

**Two Dimensional Analysis of
Groundwater Bacteria at the Edge of a Dilute Plume of
Dissolved Petroleum**

by

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ABSTRACT

This study investigated groundwater samples from the Guadalupe Dunes restoration project. The main goal of this project was to seek the identification of bacterial communities associated with the natural attenuation of petroleum. Groundwater samples from wells K5-7, H6-2, and I6-2 at the Guadalupe Dunes were analyzed as part of Unocal's natural attenuation study. These wells were located at the trailing end of a dissolved petroleum plume. The well to plume distance from closest to furthest was K5-7, I6-2, and H6-2, respectively. Each of the wells contains incremental screening depths AA-D, with AA being the deepest from ground surface level. Physical data obtained from the screening depths within each well includes petroleum, methane, sulfate, oxygen, ferrous iron, and carbon dioxide. DNA was also isolated from the groundwater samples so as to obtain Terminal Restriction Fragment (TRF) patterns for each well's individual screening depth. The bacterial community structure, identified by TRF pattern analysis, was then correlated with physical data to determine the organisms present during natural attenuation. It was found that of the three wells, only H6-2 showed sufficient physical data to continue with analysis and correlation with TRF patterns. Ultimately, PCA analysis of TRF peaks showed high potential for TPH degradation at the deepest interval H6-2AA.

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1.0 INTRODUCTION

1.1 Background & Goals

Groundwater contamination of petroleum hydrocarbons from spills, leaks, and improper disposal has become a worldwide issue (4, 11). As a result, Monitored Natural Attenuation (MNA) has become the leading process to reduce groundwater contamination of petroleum hydrocarbons. MNA, the use of natural processes to reduce petroleum concentrations has resulted in effective, low cost, remediation with minimal environmental impact (9, 15, 16). MNA processes include bioremediation, sorption, volatilization, dispersal, and diffusion (9, 12, 14, 15, 19). The focus of this study was bioremediation, the principle process of MNA. Bioremediation utilizes indigenous soil microbes to degrade contaminants, thereby completely converting hydrocarbons to carbon dioxide, water, and methane (19, 21).

MNA was considered a viable remediation strategy for this study when evidence established that sustainable biodegradation was occurring (22). Evidence for sustainable biodegradation of petroleum hydrocarbon included detection of hydrocarbon intermediates, depletion of electron donors, microcosm studies, and a description of the microbial community involved in biodegradation (3, 18, 19, 22). Therefore, by studying the physical and bacterial characteristics of contaminated sites, it was possible to predict the efficiency and sustainability of bioremediation dependent MNA for specific sites (3, 4, 14, 19).

This study took place at the Guadalupe Dunes, a former oil field, which spans 2,700 acres within the larger Guadalupe-Nipomo Dunes Complex on the central California coast. Oil exploration and production began on the site in 1947, continued to

its peak in 1988, with 215 producing wells, and ceased in 1994 (10, 17). In an effort to thin the oil and make pumping more efficient, a light petroleum distillate, referred to as diluent, was pumped into the wells (9, 10). Unfortunately, tanks and pipelines used to distribute the diluent developed leaks, inadvertently releasing hydrocarbons into the environment. Over a period of time, the diluent accumulated at the water table in the dune sand aquifer (10). The rapid ground water flow rate, combined with the permeable sand matrix, eventually resulted in groundwater contamination beneath much of the site (10). Consequently, MNA, in particular bioremediation, was chosen as a suitable remediation strategy of this site because of the depth to groundwater, the fragile dunes ecosystem, and the presence of many endangered species (17).

This project was a subset of Unocal's natural attenuation study at the Guadalupe Dunes. As part of a much larger study regarding the use of MNA, it was the goal of this project to show a correlation between bacterial community structure and physical data. The project examined the vertical and horizontal bacterial communities at distances from the plume, in the hopes of further determining the organisms involved in natural attenuation.

1.2 Site Description & Sampling

The Guadalupe Dunes contained three aquifers; the dissolved petroleum was limited to the uppermost dune sand aquifer (21). This study examined groundwater samples from wells K5-7, I6-2, and H6-2, located at the trailing end of a dissolved petroleum plume (Figure 1.0). The horizontal gradient of well to plume distance from closest to furthest was K5-7, I6-2, and H6-2, respectively. The nested wells, H2-2, I6-2,

and K5-7, consisted of 4-5 smaller wells, labeled AA, A, B, C, and D within each large nested well. For example, well H6-2 contained smaller wells labeled H6-2AA, A, B, C, and D. The nested wells had 0.3 m screens placed at 5 ft. intervals.

