THE SEARCH FOR BACTERIA INVOLVED IN ANAEROBIC PETROLEUM DEGRADATION

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TITLE: **THE SEARCH FOR BACTERIA INVOLVED IN ANAEROBIC PETROLEUM DEGRADATION**

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ABSTRACT

THE SEARCH FOR BACTERIA INVOLVED IN ANAEROBIC PETROLEUM DEGRADATION

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This work was a study of the natural attenuation of petroleum in groundwater at the Former Guadalupe Dunes Oil Field near Guadalupe, CA. Natural attenuation is the physical, chemical and biological processes acting to reduce contaminant concentration and or toxicity in the environment. In nearly all cases, microbial reactions are the dominant processes driving the natural attenuation of petroleum. For regulatory agencies to consider natural attenuation as an acceptable remediation method, evidence must be obtained that the contaminant plume is contained, and that conditions will not change in the future to allow the plume to grow thereby affecting ecologically sensitive areas down gradient. One of the key factors in determining the stability of natural attenuation is an understanding of the environmental factors that influence the bacterial degradation of petroleum and the presence and abundance of electron acceptors in the groundwater dominates the bioremediation process.

It is well established that electron acceptor depletion follows a thermodynamic gradient with oxygen being depleted first followed by nitrate. The bacteria that use these two electron acceptors are thought to completely oxidize petroleum to carbon dioxide and water, while also rapidly removing oxygen and nitrate from the contaminated environment. In the Guadalupe Dunes, a large volume of free-phase petroleum at the air-groundwater interface has resulted in anaerobic groundwater plumes of dissolved petroleum. Iron (Fe^{3+}) and sulfate also become depleted as petroleum is degraded in the

Guadalupe Dunes aquifer. Iron, the next thermodynamically preferred electron donor, is relatively abundant in the Guadalupe soil and current geochemical evidence from the site demonstrates the correlation between reduced Fe and TPH concentration across the site. Sulfate may also contribute to the natural attenuation at Guadalupe Dunes and is present at variable concentrations across the site. It is unclear whether bacteria capable of iron- or sulfate-reduction can degrade petroleum completely to CO_2 or if some organic compounds, such as acetate, are produced.

In the center of a plume where contaminant concentrations are highest, the only electron acceptor available may be CO_2. Methanogenic bacteria use CO_2 as electron acceptor and produce methane as a waste product. However, methanogenic bacteria do not utilize electrons directly from petroleum hydrocarbons. The most common substrates for methanogensis are hydrogen (H_2) and acetate, while formate and ethanol can also be used. Since methane is being generated in great quantities in the petroleum plumes as well as source zones at Guadalupe, it is clear that these methanogenic substrates are being created. Although iron reducing, sulfate reducing, and fermenting bacteria that use organic compounds as electron acceptors are a logical choice; the bacteria that create methanogenic substrates from petroleum are poorly characterized.

Identifying bacteria in the groundwater plumes and confirming their metabolic activity may provide the best evidence for long term plume stability at Guadalupe. This thesis intended to fill previously identified information gaps critical to understanding the stability of natural attenuation in the Guadalupe Dunes aquifer. For the purpose of this thesis, groundwater samples were collected at varying distances from the source of free-phase petroleum plumes and were analyzed for nutrients and bacterial DNA was isolated.

Molecular tools, including the use of Polymerase Chain Reaction (PCR) and Terminal Restriction Fragment Length Polymorphism (TRFLP), were used to characterize populations of bacteria in the Guadalupe Dunes. This research provided a glimpse of the dominant physiological processes occurring in contaminated groundwater including methanogenic, fermentative, and sulfodogenic conditions. In particular this thesis proposed a model for methanogenic natural attenuation (Chapter 1), defined the role of sulfate reducing bacteria (SRB) in groundwater (Chapter 2), and used novel analysis methods to characterize populations of Bacteria, Archaea, methanogens, and methanotrophs across the site and assessed their relationship to hydrogeochemical conditions (Appendix). This master's thesis resulted in research which has been presented at four national and one international meetings and a manuscript is currently being prepared for publication, based on the research presented in chapter 1.

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The faculty in the Biological Sciences Department at California Polytechnic State University is responsible for much of my personal and professional development. It has provided me with a safe, nourishing environment to grow throughout my undergraduate and later graduate school experience. Dr. Susan Elrod is responsible for much of my classroom and laboratory experience as undergraduate, and was the driving force in my graduate work. For this I am forever grateful. Dr. Raul Cano, a mentor who believed in me when I myself was not sure what I was capable of. Dr. Christopher Kitts, there are not words to describe the respect and admiration I have. The amount of time and energy invested into each of his students is truly exceptional and unique. I have learned how to question, analyze, and view not only science but life from his inspiration.

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Unocal and EBI not only made this project possible but ensured my success. Because of the nature of this project, I have had an incredible opportunity to work in

academics with an application based perspective. I will not only take the knowledge of these techniques, their analyses and application, but will also haul all of my wonderful memories with me into all my future endeavors.

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CHAPTER 1.

IDENTIFICATION OF BACTERIA CORRELATED WITH METHANOGENIC NATURAL ATTENUATION OF PETROLEUM IN GROUNDWATER

Abstract

This study characterized the bacterial community present in groundwater samples from an ongoing Guadalupe Dunes restoration project to identify bacteria associated with natural methanogenic petroleum degradation in the dunes aquifer. Groundwater samples were collected at varying distances along a dissolved phase petroleum plume to form a horizontal profile, while a vertical profile of 5 depths was obtained from a nested well. DNA was isolated from the groundwater samples which were also analyzed for a variety of hydrogeochemical parameters. Terminal Restriction Fragment (TRF) Length Polymorphism (TRFLP) was used to characterize populations of Bacteria and Archaea. Due to sampling that averaged across 15 feet in the vertical direction, the Bacteria and Archaea groundwater communities in the horizontal profile separated into distinct clusters without any correlation to hydrogeochemistry. However, the bacterial community structure in the vertical profile was correlated to groundwater chemistry. Analysis of relative TRF abundance revealed 8 TRF peaks that correlated with anaerobic, lower redox and higher TPH concentrations. These TRF peaks represented Chloroflexi and Spirochete bacteria, as confirmed by clone library sequences. In addition, the relative abundance of these dominant TRF peaks correlated with the Archaea community structure, reflecting a relationship between the two communities. These results suggest that Chloroflexi and Spirochetes are involved in a consortium responsible for the methanogenic natural attenuation of petroleum in the Guadalupe Dunes aquifer.

Introduction

Contamination of groundwater by petroleum hydrocarbons is a worldwide problem, the result of spills, leaks, and improper disposal (12, 24). The monitoring of natural processes to reduce contaminant concentration, a practice called natural attenuation, is a commonly approved treatment for hydrocarbon contaminated groundwater (29, 42, 43). Natural attenuation is considered an effective, lower cost, remediation strategy for dissolved phase petroleum contaminants, which has minimal environmental impact (19, 33, 34). While bioremediation, the use of indigenous soil microbes to degrade contaminants, is the principal natural attenuation process, other physical and chemical processes are involved including sorption, volatization, dispersal and diffusion (19, 25, 29, 33, 43). However, unlike these other natural attenuation processes, biodegradation can completely convert hydrocarbons to carbon dioxide, water, and methane (10, 43).

From a regulatory standpoint, natural attenuation is considered a viable remediation strategy when evidence establishes that sustainable biodegradation is occurring (45). Such lines of evidence include detection of hydrocarbon degradation intermediates, depletion of electron acceptors, microcosm studies, and a description of the microbial community involved in biodegradation (10, 40, 43, 45). Bacterial communities from many petroleum contaminated sites have been successfully characterized by culture-independent molecular techniques using 16S rRNA gene sequences (1, 2, 3, 8, 10, 12, 14, 16, 28, 29). An understanding of the microbial community, the function of the organisms present, and the relationship between these

organisms and the hydrogeochemistry could help predict the site-specific efficiency and sustainability of bioremediation dependent MNA. (10, 12, 29, 43).

This report is part of a natural attenuation project at the former Guadalupe Oil field, which occupies approximately 2,700 acres within the larger Guadalupe-Nipomo Dunes Complex on the central California coast. Oil production began on the site in 1947 and peaked in 1971. The number of oil wells reached a maximum at 215 by 1988. Oil production ceased in 1990 and all wells were abandoned by 1995 (21, 36). To thin the oil and make pumping more efficient, a light petroleum distillate, referred to as diluent, was pumped into the wells (19, 21). Leaks in the tanks and pipelines used to distribute the diluent developed, inadvertently releasing hydrocarbons into the environment. Diluent accumulated at the water table in the dune sand aquifer and the rapid ground water flow rate combined with the permeable sand matrix eventually resulted in groundwater contamination beneath much of the site (21). Because of the depth to groundwater, the fragile dunes ecosystem and the presence of many endangered species, natural attenuation was investigated as one of a suite of remediation strategies at the site (36).

Site-wide hydrogeochemical data indicated that methanogenesis, and to a lesser extent iron reduction, were the dominant anaerobic redox processes. The intent of this study was to identify bacteria strongly correlated with methanogenic petroleum degradation in the Guadalupe Dunes, and to propose a mechanistic model for carbon flow in the dune sand aquifer. Ultimately this model could be used to assess the sustainability of MNA in the Guadalupe Dunes, and to develop tools for predicting and monitoring natural attenuation at this and other sites. To this end, groundwater samples from a single

plume were collected in three dimensions and hydrogeochemical data was analyzed to summarize the petroleum degradation parameters in the plume.

Materials and Methods

Site Description and Sampling

The Guadalupe Dunes is bordered by the Pacific ocean to the west, San Luis Obispo County to the north, and the Santa Maria River and Santa Barbara County to the south. There are 3 aquifers beneath the Guadalupe Dunes and dissolved petroleum is limited to the uppermost dune sand aquifer. The direction of the groundwater flow (0.3m/day) is in an east to west direction resulting in total petroleum hydrocarbons (TPH) beneath much of the site with a flux towards the ocean and the Santa Maria River (21, 36). This study examined the largest groundwater plume associated with largest diluent source (Fig. 1).

For the horizontal profile, groundwater was collected from 11 conventional monitoring wells (5-10 cm in diameter) down gradient from the plume. Well screens were 4.6-6.1 m long and entirely submerged in groundwater, resulting in the average screen of 5.3 m (Table 1). The vertical profile consisted of one large nested well, H2-2, which housed 5 smaller wells labeled H2-2A, B, C, D, and E. Each nested well was 2.5 cm wide and had 0.30 m screens placed at increasing intervals with the H2-2E screen starting at 25.3 m and each successive nested well screen 0.85 m lower to the H2-2A screen at 36.3 m. Anaerobic groundwater samples were collected in sterile glass bottles, by letting the bottles 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). Care was taken to completely remove any headspace in the bottle.

Groundwater samples were transferred to the laboratory and stored for less than 24 hours at 4°C until chemical and biological processing occurred.

Chemical Measurement and Analysis

Chemical measurements were performed by Zymax Envirotechnology in San Luis Obispo, California (18). TPH concentrations varied from non detect (<0.050 mg/L) to 25.0 mg/L (Table 1). The sThe total concentration of benzene, toluene, ethlybenzene, and xylene was summarized in a new variable – BTEX. Prior to performing statistical analyses, all concentration variables were log transformed to account for non-normal distributions common in concentration measurements (17). In order to look for trends and to organize the data for comparison to bacterial community information collected from TRF patterns, the hydrogeochemical data was analyzed using Principle Components Analysis (PCA) with Minitab 14 (Minitab Inc., State College PA) using a correlation matrix.

Table 1. Physical and hydrogeochemical data

Well Number	Distance to Source	Screen Length	TPH	BTEX	CH_4	NH4	Fe^{2+}	CO_2	O_2	SO_4	NO_3
EPA Method	-	-	EPA 8015 DIESEL	EPA 8260	EPA RSK SOP-175	EPA 350.1	EPA 6020	EPA RSK SOP-175	EPA RSK SOP-175	EPA 300.0	EPA 300.0
Units	m	m	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
204-A	140	6.1	2.3	ND	0.18	0.5	8.4	16.37	0.46	23	ND
206-C	51	6.1	4.25	ND	0.23	1.7	15	15.07	0.15	43	ND
207-B	744	6.1	0.46	ND	0.003	ND	0.39	5.52	6.41	59	7.52
209-C	44	6.1	14.5	28.15	7.83	3.7	38	17.72	0.28	2.8	ND
209-D	324	6.1	7.95	24.65	0.08	0.04	93	19.08	2.95	11	0.71
F4-1	343	4.6	1.35	ND	ND	0.25	1.3	6.99	7.01	38	0.65
G3-1	134	4.6	10.5	22.4	5.88	0.83	16	13.46	1.5	20	ND
G3-2	211	4.6	12.5	17.9	6.09	0.49	12	24	0.66	7.4	ND
G4-3	131	4.6	7.05	134	2.85	0.31	2.7	16.31	2.1	4.4	ND
H11-1	124	4.6	3	ND	ND	ND	0.38	11.8	1.5	24	0.33
H1-3	41	4.6	10.4	23.2	0.82	0.42	11	14.98	0.2	21	ND
H2-2A	42	0.3	12	0.0201	2.79	-	4.8	2.79	1.76	1.76	ND
H2-2B	42	0.3	13	0.0278	7.61	-	14	7.61	0.66	0.66	ND
H2-2C	42	0.3	25	0.0238	8.71	-	37	8.71	0.15	0.15	ND
H2-2D	42	0.3	19	0.0225	6.93	-	65	6.93	0.09	0.09	ND
H2-2E	42	0.3	24	0.028	9.61	-	54	9.61	0.12	0.12	ND

ND, Non Detect
-, Not Measured

TRFLP Profiling

Four liters of groundwater from each well were filtered through 0.2 μm nylon membrane filters. The filters were frozen and half of each filter was crushed and homogenized under liquid nitrogen. Samples were extracted using the MoBio Ultraclean® soil DNA kit (MoBio® Laboratories Inc., Solana Beach, CA) following manufacturers protocol. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax spectrophotometer (Molecular Devices, Palo Alto CA).

PCR was performed using 16S rRNA gene primers for Bacteria and Archaea. Forward primer 46-Ba2F (5' GCY TAA CAC ATG CAA GTC GA-3') and reverse primer 536-K2R (5'- GTA TTA CCG CGG CTG CTG G-3') were used to amplify a 490-bp section of the Bacteria 16S gene (20). Forward primer ARCH-21F (TTC CGG TTG ATC CYG CCG GA) and reverse primer ARCH-958R (YCC GGC GTT GAM TCC AAT T) were used to amplify 920-bp of the Archaea 16S gene (9, 14). Both forward primers were fluorescently labeled with a Cy5 phosphamide dye (ProLigo LLC, Boulder, CO). Bacteria PCR reactions were carried out using 1 μL of diluted extraction product (1 ng/μL), 5 μL of 10x Buffer, 3 μL of 10 mM DNTP, 2 μL 20 μg/mL BSA, 7 μL 25 mM $MgCl_2$, 1 μL primer, 1 μL primer, 29.7 μL water, and 0.3 μL 5 U/μL TaqGold®. Reaction temperatures and times were 96 °C for 10 min; 30 cycles of 94 °C for 1 min, 46.5 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. Archaea PCR reactions were carried out using 1 μL of diluted extraction product (1ng/μL), 5 μL of 10x Buffer, 3 μL of 10 mM DNTP, 2 μL 20 μg/mL BSA, 7 μL 25 mM $MgCl_2$, 1 μL primer, 1 μL primer, 29.7 μL water, and 0.3 μL 5 U/μL TaqGold®. Reaction temperatures and times were 96

°C for 10 min; 30 cycles of 94 °C for 1.5 min, 55.0 °C for 1.5 min, 72 °C for 1 min; and 72 °C for 10 min.

