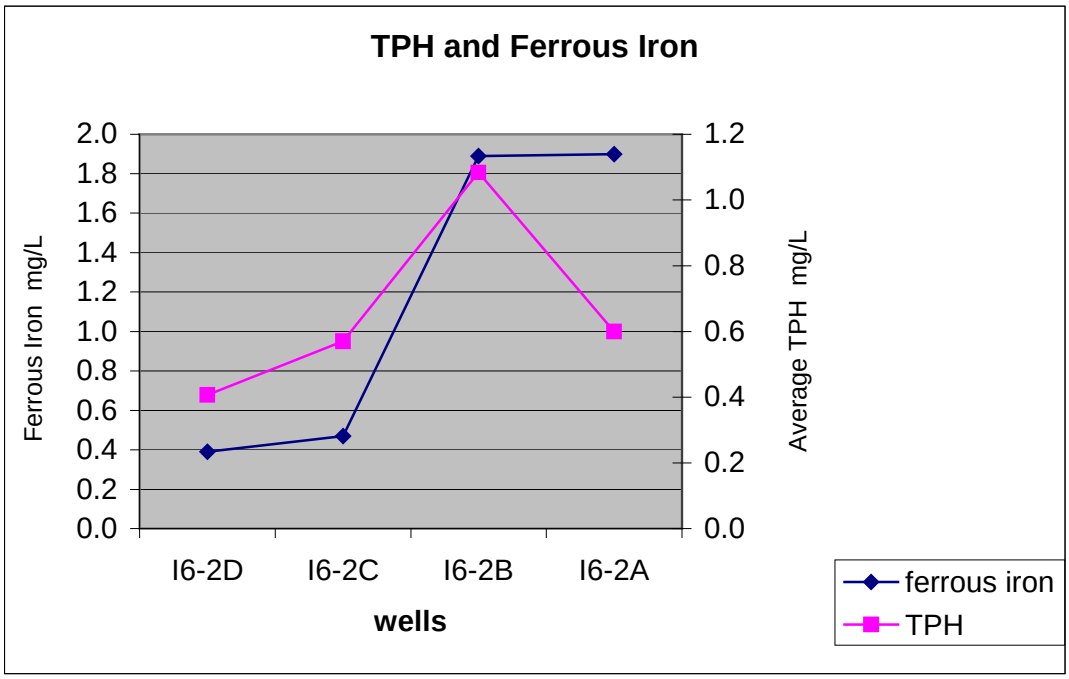
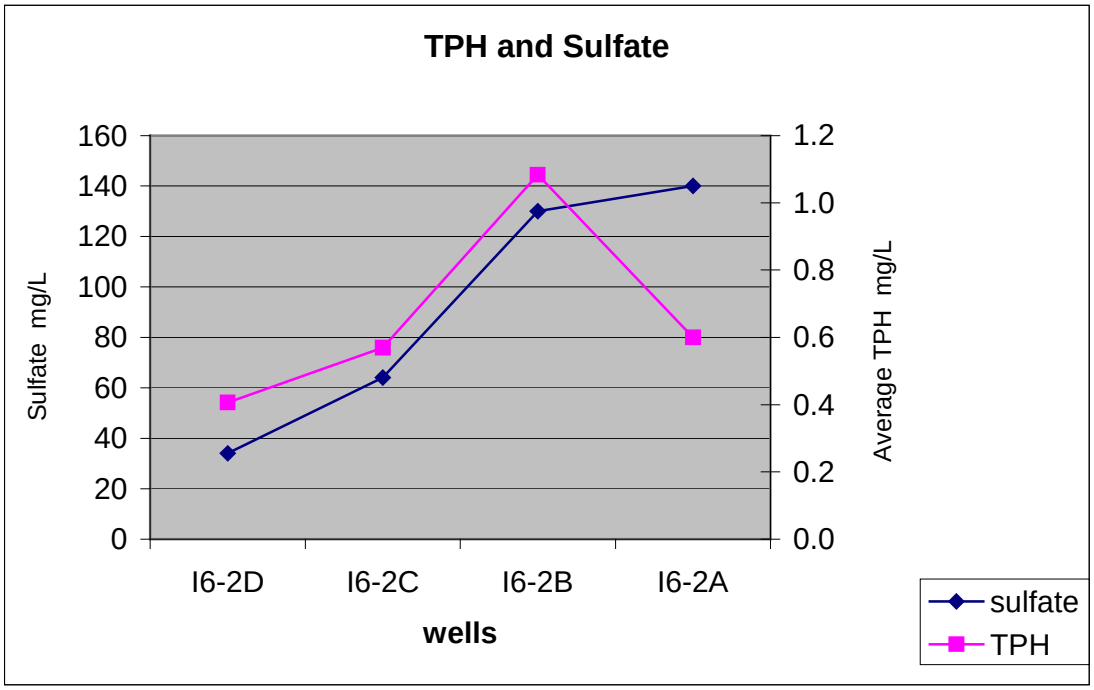


