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I am submitting herewith a thesis written by Anita Eva Biernacki entitled "Identifying the Signature of the Natural Attenuation of MTBE in Groundwater using Molecular Methods and 'Bug Traps.'" I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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IDENTIFYING THE SIGNATURE OF THE NATURAL
ATTENUATION OF MTBE IN GROUNDWATER USING
MOLECULAR METHODS AND “BUG TRAPS”

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Anita Eva Biernacki
August 2004

I dedicate this to my family and friends for their unconditional love and support.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. David C. White, my major professor, for his guidance and encouragement. I would like to thank my committee members, Dr. Susan Pfiffner and Dr. Mark Radosevich for serving on my committee and their support and assistance. Also I would like to thank Aaron Peacock for allowing me the opportunity to work on this project as well as his mentorship. The International Petroleum Environmental Consortium provided the financial support for this project. Many thanks to Dr. Kerry Sublette for providing the Bio-Sep® technology and his expertise. Additionally I would like to thank everyone at Microbial Insights, especially Greg Davis and Dora Ogles, and everyone at the Center for Biomarker Analysis, in particular Julia Gouffon, Janet Chang, Amanda Smithgall, James Cantu, and Renee Thomas for their assistance, encouragement, and friendship.

Abstract

Natural attenuation through intrinsic bioremediation is the risk-based management approach commonly used for gasoline (BTEX) contamination sites. This approach has not yet been utilized for the fuel oxygenate methyl tertiary-butyl ether (MTBE). MTBE is more resistant to biodegradation than BTEX. MTBE is more abundant than benzene in oxygenated gasoline, has a greater water solubility than BTEX, and sorbs weakly to soil. These properties complicate developing a risk based management option to be implemented as easily as for BTEX. The purpose of this project is to contribute to a growing database containing information on MTBE contaminated sites nationwide with a variety of environmental conditions. Characterizing a highly monitored MTBE biodegradation site will determine the possible microbial “signature” of the natural attenuation of MTBE. To correlate microbial community shifts with changes in MTBE product patterns, “Bug traps” consisting of Ambersorb® Bio-Sep® beads and regular Bio-Sep® beads were deployed into the polluted groundwater and into control wells at a highly monitored service station to concentrate the microorganisms for analysis. They were retrieved after 30 and 60 days of deployment. Phospholipid fatty acid and nucleic acid analysis (denaturing gradient electrophoresis (DGGE) and quantitative PCR) were used to analyze the microbial community in the groundwater.

The samples from the plume showed a difference from the control samples. Gram-negative communities in the samples closest to the plume did not show a

lack of limiting nutrients (i.e. carbon) as did other wells further from the plume and were in log growth phase. Also the Gram-negative community in the highest contaminated well showed the highest adaptation to the environmentally stressful conditions through decreased membrane permeability. The site showed microbial and geochemical evidence for methanogenesis, which may have been responsible for the observed degradation of MTBE and BTEX. Sulfate-reduction was also evident throughout the site and may also have been a responsible process for the observed biodegradation. Iron-reduction (*Pelobacter*, *Geobacter*) was only evident in wells within the plume and downgradient of the plume and may have played a role in degradation. In addition notable organisms that were identified in other studies of MTBE biodegradation included *Methylosinus trichosporium OB3b*, and environmental clones associated to the *Flexibacter-Cytophaga-Bacteroides* phylum associated with hydrocarbon intrinsic bioremediation. The results of this study provided evidence for anaerobic biodegradation of MTBE.

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1. Literature Review and Introduction

1.1 Introduction

The purpose of this project is to contribute to a growing database containing information on MTBE (methyl tertiary-butyl ether) contaminated sites nationwide with a variety of environmental conditions. Characterizing a highly monitored MTBE biodegradation site will determine the possible microbial “signature” of the natural attenuation of MTBE. The Science Advisory Board (2000) of the U.S. EPA has stated the importance of an MTBE database to parallel the Benzene, Toluene, Ethylene, Xylene (BTEX) database that has already been developed. Reference to the database may provide insight as to whether or not intrinsic bioremediation is occurring and allow for risk-based management to be implemented.

To correlate microbial community shifts with changes in MTBE product patterns, Bio-Sep® “bug traps” were deployed into the monitoring wells of polluted and non-polluted groundwater at a gasoline service station to concentrate microorganisms for analysis. Phospholipid fatty acid and nucleic acid analysis (denaturing gradient electrophoresis (DGGE) and quantitative PCR) were used to analyze the microbial community in the groundwater. The shifts in the microbial community at the site can provide a direct measure of MTBE biodegradation while the site geochemistry will give an indirect measure of potential MTBE biodegradation.

1.2 Background on MTBE

Over the years, many gasoline additives have been developed in order to increase gasoline efficiency. In the 1970's, alcohols and ethers represented a new class of octane-boosting additives designed to replace the previous high emissions lead compounds. These new additives lowered air-polluting emissions by adding oxygen to the gasoline. MTBE was first used in 1979 as a fuel oxygenate, the 1990 Clean Air Act made MTBE more common throughout the country, and by 1996 MTBE became almost universal in California (Davidson, 2000). This increased usage along with high occurrences of leaking underground storage tanks and recreational watercraft operation has led to MTBE contamination of surface and ground waters. It has been estimated that 250,000 of the approximately 385,000 confirmed leaking underground storage tank releases in the United States involve MTBE (National Groundwater Association, 2000). MTBE has a low Henry's law constant indicating that it partitions into water rather than air. MTBE is very water soluble, moderately volatile, sorbs weakly to the soil, and can make its way into the groundwater where it poses a great threat to safety. In urban aquifers MTBE has been found to be the second most common contaminant (Caprino, 1998).

MTBE's characteristics cause it to be much more persistent in the groundwater than other gasoline constituents such as BTEX. MTBE moves with the gasoline until it reaches a body of water through runoff or until it makes its way through the soil into the groundwater. Gasoline is very insoluble in water

and less dense than water. MTBE on the other hand has a high solubility of 50,000mg/L in water (EPA, 1998). This causes a separation of the MTBE from the gasoline into the water. At spill sites, MTBE migrates further than BTEX due to its mobility and inability to biodegrade as easily. MTBE has been classified by the U.S. Environmental Protection Agency as a possible human carcinogen (University of California, 1998), thus its presence and biodegradation in the environment should be monitored closely and studied.