Both Bacteria and Archaea PCR amplifications were performed in triplicate and then combined using a MoBio Ultraclean® PCR Cleanup Kit (MoBio® Laboratories Inc., Solana Beach, CA) following the manufacturer's protocol. The cleaned PCR products were quantified using a fluorometer (Bio-tek Instruments INC., Winooski, VT) tuned to the Cy5 labeling dye. Bacteria DNA (75 ng) was digested with 4 U *Dpn*II (New England Biolabs Inc., Beverly, MA), 4 μL of manufacturer's recommended buffer and water to 40 μL. Archaea DNA (75 ng) was digested with 1 U of *Hae*III enzyme (New England Biolabs Inc.), 4 μL buffer, and water to 40 μL. The Bacteria and Archaea reactions incubated for 4 hours at 37°C and deactivated for 20 min by incubation at 65°C or 80 °C respectively.

Following digestion, Bacteria and Archaea DNA was purified by ethanol precipitation. The purified DNA was dissolved in 20 uL CEQ™ Sample Loading Solution and 0.25 μL CEQ™ DNA size standard-600 (Beckman Coulter Inc, Fullerton, CA) and run on a CEQ™ 8000 (Beckman Coulter Inc, Fullerton, CA). Fragment results were analyzed with CEQ™ 8000 Genetic Analysis System. TRF peaks were aligned using the AFLP align function of the CEQ™ 8000 software.

TRFLP Analysis

Terminal Restriction Fragment (TRF) length in nucleotides, and TRF peak area were exported from the CEQ8000 into Excel (Microsoft, Seattle WA). To facilitate statistical analysis, the area under each TRF peak in a TRF pattern was calculated as a percentage of the total peak area in the pattern and expressed as parts per million (ppm).

TRF peaks with an area of less than 10,000 ppm (<1.0% of the total for that sample) were excluded from analysis. Bacteria TRF peaks 80-85 were determined to represent primer dimers (uncut samples were compared to cut samples before dropping peaks at 80-85 bp) and excluded from TRF analysis. The exclusion of these peaks was not significant as they contributed less than 2.5% of the total peak area. 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, 7). Transformed data were analyzed in Minitab 14 (Minitab Inc., State College PA), Excel (Microsoft, Seattle WA), and Primer V (Plymouth Marine Laboratory, Plymouth UK).

Due to the heterogeneous nature of bacterial community structure data, visually discerning relationships between samples can be difficult; as a result PCA and non-parametric Multi Dimensional Scaling (MDS), two multivariate ordination techniques were used in this study. Both methods account for the relative abundance of each TRF peak when comparing two patterns, however, PCA is based on Euclidean distance whereas MDS is based on a Bray-Curtis similarity matrix. Euclidian distance can give anomalous results with data containing large numbers of zeros, is prone to bias by large values, and may distort the relationships between samples when the data does not follow a normal distribution (30). While Euclidian distance is a standard method for evaluating TRF data (5), Bray-Curtis similarity was specifically designed for use with species abundance data sets and is less susceptible to bias introduced by large numbers of zero abundance data (4). TRF data often contains zero values (a peak present in one pattern that is not seen in another), covers 2.5 orders of magnitude variation in TRF peak area,

and generally does not follow a normal distribution. Therefore, Bray-Curtis similarity is a more accurate similarity measure when comparing TRF patterns (30, 33).

In MDS, the relationship between the original data and the model output is a map based on the relationships existing in the Bray-Curtis similarity matrix. While MDS accounts for total similarity, PCA breaks distances into new variables such that two samples that are in the same position with respect to the PC1 and PC2 axes but are not closely related on the PC3 axis will erroneously appear closely related in a two dimensional PCA plot. The distances on the MDS map are unitless and based on relative similarity such that samples more similar will appear closer on the MDS map. As the number of samples increases and the similarity matrix becomes larger, it becomes more difficult to exactly transpose the Bray-Curtis matrix into two-dimensional space. Some distortion, known as stress, between the similarity matrix and the MDS distance outputs occurs. Therefore, the ultimate goal of the MDS algorithm is to configure the samples such that this stress value is minimized. In general, a stress value of less than 0.05 provides an excellent representation of the data, while a value of 0.01 is ideal. Stress values less than 0.1 corresponds to good ordination with no misleading interpretation, while stress levels less than 0.2 provide a helpful two dimensional picture but any conclusions should be verified with an alternative technique like cluster analysis. Stress values less than 0.3, should be treated with hesitation as the points are close to being randomly assigned and thus the ordination should be reexamined in three or four dimensions (7).

Cloning and Sequencing of 16S rDNA

One vertical profile sample, well H2-2D, was used for constructing a Bacteria 16S rRNA gene clone library. Bacteria communities were amplified as stated above, except an unlabeled forward primer was used. PCR products from these samples were purified using the MoBio PCR Clean-Up kit (MoBio® Laboratories Inc., Solana Beach, CA). Cleaned PCR product was then ligated into the pCR 4 TOPO vector provided in the original TA cloning kit as directed by the manufacturer (Invitrogen, Carlsbad, Calif.). Ligated vector was then used to transform one shot TOPO 10 competent cells (Stratagene, La Jolla, Calif.) according to the manufacturer's protocol. Cells were plated onto medium containing 100 μg/mL ampicillin and grown overnight. White colonies were picked and grown overnight in Terrific Broth (MoBio® Laboratories Inc., Solana Beach, CA) containing ampicillin. Cells were pelleted, and plasmids were extracted using Quantum Prep HT/96 plasmid miniprep kits as directed by the manufacturer (Bio-Rad, Hercules, Calif.). Sequencing reaction mixtures (10 μL) contained the following: DNA, 4μL; primer, 1.6 10^{-5} mmol; ABI Big Dye (Applied Biosystems), 4 μL; PCR water, 0.4 μL. Samples were run on an ABI 377 DNA sequencer, and the resulting sequences were analyzed using SeqManII (DNAStar, Madison, Wis.). Sequences were tentatively identified using the BLAST 2.2 search on the National Center for Biotechnology Information webpage.

Database Matching of TRF Peaks

Two databases were used in this study: one was composed of predicted TRFs from the clone library, while the other contained TRF lengths predicted from approximately 30,000 16S rRNA gene sequences out of the Ribosomal Database Project

(23) and GenBank. Both databases were trimmed to the correct size using *in silico* PCR with primers Ba2F and K2R and digested *in silico* with *Dpn*II specific cut sites.

Observed TRF peaks were compared to the predicted *in silico* results from the H2-2D clone library. Observed TRF peaks that could not be identified from the clone library were then compared to gene sequences from the RDP and GenBank TRF database. Observed TRFs were considered valid if they were within 1 bp of the predicted TRFs from the databases (19).

Nucleotide Accession Numbers

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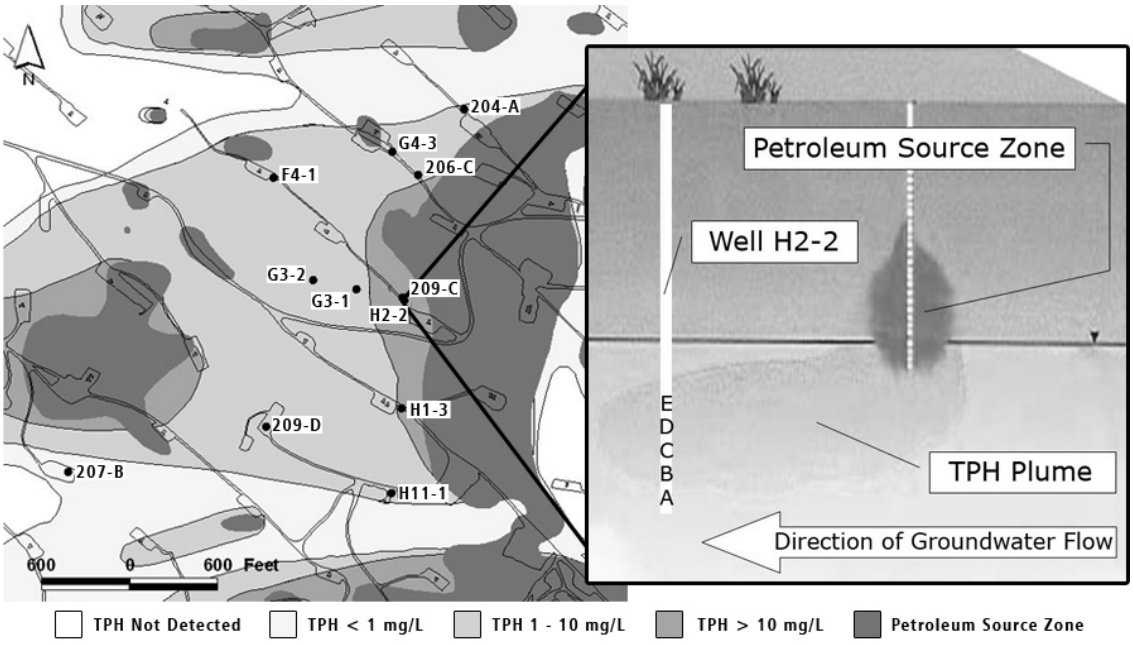
Results

Hydrogeochemistry of a Horizontal Groundwater Profile

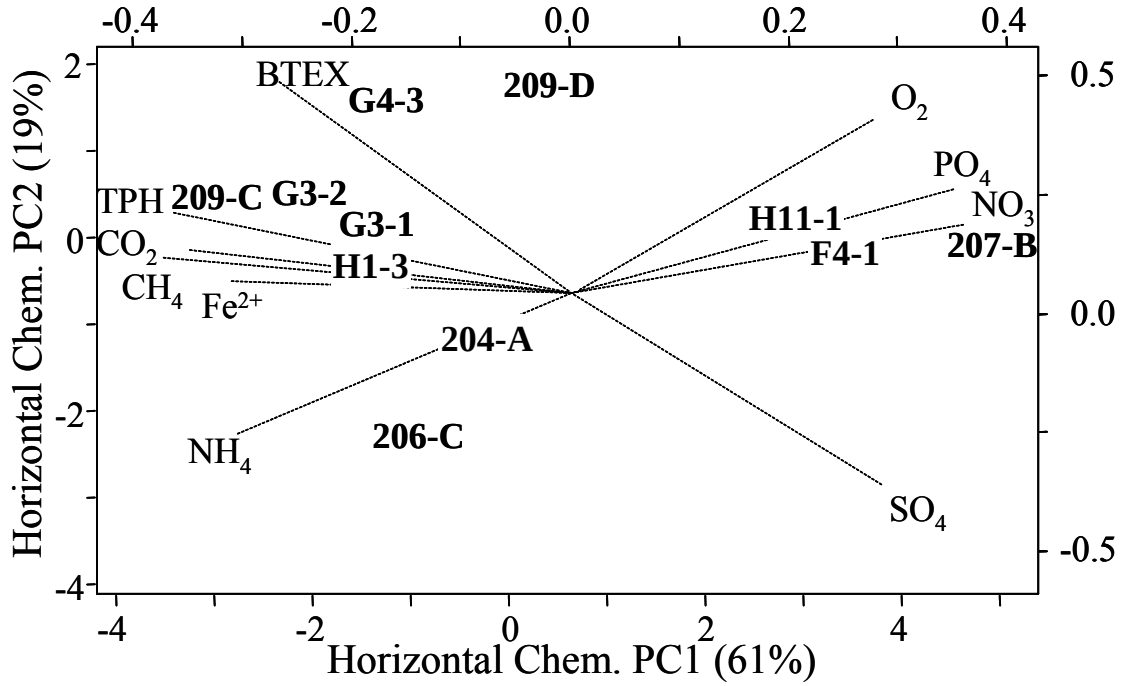
Groundwater was collected from the 11 sample wells and processed for chemical and biological analyses (Fig. 1). Chemical analysis included the following variables: dissolved total petroleum hydrocarbons (TPH), benzene, ethlybenzene, toluene, xylene, methane, hydrogen, ammonium, dissolved iron, oxygen, carbon dioxide, nitrate, sulfate, and phosphate. Chemical data was analyzed to look for trends across the horizontal plume profile, as well as to summarize the data for comparison to bacterial community information collected from TRF patterns.

Fig. 1. Plume and sample well locations for both the horizontal and vertical profiles. Vertical well H2-2, with samples A-E, illustrated with sample depths and distance from source.



Principle Components Analysis (PCA) was used to create a new variable, Horizontal Chem. PC1, which consolidated a portion of the variation in the original data set. Horizontal Chem. PC1 represented a plume wide TPH degradation parameter accounting for 61% of the variation in the measured hydrochemistry. Horizontal Chem. PC1 revealed a TPH and redox gradient within the chemical variability of the aquifer (Fig. 2). The 11 sample wells are spread across the PC1 axis with wells 207-B, F4-1, and H11-1 affiliated with a positive Chem. PC1 value, associated with higher redox, more aerobic, lower TPH samples from wells farther from the source zone. On the opposite side of the gradient, and associated with a negative Chem. PC1 value, wells 209-C, G3-2, G3-1, and H1-3 are more anaerobic samples with lower redox, and higher TPH concentrations, located closer to the TPH source zone. Horizontal Chem. PC1 is therefore an appropriate, global variable for examining the relationship between bacterial community structure and groundwater chemistry.

Fig. 2. Principal Component Analysis (PCA) biplot of groundwater parameters from the horizontal profile. Sample wells are indicated in bold, while the loadings for groundwater variables are displayed as vectors (dotted lines, secondary X and Y axes). High negative Chem. PC1 values (e.g. wells 209C, G3-2, G3-1, H1-3, G4-3) represent anaerobic, lower redox, and higher TPH environments, where as high positive Chem. PC1 values (e.g. wells H11-1, F4-1, and 207-B) represent more aerobic, higher redox, and lower TPH values.

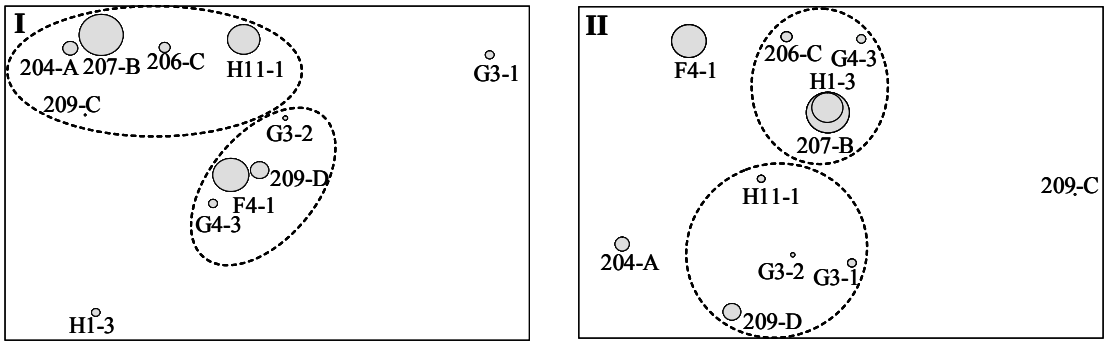


Microbial Community Structure of a Horizontal Profile in Groundwater

Bacteria and Archaea community structure was determined by TRFLP using primers specific for respective 16S rRNA genes. The relationship between bacterial community structures in the different wells was visualized by Multi Dimensional Scaling (MDS) of TRF data using a Bray-Curtis Similarity matrix. Sample similarity is represented by distance between samples on the MDS plot (Fig. 3-I). Two major clusters were visible in the Bacteria TRF data and the apparent sample grouping was confirmed with Bray-Curtis Cluster Analysis at a similarity level of 35%. The clustered samples were not related by distance from source material and an overlay of Horizontal Chem. PC1 (Fig. 2) showed no clear relationship between this global groundwater chemistry variable and the clustering of TRF data.