Figure 5. Line Plot Comparing the levels of TPH and Sulfate in Wells I6-2D to I6-2A.



Association of TRF Peaks with Hydrogeochemical Data

Figures 6 and 7 show overlapping patterns for each well sample from the digestions with *Hae*III and *Dpn*II. The TRF patterns alone are not sufficient to identify the bacteria present in well I6-2. Data from the TRF peaks and the hydrogeochemical data of the well must be analyzed together to produce conclusive results. *Hae*III and *Dpn*II enzymes each cut at unique sites of the DNA and therefore, produce different fragment lengths. If TRF peaks from two separate enzymes are suspected of coming from the same organism, similarities should be seen in peak area and correlations to chemical data.

After the percentage of total peak area (ppm) was calculated, the relative abundance of each TRF peak was correlated with the chemical data from the wells. The list was sorted in ascending order based on correlation with TPH, CO_2, and Fe^{2+} data; this was done separately for data from both *Hae*III and *Dpn*II enzyme digests. Peaks with a strong negative or positive correlation were kept for further analysis.

Data from *Hae*III and *Dpn*II were then compared to identify peaks from different enzymes that were close in peak area and correlations to chemical data. The chosen peaks (Table 3) all have a strong negative correlation with the chemical data of the wells. High negative correlation values indicate that more bacteria will be found in an environment with low TPH and high oxygen levels. This data supports our previous hypothesis that the TPH-degrading bacteria in the wells are mainly aerobic. Ultimately, four combinations were arranged that paired TRF peaks from *Hae*III and *Dpn*II. Each pair of peaks displayed similarities in both their chemical and statistical data.

The Z score was calculated for each TRF peak to validate our selection. The Z score represents the number of standard deviation units from the mean; it is calculated by taking the mean and standard deviation of all TRF peak area values from a well (e.g. I6-2A) and determining how far one value is from the mean. Each arranged pair is graphed according to their Z scores (Figures 8 –11). If a similar trend is seen in the *Hae*III and *Dpn*II plots, it can be concluded that the two TRF peaks are likely to represent the same microbe.

Figure 6. TRF patterns from digestion with *Hae*III enzyme. Patterns from I6-2A (*Hae* A), I6-2B (*Hae* B), I6-2C (*Hae* C), and I6-2D (*Hae* D) are overlapped for comparison.