1.3 Remediation of MTBE

The fate of MTBE in groundwater is influenced by dispersion, dilution, and degradation. Dispersion happens with the expansion of the contaminant both in the direction of groundwater flow and perpendicular to it. Dilution involves the mixing of MTBE-contaminated water with uncontaminated water. Dispersion and dilution reduce the concentration of MTBE, but overall the total amount of MTBE *in situ* is the same. Degradation breaks down the MTBE into by-products such as tertiary butyl alcohol (TBA) and thus decreases both the concentration and total mass in the environment. TBA is also a component of gasoline and degrades at a slower rate than MTBE. Surface and groundwater contamination with MTBE can be treated either *ex situ* through pumping and treating the water or *in situ* by treating the water in the environment. The option chosen for remediation is dependent on the risk of the site. Different options would be chosen based on whether or not a site needs active control, has a somewhat

longer time frame for remediation, or is low risk with an even longer time frame possible for remediation.

1.3.1 Ex Situ Remediation

Ex situ remediation involves removal of the contaminated water through pumping and treating it with the use of engineered reactors and then replacing the water back into the environment. Engineered remediation processes employed for MTBE cleanup are adsorption systems, air-stripping systems, and advanced oxidation processes (The California MTBE Research Partnership, 2000).

In adsorption systems, the MTBE is adsorbed onto a highly adsorptive surface, usually Granular Activated Carbon (GAC), a charcoal-like material with high surface area and a large capacity for adsorbing organic compounds. MTBE's characteristics of low sorption and high water solubility do not allow it to be adsorbed efficiently (The California MTBE Research Partnership, 2000). Natural organic matter and other synthetic organic chemicals compete with MTBE for adsorption. The GAC can be 99% efficient at removing MTBE, but after a very short time, the GAC becomes saturated with MTBE and no more treatment can occur. Thus the GAC would have to be replaced or regenerated through thermal oxidation or steam cleaning quite often, thereby greatly increasing operational costs. This is why GACs are usually used for removing lower MTBE concentrations.

Ambersorb® resin, a commercially fabricated synthetic sorbent manufactured by Rohm and Haas, (Philadelphia, PA) is currently the industry's most powerful resin for MTBE removal (Malley et al., 1993), but it is expensive and is not easily regenerated. Data show that Ambersorb® is not affected by pH, temperature, oxidants, and natural organic matter, or TBA.

Air stripping systems operate by trying to transfer the MTBE into the gas phase. This is usually done through diffused bubble aeration by bubbling air through the water, or packed tower aeration by passing it through a thin film of water. This method is efficient for gasoline components that have a higher volatility than MTBE, but has a reported removal of 60% of MTBE when a large amount of air was used (Malley et al., 1993). Once the MTBE is moved from the water phase into the air phase, the air must be treated before it can be released. It is usually treated by thermal oxidation, which combusts the MTBE, or by sorption of it in the gas phase onto GAC. This method for treating MTBE contamination is also expensive and complex.

Other types of methods used for *ex situ* removal of MTBE are advanced oxidation processes (AOP). These processes chemically or physically oxidize MTBE to CO₂ and H₂O through the use of hydrogen peroxide, ultraviolet light, or ozone oxidants. Research has shown the possibility of 99% removal of MTBE from the water through advanced oxidation processes (Kinner, 2001). High energy electron beam irradiation and sonication/hydrodynamic cavitation are two emerging AOPs that are currently under study for their ability to remove MTBE

from drinking water (Stocking et al., 2000). The challenge of all AOPs involves the formation and fate of oxidation by-products such as TBA and tertiary-butyl formate (TBF), as well as non-selective radical oxidation. Ongoing research on these methods is helping to further this application as well as helping to reduce the expensive cost of operation.

1.3.2 *In Situ Remediation*

In situ remediation involves treating the MTBE in the environment. It does not require the water be removed from the environment, as is the case in *ex situ* remediation. Capturing all of the contaminated groundwater for *ex situ* treatment may prove difficult, since plume migration is not always known and complete plume contaminant /capture is difficult to ascertain. *In situ* remediation occurs through the biodegrading ability of certain microorganisms that live in the environment. Biodegradation is the breakdown of toxic chemicals into either less toxic or nontoxic compounds.

Natural attenuation involves numerous *in situ* processes such as biodegradation, dilution, dispersion, volatilization, irreversible sorption, transformation, radioactive decay, chemical or biological stabilization, and destruction of contaminants in soils and groundwater (ASTM, 1998). These processes do not involve human intervention and they decrease the mass, toxicity, mobility, volume, and/or concentration of the contaminants (EPA OSWER, 1998). Studies have shown that natural attenuation of MTBE is

possible in groundwater (Borden 2000: Butler et al. 2000), Landmeyer (2000), and Wilson (2000)), but the extent of attenuation differed greatly among the sites. Other studies, such as Hunter (2000) and Weaver (2000), showed a lack of natural attenuation. This may have been due to the high groundwater velocity. Schirmer et al. (2000) carried out a study where MTBE was injected into the Borden Aquifer. It was hypothesized that the decrease of MTBE to 3% of the initial mass over eight years was due to biodegradation. Borden (2000) did an extensive characterization of a shallow coastal plain aquifer. Biodegradation rates were highest at the source, but existing models were inadequate for predicting natural attenuation and could have underestimated the plume size over time. Natural attenuation can be an effective and inexpensive remediation technique at petroleum release sites mainly due to intrinsic biodegradation, but it is highly dependent on individual site characteristics and may not be possible at some sites. At sites where it is possible, however, long term monitoring is required to show that the contaminant concentration is decreasing at a rapid enough rate to prevent contact with an environmental receptor. Additional research on these sites is needed to help uncover the factors that influence natural attenuation.

Enhanced *in situ* bioremediation involves processes with the aid of human intervention such as bioaugmentation and biostimulation. Bioaugmentation involves the introduction of microbial cultures into the environment. Scow et al. (2000) introduced PM1, an MTBE degrading strain, into the environment and

showed through microcosm studies that the MTBE biodegradation was significantly higher in these spiked samples than in the microcosms with no inoculation of PM1. Salanitro (2000) also observed more rapid degradation of MTBE when microcosms were inoculated with a MC-100 strain. Current studies of bioaugmentation for MTBE contamination have shown promise, but more research is needed. Another process under study is that of biostimulation. Mackay et al. (1999) stimulated the native aerobic MTBE-degrading organisms present at an MTBE plume at Vandenberg Air Force Base, CA through the addition of dissolved oxygen and observed *in situ* biodegradation of the MTBE. Additional research is needed to investigate the many factors involved with these methods. In particular, the metabolic pathways for MTBE degradation need to be elucidated so more effective treatment strategies can be developed.