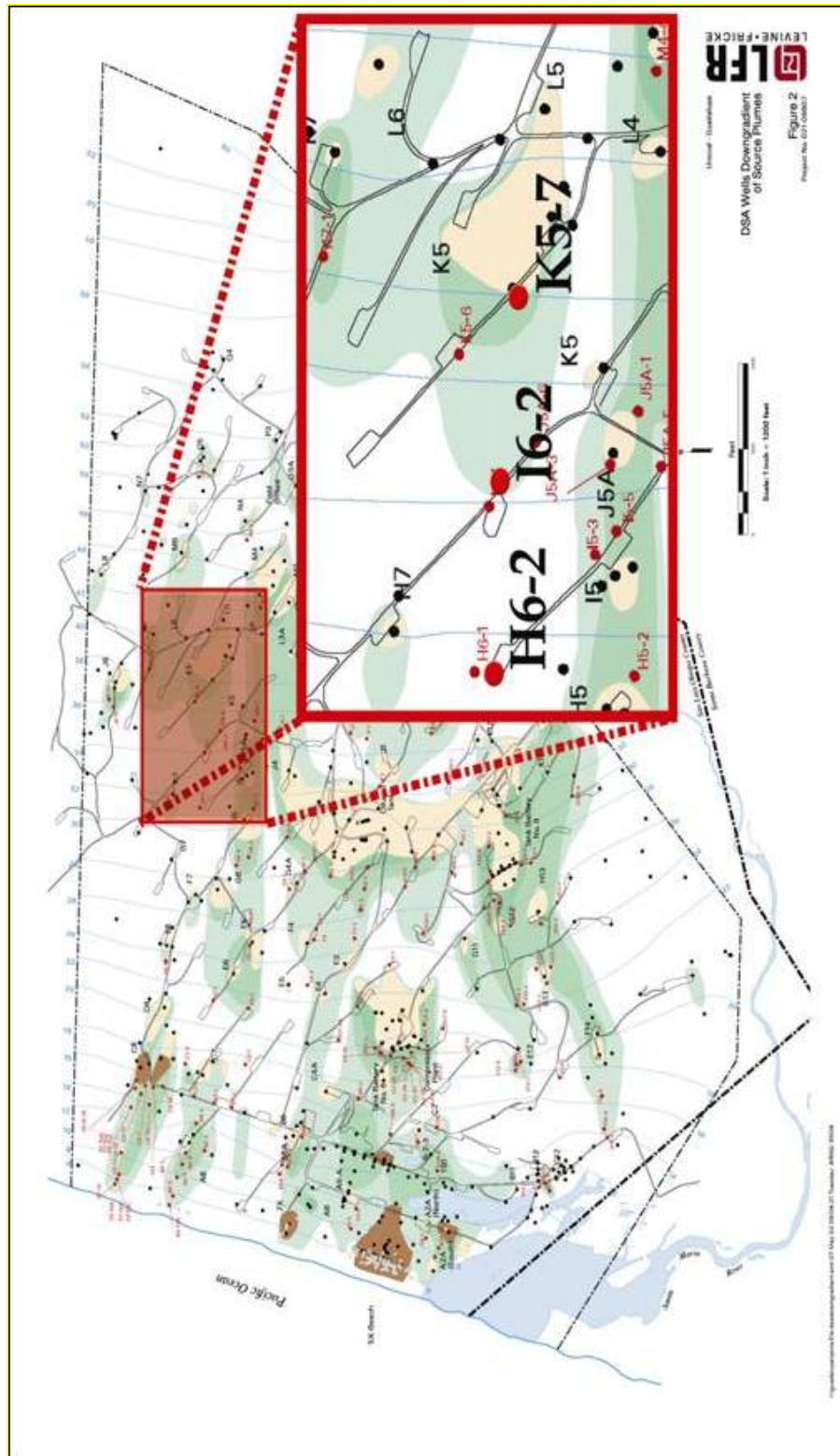


Figure 1.0: Plume and sample well locations for horizontal and vertical profiles

2.0 MATERIALS & METHODS

2.1 Sampling Procedure

Anaerobic groundwater samples were collected in sterile glass bottles by Unocal employees. Each sample was collected by letting the bottle overflow, after approximately 4 well volumes of standing water was removed from the well by a submersible pump (horizontal profile) or inertial pump (vertical profile) (21). It should be noted that any headspace in the bottle was carefully removed (21). The groundwater samples were subsequently transferred to Zymax Envirotechnology in San Luis Obispo, California, where they were stored for less than 24 hours at 4 °C until chemical and biological processing occurred (7, 21).

2.2 Chemical Measurement & Analysis

Chemical measurements were performed by Zymax Envirotechnology. The total concentration of TPH, methane, sulfate, oxygen, ferrous iron, and carbon dioxide were measured for each depth of each well. TPH concentrations varied from no detection to 1.46 mg/L. Prior to performing statistical analysis, the natural logarithm of the physical data was taken to account for non-normal distributions common in concentration measurements (6). Then, the physical data was analyzed using Principle Component Analysis (PCA) along with Minitab (Minitab Inc., State College PA). Using these two analytical methods, a correlation matrix was used to look for trends and to organize the data for comparison to bacterial community information collected from TRF patterns (21).

Table 1. Natural Logarithm of Physical Data										
WN	IFe	O2	CH4	CO2	dFe	NO3	SO4	TPH	Depth	
H6-2D	-5.30	2.27	-7.60	0.07		1.53	2.83	-3.69	1.33	
H6-2C	-5.30	2.14	-7.60	0.71		1.84	2.94	-3.69	1.91	
H6-2B	-5.30	1.86	-7.60	1.10		2.09	3.58	-3.69	2.27	
H6-2A	-0.94	1.23	-7.60	1.35		-0.24	3.95	-2.84	2.53	
H6-2AA	0.75	1.17	-7.60	1.33		-3.00	4.61	-2.70	2.80	
I6-2D	-0.94	1.94	-3.54	1.00	-0.51	-3.00	3.53	-0.90	1.00	
I6-2C	-0.76	1.65	-4.96	1.67	-0.49	-3.00	4.16	-0.56	1.82	
I6-2B	0.64	1.61	-5.81	1.64	0.74	-3.00	4.87	0.08	2.28	
I6-2A	0.64	1.64	-5.81	1.49	1.13	-3.00	4.94	-0.51	2.58	
K5-7D	-5.30	1.77	-5.30	1.18	-2.72	-3.00	3.53	0.96	0.94	
K5-7C	-5.30	1.67	-4.07	1.42	-2.12	-3.00	3.91	-0.69	1.66	
K5-7B	-1.61	1.65	-2.04	1.69	-1.17	-3.00	3.89	-0.15	2.09	
K5-7A	-2.30	1.83	-5.81	1.82	-1.66	-3.00	4.29	1.46	2.37	
K5-7AA	-1.61	1.90	-6.21	1.39	-0.87	-3.00	4.94	0.26	2.62	

*Depth was calculated by subtracting screening interval elevation from groundwater elevation measurements.

2.3 DNA Extraction

Four liters of groundwater from each well were filtered through 0.2 micrometer nylon membrane filters (21). The filters were frozen and half of each filter was crushed and homogenized under liquid nitrogen (21). Samples were extracted using MoBio Power Soil DNA Extraction Kit (MoBio® Laboratories Inc., Solana Beach, CA) following the manufacturers protocol (8). For each sample, there were up to five extractions.