A similar MDS analysis of the Archaea TRF data also produced two clusters of samples at a 35% similarity (Fig. 3-II). The samples with similar Archaea TRF patterns did not have similar Bacteria TRF patterns indicating of a lack of relationship between the two communities. Similar to the Bacteria TRF data, the Archaea community structure represented in the TRF patterns exhibited no relationship to Horizontal Chem. PC1. Using the same analyses, the clustering of Bacteria and Archaea TRF data did not correlate to any of the individual groundwater variables measured. When PCA was used to analyze TRF data there was also no correlation between any of the principle component scores from the TRF data and Horizontal Chem. PC1 (data not shown). It would appear as though neither the Bacteria nor Archaea community structure had a relationship to groundwater chemistry.

Fig. 3. Multi Dimensional Scaling (MDS) of TRFLP data (stress value of 0.16) from the horizontal plume with horizontal Chem. PC1 values shown by bubble size (Fig. 2). as noted by the sample associated bubbles. Bubble area represents Chem. PC1 for each well and ranges on a scale from -3 to +5 (small to large). Clusters confirmed at 35% similarity by Bray-Curtis Cluster Analysis are indicated by dotted lines. Panel I, Bacteria TRFLP data with Horizontal Chem. PC1 (Fig. 2). Panel II, Archaea TRFLP data with Horizontal Chem. PC1, both suggest a lack of relationship between microbial community and hydrochemistry (Chem. PC1 bubbles), as well as a lack of relationship between Bacteria and Archaea communities (dissimilar clustering).



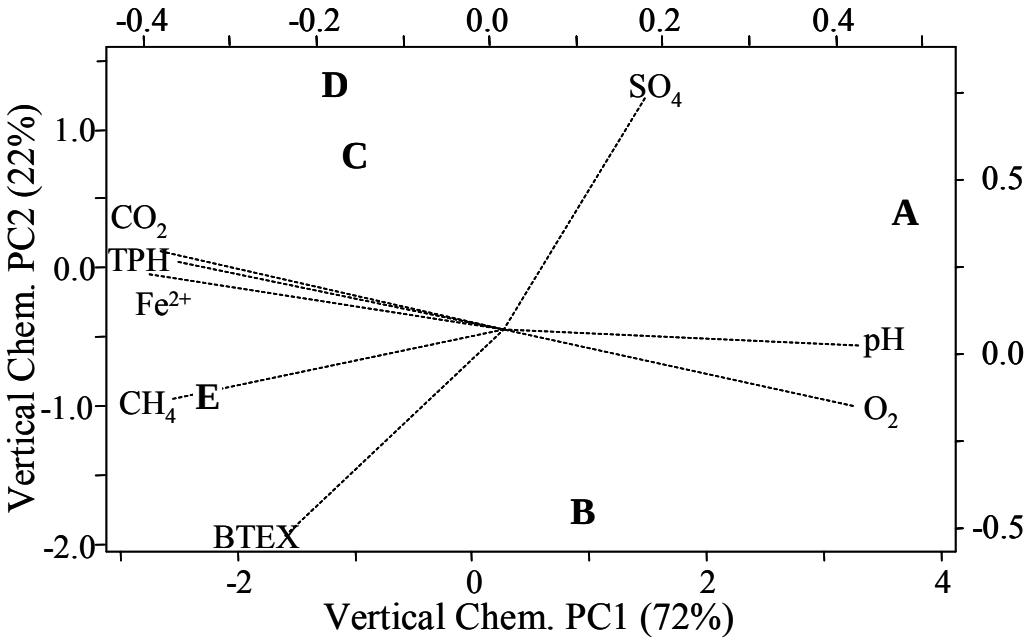
This apparent lack of relationship between bacterial community structures and groundwater chemistry was unanticipated since the redox gradients observed in Fig. 2 should be a reflection of changes in the dominant microbial activity across the plume. However, the disconnect between groundwater chemistry and bacterial community structure (TRF data) could be an artifact of the methods used for sample collection. The samples for this study represented a mix of groundwater collected across approximately 4.6-6.1 m well-screens that were fully developed, (up to four well volumes pumped prior to sampling) a standard field sampling procedure which disrupted any established vertical gradient. By collecting samples across two dimensions with an average in the third dimension, much of the original structure was lost. Perhaps sampling in the third dimension could provide more relevant results and better elucidate the relationship between bacterial community structure and groundwater chemistry.

Hydrogeochemistry of a Vertical Profile in Groundwater

To better reveal the relationship between the bacterial communities and the hydrochemistry of the dunes aquifer, a companion study using a vertical profile was conducted. In this complementary study, groundwater was collected from 5 different depths from a single well cluster, H2-2, located in the same hydrocarbon plume as the previous horizontal study (Fig. 1). The inherent variability associated with the horizontal profile was reduced by collecting a sample every 0.85 m across a 0.3 m screen rather than across 4.6 or 6.1 m screens. Split samples were analyzed for dissolved TPH, BTEX, methane, hydrogen, dissolved iron, oxygen, carbon dioxide, nitrate, sulfate, and pH. Hydrogen and nitrate concentrations were below detectable levels for all wells, as a result both variables were excluded from the subsequent analysis.

Similar to the horizontal profile, the vertical profile contained a redox and TPH gradient (Fig. 2). Samples A and B with positive Chem. PC1 values; were aerobic samples with high redox and low TPH concentrations that were located near the bottom of the plume. Samples, C, D, and E, on the other hand, with negative Chem. PC1 values, were anaerobic samples with lower redox and higher TPH concentrations. Vertical Chem. PC1, a consolidation of the 10 groundwater variables accounted for 72% of the variation in the hydrochemical data and represented a single global variable reflecting TPH degradation.

Fig. 4. PCA biplot from the vertical profile. Sample wells are indicated in bold, while the groundwater parameters are displayed in the loadings (dotted lines). Negative Chem. PC1 (samples C, D, and E) represent anaerobic, low redox, and high TPH environments while positive Chem. PC1 (samples A and B) is associated with more aerobic environments, higher redox and lower TPH concentrations.



Bacterial Community Structure of a Vertical Profile in Groundwater

Similar to the horizontal profile, the vertical distribution of Bacteria was profiled by TRFLP. The largest TRF peaks in samples A (165) and B (236) were unique, while samples C, D, and E shared the same large TRF peaks (74, 77, 461). In general, samples C, D and E appeared quite similar. This was confirmed by MDS analysis of the TRF patterns (Fig. 5-I). In addition, superimposing Vertical Chem. PC1 values (bubble area) on the MDS map, showed a clear relationship between the bacterial community structure (TRF data) and hydrochemistry (Vertical Chem. PC1).

In order to better understand which TRF peaks, and thus Bacteria, are associated with the sample clustering of C, D, and E, PCA was performed (Fig. 5-II). PCA provides a simple method for determining the relative contribution of specific variables to TRF pattern similarity and like the MDS analysis produced a similar view of the TRFLP data. PCA created a new variable, Bacteria PC1, which accounted for 72% of the variation in Bacteria TRFLP data (Fig 5-III). Vertical Chem. PC1 and Bacteria PC1 were significantly correlated (R^2 =0.79, p-value =0.044). Since Bacteria PC1 and Vertical Chem. PC1 each represent 72% of the data variation, it is unlikely that this correlation is an artifact.

Eight TRF peaks with PC1 loadings less than -0.2 (471, 74, 236, 136, 77, 343, 470, and 494) represented dominant members of the Bacteria community in samples C, D, and E. These 8 TRF peaks may represent bacterial types associated with more anaerobic TPH degradation conditions, as the relative abundance of these 8 TRF peaks was significantly correlated to the concentration of 3 out of the 10 hydrogeochemistry variables measured (Table 2). These TRF peaks are negatively correlated with oxygen

and positively correlated with carbon dioxide and reduced iron. They represented up to 80% of the bacterial community in sample D, where the oxygen concentration was the lowest (0.09 mg/L) while comprising less than 2% of the community in sample A where oxygen concentrations reached 1.76 mg/L (Fig. 5-IV). The organisms associated with these 8 TRF peaks may represent the anaerobic bacteria that we were looking for, considering their strong association with anaerobic TPH degradation conditions.

A library of 76 clones of 16S rRNA genes from sample D was sequenced in order to identify these 8 dominant TRF peaks. All of the sequences were homologous to uncultured bacteria exposed to petroleum hydrocarbons or other environmental contaminants. The majority of clones matched known organisms: 55% Chloroflexi subdivision 1, 4% Spirochetes, 4% Actinobacteria, and 3% Clostridia. An additional 8% of the sequences matched a sequence (2) most closely associated with the Chloroflexi. Predicted TRF lengths were calculated for each sequence and used to identify 6 of the 8 TRF peaks above (Table 3). The remaining TRF peaks (343 and 494) were tentatively identified by comparison to a TRF database generated from 16S rRNA sequences collected from Genbank and the Ribosomal Database Project (23). TRF 343 most commonly represented unidentified green-non-sulfur bacteria (Chloroflexi), while TRF 494 most commonly represented uncultured Spirochetes. Chloroflexi were the most dominant phylotype in both clone abundance (58% of the library) and TRF abundance (75%).

Fig. 5. Summary of Bacteria TRFLP analysis from the vertical profile. Panel I, MDS of Bacteria TRFLP data (stress level 0.0) with Chem. PC1 indicated by symbol bubble area. Clusters confirmed at 50% similarity by Bray-Curtis Cluster Analysis are indicated by dotted lines. Panel II, PCA of Bacteria TRFLP data with organisms associated with anaerobic TPH samples (loadings <-0.2) indicated by solid lines. Panel III Regression analysis confirmed the significant relationship between Bacteria PC1 and Chem. PC1 (R^2=0.79, p-value= 0.044). Panel IV, the relative abundance of TRFs with loadings <-0.2 negatively correlated to log of the oxygen concentration (R^2=0.85, p-value= 0.026).

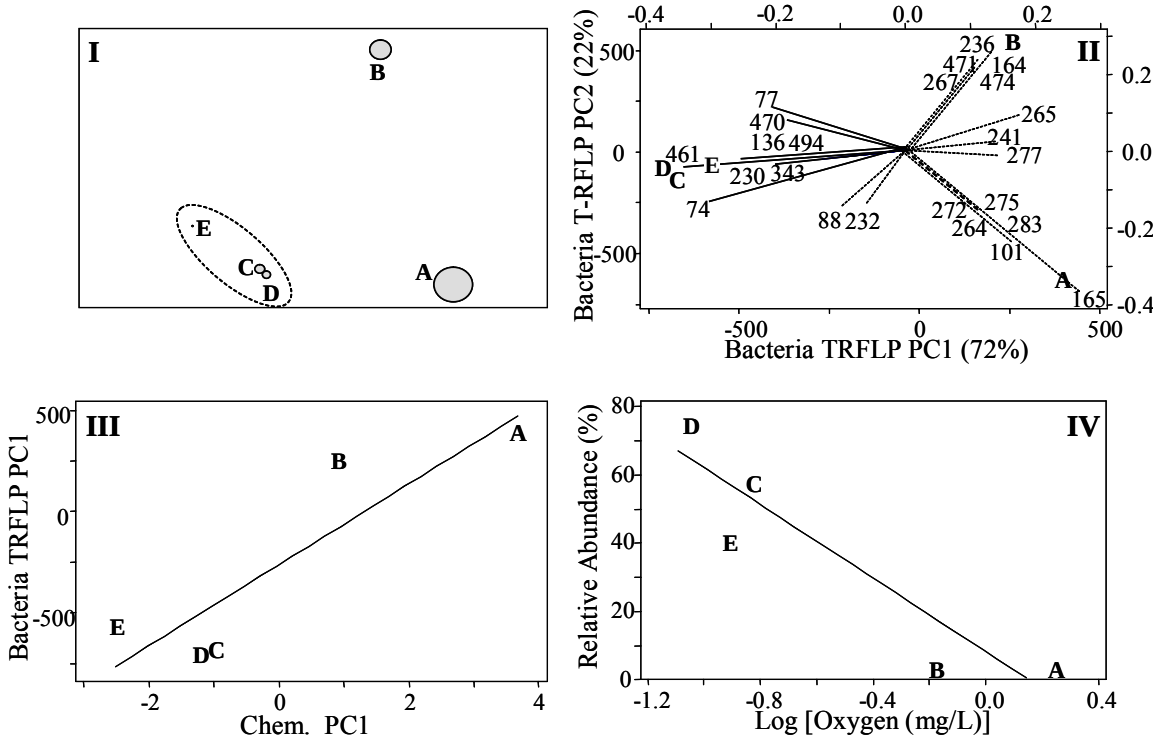


Table 2. Correlation between the relative abundance of the 8 dominant TRF peaks and individual groundwater parameters. Statistically significant ($p < 0.05$) R^2 values are in bold.

	R^2	P-value
O_2	**0.85**	0.026
CO_2	**0.79**	0.043
Fe^{2+}	**0.78**	0.046
TPH	0.63	0.111
pH	0.58	0.137
CH_4	0.29	0.346
SO_4	0.09	0.06
BTEX	>0.01	0.964

Table 3. Identification of 8 dominant TRF peaks using H2-2D clone library sequences or TRF database. Clone sequences were identified by BLAST match to Genbank.

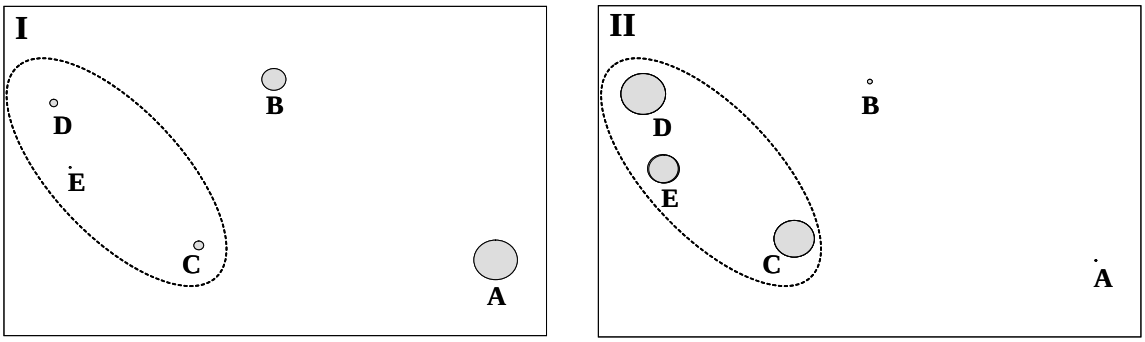
TRF (nt)		Matching Clones	Best BLAST Match	Percent Match
Observed	Predicted			
74	75	54, 68	Uncultured Bacterium (Chloroflexi), clone G1F9 (AF407200)	96
136	136	75	Uncultured Chloroflexi, clone 44a-B1-15 (AY082460)	96
230	230-231	14, 20, 77	Uncultured Bacterium (Chloroflexi), clone G1F9 (AF407200)	95
343	344*	No clone match	Unidentified green non-sulfur bacteria	N/A
461	460-462	3, 5, 12, 35, 36, 40, 44, 51, 63, 65, 74	Uncultured Bacterium (Chloroflexi), clone Eub 4 (AF423184)	96
470	469-470	22, 26, 27, 28, 37, 58, 96	Uncultured Chloroflexi, clone SJA-117 (AJ009488)	96
77	76	10, 84	Uncultured Spirochete, clone SJA-69 (AJ009476)	97
494	494*	No clone match	Uncultured Spirochetes	N/A

*, Predicted from TRF Database
N/A, Not Applicable

Archaea Community Structure of a Vertical Profile in Groundwater

To better characterize bacteria involved with methanogenesis, TRFLP was used to define the Archaea community structure. Although many of the Archaea TRF peaks were not represented in the TRF database, approximately half of the identifiable Archaea TRF peaks matched uncultured methanogens while the remainder matched uncultured Archaeon. The relationships between samples were visualized with MDS and a good correlation to groundwater chemistry was seen by superimposing Chem. PC1 (bubbles) on the MDS map (Fig. 6-I). MDS also revealed a relationship between the relative abundance of the 8 dominant Bacteria TRF peaks and the Archaea community structure (Fig. 6-II).