1.3.3 MTBE Biodegradation

There is limited information on the biological fate of gasoline oxygenates. Early studies of MTBE in biosludges, sediments, and groundwater did not give any evidence of biodegradability (Fujiwara et al., 1984; Jensen and Arvin, 1990). The ether bond and the tertiary carbon atom in MTBE hinder efficient biodegradation (Suflita and Mormile, 1993). Although a lot of energy is needed to break the ether bond, thermodynamically MTBE degradation is possible (Finneran and Lovley, 2001). MTBE biodegradation pathways, though, are still

not clear. Figure 1-1 represents a general pathway based on Church et al. (2000) and Steffan et al. (1997). MTBE can be used as an energy source when the microorganisms are able to remove electrons and hydrogen ions and use them in the electron transport chain. Under aerobic conditions, the terminal electron acceptor (TEA) is oxygen, this process produces the most energy. Under anaerobic conditions the TEA can be many compounds (e.g. nitrate, sulfate, Fe(III), or methane ($\text{CO}_3^{-2}/\text{H}_2$, etc.)) To date, several different degradation scenarios including aerobic, anaerobic, mixed consortia, pure culture, and cometabolic processes have been demonstrated.

Aerobic and anaerobic bacterial consortia as well as mono cultures have been found to degrade MTBE (Garnier, 2000). An aerobic mixed bacterial culture (BC-1) represented the first report of aerobic degradation of MTBE (Salanitro et al. 1994). BC-1 was developed from microorganisms in a chemical plant bioreactor sludge that degraded MTBE to CO_2 (40%) and cell mass (40%). Other enrichment cultures have been found to degrade MTBE including Cowan and Park's MTBE-degrading enrichment culture (1996) from a petroleum refinery's wastewater treatment plant. Landmeyer et al. (2001) used a biofilm from an MTBE-contaminated site to demonstrate MTBE biodegradation in liquid culture. Bradley et al. (2001) showed *in situ* degradation occurring in sediments from stream and lakebeds. Church et al. (1999) and Wilson et al. (2002) showed subsurface sediments had a great potential for MTBE biodegradation. Many pure cultures capable of MTBE biodegradation have been isolated. Scow et al.

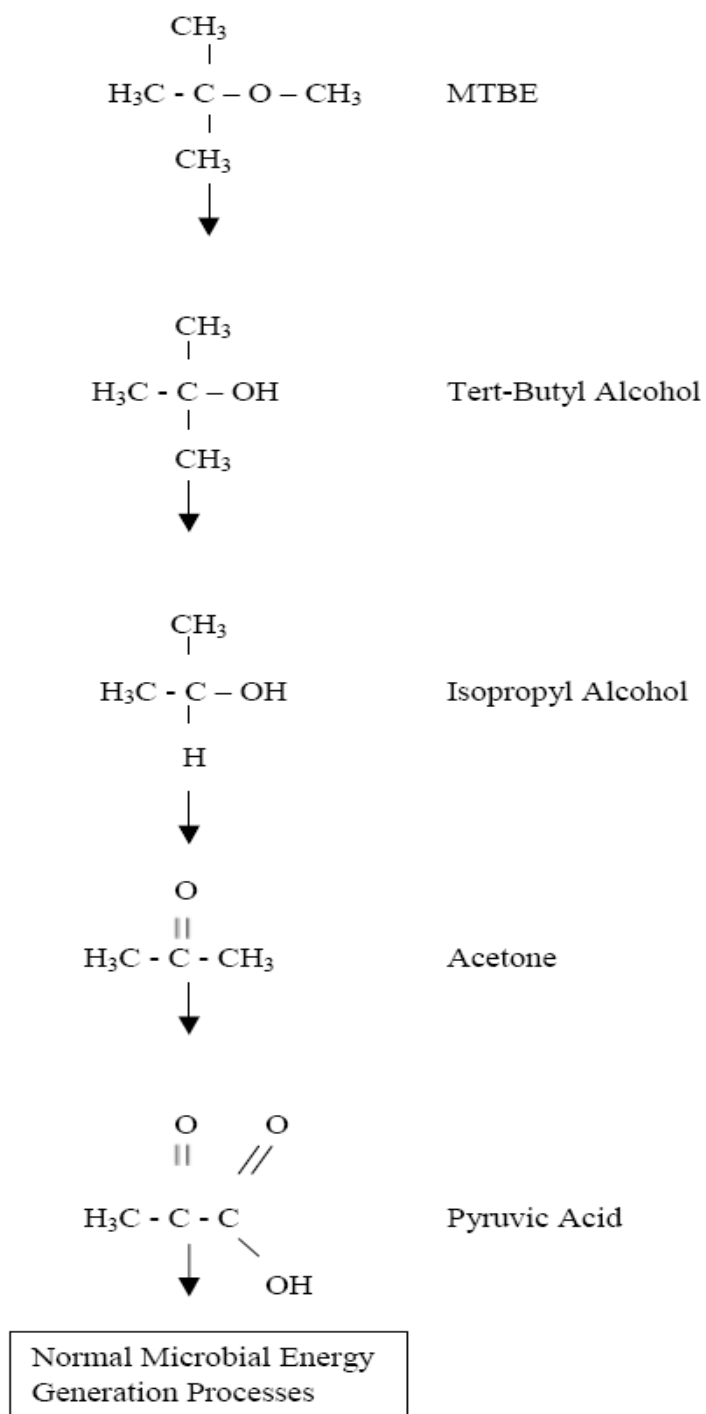


Figure 1-1 Generalized pathway of MTBE biodegradation based on Church et al., (2000) and Steffan et al., (1997).

(2000) isolated PM-1, an MTBE degrading strain. Mo et al. (1995) isolated 15 bacterial strains that used MTBE as their sole carbon source. Hanson et al. (1999), Hernandez-Perez et al., (2001), Francois et al., (2002), and Hatzinger et al., (2001) all isolated MTBE degrading isolates.

The possibility for anaerobic MTBE biodegradation is important because this is the predominant redox state of contaminant plumes, often these anaerobic conditions arise because of oxygen depletion during aerobic biodegradation. Anaerobic MTBE degradation has been doubted and several researchers failed to find supporting evidence for it, yet others have observed anaerobic degradation of MTBE. Suflita and Mormile (1993) and Kropp et al., (2000) found evidence for MTBE degradation under methanogenic conditions, Mormile et al., (1994) under nitrate and sulfate reducing conditions, and Landmeyer et al., (1998) with less than 3% mineralization under iron-reducing conditions did not find anaerobic MTBE biodegradation. It has been observed that increased carbon branching, as seen with MTBE, decreases its ability to be anaerobically degraded. Finneran and Lovley (2001) have shown anaerobic MTBE degradation in one replicate of a microcosm that contained ferric oxide and humic substances. Bradley et al., (2001b) showed anaerobic MTBE biodegradation by microorganisms from streambed sediments with $23 \pm 5\%$ to $75 \pm 14\%$ of MTBE degraded under nitrate-reducing conditions. Borden et al., (1997) showed MTBE degradation under a mix of aerobic and nitrate-reducing conditions in a shallow coastal plain aquifer. Also the Borden Test Site had a

reduction of MTBE from 1000 ug/L to <200 ug/L over a period of 7 years (Borden et al., 2000). Complete MTBE and TAME (tert-amyl methyl ether) degradation by enrichments from contaminated estuarine sediments under sulfate-reducing conditions was shown by Somsamak et al., (2001). Yeh and Novak, (1994) showed that one of three methanogenic replicates of microcosms containing soil and water contaminated with fuels displayed MTBE biodegradation when furnished starch, inorganic nutrients, cysteine sulfide, and sodium molybdate (to inhibit sulfate reduction). Different cultures and conditions induce varying MTBE biodegradation rates.