2.4 Gel Electrophoresis

A 1% agarose gel was prepared in TBE buffer, pH 8.5 (89.2 mM boric acid, 2.47 mM disodium EDTA) (8). Ten microliters of each DNA sample and two microliters of 5X loading buffer was mixed and then loaded into the gel. The gel was run at 100V for approximately 20-25 minutes. After staining with ethidium bromide for 15 minutes, the gel was observed, using the CEQ-UV spectrophotometer, to check for successful DNA extraction.

2.5 PCR

PCR was performed using 16S rRNA gene primers, Ba2F and K2R. The forward primer, Ba2F, was labeled with the Cy5 fluorescent tag. The PCR reactions were carried out in triplicate for each sample, using the protocol found in table 2. The PCR reagents were 5 and 3 μ L template DNA, 5 μ L of 10x Buffer GOLD, 3 μ L of dNTP (10 mM, 2.5 mM of each: A, T, C, G), 2 μ L BSA (20 μ g/mL), 7 μ L of MgCl₂ (25 mM), 1 μ L of Ba2F (10 μ M), 1 μ L K2R (10 μ M), water to bring to final volume (50 μ L), and 0.3 μ L

AmpliTaq Gold® (5 U/μL) (8). Reaction temperatures and times were 94 °C for 10 minutes; 30 cycles of (94 °C for 1 minutes, 46.5 °C for 1 minute, 72 °C for 2 minutes), and 72 °C for 10 minutes (8).

2.6 Clean-up of PCR Products

A 1% agarose gel was run with 3 & 5 μL of each PCR reaction product and 1 μL of 5x loading buffer. After visualizing the gel, successful PCR reactions obtained in triplicate for each sample were combined using a MoBio Ultraclean® PCR Cleanup Kit (MoBio® Laboratories Inc., Solana Beach, CA) following the manufacturer's protocol; this removed any unused reactants and concentrated the amplicons (13, 21).

2.7 Quantification of Labeled PCR Product and Enzyme Digest

Using the Bio-Tek Fluorometer (Bio-tek Instruments INC., Winooski VT), the PCR product concentration for each sample was determined by measuring the Cy5 incorporated fluorescent label from the forward primer (8, 21). The PCR products were then diluted with water in order to add 75 ng of DNA to the enzyme digest, making a final volume of 40 μL.

Each sample was then digested with 1 μL *Dpn* II (10,000 U/mL) and 4 μL buffer. The samples were then incubated for 4 hours at 37 °C, deactivated for 20 minutes by incubation at 65 °C, and kept at 4 °C for infinity.

2.8 Ethanol Precipitation of Digest

Upon digestion, the samples were purified by ethanol precipitation. To each digest, 100 μ L (2.5x digest volume) of cold 95% ethanol, 2 μ L of 3 M sodium acetate [pH 4.6 (5% digest volume)], and 1 μ L glycogen (20 mg/mL) were added and mixed. The mixture was incubated at 4 °C for 30 minutes and then centrifuged for 15 minutes at 5,300 RPM to pellet the DNA (program 2). The ethanol was removed and the pellet was washed with 100 μ L of cold 70% ethanol, centrifuging again for 5 minutes at 5,300 rpm (program 3). In order to remove the ethanol, the PCR tray was inverted on a paper towel and centrifuged for 1 minute at 700 RPM to dry the pellet (program 4). The PCR tray was then left to dry under a vacuum hood for approximately 15 minutes to remove excess ethanol (8).

2.9 Terminal Restriction Fragment Length Polymorphism (TRFLP) & Analysis

The purified DNA was dissolved in 20 μ L formamide and 0.25 μ L CEQTM DNA size 600 base pair standard (Beckman Coulter Inc, Fullerton, CA). Then one drop of mineral oil was placed on to the top of each well to prevent evaporation. The samples were subsequently run on a CEQTM 8000 Genetic Analysis System.

TRF peak analysis began with the alignment of the peaks, using the AFLP align function of the CEQTM 8000 software. Upon collection of this information, the Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQTM 8000 into Excel (Microsoft, Seattle, WA). To facilitate statistical analysis, the area under each TRF peak in a pattern was calculated as a percentage of the total peak area in the pattern and expressed as parts per million (ppm) (21). TRF peaks

with an area of less than 10,000 ppm ($<1.0\%$ of the total for that sample) were excluded from analysis (21). Normalized TRF data sets were transformed by taking the square root of the area under each TRF peak to de-emphasize large TRF peaks while still taking relative abundance into account (5, 21). The transformed data was then analyzed in Minitab 14 (Minitab Inc., State College PA) and Excel (Microsoft, Seattle WA).