Fig. 6. MDS of Archaea TRFLP data (stress level 0.0) with clusters confirmed at 60% similarity by Bray-Curtis Cluster Analysis indicated by dotted lines. Panel I, with Chem. PC1 indicated by bubble area. Panel II, relative abundance of the 8 Bacteria (Chloroflexi and Spirochetes) indicated by bubble area.



Discussion

The intent of this study was to identify bacteria correlated with methanogenic TPH degrading environments in the Guadalupe Dunes, and to propose a mechanistic model for methanogenic TPH degradation in the dune sand aquifer. Analysis of TRF patterns showed no correlation between Horizontal Chem. PC1 and Bacteria or Archaea community structure in samples collected from conventional monitoring wells along a plane transect (Fig 3). In addition, Bacteria and Archaea communities did not appear to be related. Because TRF patterns do not accurately quantify active community members (41), perhaps the dominant TRF peaks did not represent the organisms associated with TPH degradation resulting in a lack of correlation to hydrogeochemistry. However, TRF patterns have successfully documented succession in active environments; from batch reactors to land treatment (2, 8, 19) so this option does not seem likely. In addition, this explanation does not account for the lack of relationship between Bacteria and Archaea communities.

Alternatively, a mixing of groundwater with varying chemistry and community structure during sample collection better explains the lack of correlation between hydrogeochemistry and bacterial community structure, as well as the lack of relationship between Bacteria and Archaea communities. Groundwater samples in the horizontal profile were collected across 4.6-6.1 vertical meters resulting in an average representation of the vertical dimension. Furthermore, standard practice on the site was to pump four well volumes from each well before sampling, completely expunging any established vertical gradient in the well (30). Because hydrogeochemical data are measured quantitatively and TRF data are transformed into relative abundance, an average across

this large a sampling area will not result in similar distributions from these two different data sources. This result suggests that when studying the spatial distribution of bacteria in groundwater one should avoid sampling wells with long screen intervals.

In contrast to the horizontal profile, vertical profile samples were collected from nested wells with 0.3 meter screens, which provided information on the local rather than average groundwater chemistry. This sampling strategy explored a single dimension and better preserved the relationship between hydrogeochemistry and bacterial community structure. PCA of the hydrogeochemistry variables again revealed a redox and TPH gradient in the five samples and also created Chem. PC1, a global variable that summarized 72% of the variation in hydrogeochemistry (Fig 4). In this case, MDS analysis of Bacteria TRF patterns showed a strong correlation to hydrogeochemistry as represented by Chem. PC1 (Fig 5-I).

Unlike MDS, PCA provides a simple method for determining the relative contribution of specific TRF peaks to overall TRF pattern similarity. Although the TRFLP data does not strictly conform to the assumptions of PCA (30), essentially the same relationship between the samples was provided by both PCA and MDS (Fig 5-II). Samples with lower redox and higher TPH concentrations were dominated by 8 TRF peaks corresponding to Bacteria in the phyla Chloroflexi and Spirochetes. Considering their strong association with methanogenic TPH degradation conditions, Chloroflexi and Spirochetes could be associated with the anaerobic natural attenuation of petroleum, The Spirochetes represented 2 of the dominant TRF peaks and had a much lower representation in the clone library, while 6 out of the eight dominant TRF peaks and more than half of the clone library were identified as Chloroflexi subdivision 1, indicating a

dominant role for these organisms. In fact, when Chloroflexi/Spirochete associated TRF peaks were observed in the horizontal profile there was a trend (data not shown) relating these TRF peaks to hydrogeochemistry (carbon dioxide, methane, and TPH), suggesting these organisms may act as a biological indicator for anaerobic degradation in the Guadalupe Dunes.

TRF peaks 74, 136, 230, 461, and 470 unambiguously matched (sequence similarity greater than 92%) sequences corresponding to Chloroflexi. These TRF peaks matched cloned sequences from a wide variety of anaerobic environments undergoing remediation. TRF peaks 74 and 230 matched a clone from monochlorobenzene contaminated groundwater (2) as well as a clone designated as Chloroflexi with a slightly lower similarity (91%) retrieved from methane hydrate-bearing marine sediments (26). TRF 136, matched a clone from anaerobic acid mine drainage (M. Labrenz, and J.F. Banfield, unpublished data). While TRF 461, best matched clones from a toluene-degrading methanogenic consortium (12) it also matched (94%) Chloroflexi clone SJA-117 from a methanogenic, trichlorobenzene-transforming microbial consortium (44). In fact, 3 of the dominant TRF peaks, TRF 461, 470, 77, matched clones from this methanogenic consortium, with TRF 470 corresponding to clone SJA-117 and TRF 77 corresponding to Spirochete clone SJA-69 (44).

Sequences from the Guadalupe Dunes aquifer were similar to sequences from environments where a combination of anaerobic conditions, high organic content and methane production were common. The Chloroflexi, previously known as green non-sulfur bacteria (13) are a ubiquitous yet rarely studied phylum of Bacteria with a potentially significant role in environmental chemistry including a role in macromolecule

degradation (16, 27, 31). All Chloroflexi clones in this study belonged to subdivision I, which contains non-photosynthetic members from contaminated environments (6, 16, 44). The phenotypic properties of subdivision I have recently been characterized with the isolation of the first cultured representative, UNI-1, from anaerobic sludge (37, 38). This strictly anaerobic fermentative strain used a variety of short chain fatty acids to produce hydrogen, acetate, and carbon dioxide and was found to form syntrophic relationships with hydrogenotrophic methanogens (38).

Both Chloroflexi (6, 37, 38, 39, 44, 46) and Spirochetes (11, 15) have been identified in methanogenic consortia from diverse habitats. In the Guadalupe Dunes aquifer, Chloroflexi and Spirochetes, represented by the dominant TRF peaks identified in the most anaerobic samples, were associated with specific Archaea community structures (Fig 6-II). This may indicate that some types of Archaea (methanogens) were more abundant when these Bacteria were present. However, differences in the Archaea community structure were also related to the aquifer hydrogeochemistry so the functional link between these Bacteria and methanogens is still tentative.

Because methanogens have a limited range of substrates, the production of methane from petroleum hydrocarbons is undoubtedly the result of a complex consortium of bacteria (12). While there are no reports suggesting that Chloroflexi, and to a lesser extent Spirochetes, are involved in the initial oxidation of petroleum hydrocarbons, the results suggest that a Chloroflexi/methanogen consortium may be involved in the final stages of methanogenesis from petroleum. OP11 bacteria, previously suggested as filling this niche, were not found at Guadalupe (10). We propose that the Chloroflexi and Spirochetes ferment partially oxidized products of anaerobic hydrocarbon degradation

(e.g. short chain organic acids) to acetate or hydrogen gas (34). Non-detectable H_2 concentrations in the wells confirm the tight association between these fermentative Bacteria and methanogens. This fermentation is energetically favorable only when the concentrations of acetate and hydrogen are kept low by acetotrophic or hydrogenotrophic methanogens (10, 12, 34, 35). Therefore, sustained methanogenic hydrocarbon degradation is dependent on the syntrophic relationship between Chloroflexi/Spirochetes and methanogens.

To test this hypothesis, Chloroflexi should be isolated from petroleum contaminated aquifers and functionally characterized since they were by far the most dominant group. In addition, specific primers for Chloroflexi subdivision I should be used to quantify the absolute abundance of these bacteria with respect to hydrogeochemistry. With the development of a rapid assay for Chloroflexi these organisms may serve as a field biomarker for anaerobic natural attenuation in petroleum contaminated aquifers.

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CHAPTER 2.

EVALUATION OF PCR PRIMERS FOR SULFATE REDUCING BACTERIA

Introduction

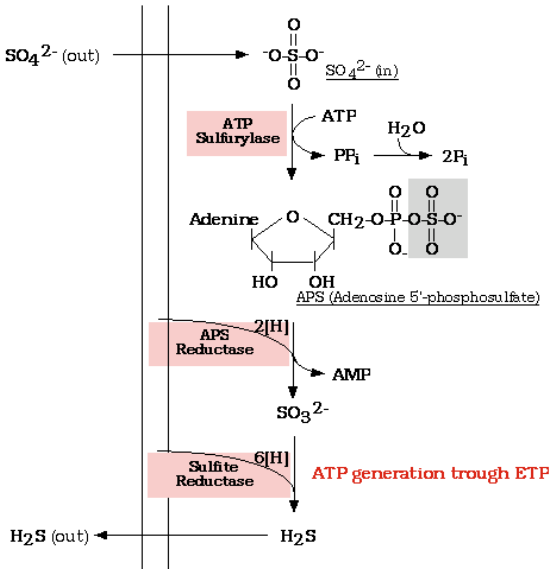
Thirty years ago, Unocal Corporation owned and operated a 2,400 acre active oil field in Guadalupe, CA. During this period of operation, crude and refined oil products were released into the soil subsurface and groundwater. Currently, as a result of a clean up and abatement order, Unocal is actively pursuing a variety of remediation efforts at the site. Natural attenuation, or the reduction of contaminants *in situ* by natural microbial processes, is one of the technologies being utilized.

Ultimately, an understanding of the microbial community structure and function could provide a mechanistic model for evaluating the effectiveness and sustainability of natural attenuation in Guadalupe Dunes aquifer. In an attempt to better elucidate the relationship between microbial community structure and function, primers specific for key physiological roles were evaluated. Sulfate Reducing Bacteria (SRB) could play an important role in the Guadalupe Dunes ecosystem since groundwater at the site was shown previously to contain detectable amounts of sulfate and hydrogen sulfide, a product of sulfate reduction.

SRB constitute a phylogenetically diverse group of anaerobes, which are capable of degrading a wide range of petroleum products including alkanes, toluene, benzene, and polyaromatic hydrocarbons (2). All of these organisms are capable of obtaining energy from the reduction of sulfate to sulfide using three enzymes: ATP sulfurylase, APS reductase, and dissimilatory sulfite reductase. Dissimilatory sulfite reductase (DSR), the enzyme that catalyzes the energy generating step by reducing sulfite to sulfide, is integral to sulfate reduction and thus highly conserved among SRB (Fig. 1) (4,5). Four Bacterial

and one Archaeal DSRs have been characterized and all share a common $\alpha_2\beta_2$ or $\alpha_2\beta_2\gamma_2$ structure (8).

Fig. 1. Pathway for dissimilatory sulfate reduction (4).



The DSR1f and DSR4r primer set amplified a 1.9-kb DNA fragment encoding a highly conserved portion of alpha and beta subunits of the sulfite reductase gene. This primer pair was specific for SRB (Table 1) and successfully amplified 22 representative bacteria from the four main groups of SRB (delta-*Proteobacteria*, *Nitrospira* division, *Thermodesulfobacterium* division, and the Gram-positive division of sulfate reducing organisms). This primer pair did not amplify genes encoding assimilatory sulfite reductase, genes encoding sulfite reductase from an organism capable of respiring sulfite but not sulfate, or the genes for the reverse sulfite reductase, which have a similar subunit composition. (8)

Table 1. PCR amplification of genomic DNA from reference organisms (8).

Species or isolate[a]	Source or strain	PCR products obtained with primer pair[b]				Hybridization with DSR probe
		I	II	III	IV	
SRs of the δ-*Proteobacteria*						
Desulfovibrio vulgaris	ATCC 29579	+	+	+	+	+
Desulfovibrio desulfuricans	ATCC 27784	+	+	+[2]	+	+
Desulfovibrio africanus	ATCC 19996	(+)	−	+	−	+
Desulfovibrio sp. strain PT-2	D. A. Stahl	+	+	+	+	+
Desulfovibrio oxyclinae	Y. Cohen	+	+	+	−	+
Desulfovibrio sp.	D. Gevertz	+	+	+	−	+
Desulfovibrio sp. strain G11	M. J. McInerney	+	+	+	+	+
Desulfoarculum baarsii	M. J. McInerney	−	−	+	−	+
Desulfobacterium niacini	DSM 2650	−	+	+	−	+
Desulfobacterium vacuolatum	DSM 3385	−	+	+	−	ND
Desulfococcus multivorans	ATCC 33890	−	+	+	−	+
Desulfonema ishimotoi Jade 02	F. Widdel	(+)	+	+	−	+
Desulfonema ishimotoi Tokyo 01	F. Widdel	−	+	+	(+)	+
Desulfonema limicola	ATCC 33961	−	−	+	−	+
Desulfobotulus sapovorans	ATCC 33892	+	−	+	−	+
Desulfomonas pigra	ATCC 29098	+	+	+	+	+
Desulfobacter latus	ATCC 43918	+	−	+	−	+
Desulfomicrobium baculatus	DSM 1743	+	−	+	−	+
Desulfobulbus propionicus	ATCC 33891	−	−	+	−	+
Nitrospira division SR						
Thermodesulfovibrio yellowstonii	R. Devereux	−	−	+	−	+
Thermodesulfobacterium division SR						
Thermodesulfobacterium commune	ATCC 33708	−	−	+	−	+
Gram-positive division SR						
Desulfotomaculum ruminis DL	ATCC 23193	−	+	+	−	+
δ-*Proteobacteria* with "reverse" SR						
Beggiatoa sp. strain MS-81-1c	D. Nelson	−	−	−	−	−
Beggiatoa sp. strain OH-75-2a	D. Nelson	−	−	−	−	−
Beggiatoa sp. strain 81-6	D. Nelson	−	−	−	−	−
Chromatium vinosum	ATCC 17899	−	−	−	−	−
Thiobacillus denitrificans	ATCC 25259	−	−	−	−	−
δ-*Proteobacteria* sulfite-respiring bacterium						
Shewanella putrefaciens	ATCC 8071	ND	ND	−	ND	−

[a] SR, sulfate reducer.

[b] +, PCR product of the expected size; −, no PCR product; (+), low yield of PCR product; +[2], two similar-sized PCR products; ND, not determined. PCR amplification of genomic DNA from 22 sulfate-reducing bacteria, 5 bacteria considered to possess a reverse-type sulfite reductase, and 1 bacterium having the capacity to respire sulfite with the DSR primers. The primer pair DSR1F-DSR4R (III) amplified the expected ~1.9-kb fragment for all sulfate reducers tested. Primer pairs DSR1F-DSR3R (I), DSR2F-DSR4R (II), and DSR2F-DSR3R (IV) generated the expected ~1.1-kb, ~1.4-kb, and ~0.5-kb fragments for only some of the sulfate-reducing bacteria analyzed. Sufficient quality of each genomic DNA for successful PCR amplification was demonstrated using conserved 16S rDNA-targeted primers (data not shown). Amplification products of all sulfate reducers with primer pair III hybridized specifically with a DNA probe complementary to a conserved region of the α subunit of the DSR.

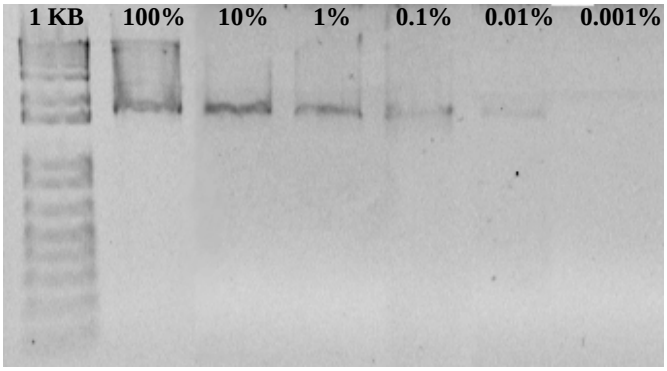
In addition, the DSR1f and DSR4r primers have successfully amplified SRB populations from a wide variety of environments including anoxic microbial mats (5), hot springs (3), sediments (6, 7), and metal contaminated groundwater (1). In order to confirm the role of SRB in the Guadalupe Dunes, the DSR1f /DSR4r primer pair was optimized for groundwater samples. Groundwater DNA was isolated from 25 wells located along a TPH and sulfate gradient and PCR, and if appropriate TRFLP, were performed to better characterize SRB populations in the Guadalupe Dunes aquifer.