Microbial cometabolism of MTBE has also been observed. Several aerobic bacteria and fungi have been observed to cometabolically degrade MTBE. Several primary substrates, such as alkanes, aromatics, and cyclic compounds, have been found to be cometabolically degraded along with MTBE. Most MTBE cometabolism occurs with aerobic bacteria growing on short chain alkanes (Hyman, 2000). Simple branched alkanes can represent 10% v/v of gasoline and it has been hypothesized (Hyman 2000) that microorganisms that can break down these structural analogs of MTBE may concurrently exhibit cometabolic MTBE degradation. For example, bacteria that degrade butane can metabolize the petroleum hydrocarbons and the resulting cometabolic enzymes that are produced, such as butane monooxygenase, are able to destroy MTBE without supplying energy to the bacteria (Morse et al., 2002). Stringfellow (2000) has shown *in situ* MTBE biodegradation in the presence of iso-pentane as the

primary substrate in laboratory scale bioreactors and this potential is being tested in the field. Further study is needed to elucidate the pathway for cometabolic MTBE biodegradation.

1.4 Why identify the Signature of the Natural Attenuation of MTBE Biodegradation?

Natural attenuation is a good solution for many low-risk MTBE-contaminated sites. A defensible risk-based management approach, such as natural attenuation, would benefit the petroleum industry, and limited resources could be used more efficiently to remediate sites that pose more serious threats to human health. Biodegradation accountably reduces the contaminant mass. To implement risk-based management of MTBE, it is necessary that the MTBE naturally attenuate at a rapid rate to prevent contact of the MTBE plume with an environmental receptor. Thus, to make a convincing case for this approach with contaminated groundwater, it is necessary to establish that a contaminant naturally biodegrades at a sufficient rate to prevent plume migration.

There is currently an insufficient scientific basis for a risk-based management approach, but there is evidence that suggests that MTBE is susceptible to biodegradation under certain conditions. The Science Advisory Board (2000) of the U.S. EPA has stated the importance and need of establishing a database describing the signature of the natural attenuation of MTBE. The footprint or signature can be investigated through monitoring multiple representative and

highly characterized MTBE biodegradation sites. Knowing the signature of MTBE biodegradation would help to identify eligible sites for implementation of bioremediation through natural attenuation.

1.5 Site Description

Retail British Petroleum sites were surveyed (Kolhatkar et al., 2000) for evidence of MTBE natural attenuation and a geochemical “footprint” of MTBE degrading potential. A site was chosen for this study that had groundwater monitoring wells down-gradient from the plume, within the plume, and distant and parallel from the plume and also exhibited a decrease in the contaminant concentration from initial site characterization in the years previous to this study. Using the approach of Buscheck and Alcantar (1995), first order biodegradation rate constants were estimated for MTBE, TBA, and benzene and suggested that degradation was occurring in the subsurface under anaerobic conditions controlling the rates of migration of MTBE and TBA. The geochemical data to date indicated that a plateau in the degradation rate had been reached with steady state degradation of the MTBE now occurring.

1.6 Research Objectives

This study sought to identify the signature of MTBE biodegradation within the microbial ecology of the contaminated aquifer groundwater, which would give support of the intrinsic bioremediation occurring. Geochemistry has been used

primarily as an indirect measure of intrinsic bioremediation. Geochemistry or contaminant chemistry measurements at a site have been considered indirect proof of degradation, since they do not definitively show what is happening to the contaminant to make it disappear. It could simply be diluted in concentration but not in overall mass. One objective of this study is to relate the signature of the microbial ecology of each sampled well to any evident MTBE degradative activity within each monitoring well through its geochemistry and a measured decrease in contaminant concentration. The correlation between the geochemical parameters and the microbial community at a site will give stronger evidence for bioremediation than either measurement alone. The methods employed to determine the microbial community consisted of phospholipid fatty acid analysis, nucleic acid analysis via DGGE, and quantitative PCR.

Currently, the methods used for monitoring the microbial community at a site differ depending on the agency or company responsible for monitoring, and it is not a uniform process between sites. A second objective of this study is to help develop an efficient, accurate, and cost-effective means of monitoring intrinsic MTBE bioremediation in groundwater. Bio-Sep® beads were the type of monitoring system that was used to determine the first objective of identifying the microbial community. In this study retrievable Bio-Sep® bead traps were placed into the monitoring groundwater wells at the site. A total of eight wells were sampled at the site, including two control wells upstream of the MTBE plume. Quadruplicate Bio-Sep® traps (with 2 replicates containing Amborsorb® and 2

containing regular control Bio-Sep® beads) were sampled from each well. The traps were deployed simultaneously for 30 or 60 days to provide a time-integrated picture of the groundwater microbial community. The two incubation times were chosen to examine possible changes over time. Ambersorb® incorporated Bio-Sep® beads were also evaluated for their similarities and differences from original Bio-Sep® beads.

Discerning the microbial signature of MTBE degradation at this site will contribute to a growing database of information for the development of an integrated model of natural attenuation of MTBE-contaminated sites. Such a database of information has already been developed for BTEX contamination, and it provides the scientific basis for the Risk Based Corrective Action (RBCA) approach. A similar approach is needed for MTBE contamination. The RBCA approach acknowledges that different contaminated sites have varying levels of risk associated with them. This approach takes into consideration different remedial actions for the various types of risk present. Geochemical, phenotypic, and nucleic acid analyses are the methods used in this study that will help elucidate the signature of the natural attenuation of MTBE.

1.7 Bio-Sep® Beads

Monitoring devices called “bug traps” consisting of Bio-Sep® beads were used to collect and determine the microbial community. A description of how the

Bio-Sep® beads were assembled into “bug traps” is in the materials and methods section. The Bio-Sep® beads that compose the “bug trap” consist of 2-3mm spherical beads made from 25% polymer (Nomex) and 75% powdered activated carbon (Figures 1-2 and 1-3). They were developed by DuPont® in 1996. Co-inventors were Dr. Tim Bair and Dr. Carl Camp. In 1998 the patent from Dupont® was awarded to the University of Tulsa. Dr. Kerry Sublette is the inventor or co-inventor of Bio-Sep® adsorbent biocatalyst porous beads (US patent 6,471,864). The median pore diameter of a bead is 1.9 μm , but pore sizes can be greater than 20 μm within the beads. This results in an internal adsorptive capacity greater than 600 m^2/g . The beads outer surface is an ultra filtration-like membrane with pores ranging from 1-10 μm . Bacteria are able to enter through this outside membrane and are trapped in the porous matrix inside of the bead. The bacteria are still able to move within the bead or to the outside environment, but tend to stay in the most favorable environment. The adsorptive surface of the bead, as well as the adsorption of a carbon energy source onto it, makes it a suitable immobilization matrix for bacteria. Once the bacteria become entrapped, it is more difficult for them to leave and they multiply until much of the internal volume is packed with high bacterial biomass density. As the bacteria degrade the carbon source within the bead, they regenerate the adsorptive surface and allow for more carbon to be adsorbed onto the bead.