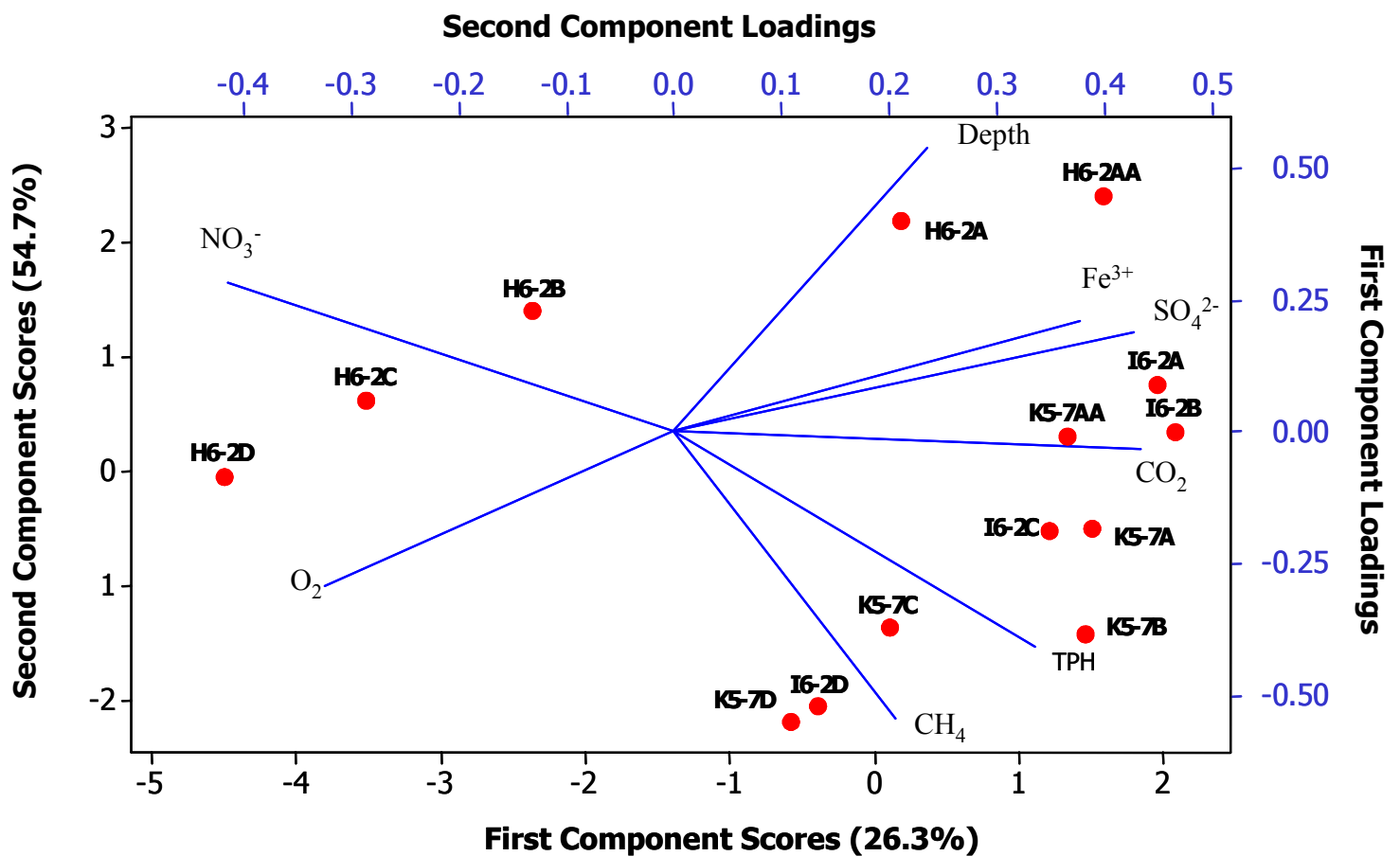
2.10 Principal Components Analysis (PCA)

Due to the heterogeneous nature of bacterial community structure data, visually discerning relationships between samples can be difficult; as a result, PCA was used in this study (21). PCA took multiple variables within the physical and biological data and created new variables that combined the old ones, but covered more of the total variation. PCA was based on the Euclidean distance, a standard method for evaluating TRF data (21). The greatest variance by any projection of the data set came to lie on the first axis (called the first principal component) and the second greatest variance came to lie on the second axis (called the second principal component). Therefore, PCA was used as an analytical tool for interpreting physical and/or bacterial community structure within wells K5-7, I6-2, and H6-2.

3.0 RESULT & DISCUSSION

Groundwater samples taken from wells K5-7, H6-2, and I6-2 were processed for chemical and biological analyses (Figure 2.0). Chemical analyses include the following variables: dissolved total petroleum hydrocarbons (TPH), methane, sulfate, oxygen, ferrous iron, and carbon dioxide. Chemical data was analyzed to look for trends across horizontal and vertical parameters and also to summarize the data for comparison to bacterial community information collected from TRF patterns (21).

Figure 2: PCA biplot of groundwater parameters for wells K5-7, I6-2, & H6-2. Sample wells, with specified screening intervals are indicated in red dots, while the loadings for the groundwater variables are displayed as vectors (solid blue lines, secondary X and Y axes). High negative Chem. PC1 values (eg. well H6-2D, C, and B) represent aerobic, higher redox, and lower TPH environments, whereas high positive Chem. PC1 values (eg. wells I6-2A, I6-2B, K5-7AA) represent more anaerobic, lower redox, and higher TPH values (21). Percent variation covered by each principle component is in parenthesis.