Primer Optimization

Based on the results by Wagner, et al., PCR was performed using reverse primer DSR4r (5'- GTG TAG CAG TTA CCG CA-3'), and forward primer DSR1f (5' ACS CAC TGG AAG CAC G-3') (8). *Desulfovibrio vulgaris* (ATCC 29579) genomic DNA was used as a positive control (ATCC, Manassas, VA). Positive control reactions were carried out using 2 μL of *D. vulgaris* DNA (1 ng/μL), 5 μL of 10x Buffer, 3 μL of 10 mM DNTP, 2 μL 20 μg/mL BSA, 5 μL 25 mM MgCl2, 1 μL DSR4r, 1 μL DSR1f, 28.7 μL water, and 0.3 μL 5 U/μL TaqGold®. Final PCR conditions were 94 °C for 10 min; 30 cycles of 94 °C for 1 min, 55.5 °C for 1 min, 72 °C for 1.5 min; and 72 °C for 10 min.

The primers were evaluated for specificity and sensitivity. Primers were found to be 100% specific for all organisms tested. They positively amplified *Desulfovibrio vulgaris* and failed to amplify 10 negative controls including the following soil associated bacteria *Bacillus subtilis*, *Pseudomonas aeroginosa*, *Methylomonas methanica*, *Methanococcus jannaschii*, and *Escherichi coli* (data not shown). In order to emulate amplification of SRB DNA from a bacterial community, primer sensitivity was detected by performing serial ten fold dilutions in *E. coli* DNA (Fig. 2). Based on these results primers could detect *dsr*AB gene copies at a 1:1000 dilution.

Fig. 2. Primer sensitivity to 0.01% determined by performing serial ten fold dilutions of *D. vulgaris* (SRB) in *E. coli* DNA.

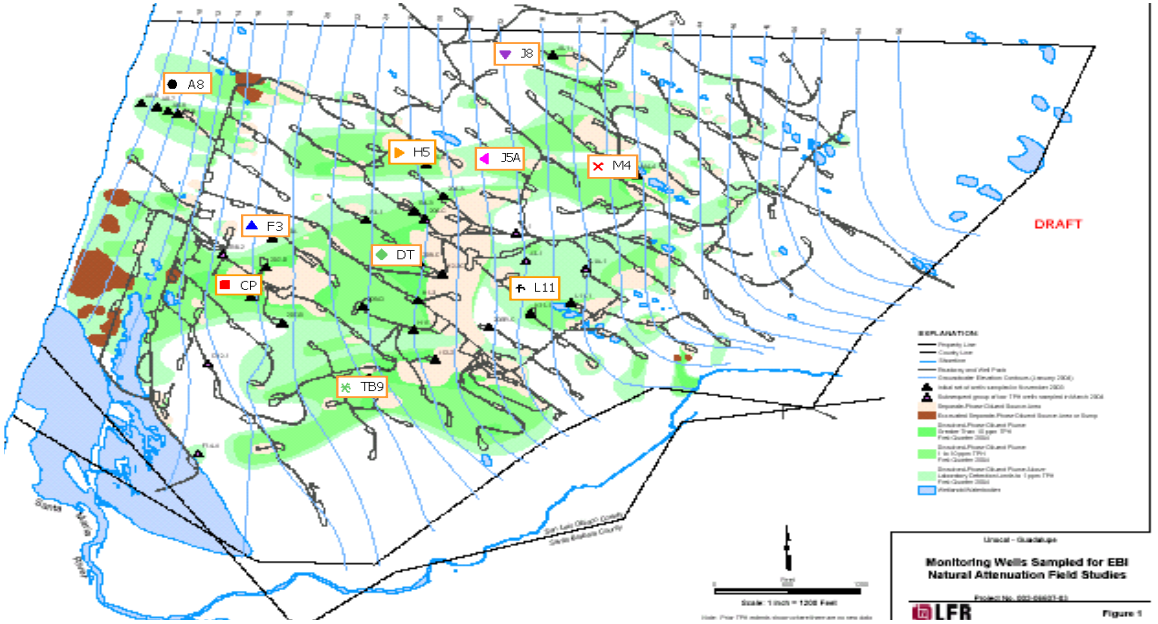


Application to the Guadalupe Dunes

Sample Collection

25 groundwater samples from 10 plumes were collected at Unocal's former Guadalupe Dunes oil field (Fig.. 3). The hydrogeochemical data was analyzed as discussed in Chapter 1 (p. 7) by Zymax Laboratories (San Luis Obispo, CA).

Fig. 3. Guadalupe Dunes indicating the locations of the ten dissolved phase groundwater plumes (colored symbols) and wells (black triangles) that were sampled and analyzed. This map also shows the natural flow of groundwater at this site.



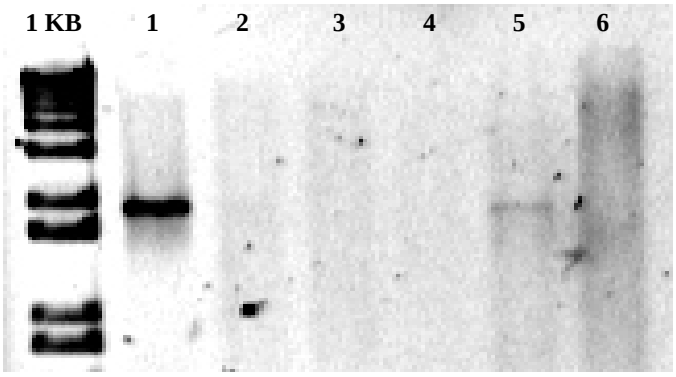
Extraction of Groundwater DNA

Four liters of groundwater from each well were filtered through 0.2 μm nylon membranes filters. The filters were frozen and half of two filters were crushed and homogenized under liquid nitrogen. Samples were extracted using the protocol outlined in Chapter 1 (p. 8) and was found to contain 1.659-120.44 ng/μL of DNA.

Amplification of *dsr*AB

Groundwater DNA from 25 wells was successfully amplified with 4 primer sets (General Bacteria, Archaea, methanotroph, and methanogen); however, upon initial testing no SRB positive wells were detected. In order to make the primers less stringent, a modified protocol was developed for the field samples. This protocol included the use of 7 μL undiluted DNA, increased cycle time from 30 to 40 cycles, and a lowered annealing temperature to 54°C. After performing these modifications, one positive SRB well (207 C) was detected. Given the lack of positive results, TRFLP was not attempted with *dsr*AB specific primers.

Fig. 4. Gel electropherogram results from the Guadalupe Dunes. Lane 1 contain the positive control. Lanes 2-6 contain PCR product from groundwater. Only Lane 5 (well 207C) contained detectable SRB.



Conclusions

The one positive SRB well could be attributed to a lack of primer specificity for certain SRB populations, a low level of SRB in the Guadalupe Dunes, or the existence of a larger biofilm-associated population of SRB in the dunes aquifer. Primer sensitivity and specificity was evaluated prior to use and in the literature (8), and is most likely not responsible for the lack of amplification. In fact, our lab recently used these primers to confirm the presence of SRB in sample GSYC-4-B14-091504 from a Guadalupe groundwater injection well. (personal communication: Alice Hamrick). Perhaps the SRB present in the soil at Guadalupe are more tightly associated with soil biofilms and are thus not detected as free-swimming bacteria in groundwater.

Based on the results of this study, SRB are not dominant members of the free-swimming microbial community in groundwater (Fig. 3). Extrapolation of the primer sensitivity results (Fig. 2) suggested that the SRB population represents approximately 0.1 to 0.01% of the microbial population in well 207C and less than 0.01% in the remaining wells. Furthermore, no 16S Bacteria TRF peaks were detected that corresponded to sulfate reducing bacteria, confirming the low abundance of SRB population in the aquifer.

If future studies will be conducted to evaluate the role of SRB in the Guadalupe Dunes they should include samples from the saturated soil to determine their presence in biofilms. While SRB are most likely not dominant in the groundwater, their role in the soil has yet to be confirmed.

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APPENDIX.

BACTERIAL FACTORS AFFECTING THE SUSTAINABILITY OF NATURAL ATTENUATION: 2003-2004 FINAL UNOCAL REPORT

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Executive Summary

The overall goal of 2003/04 study was to fill previously identified information gaps critical to understanding the stability of natural attenuation at Guadalupe and devise a theoretical, mechanistic model for the bacterial contribution to natural attenuation at Guadalupe. The 2002/03 study provided a tantalizing glimpse of the dominant physiological processes occurring in contaminated groundwater. In particular, petroleum concentration and vertical position in a plume were correlated with specific Terminal Restriction Fragment (TRF) peaks that may correspond to organisms with either sulfate reducing or fermenting physiologies. These organisms may provide substrates for methane generation, fueling the methanogens that were also present in the plume interior. This study included and extensive sampling scheme followed by exhaustive chemical analyses, and the bacterial community was analyzed with both qualitative and quantitative tools. Several methodological shortfalls were discovered during analysis of the data. First, accurate quantification of bacteria in groundwater samples is not possible due to varying amounts of suspended solids in the samples. Second, because samples were taken from wells with 10 to 20 foot screens after full well development, the resulting water represented a vertical average of groundwater at that position. The result of this sampling scheme was that no correlations were seen between bacterial types and groundwater physical/chemical measurements. The only exception was that the type of methanotrophic bacteria present appeared to depend on the concentration of dissolved O_2. This study resulted in two master's theses and some of the results have been presented at national and international

meetings. A manuscript is currently being prepared for publication, including results from the 2002/03 study.

Objectives and Milestones

1) Develop a sampling scheme (20-30 samples total) in collaboration with Yarrow Nelson and appropriate Unocal representatives whereby a range of DPD concentrations and distances from contaminant sources are covered, both horizontally and vertically. Samples should be collected in small lots (5-10 at a time) that can then be processed rapidly.
2) Analyze these new samples for TPH, electron acceptors (or end products) and methanogenic substrates using outside labs (Zymax, Creek Environmental and/or BC labs).
    a. TPH (SIMDIS), O_2, NO_3, SO_4, Fe^{2+}, acetate, formate, H_2S, H_2, CO_2, CH_4
3) Analyze these new samples for TPH and polar composition using GCxGC technology to look for differential degradation of petroleum components.
    a. Utilize Chris Reddy's lab at Woods Hole
4) Analyze these new samples as well as a select set of previously collected sample DNA (perhaps from C8) with an array of TRF tools available at the EBI.
    a. General Bacterial TRF
    b. Archaeal TRF
    c. Sulfite reductase TRF
    d. Actinobacterial TRF

5) Analyze these new samples as well as a select set of previously collected sample DNA (perhaps from C8) with molecular and conventional quantification tools available at the EBI.

   a. Q-PCR of Archaea (methanogens)

   b. Q-PCR of sulfite reductase (sulfate reducers)

   c. Direct count of bacterial cells

6) After TRF analysis, select DNA from specific samples (3 to 5) to create PCR clone libraries for accurate identification of important TRF signals.

Progress against Objectives

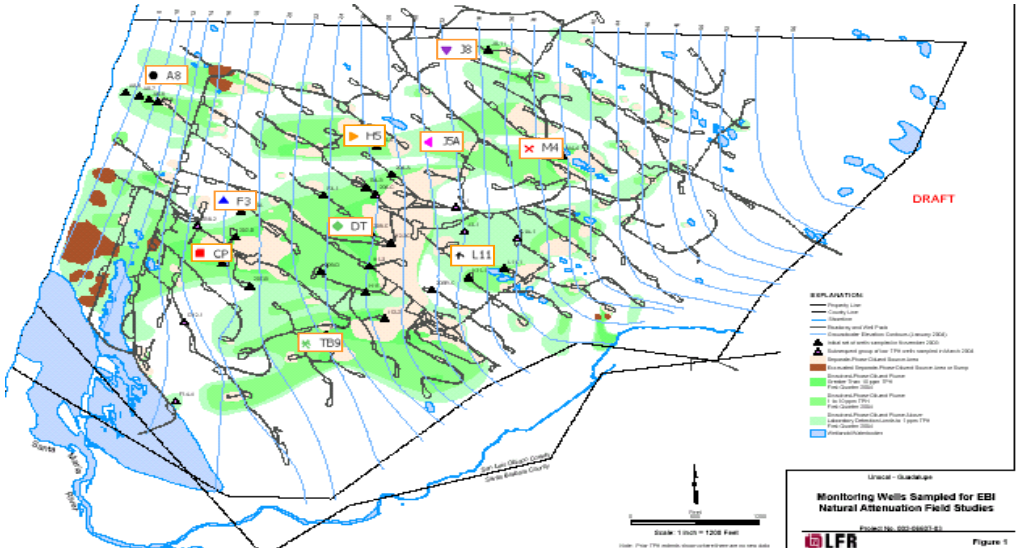
Sampling Schematics

In October of 2003, 30 groundwater wells were selected for sampling from the Guadalupe dunes restoration site west of Santa Maria, California (Fig. A1). Two samples, E3-2 and K5-6, were not collected. Therefore 28 samples, from ten different plumes of dissolved phase diluent, were received for processing in November, 2003. In April 2004, an additional 6 wells were selected (F14-4, LIA-1, J2-1, TB8-2, D12-1, and I3-1) as a low TPH complement to the original well set. Ultimately, a total of 34 samples were processed for chemical and biological analyses to provide a horizontal profile across the Guadalupe Dunes aquifer.

Groundwater was collected from each well and processed for chemical and biological analyses. Split samples were analyzed for pH, alkalinity, conductivity, and redox, concentrations of acetate, formate, hydrogen, dissolved total petroleum hydrocarbons (TPH), TPH after silica gel, benzene, ethlybenzene, toluene, xylene, sulfate, dissolved gasses (methane, oxygen, carbon dioxide, hydrogen, nitrous oxide) nitrite, nitrate, ammonium, phosphate, dissolved iron (field and laboratory methods) and dissolved oxygen (DO) by electrode (also in the field). Measurements below detection limits were recorded as half of the measurement threshold. An estimate of the distance from a free-product TPH source was also documented for every well. Unfortunately, wells H2-3C and A8-7 were not processed for gasses due to compromised samples and were thus dropped from analysis due to lack of pertinent physical and chemical information. Four liters of groundwater was run through 0.2 micron filters and the filters were extracted for DNA. The DNA was used in PCR reactions for 16S rRNA genes

(Eubacteria and Archaea), sulfite reductase (Sulfate Reducing Bacteria), particulate methane monooxygenase (Methanotrophic Bacteria), and methyl-coenzyme M reductase (Methanogenic Bacteria). Seven wells (F14-4, LIA-1, J2-1, 208R-C, TB8-2, D12-1, and I3-1) did not produce enough DNA for positive PCR results and so were not include in further analyses.