The adsorptive surface pulls hydrocarbons, and to some extent MTBE, into the beads from the groundwater. Thus, the bacteria’s preference for the internal



Figure 1-2 Cross section of Bio-Sep® bead.



Figure 1-3 Bacterial colonization of the bead interior.

bead matrix, as observed in bioreactors, makes the Bio-Sep® bead a very suitable “bug trap”. The activated carbon also protects the bacteria from any toxic contaminants that may be present and allows for contaminants to be retained in the bead in low concentrations long enough to be degraded by the bacteria. Thus the Bio-Sep® beads serve as a great tool for sampling the microbial community over time. The microbial ecology of a contaminated site is better represented by *in situ* biofilms than planktonic organisms in sampled groundwater. Traditional sampling of subsurface bacteria requires coring aquifer sediments and extracting viable microorganisms or biomarkers (lipids, DNA, etc.). Extraction efficiency of these compounds varies with the geochemistry of the sediments. This study uses a novel approach of sampling with Bio-Sep® technology. Research has shown that Bio-Sep® technology provides a suitable base for the development of biofilms representative of those in aquifer sediments and can be rapidly and efficiently collected using Bio-Sep® “bug traps”. Bio-Sep® beads have been used in bioreactors to treat contaminated groundwater and wastewater and have the capacity to accumulate up to 10 times more bacteria in bioreactors. It has been shown that bacteria cultured in the presence of the beads become immobilized in the Bio-Sep® (Sublette et al., 1996, Conner et al., 2000).

Ambersorb® was incorporated into a portion of the Bio-Sep® beads used in this study to test its highly adsorptive characteristics. Ambersorb® resin is thought to be much more adsorptive than powdered activated carbon and thus

could adsorb more of the carbon source or MTBE from the groundwater. This may attract the microbial community that is responsible for degrading the MTBE.

1.8 Molecular Analysis

1.8.1 PLFA Analysis

The microbial community present at a site of contamination largely dictates the capacity for biodegradation, especially natural attenuation, of a contaminant. Understanding the changes in the microbial community that occur when a contaminant is introduced to an environment is very important when assessing a risk-based management program. Recently, many new advances and improvements in molecular technology have allowed the opportunity to more accurately study the microbial community in the natural environment. Phospholipid fatty acid (PLFA) analysis is one such method that supplies a quantitative measure of viable biomass, community composition, and nutritional/physiological status of the *in situ* microbial community (White and Ringelberg, 1996; Frostegard et al., 1996; Stephen et al., 1999). PLFA analysis has been widely used in the characterization of microbial communities at many contaminated sites and has shown changes in bacterial and fungal biomass and composition. One example is that of Morse et al., (2002) which involved PLFA analysis and 16S rDNA profiling by DGGE to characterize the microbial community of an MTBE oxygen injection site.

Since the degradation of MTBE in the groundwater is dependent greatly on the microbial community of the groundwater, determining the microbial community in non-contaminated areas (e.g., upgradient of the contaminant plume) and in contaminated areas will help elucidate the signature of MTBE biodegradation and was the focus of this project. The microbial community biomass, metabolic status, and community structure was investigated through phospholipid fatty acid (PLFA) analysis (White et al., 1997; Stephen et al., 1999.)

Culture based techniques for whole microbial community composition studies and direct microscopic counts have been consistently shown to be inaccurate, with direct counts representing only 0.1 to 10% of the existing community (Tunlid and White, 1992; White et al., 1979). Signature lipid biomarker analysis, also known as PLFA, is a method that estimates the total microbial community without the difficulty of having to isolate viable organisms from the environment. Lipids are cellular components of all cells and have rapid turnover, which makes them biomarkers. The total amount of phospholipid ester linked fatty acids (PLFA) present give a quantitative measure of the viable biomass because all living bacteria have an intact membrane containing PLFA, where the phosphate head group gets hydrolyzed within minutes to hours after cell death (White et al., 1979).

The microbial community structure is also determined through signature lipid biomarker analysis because certain groups of organisms produce unusual lipids (White et al., 1996). Detection of these different lipids by gas

chromatography/mass spectrometry enables for either very general or modestly specific identification of microbes, depending on the types of PLFA present. Relatedness between different microbial communities can also be quantitatively measured through hierarchical cluster analysis of PLFA patterns of total microbial communities (White and Ringelberg, 1997). The PLFA patterns can be used to show changes in the community composition, since specific PLFA factors can be associated with certain groupings of the microbial community. Examples of PLFA that are used as biomarkers of community composition are listed in Table 1-1. Since PLFAs are products of biosynthetic pathways they indicate the phenotypic response of microorganisms to different conditions. Exposure to environmental toxicants has shown increases in the proportion of trans monoenoic PLFA when compared to its cis isomer, thus decreasing membrane fluidity (Pinkart et al. 1996; Sekkima et al., 1995). Stationary phase or shortage of oxygen can result in the increase of cyclopropane PLFA (cy17:0 and cy19:0) over their monoenoic precursors (16:1w7c and 18:1w7c) (Kieft et al., 1997).