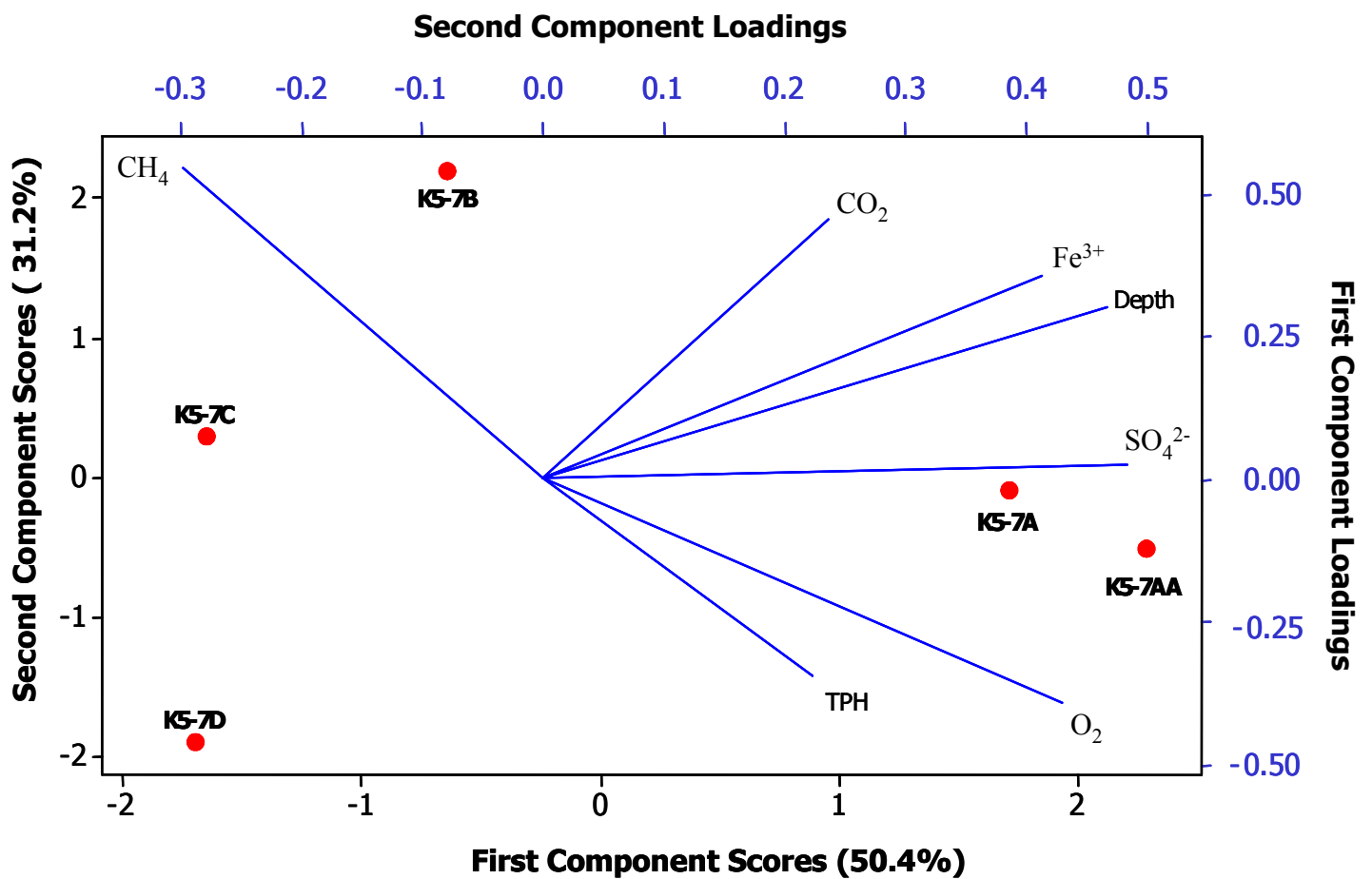


The physical variables of wells K5-7, H6-2, and I6-2, were analyzed using PCA (Figure 2). TPH, methane, carbon dioxide, and ferrous iron have loadings in the positive direction for the first component. Methane, an indication of anaerobic conditions, was in the opposite direction of oxygen and nitrate, which were in the negative direction in the first component. Overall, the physical PCA showed a shift in the gradient from aerobic (left) to anaerobic conditions (right). Likewise, the decrease of depth below groundwater corresponded to the increase of oxygen and nitrate.

Interestingly, sulfate increased with increasing depth and TPH, regardless of the well. Since sulfate may be used during anaerobic degradation of petroleum, TPH and sulfate are expected to have opposite loadings. However, it is likely that sulfate utilization is not involved in TPH degradation at this site. It is also possible that the concentration gradient was an artifact of concentrated sulfate at the bottom of the aquifer. Furthermore, sulfate bore a strong correlation with depth, another indication that sulfate could have been diffusing up from the aquifer below, making this variable independent of microorganism activity.

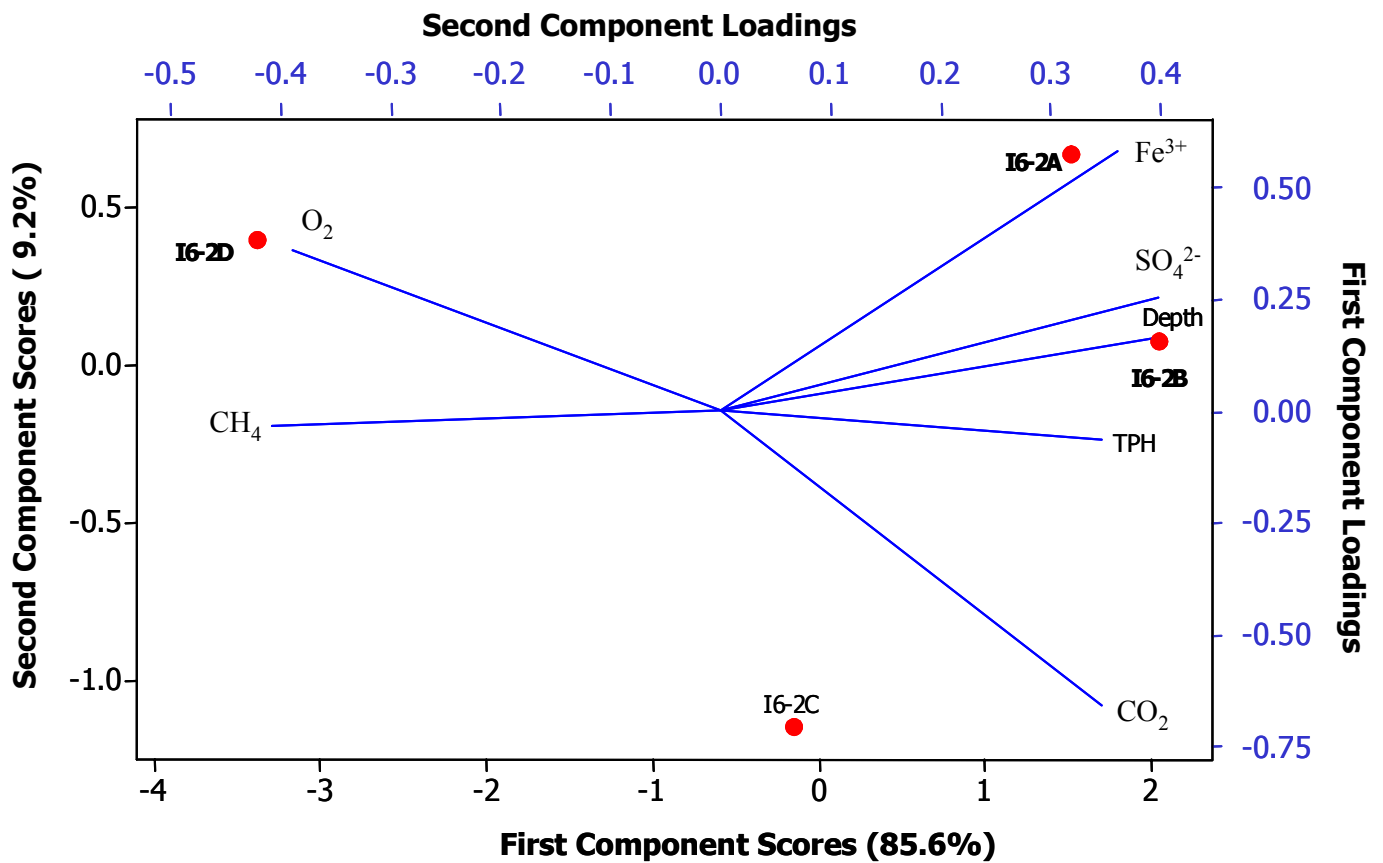
Upon examination of the physical PCA for wells K5-7, I6-2, and H6-2, it was determined that a better overall picture of natural attenuation could be achieved by examining each well separately to tease out different trends. The nested wells generally showed a correlation of physical data with depth. However, the screening intervals within each well differed otherwise.