Fig. A1. Regional map indicating plume and well locations



Physical and Chemical Analyses

The physical/chemical data was analyzed separately to look for trends and to organize the data for comparison to bacterial community information collected from TRF patterns. Principle Components Analysis (PCA) provides a rapid way to assess variation with multidimensional data such as those provided by the physical and chemical variables. PCA is a method of data reduction that transforms the data into new variables (the principal component scores), which are constructed to represent the greatest variation in the data set. The first principal component (PC) accounts for as much of the variability in the data as possible, and each succeeding PC accounts for as much of the remaining

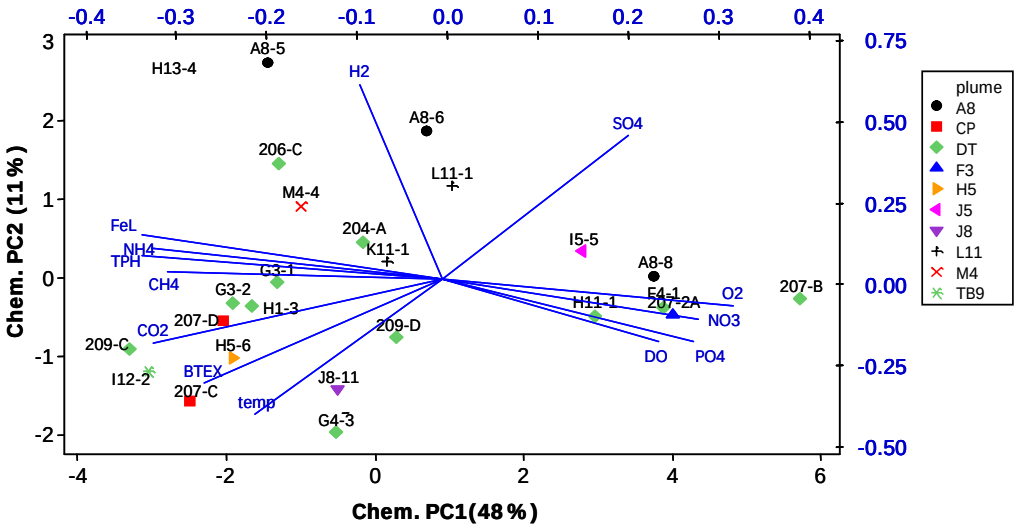
variability as possible. PC scores are generated by assigning a loading factor to each variable and summing the total of all variables multiplied by their loading factors. Plotting the first PC scores on the x-axis and the second PC scores on the y-axis creates a score plot. A meaningful result would be an obvious grouping, or gradient, of samples within the plot. Plotting the first PC loadings on the x-axis and the second PC loadings on the y-axis creates a loading plot. A loading plot indicates which variables are important to each PC score. Score plots and loading plots can be overlaid to increase the information content. A PCA biplot displays the PC scores on the primary X- and Y-axes, with the PC loadings on the secondary X- and Y-axes.

The physical/chemical data set was adjusted prior to being imported into the Minitab software package. Certain chemical variables were excluded from statistical analyses due to lack of unique information, or concern regarding the accuracy of the measurement techniques. The total concentration of benzene, toluene, ethlybenzene, and xylene was summarized in a new variable – BTEX. The final chemical variable set included the following variables: TPH, BTEX, methane, hydrogen, oxygen, carbon dioxide, nitrate, sulfate, nitrate, ammonium, phosphate, dissolved iron (laboratory method), and pH. Prior to performing statistical analyses, all concentration variables were log transformed to account for non-normal distributions common in concentration measurements. As mentioned earlier, 9 wells (H2-3C, A8-7, F14-4, LIA-1, J2-1, 208R-C, TB8-2, D12-1, and I3-1) were dropped from the study due to lack of data, leading to a final set of 25 groundwater wells.

Because all the physical/chemical variables are on different scales the PCA was performed using a using a correlation, rather than a covariance, matrix which normalizes

the data so that one variable will not dominate the analysis based solely on scale. PC1 scores from the physical/chemical data (Chem. PC1) appear to represent a compilation of parameters that characterize TPH degradation and include a gradient of electron donor to electron acceptor variables that demonstrate a gradient in the physical and chemical variability of the aquifer (Fig. A5). Chem. PC1, a consolidation of the 13 physical and chemical variables, represents a TPH degradation parameter and accounts for 48% of the variation in the physical and chemical data, while PC2 explains another 12% of the message in the data set. Plume specific variability is noted in the figure legend and further illustrates the large spread in the physical and chemical parameters across the ten plumes.

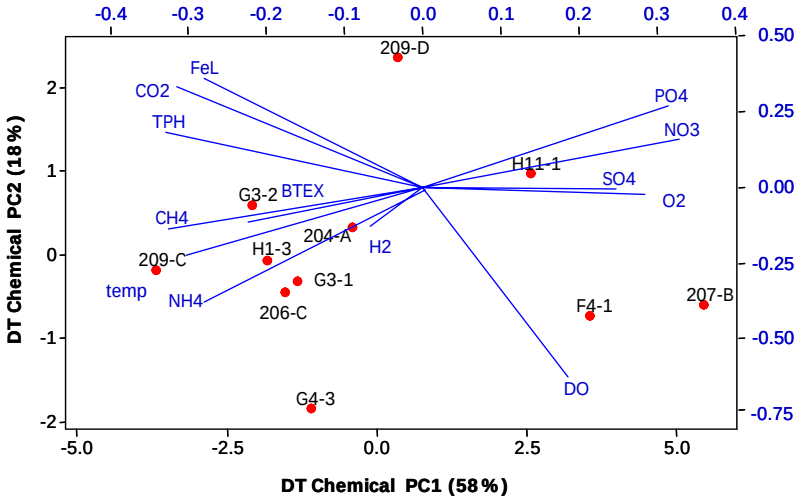
Fig. A2. Principal Components Analysis (PCA) of groundwater parameters for all wells



Different plumes may have been formed from source material with differing compositions, thereby contributing to the overall variability in the data set. In an attempt to eliminate the noise from plume variation, a PCA was performed using well information from the DT plume (Fig. A3). DT Chem. PC1 accounts for 58% of the variability, as opposed to the 48% accounted for in PC1 of all plumes, suggesting that

plume-to-plume variation of groundwater chemistry was significant. When correlating bacterial community data (TRF patterns) to groundwater chemistry, both site wide and DT plume specific analyses were performed.

Fig. A3. PCA of groundwater parameters for wells from the DT plume

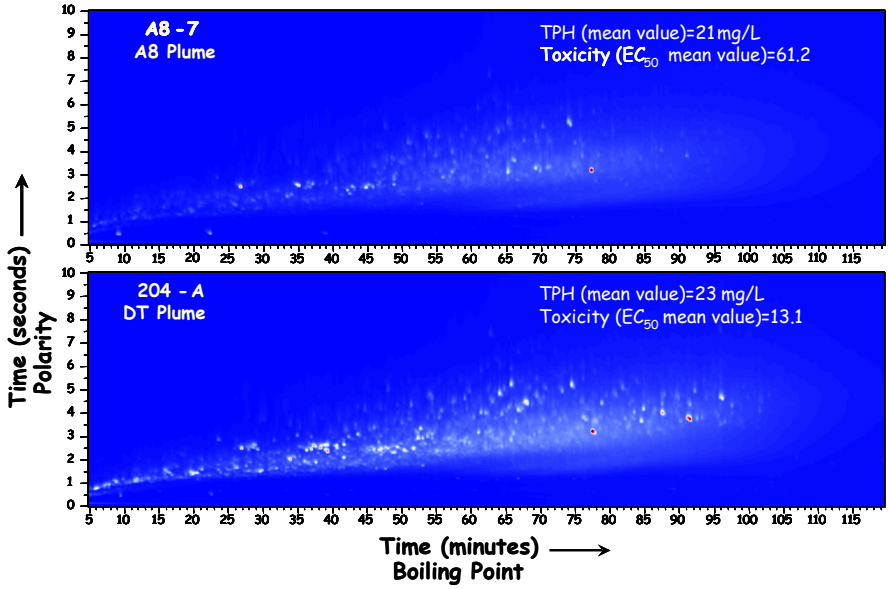


GCxGC analysis

Two dimensional gas chromatography (GCxGC) was performed by the Department of Marine Chemistry and Geochemistry at Woods Hold Oceanographic Institution in the laboratory of Christopher Reddy. GCxGC provides a high resolution analysis of complex mixtures, which is based on the resolving power of two orthogonal chromatographic columns. Resolution in the first dimension, X axis, is based on boiling point, while resolution in the second dimension is based on polarity. TPH samples from several groundwater wells were evaluated to determine the variability in hydrocarbon components in the dunes aquifer. Plume-to-plume variation was clearly demonstrated between two samples with relatively similar TPH concentrations from the A8 and DT plumes (Fig. A4). The GCxGC data supports the chemical principal component analysis

concluding that plume-to-plume variation is significant (Fig. A2 and A3) within the dunes aquifer.

Fig. A4. GCxGC images illustrating plume to plume variation



TRF analyses

DNA Extraction and PCR Results

DNA was extracted from groundwater samples following filtration through 0.2 micron filters, a total of 4 to 8 filters were required to filter all four liters of each sample. DNA was extracted by crushing two filters chosen at random. Of the original 34 wells, DNA was obtained from 28 wells. No PCR amplification products were recovered from well 208-RC using any of the 5 PCR primer sets used in the study. A total of 27 wells produced PCR products with Eubacteria 16S and Archaea 16S and Methanogen primers. Methanotroph primers successfully amplified 24 wells, excluding wells I5-5, 204-A, and M4-4.

Statistical Methods for TRF Data Analysis

Because PCA requires that variables follow a normal distribution which is not common with species abundance data (TRF patterns) other methods of analysis were

investigated in this study. PCA is based on Euclidean distances and may distort the relationships between samples when the data does not follow a normal distribution. In contrast, the ordination technique known as non-metric Multidimensional Scaling (MDS) is based on a Bray-Curtis similarity matrix, requiring fewer model assumptions. Both Euclidean Distance and Bray-Curtis Similarity take the relative abundance of each TRF peak into account when comparing two patterns (Fig. A5). Euclidian distance is prone to bias by large values and can give anomalous results when data sets contain large numbers of zeros. TRF data often contains zero values (a peak present in one pattern that is not seen in another) and covers 2.5 orders of magnitude variation in TRF peak area. Because the physical/chemical data could be transformed to maintain a normal distribution, PCA was an appropriate ordination method. However, Bray-Curtis similarity was specifically designed for use with species abundance data sets and is less susceptible to bias introduced by large numbers of zero abundance data (1). Bray-Curtis similarity is therefore a preferable similarity measure when comparing TRF patterns.

Fig. A5. Formulas for the measures used in TRF analysis, d1 and d7 refer to data from different TRF patterns.

Euclidean Distance

$$\sqrt{\sum_{TRF=60}^{600}\left(Area_{TRF,d1} - Area_{TRF,d7}\right)^2}$$

Bray-Curtis Similarity

$$100\left(1 - \frac{\sum_{TRF=60}^{600}\left|Area_{TRF,d1} - Area_{TRF,d7}\right|}{\sum_{TRF=60}^{600}\left|Area_{TRF,d1} + Area_{TRF,d7}\right|}\right)$$

In MDS, the relationship between the original data and the model output is a map based on the relationships existing in the Bray-Curtis similarity matrix. The distances on the map are unitless and based on relative similarity. So for instance, if sample A is more similar to sample B than it is to sample C, then sample A will be closer to sample B and farther from sample C on the MDS output. In contrast, samples that may be in the same position with respect to the PC1 and PC2 axes and are not closely related on PC3 will appear to be closely related in a two dimensional PCA plot. MDS accounts for total similarity and does not break distance into sub-variables.

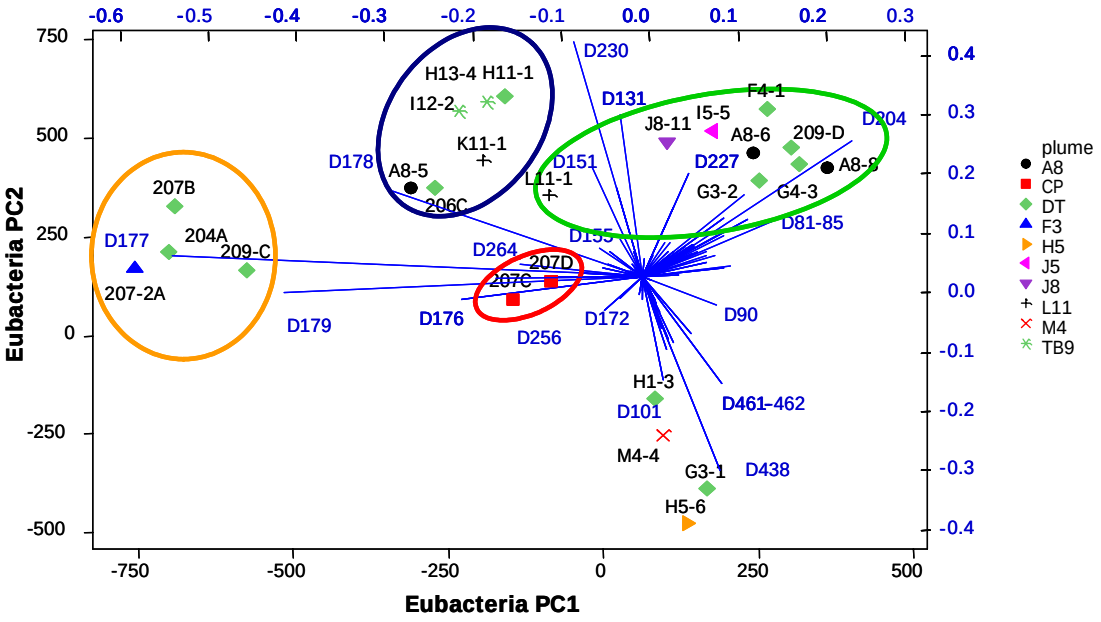
As the number of samples increases and the similarity matrix becomes larger, it becomes more difficult to exactly transpose the Bray-Curtis matrix into two-dimensional space. Some distortion, known as stress, between the similarity matrix and the MDS distance outputs occurs. Therefore, the ultimate goal of the MDS algorithm is to configure the samples such that this stress value is minimized. In general, a stress value of less than 0.05 provides an excellent representation of the data, while a value of 0.01 is

ideal. Stress values less than 0.1 corresponds to good ordination with no misleading interpretation, while stress levels less than 0.2 provide a helpful two dimensional picture but any conclusions should be verified with an alternative technique like cluster analysis. Stress values less than 0.3, should be treated with hesitation as the points are close to being randomly assigned and thus the ordination should be reexamined in three or four dimensions.(3)

General Bacterial TRF

PCA demonstrated the variation in the 16S Eubacteria TRF patterns across the site and illustrates an apparent clustering of the samples (Fig. A6). The 4 clusters were confirmed, at a similarity level of approximately 28%, using Cluster Analysis based on average linkage and a Euclidean distance matrix (data not shown). Wells not included in a cluster (circled data points) represent outliers based on the cluster analysis. Samples did not cluster based on the plume of origin, samples from the largest plume (DT) were present in most of the clusters. In addition, PC1 and PC2 scores from the TRF data did not correlate to Chem. PC1 ($R^2 < 0.1$) indicating that the clusters had no apparent relationship groundwater chemistry (data not shown).

Fig. A6. PCA demonstrating the variation of the 16S Eubacteria TRF patterns across the site. Clusters confirmed by Euclidian Distance Cluster Analysis are indicated by the colored shapes.



In addition to PCA, 16S Eubacterial TRF data was also evaluated using MDS (Fig. A7). One important consideration with MDS is that more attention is given to representing the overall structure of similarities rather presenting a view of the two components that cover most of the variation, as is the case with PCA (3). Thus, some samples with very low similarity to any others (H5-6, G3-1, M4-4, and H1-3) force the remaining samples to appear more closely related. With PCA plots it is possible that the additional principle components cover the added variation and so such differences are missed and these outlier samples appear closely related when they are not. With a stress level of 0.16, MDS of the Eubacteria TRF data gives a useful two dimensional representation of the relationships between wells; however it is important to use a complementary Cluster Analysis to confirm the sample clustering (Fig. A8). The

clustering of wells based on TRF data was confirmed at a similarity of 35% as is indicated on the cluster dendogram below (Fig. A8).