1.8.2 Nucleic Acid Analysis

Investigating the changes in the microbial community that occur due to a contaminant may help to elucidate the process of contaminant biodegradation, in this case MTBE. Nucleic acid analysis has become more widespread among scientists interested in assessing the diversity of microbial communities and their responses to environmental change. Nucleic acid analysis effectively

Table 1-1 Selected PLFA

PLFA	Functional Group	Reference
i15:0, a15:0, i17:0, a17:0	Gram-positive bacteria	Ringelberg et al., 1997
i15:0, a15:0, i16:0, i17:0, a17:0	Gram-positive bacteria	Frostegard & Baath, 1996
i15:0, a15:0, i16:0, i17:0	Gram-positive bacteria	Bardgett et al., 1996
10me16, i15:0, a15:0, i16:0, i17:0, a17:0	Gram-positive bacteria	Zogg et al., 1997
i15:0, i16:0, 10me16:0	Gram-positive bacteria	Zak et al., 1996
16:1w7c, 18:1wc, cy17:0, cy19:0	Gram-negative bacteria	Ringelberg et al., 1997
16:1w9c, 16:1w7t, cy17:0, 18:0w7, cy19:0	Gram-negative bacteria	Frostegard & Baath, 1996
cy17:0, 18:1w7, cy19:0	Gram-negative bacteria	Bardgett et al., 1996
16:1w5c, 16:1w7c, 16:1w7t, 16:1w9c	Gram-negative bacteria	Zogg et al., 1997
18:1w5c, 18:1w7c, cy17:0, cy19:0		
16:1w7c, cy17:0, 18:1w7c, 18:1w7t, cy19:0	Gram-negative bacteria	Zak et al., 1996
10me18:0	Actinomycetes	Ringelberg et al., 1997
Mid-chain branched fatty acids	Actinomycetes	White et al., 1997
10me16:0	Sulfate-reducers (Desulobacter)	Ringelberg et al., 1997
i17:1w7c, i15:1w7c, i19:1w7c	Sulfate-reducers (Desulfovibrio)	White et al., 1997
i17:1w7c	Sulfate-reducers (Desulfovibrio)	Ringelberg et al., 1997
16:1w8c, 18:1w8c	Methane oxidizers	Ringelberg et al., 1997
18:2w6, 18:3w6, 18:3w3	Fungi	Ringelberg et al., 1997
18:1w9, 18:2w6, 18:3w3	Fungi	Zogg et al., 1997
18:1w9, 18:2w6,	Fungi	Zak et al., 1996
20:3w6, 20:4w6	Protozoa	Ringelberg et al., 1997
20:2w6, 20:3w6, 20:4w6	Protozoa	White et al., 1997
18:1w9c, 18:1w11c, 18:3w3, 20:5w3	Higher plants	Ringelberg et al., 1997

Taken from Kirchner (1999)

complements PLFA analysis and has been very successful in the quantitative and specific analysis of indigenous microbial communities. Nucleic acid analysis allows for a more specific identification of the microbial community at a taxonomic level since PLFA profiles do not have enough variety to distinguish among species (Kaneda, 1991). PLFA profiles give insight into the nutritional status of microbial communities at a site.

It has been shown that rDNA gene amplification detects different dominant organisms than microbial culturing techniques and the majority of sequenced organisms recovered using molecular techniques have not yet been cultured (MacNaughton et al., 1999b). Through the use of DGGE, PCR-amplified segments of the 16S rDNA genes can be separated based on differences in their sequences. The DGGE gel contains a denaturing chemical gradient that increases towards the base of the gel, separating the DNA in a sample based on melting properties (Figure 1-4). The order of the four bases in a DNA sequence determines how much heat is required to break apart the two strands of DNA. Chemical denaturants break apart the DNA strands. Increasing the denaturant concentration in a gel matrix from top to bottom causes double stranded PCR products to break apart during electrophoresis. As the DNA denatures, it goes from a linear form to a Y-shaped (or T-shaped) molecule and thus slows down its migration. The gel pore size is uniform and thus as the molecule gets bigger due to breaking apart it slows its movement in the gel. The various PCR products denature at different times (in different places in the gradient) because they differ

Principle of Community Analysis by Denaturing Gradient Gel Electrophoresis

DGGE can be used to generate a profile of a bacterial community following PCR amplification of a fragment of the universal 16S rDNA gene.

PCR products are "clamped" at one end. Other end is free to denature.

At a critical combination of temperature and denaturant, a given gene fragment will denature.

Mobility of denatured fragments is greatly reduced compared to double stranded fragments.

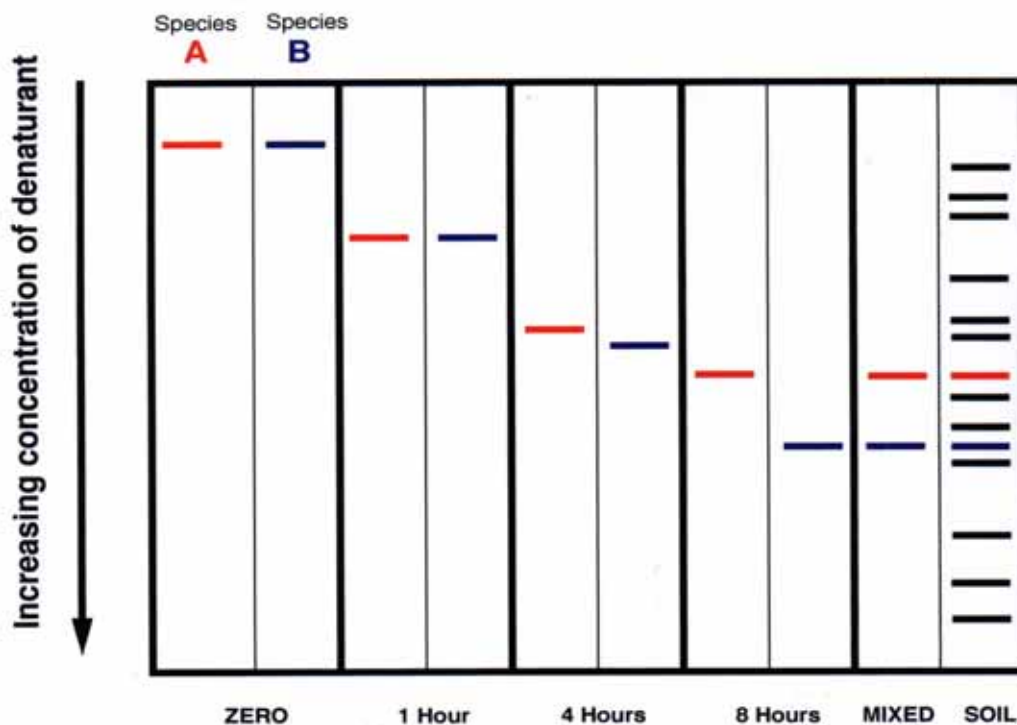


Figure 1-4 Principle of community analysis by DGGE.

in their DNA sequences. Each band on a DGGE gel represents a different organism that can be identified by excising the band from the gel and then sequencing the DNA recovered from the band (Kowalchuk et al., 1997; Macnaughton et al., 1999a,b). Sometimes bands with different DNA sequences migrate to the same place in the gel by random chance, so there is a need to sequence each band of interest in order to determine its phylogenetic affiliation. The sequence is then aligned to a database of known sequences, such as the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>), to find the organism to which it most closely aligns. The strength of the match depends on the similarity to known sequences and the length of the sequence recovered. The Ribosomal Database Project and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) have an extensive sequence library thanks to the efforts of many researchers and culture collections.