Figure 3: Well K5-7 Physical PCA Analysis. The sample well's specific screening intervals are indicated in red dots, while the loadings for the groundwater variables are displayed as vectors (solid blue lines, secondary X and Y axes). Percent variation covered by each principle component is in parenthesis.



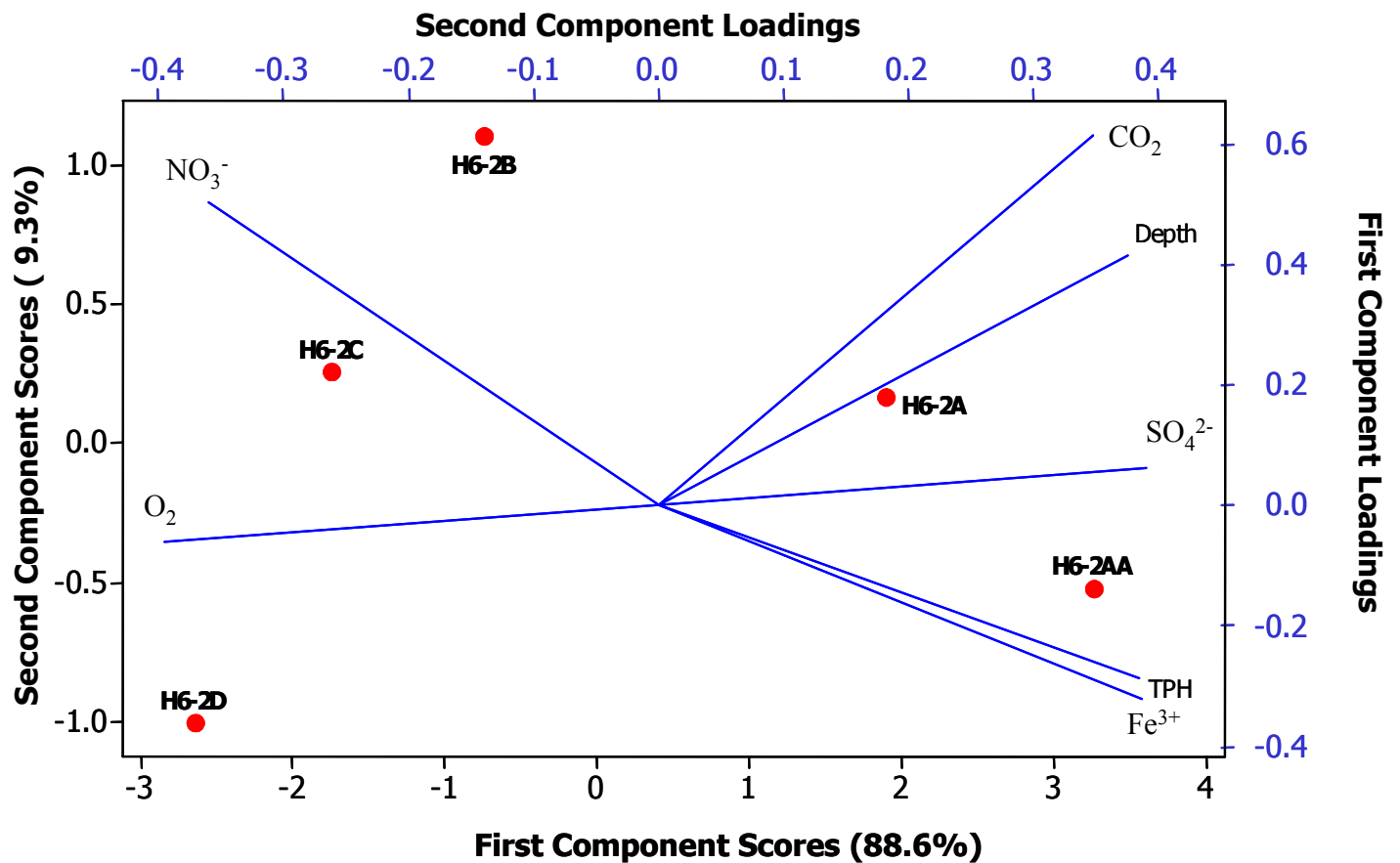
The physical data from PCA analysis of well K5-7 showed that the expected aerobic gradient did not exist in this well. There are various components that discredited the data. For example, methane and TPH have opposite loadings, while TPH and oxygen both have positive loadings in the first component. No further bacterial analysis was performed for well K5-7 because of these conflicting trends in the physical data.