Fig. A7. MDS of the 16S Eubacteria TRF data demonstrating the variation across the site (stress level of 0.16). Clusters confirmed by Bray-Curtis Cluster Analysis (Fig. A8) are indicated by colored shapes.

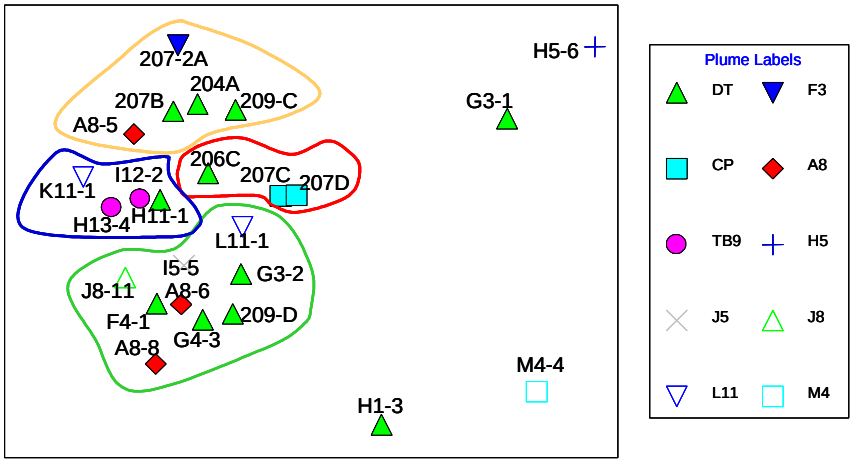
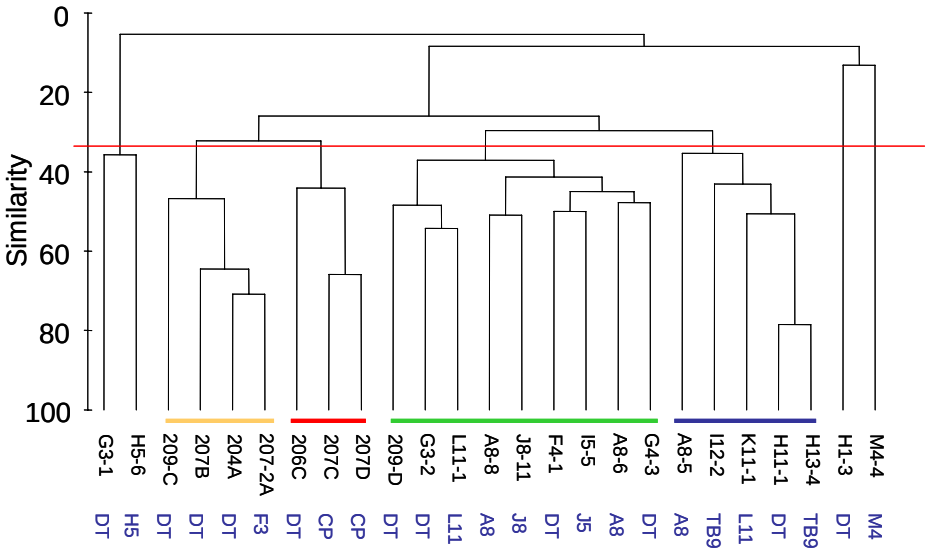


Fig. A8. Cluster Analysis output based on Bray-Curtis similarity matrix of the Eubacteria 16S TRF data.



MDS and PCA provided a similar interpretation of the biological data. The main advantage of PCA is that it includes the loadings, which illustrate which TRF peaks influence sample clustering (2). MDS on the other hand, is useful as it more accurately portrays the relationships between samples. For instance, wells H5-6, G3-1, M4-4, and H1-3 are not related to each other or any of the other wells on the MDS output (Fig. A7), but do appear more closely related to each other on the PCA output (Fig. A6). In addition, well 206-C clustered differently between methods, it is in the blue cluster in the PCA while it fits in the red cluster with MDS

When any statistical tool is employed, it is imperative to compare the statistical model with the actual data to verify the model accurately reflects the results. Upon viewing TRF patterns for each groundwater sample it is evident that the clusters defined by the PCA and MDS output (Fig. A6 and A7) are driven and supported by the dominant TRF peaks illustrated by the PCA loadings (Fig. A9) In addition, most of these TRF peaks matched to known petroleum degraders (Table A1).

Fig. A9. Representative TRF peaks from the 4 clusters and the outlier group.

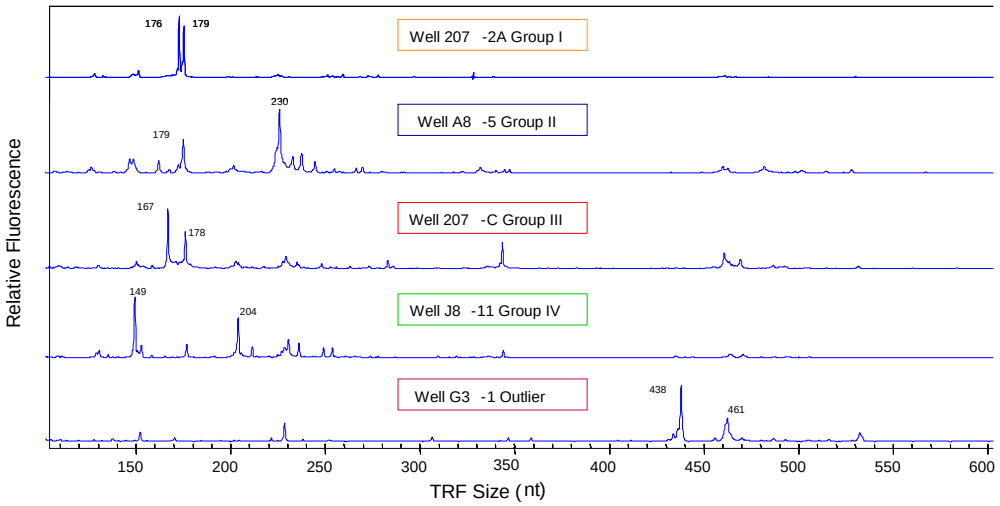


Table A1. Database matches for key TRF peaks from the main 16S Eubacterial TRF clusters.

T-RFLP Groups	Dendogram ID	Wells	Unique T-RFLP peaks	Organisms associated with T-RFLP
I	1 3 4 7	204-A 207-2A 207-B 209-C	176-179	ε- Proteobacteria isolated from a petroleum contaminated aquifer
II	2 9 18 19 22 25	206C A8-5 H11-1 H13-4 I12-2 K11-1	230	γ-Proteobacteria of known petroleum degrading organisms including *Pseudomonas*, *Acetobacter*, and *Azoarcus sp.*
III	5 6	207-C 207-D	167	Unidentifiable Eubacteria from soil samples
IV	8 10 11 12 13 15 16 23 24 26	209-D A8-6 A8-7 A8-8 F4-1 G3-2 G4-3 I5-5 J8-11 L11-1	204	α- and β-Proteobacteria including *Thiobacillus ferrodoxin* *Rhodopseudomonas sp.* and *Paracoccus sp.*

No relationship existed between 16S Eubacterial TRF patterns and any groundwater variable including TPH concentration, plume location, and Chem. PC1 (data not shown). Perhaps the best explanation for the lack of a coherent message in the correlations of TRF data with physical and chemical data is the fact that the samples were collected as a mixture from 15 to 20 foot well screens and represent an average of all vertical variations in each well. Current studies using vertical profiles will help reduce the inherently large variability in the data set and allow for a clearer representation of the bacterial response to the chemical parameters in the dunes aquifer.

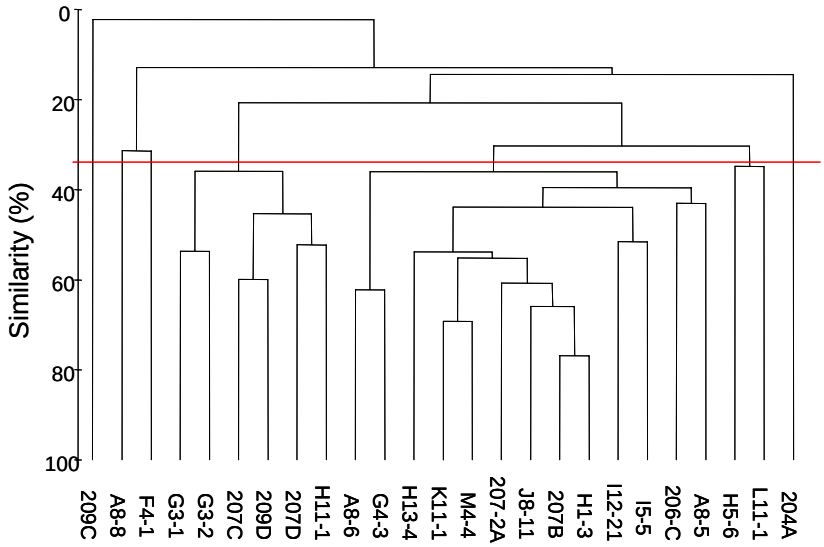
A companion study, currently in progress, using a vertical profile may better represent the effect of the groundwater chemistry on the bacterial community structure. In addition to the vertical profile, organisms of interest are currently being cloned and sequenced to provide better identification of TRF peaks. Culturing is also beginning, in attempt to isolate fermentative, iron reducing, and sulfate reducing bacteria capable of

using diluent as a sole carbon source. Culturing will provide causality to biological data and allow us to also enrich for organisms which may not previously be detected using TRF.

Archaea TRF

Bray-Curtis Cluster Analysis of Archaea TRF patterns across the site showed one major cluster and two minor clusters along with 4 outliers at 35% similarity (Fig. A10). This contrasts sharply with the Eubacteria cluster analysis (Fig. A8), even to the point of different outlier wells. The Archaea community structure exhibited no relationship to any physical or chemical parameter, TPH concentration, plume, and Chem. PC1 for instance, of the groundwater (data not shown). Most identifiable Archaea TRF peaks corresponded to database entries of methanogenic bacteria isolated from petroleum affected soils (data not shown).

Fig. A10. Dendrogram output from average linkage Bray-Curtis Cluster Analysis of the Archaea TRF demonstrating no significant clustering due to TPH concentration or plume location.



Sulfite reductase TRF

Primers specific for the gene that catalyzes the energy generating step in sulfate reduction, *dsr*AB, were optimized. Although there is geochemical evidence of sulfate reduction in the dunes aquifer, only one groundwater well (207C) gave a positive result for *dsr*AB. According to sensitivity studies, it is estimated that the SRB community is approximately 1:1000 of the total bacterial community in well 207C. Due to the lack of positive results, TRF specific for SRB were not performed using this groundwater DNA.

The one positive SRB well could be attributed to a lack of primer specificity for certain SRB populations, a low level of SRB in the groundwater samples, or the existence of a larger biofilm-associated population of SRB in the dunes aquifer. Primer sensitivity and specificity was evaluated prior to use and in the literature (4). The primers were able to detect to 1:1000 from community DNA and were found to be specific for the delta-*Proteobacteria*, *Nitrospira* division, *Thermodesulfobacterium* division, and the Gram-positive division of sulfate reducing organisms. Sulfate reduction has proven to be a dominant geochemical process in the aquifer, as detected by the presence of sulfide, and in fact recent work using these primers has confirmed the presence of SRB in injection wells at Guadalupe (data not shown). Current culture enrichment studies using soil are being conducted to confirm the presence, as well as identity, of SRB communities. In addition, biofilm studies are being designed to further evaluate the role of SRB in the dunes aquifer.

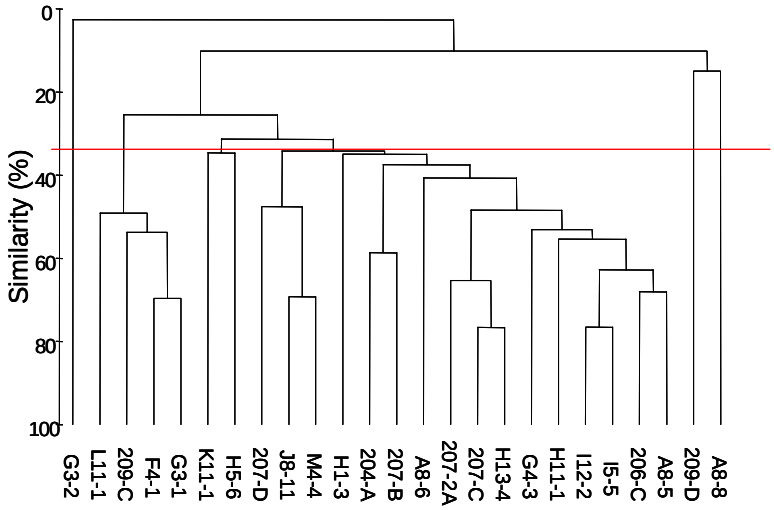
Actinobacteria TRF

Actinobacteria TRF were not performed, instead methanotroph and methanogen primers were evaluated to elucidate the relationship between these two genera.

Methanogens

Bray-Curtis Cluster Analysis of methanogen TRF patterns across the site showed one major cluster and two minor clusters along with 3 outliers at 35% similarity (Fig. A11). These clusters do not have any relationship to Archaea clustering (Fig. A10). Additional statistical analyses indicated that there is not a definite correlation between any of the physical and chemical parameters measured in the groundwater and the presence of methanogenic bacteria, indicating that different types of methanogens do not dominate under different groundwater conditions. DT plume data confirmed this finding when regression analysis failed to find any correlation to Chem. PC1 or any physical/chemical measurement (data not shown) Thus, plume-to-plume variation in both TRF and physical/chemical data was not responsible for the lack of correlations.

Fig. A11. Dendrogram output from average linkage Bray-Curtis Cluster Analysis of the methanogen TRF demonstrates no significant clustering due to TPH concentration or plume location.



Methanotrophs

The methanotroph TRF data produced 3 sample clusters at a similarity level of approximately 30%, using Cluster Analysis based on a Bray Curtis Similarity matrix (Fig. A12). Well 206-C was not included in a cluster (circled data points) defining it as an outlier. Samples with a similar distribution of different types of methane monooxygenase genes, presumably from different species of methanotrophic bacteria, clustered together in this analysis. PCA of the same data (Fig. A13) generally confirmed the clustering suggested above with the exception of well 209-D, illustrating the possibility of discrepancy between Euclidean and Bray-Curtis distance measurements. According to the PCA, well 209-D falls into the orange cluster, while based on the Bray-Curtis cluster analysis, well 209-D would cluster with the green group. The intermediate position of well 209-D between these two clusters is not surprising since this well occupies an intermediate position in the physical/chemical gradient measured in this study.

Fig. A12. Dendrogram output from average linkage Bray-Curtis Cluster Analysis of methanotroph TRF patterns across the site. Clusters defined at 30% similarity are labeled with colors at the bottom.