Comparing and contrasting band patterns and sequences from different wells at a site can be used to monitor any changes in the microbial community after exposure to a contaminant such as MTBE. In this study PCR amplification of the 16S rDNA was performed and followed by DGGE in order to elucidate the microbial community present at the site of MTBE contamination.

1.8.3 Quantitative DNA Analysis

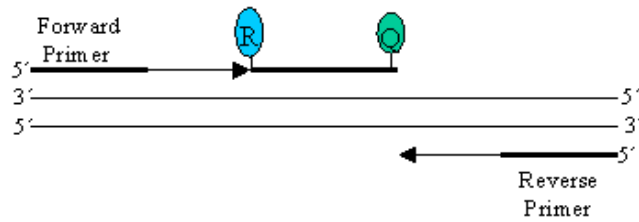
Quantitative DNA analysis, such as quantitative PCR, is a very useful technique that is applied in environmental microbiology. Quantitative DNA

analysis was performed in this study to obtain a measure of the total eubacterial biomass, iron-reducing/sulfate-reducing biomass, and methanogen biomass in the microbial community.

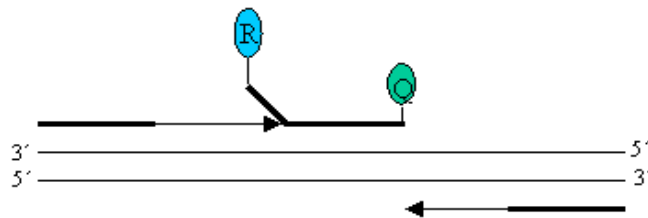
When observing the microbial community it is important to be able to quantify the amount of bacteria present in order to assess changes. Quantitative PCR analysis is a cutting-edge technology that allows for bacterial quantification in a sample. Through the use of different primer probe combinations, one can quantify total eubacterial biomass, group specific bacterial biomass, or functional genes (Applegate et al., 1995; Stapleton et al., 1998). The first two targets are phylogenetic and can be useful when moderately or closely related bacteria have a similar function in various environments. The last target is useful when what bacteria do physiologically is not tied to who they are phylogenetically, but instead is encoded by certain genes (e.g., genes encoding key enzymes in catabolic degradation pathways).

Quantitative PCR involves the same concept as traditional PCR where a single gene copy can be amplified by a million-fold within a few hours. It differs however, in that special instrumentation and often special chemistry is used to monitor the amplification through the log phase to allow quantification. One method of quantitative PCR uses a set of primers and a fluorescently labeled probe. The probe is a linear oligonucleotide that is complementary to a target nucleic acid sequence in between the two primer binding sites (Fig. 1-5). It binds to the DNA strand, and the 5' exonuclease activity of the *Taq* polymerase

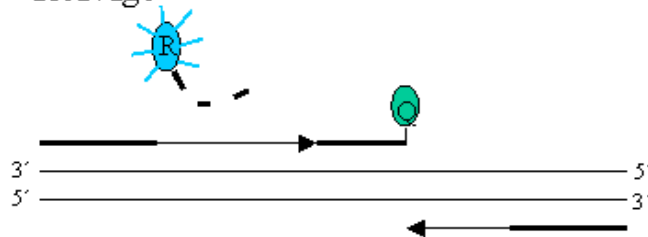
Polymerization



Strand Displacement



Cleavage



Polymerization Completed

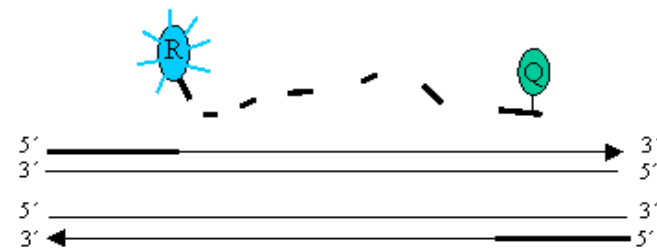


Figure 1-5 Real-time quantitative PCR.
(Taken from <http://www.ukl.uni-freiburg.de/core-facility/taqman/taqindex.html>)

(Clonetech, Palo Alto, CA) cleaves the probe. The cleavage releases the 5' reporter dye from the 3' quencher dye allowing it to fluoresce. Each cycle results in more reporter dye molecules being cleaved, thus allowing for an increase in the amount of fluorescence in proportion to the amount of PCR product produced. A specially equipped instrument is used to detect this fluorescence increase.

In this experiment the eubacteria, iron-reducing/sulfate-reducing bacteria, and methanogenic bacterial biomass were quantified through the use of quantitative (real-time) PCR. The eubacterial biomass was targeted through the use of a primer and probe that was designed by Harms et al., (2003) to be a broad-based test for the estimation of the total bacterial biomass present. The number of bacterial cells quantified through this assay is an estimate since some bacterial species or strains may not hybridize to the designed primers and probes and will not be detected. The probe developed by Stults et al., (2001) is complementary to many iron and sulfate-reducing genera within the delta Proteobacteria. Primers specific to the methyl coenzyme M reductase (MCR) gene of methanogens (Hales et al., 1995) were used to target methanogens. The MCR enzyme appears to be conserved in all methanogens since it catalyzes the last step in methanogenesis.

2. Materials and Methods

2.1 Site Description

The MTBE contaminant spill site is located at a British Petroleum gasoline service station in Parsippany, NJ. It was chosen for this study due to the observed decrease in BTEX, MTBE, and TBA. Also this plume has appeared to have reached a plateau with steady-state degradation occurring. The site map and the direction of the groundwater flow can be seen in Figure 2-1. The size of the site was approximately 120 feet by 90 feet. The direction of groundwater flow was northeast. The depth to water was approximately 1-2 feet throughout the sampled wells. MW13 and MW14 were considered control wells due to their location of being distant and parallel to the plume.

2.2 Sampling Overview

Bio-Sep® traps were placed into eight monitoring wells where data suggested that intrinsic biodegradation of MTBE was occurring under anaerobic conditions and controlling the rates of migration of the MTBE. Eight traps were removed from sterile plastic bags and were suspended on nylon cord approximately 9 feet down into each well. Also Bio-Sep® traps were placed for comparison into monitoring wells with very little degradation occurring and into wells with groundwater not impacted by MTBE (distant and parallel to the plume). The monitoring wells sampled included MW-13, MW-14, MW-5, MW-6, MW-7, MW-8,