Figure 4: Well I6-2 Physical PCA Analysis. The sample well's specified screening intervals are indicated in red dots, while the loadings for the groundwater variables are displayed as vectors (solid blue lines, secondary X and Y axes). Percent variation covered by each principle component is in parenthesis.



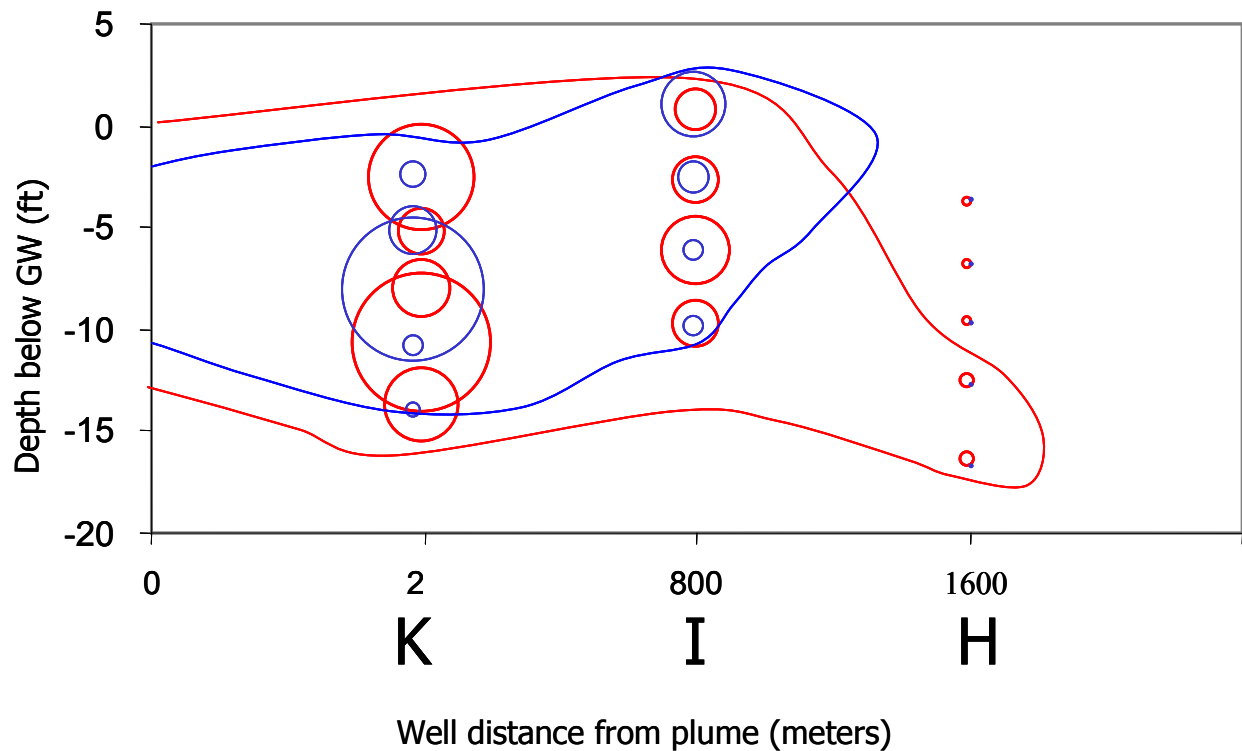
As with K5-7, PCA revealed confusing trends in the physical data from well I6-2. For example, oxygen and methane are loadings in the negative direction for the first component, while TPH is a positive loading for the first component. Methane and TPH were expected to both be in the positive direction to support a true aerobic/anaerobic gradient. Nevertheless, it is possible that methane is not being generated by TPH degradation at the site of sample collection. No further bacterial analysis was performed for well I6-2 because of these conflicting trends in the physical data.

Figure 5: Well H6-2 Physical PCA Analysis. The sample well's specified screening intervals are indicated in red dots, while the loadings for the groundwater variables are displayed as vectors (solid blue lines, secondary X and Y axes). Percent variation covered by each principle component is in parenthesis.