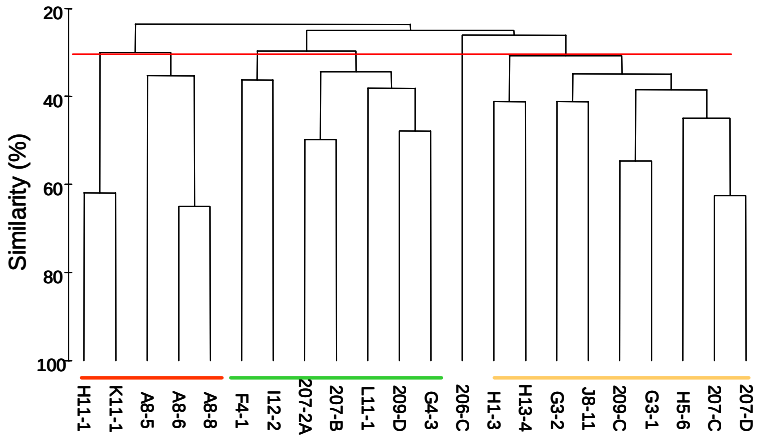
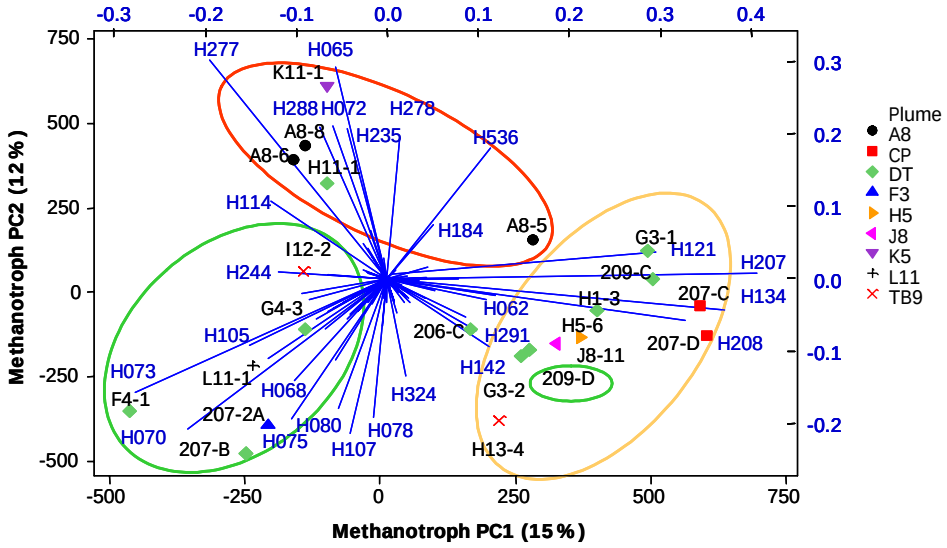


Fig. A13. PCA demonstrating the variation of the methanotroph TRF patterns across the site. Clusters defined in Fig. A12 are circled with the appropriate color.



These sample clusters were not based on the plume of origin, as samples from the largest plume (DT) were present in all clusters. However, the distribution of methane monooxygenase gene types, represented by TRF PC1, correlated ($R^2 < 0.54$) to groundwater chemistry in a regression analysis between methanotroph TRF PC1 and

Chem. PC1 (Fig. A14). Unfortunately, TRF PC1 only accounts for 15% of the variation in methanotroph TRF data, so this correlation may be an artifact. MDS analysis of the same data (Fig. A15) showed a similar trend with Chem. PC1, confirming that the distribution of methane monooxygenase genes varies with some physical/chemical variables in the groundwater.

Fig. A14. Regression of methanotroph TRF PC1 (Fig. A13) to Chem. PC1 for all wells.

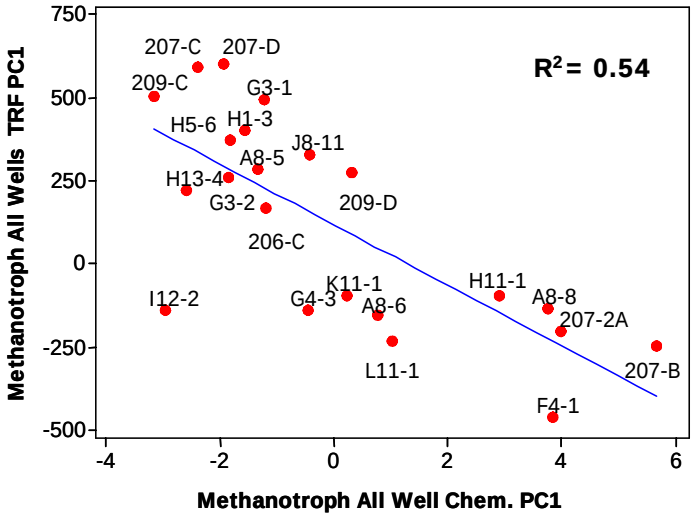
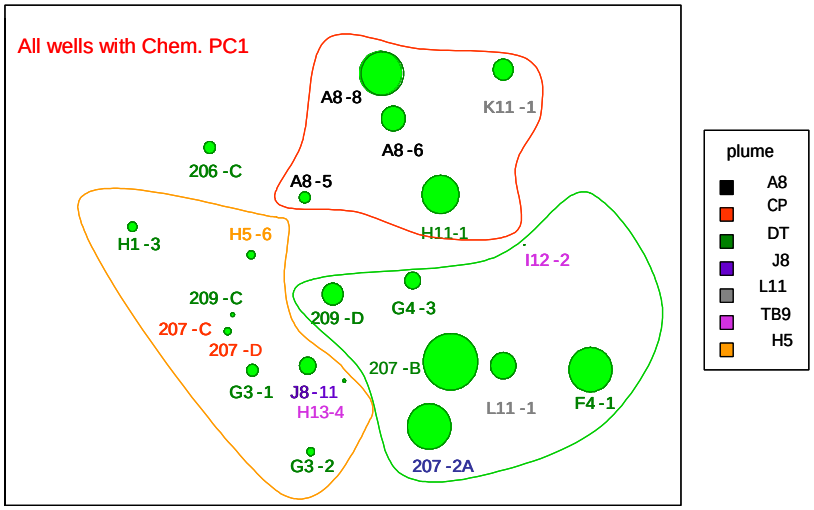


Fig. A15. MDS of methanotroph TRF data (stress value of 0.21) with Chem. PC1 indicated by the area of symbol bubbles. Note the decrease in average bubble size going from green to red to orange clusters. Plumes are indicated by the color of the text according to the legend on the right.



The correlation between methanotroph types and chemical variables was even clearer within the DT plume. Data from the DT plume was analyzed separately using PCA (Fig. A16) and MDS (Fig. A17) to examine relationships between the distribution of methane monooxygenase types (TRF data) and physical/chemical data. Because the substrates of methane monooxygenase are O_2 and CH_4 we might expect the concentration of these compounds to influence the distribution of methane monooxygenase types present. For example, some methane monooxygenase proteins may require higher substrate concentrations while others may work well at lower concentrations. TPH and Chem. PC1 will also reflect changes in O_2 and CH_4. All four of these variables gave good correlation to TRF PC1 for methanotrophs from the DT plume (Fig. A17). This was confirmed by MDS analysis of the same data (Fig. A18).

Fig. A16. PCA of methantroph TRF data in the DT plume.

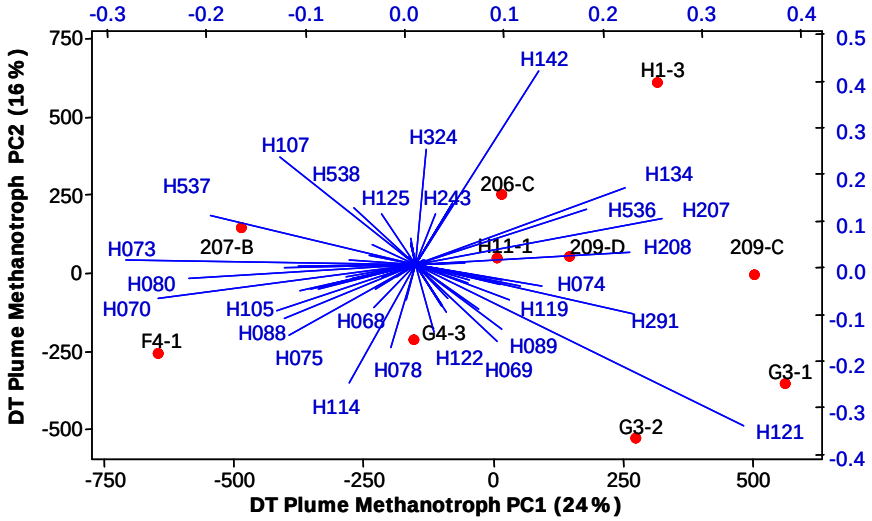


Fig A17. Regression of DT methanotroph TRF PC1 to four physical/chemical variables. Panel a, DT plume Chem. PC1; panel b, TPH log (mg/L); panel c, O_2 log (mg/L); panel d, CH_4 log (mg/L).

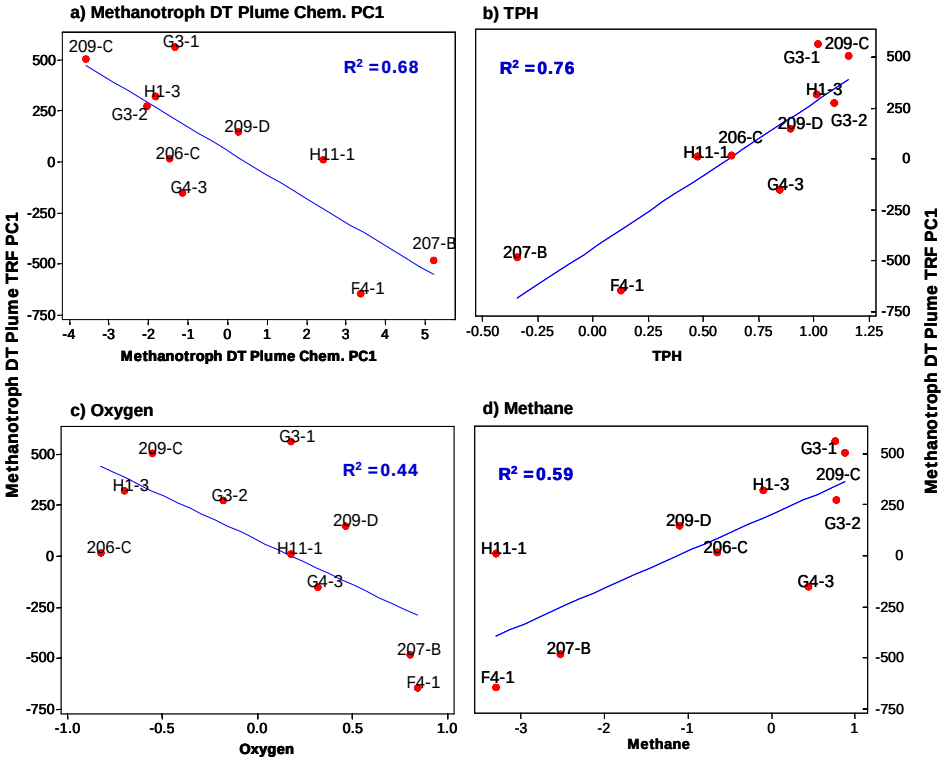
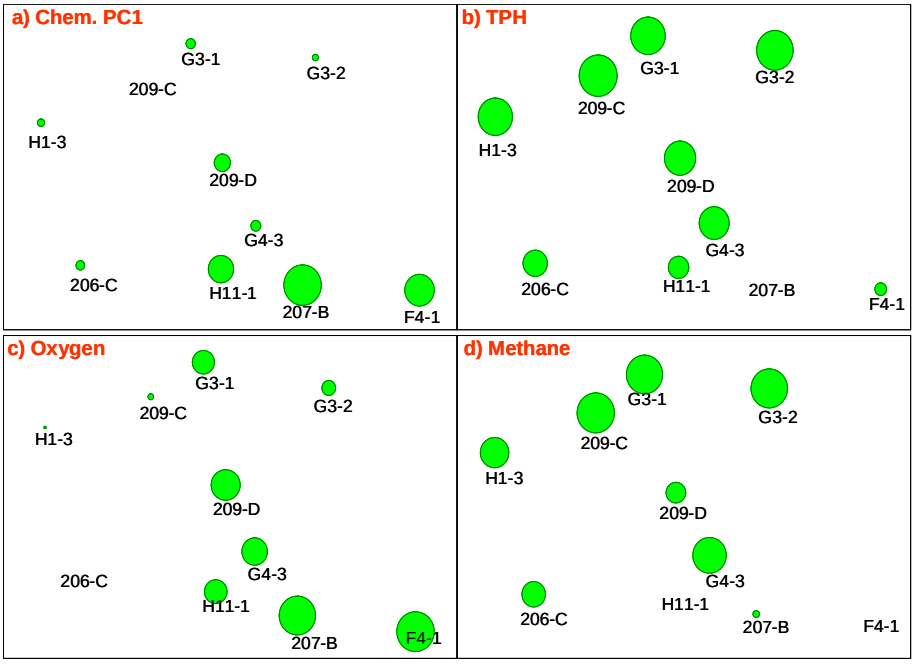


Fig. A18. MDS of methanotroph TRF data (stress level of 0.1) with physical/chemical variables indicated by the area of symbol bubbles. Panel a, DT plume Chem. PC1; panel b, TPH log (mg/L); panel c, O_2 log (mg/L); panel d, CH_4 log (mg/L). Note the change in bubble size following diagonal lines across the plots.



Quantification Tools

Quantification of specific bacteria using qPCR was deemed impossible with groundwater samples (see Microbial Tools at Guadalupe 2003/04 Report). In short, because the amount of material used to collect DNA could not be standardized due to turbidity, volume of water filtered and suspended solids, it was impossible to use the DNA to return a Cells/mL of groundwater result. In the future, qPCR applications will be limited to soil samples. Direct microscopic counts of cells suspended in groundwater produced data that was suspect for the same reasons. The number of bacterial cells/mL

of water varied in an apparently random fashion between 10^3 and 10^5 had no correlations to physical and chemical data (data not shown).

PCR Clone Libraries for Accurate Identification of Important TRF signals

As discussed in the Microbial Tools at Guadalupe 2003/04 Report, clones were not sequenced due to lack of personnel. Some DNA from the 2002/03 vertical profile NA study were cloned and sequenced and this data is being used to complete a manuscript due out in 2005. Because of the lack of correlations between 16S rRNA TRF data and physical/chemical data in the horizontal profile covered this year, the 2002/03 vertical profile seemed a more productive venue for sequence analysis.

Conclusions

- Because of the sampling design used in this study, we could not detect correlations between the taxonomic (16S rRNA) bacterial community structure and any physical/chemical variables in this study.
    - Previous studies (2002/03) showed good correlations with vertical sampling, which indicates that averaging a sample over a 10-20 foot screen destroyed correlations in the horizontal direction.
    - This was true for 16S rRNA Eubacteria taxonomy, 16S rRNA Archaea taxonomy and *mcr*A methanogen types.
    - In spite of the sampling design, the distribution of *pmo*A methanotroph types was correlated to groundwater chemistry.
- The distribution of *pmo*A gene types (methanotrophs) was shown to vary with groundwater chemistry. This indicates a very strong correlation in the type of gene required for growth since general taxonomic indicator genes (16S rRNA) did not show any correlations to groundwater chemistry. The reason for this is not clear.
    - The correlations could have been visible for methanotrophs if the driving variable was oxygen concentration. In a vertical profile of a down gradient plume, TPH, CO_2 and even methane should follow a bidirectional gradient, highest near the center of the profile. Conversely, oxygen, which diffuses from the surface, might only form a unidirectional gradient from the surface. Therefore, as samples are taken horizontally, further from the source, a larger methanotrophic zone should be part of the averaged vertical sampling volume.
- Only one well produced a positive result with the *dsr*AB gene primers.

- This could be because of low abundance for SRBs in the groundwater (perhaps these organisms are more often biofilm associated) or because these primers do not detect the type of SRBs present at Guadalupe.

- Quantitation of bacteria from groundwater was proven to be impossible due to sampling irregularities; most importantly, differing amounts of suspended solids.

- Bray-Curtis similarity and Multidimensional Scaling (MDS) were shown to confirm analyses based on Euclidian distance and PCA.
  - This validates the use of PCA for identifying trends and organisms in TRF data by the use of methods that do not rely on assumptions of normal distributions of data.

Outcomes

1) Two MS theses to be completed in 2005.
2) One manuscript based on a combination of the current data set with the 2002/03 vertical profile data set to be completed in 2005.

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