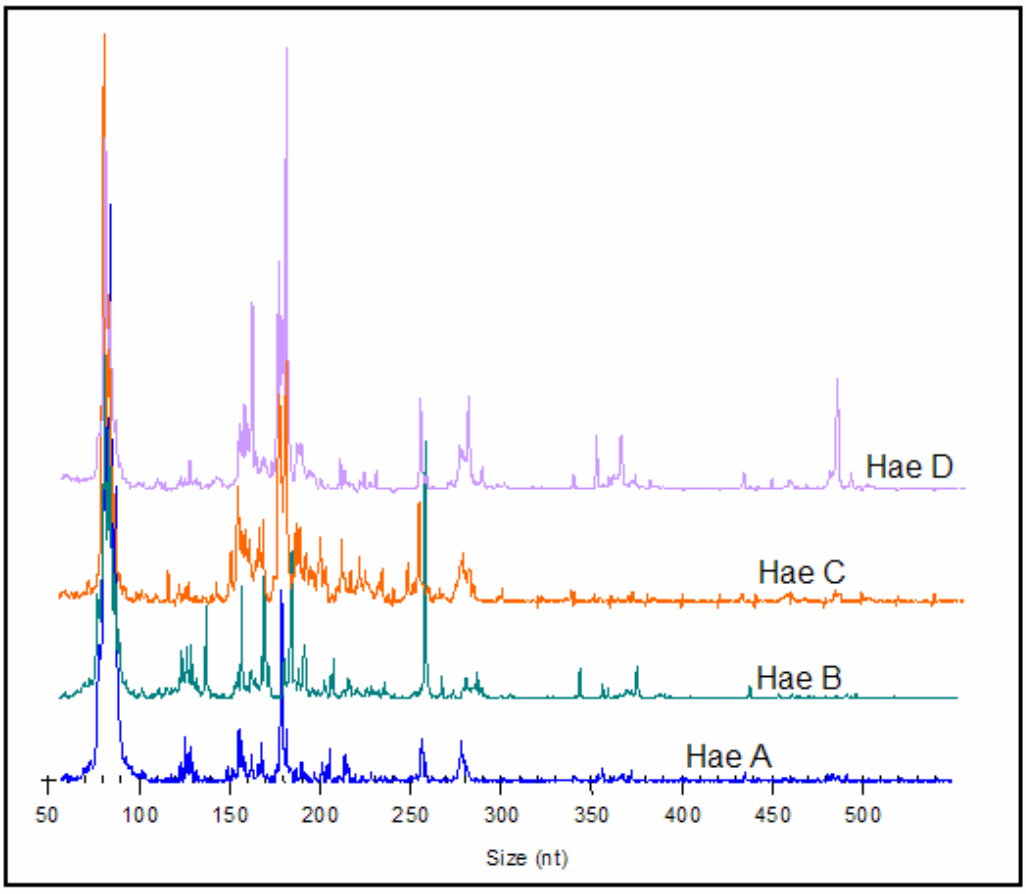


Figure 7. TRF patterns from digestion with *Dpn*II enzyme. Patterns from I6-2A (*Dpn* A), I6-2B (*Dpn* B), I6-2C (*Dpn* C), and I6-2D (*Dpn* D) are overlapped for comparison.