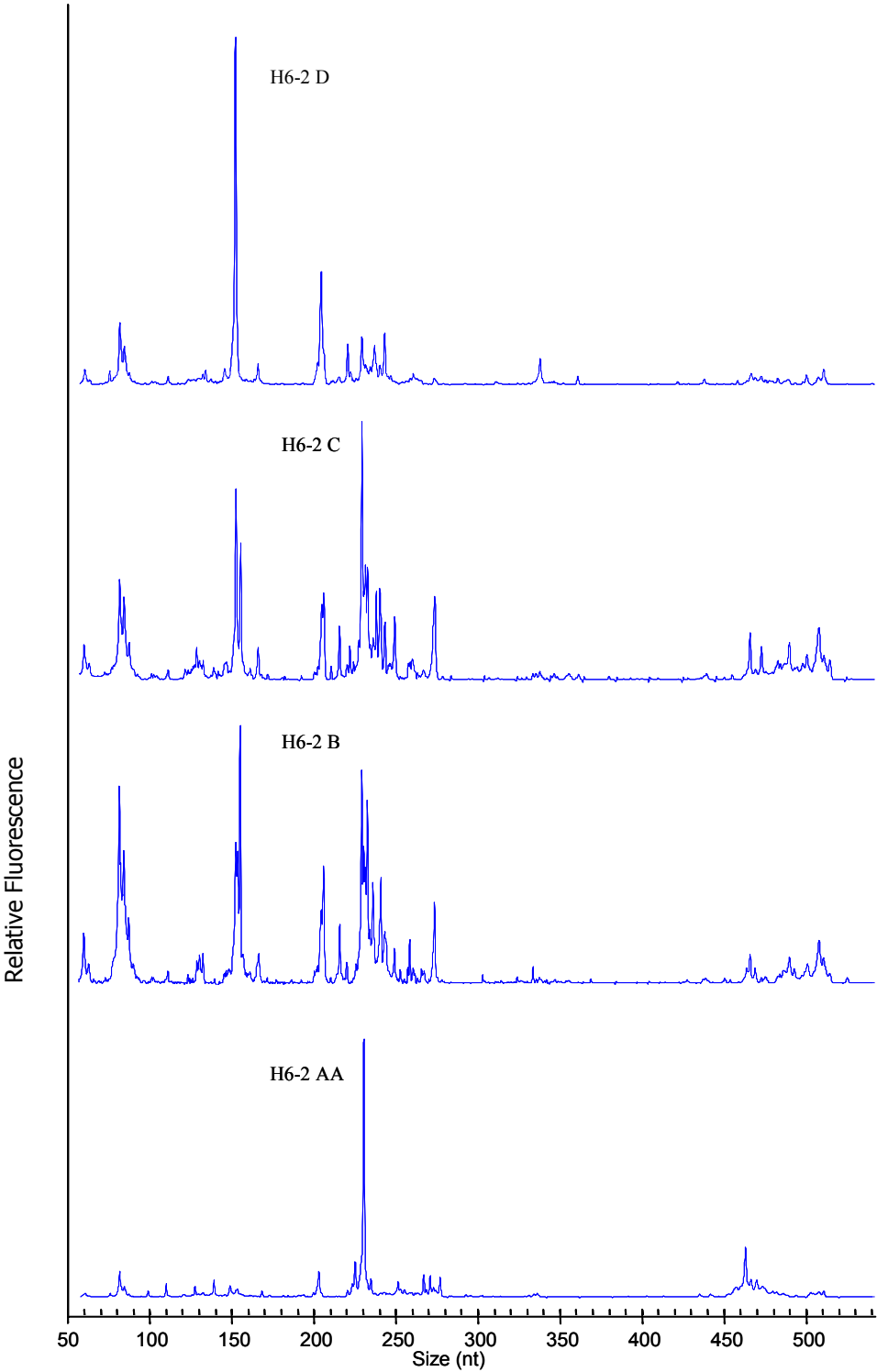
PCA of physical/chemical data from well H6-2 showed that there was no methane present, which is indicative of aerobic conditions. A good anaerobic/aerobic gradient within the well is indicated by TPH, which has a loading in the positive direction for the first component, and the oxygen, which has a loading in the negative direction for the first component. Likewise, TPH and depth are both in the positive direction of the first component. In short, the screening intervals of well H6-2 correlate with its physical PCA, showing H6-2AA to be more anaerobic and H6-2D to be more aerobic.

Figure 6: Wells KIH: Average TPH and Methane Concentrations. Average TPH concentration is shown in red, while average methane concentration is shown in blue. Circles show concentration by diameter, while lines indicate a theoretical limit of area. Screen interval H6-2D, highest bubble in well H, shows the smallest concentration of TPH (approximately 1 mg/L).



In an effort to more accurately portray average TPH and methane concentrations, a bubble plot was created for wells K5-7, I6-2, and H6-2. It should be noted that well I6-2 contained readings above 0 ft below groundwater; this was probably due to the 1 ft. screen, which is only partially submerged at this depth. Overall, the bubble plot illustrated the lack of correlation of TPH degradation and methane production. Furthermore, it appeared that the methane floated as it moved away from the plume. It could be hypothesized that methane might have been rising if it was coming out of solution as a gas. The lack of correlation between TPH and methane concentrations with increasing depth supported the decision to cease further analysis of potential physical data correlation with TRF patterns of wells K5-7 and I6-2. In contrast, well H6-2, which was furthest from the plume, showed no detectable methane and an increasing concentration of TPH with increasing depth. Therefore, well H6-2 showed an increase in the potential for TPH degradation with increasing depth, which could then be correlated to the relative abundance of specific bacterial types.

Figure 7: Well H6-2 TRF patterns.



DNA extracted from the nested wells of H6-2 was used to characterize TRF patterns. Unfortunately, no DNA was recovered from H6-2A, excluding it from TRF analysis. TRF patterns produced from nested wells H6-2D, C, and B gave similar peak patterns, all containing a predominant TRF at 150 nucleotides (Figure 7). H6-2B and C appear to contain the most similar TRF patterns. Therefore, it was hypothesized that H6-2B and C share similar bacterial community structures. Interestingly, H6-2AA's signature peak pattern was significantly different from the other depths. Evidence supporting the unique bacterial community within H6-2AA was found in Figures 5 & 6. In Figure 5, H6-2AA was separated via physical data from the other nested wells within H6-2, showing a distinct correlation with the TPH loading in the positive direction of the first component. Likewise, H6-2AA, located at the deepest depth below groundwater, showed the highest concentration of TPH. Consequently, the unique TRF at 230 suggested that the predominant bacterial community of H6-2AA might be *Pseudomonas*, a well known aerobic TPH degrader (8).

4.0 CONCLUSION

In conclusion, K5-7 and I6-2 had distributions in their groundwater chemistry which were ambiguous, making microbiological analysis impractical at this point in time. In contrast, H6-2 contained a distribution of groundwater chemistry which allowed microbiological analysis. The physical data showed correlation of TPH, carbon dioxide, ferrous iron, and sulfate with increasing depth of well H6-2. Likewise, a TRF peak of 230 nt was present at the leading edge of the plume in nested well H6-2AA. The same 230 nt TRF peak was seen in a Land Treatment Unit (LTU) during rapid TPH degradation (8). Therefore, it was deemed probable that TPH degradation was occurring at the screening depth of H6-2AA. Nevertheless, there was no conclusive evidence that organism with TRF 230 actually degraded TPH. Therefore, the next step would be to clone and sequence the 16S rRNA genes present in these samples, and perhaps culture the bacteria from the wells to test for true TPH degradation.

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