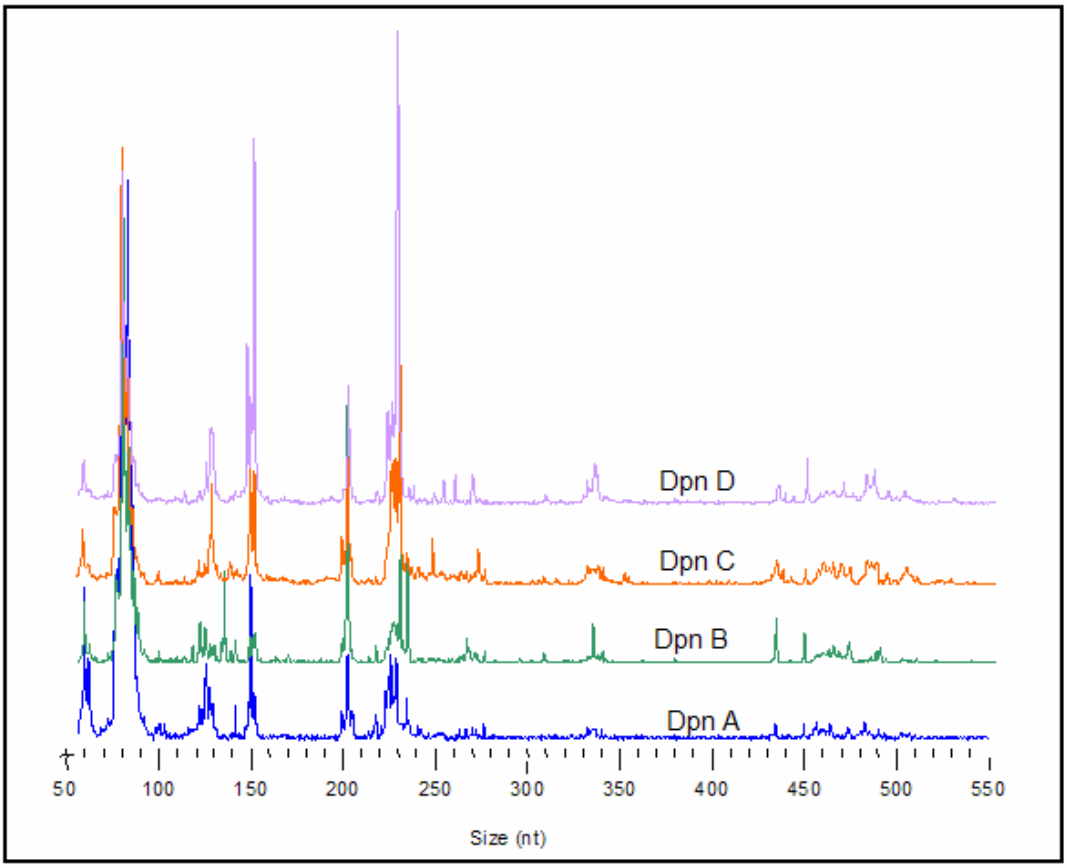


Table 3. Excel table of TRF peak data (% of total peak area) and correlation with hydrogeochemical data of the wells from digestion with the enzymes *Hae*III and *Dpn* II.

BP	**I6-2A *Hae***	**I6-2B *Hae***	**I6-2C *Hae***	**I6-2D *Hae***	**TPH**	**Fe2+**	**CO_2**
163	0.00%	1.54%	1.37%	13.50%	-0.54	-0.64	-0.90
182	2.04%	0.00%	19.30%	23.69%	-0.77	-0.99	-0.52
BP	**I6-2A *Dpn***	**I6-2B *Dpn***	**I6-2C *Dpn***	**I6-2D *Dpn***	**TPH**	**Fe2+**	**CO2**
154	3.36%	2.59%	7.84%	15.77%	-0.75	-0.86	-0.81
230	6.44%	3.39%	8.40%	24.05%	-0.75	-0.73	-0.92

Figure 8. Line Plot Comparing the Z score Values of *Hae* 182 and *Dpn* 154 in Wells I6-2A, I6-2B, I6-2C, I6-2D

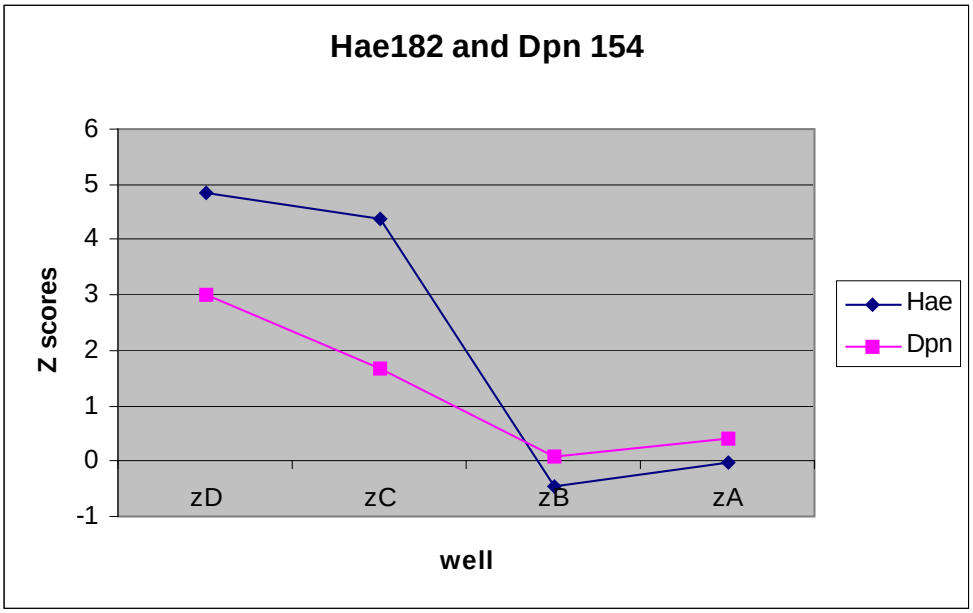


Figure 9. Line Plot Comparing the Z score Values of *Hae* 182 and *Dpn* 230 in Wells I6-2A, I6-2B, I6-2C, I6-2D

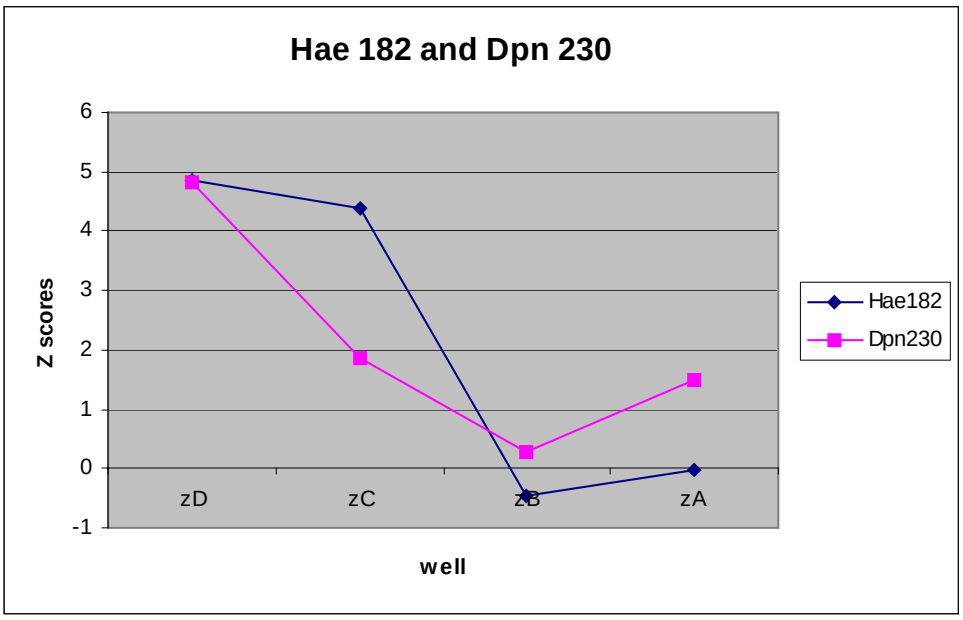


Figure 10. Line Plot Comparing the Z score Values of *Hae* 163 and *Dpn* 230 in Wells I6-2A, I6-2B, I6-2C, I6-2D

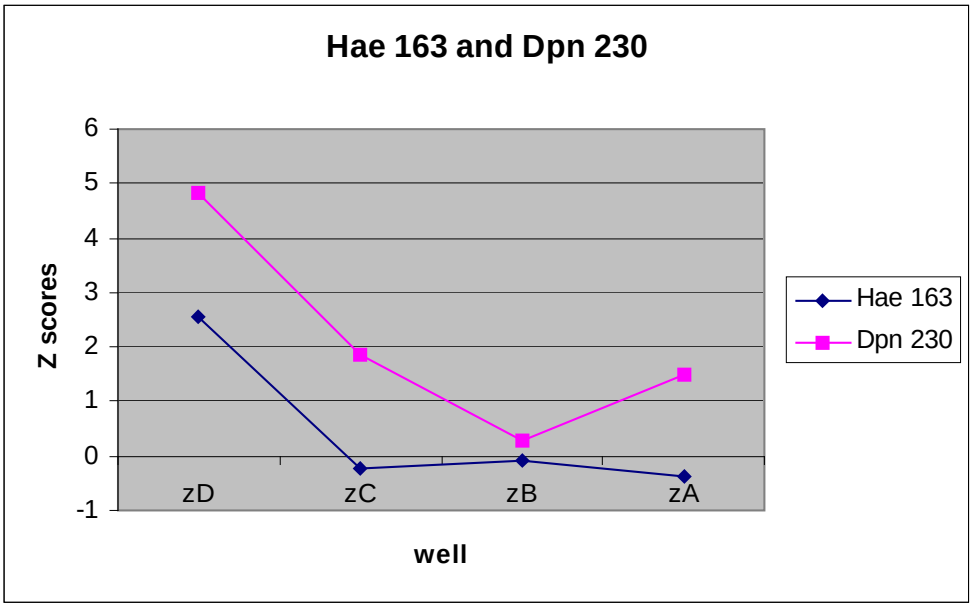
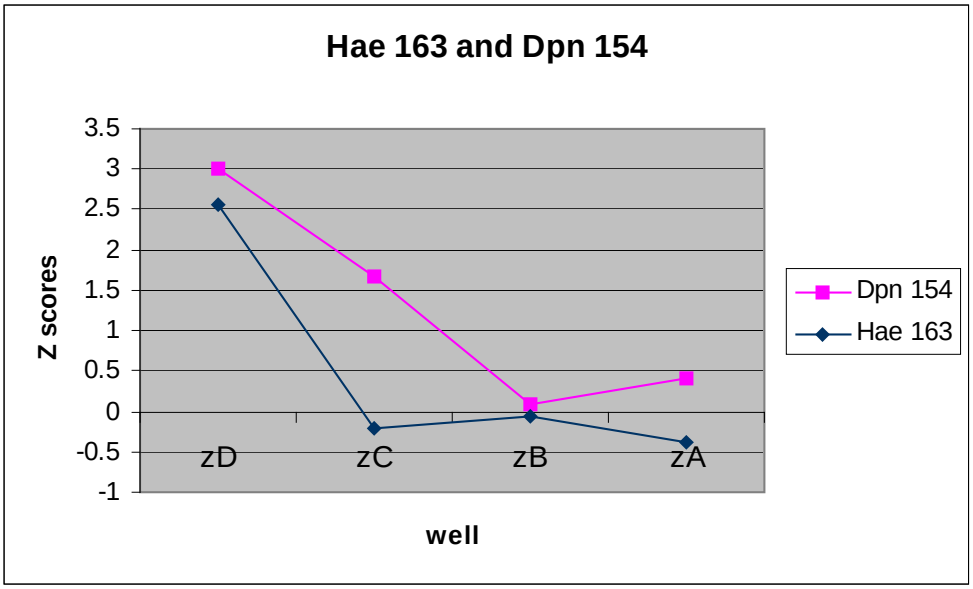


Figure 11. Line Plot Comparing the Z score Values of *Hae* 163 and *Dpn* 154 in Wells I6-2A, I6-2B, I6-2C, I6-2D



Microbial Identification

TRF peak values for each enzyme were associated with corresponding values on the EBI database to provide possible matches to TPH degrading bacteria. The EBI database is a collection of records from GenBank listing the organism isolated and the prominent peaks found after digestion with various enzymes (including *Dpn*II and *Hae*III). A few prominent genuses were chosen out of a list of bacteria matching our data. *Hae*182/*Dpn*154 is indicative of *Nitrosomonas* sp. and *Dechlorisoma* sp ; *Hae*182/*Dpn*230 is associated with *Pseudoalteromonas* sp.; *Hae*163/*Dpn*230 corresponds primarily with isolated organisms of *Acinetobacter* sp. and *Pseudomonas* sp.; *Hae*163/*Dpn*154 did not provide any conclusive matches. (Table 4). All bacteria discovered in the well samples are grouped within the phylum Proteobacteria, which are gram-negative organisms with varying metabolic abilities. *Acinetobacter*, *Pseudomonas*, and *Pseudoalteromonas* are classified in the phylum Gamma-Proteobacteria, while *Nitrosomonas* and *Dechlorisoma* are classified within Beta-Proteobacteria [2]. With the exception of *Nitrosomonas* sp. and *Dechlorisoma* sp., all other species of microorganisms found in the analysis are frequently seen in bioremediation and are known TPH degraders.

Table 4. Result of possible microbial species present in Well I6-2 after referencing TRF peaks with the EBI database

***Hae* III**	***Dpn* II**	**Tentative I.D.**
182	154	*Nitrosomonas* sp., *Dechlorisoma* sp.
182	230	*Pseudoalteromonas* sp.
163	230	*Acinetobacter* sp., *Pseudomonas* sp.
163	154	No conclusive matches

Conclusion

In previous studies on monitoring wells located closer to the source of the petroleum plume, methanogens and other bacteria were found that correlated with a more anaerobic environment [6]. In comparison, this study identified mainly aerobic bacteria in the samples due to the fact that the well examined is located on the edge of the plume where the environment contains less TPH. However, the results suggest the presence of petroleum degrading bacteria, meaning it is possible that the bioremediation process is still active at that site. In addition, more diverse groups of organisms were anticipated to be present in the samples. The unexpected results may be due to the fact that with increasing depth, no significant changes were seen in the level of TPH and other substances. The petroleum at that distance may exist in only small amounts and be mixed unevenly with the soil, causing the unpredictable distribution of microbial communities. In order to confirm this hypothesis, further analysis of other similar wells may be necessary.

Acknowledgements

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References

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