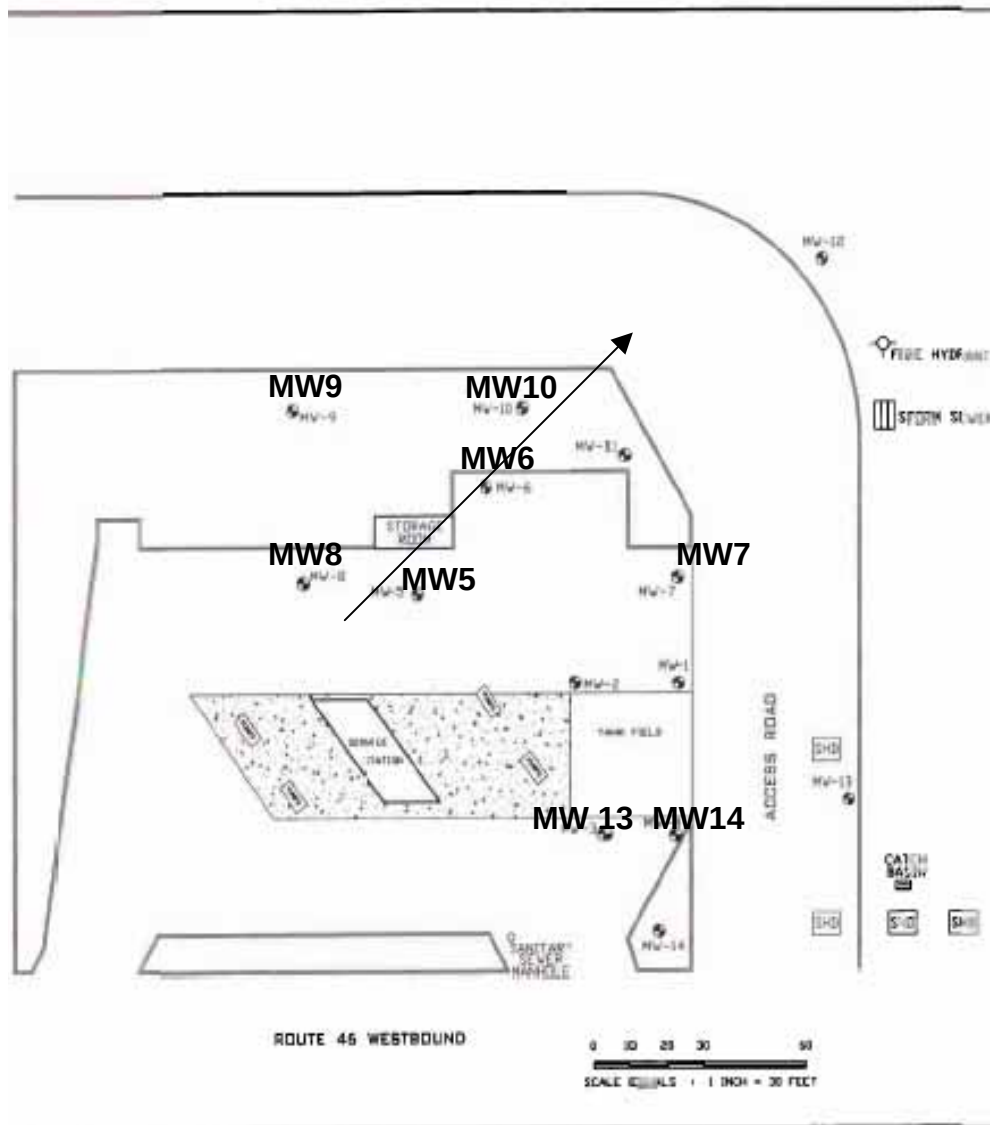


Figure 2-1 Site map showing groundwater monitoring wells and direction of groundwater flow.

MW-9, and MW-10. Four traps, duplicates of regular Bio-Sep® beads and Ambersorb® incorporated Bio-Sep® beads, were retrieved after 30 days and 60 days of exposure. Every analysis that was performed on the beads also had a negative control.

2.3 Geochemical Parameters

The following geochemical parameters were measured: dissolved hydrogen, dissolved oxygen, dissolved CO₂, alkalinity, ferric iron, ferrous iron, divalent manganese, dissolved methane, nitrate, dissolved nitrogen, sulfate, sulfide, dissolved ethane, dissolved ethene. Standard USEPA and Microseeps methods were used for measuring these parameters and were performed by Microseeps Inc. the same day as the first trap sampling. Also the MTBE, TBA, and BTEX concentrations were measured consistently since 1993 using Standard Method USEPA 8021.

2.4 Bio-Sep® Trap Devices

Bio-traps containing autoclavable Bio-Sep® polymer beads were packed into solvent-resistant, autoclavable, polyfluorylalkoxy perforated tubes with metal screw endings (Figure 2-2). Approximately 200 beads were used in each trap. Prior to assembling the trap apparatus, the Bio-Sep® beads were heated to 450°C for a minimum of six hours to remove any organic carbon contamination. The traps were attached to a nylon cord that was fastened to the top of the



Figure 2-2 Bio-Sep® and Ambersorb® “bug traps”.

groundwater well lid. Each set of four traps contained duplicates of Bio-Sep® traps and Ambersorb® incorporated Bio-Sep® traps. Traps were deployed in August and retrieved in September and October of 2003. Deployment of the traps consisted on placing them into sterile plastic bags and shipped on ice overnight to the laboratory where they were immediately analyzed.

2.5 PLFA Analysis

2.5.1 PLFA Extraction and Analysis

One-hundred and eighty beads from each trap were used for PLFA analysis. All solvents used for PLFA analysis were GC grade from Fisher Scientific, and each lot was tested for purity prior to use. The silicic acid used was Unisil 200 mesh from Clarkson Chemical Company. Potassium hydroxide and the fatty acid methyl heneicosanoate methyl ester (21:0) internal standard were purchased from Sigma Chemical Co.. All glassware was cleaned of any lipid contaminants by washing with non-phosphate detergent and baking at 450°C for 4 hours in a muffle furnace

The Bio-Sep® bead samples were extracted using the single-phase chloroform-methanol-buffer system of Bligh and Dyer (1954), as modified by White et al. (1979). Five ml of chloroform, 10ml of methanol, and 4ml of phosphate buffer were added to each sample and then they were placed on a shaker for 4 hours. Five mls each of chloroform and water was added to split the aqueous phase from the organic phase. The final ratio was 1:1:0.8

MeOH:CHCl₃:buffer. The phases were allowed to separate for a period of 12 hours, then the organic phase was pipetted into a new test tube. At 37°C in a water bath the solvent was removed using a stream of nitrogen gas (White and Ringelberg, 1998).

The total lipid was fractionated via silicic acid column chromatography (Guckert et al., 1985) into neutral lipids, glycolipids, and polar lipids. Silicic acid was made into a slurry by mixing 0.5 g with 30 mM ammonium acetate in methanol (Macnaughton et al., 1997) and added to the pipets to complete the column. Five ml of acetone and 5 ml of chloroform were used to pre-elute the silicic acid columns. The sample was dissolved with three 100uL aliquot rinses of chloroform and transferred to the column. Five ml of chloroform was added to elute the neutral lipids, 5 ml acetone to elute the glycolipids, and 10 ml of methanol to elute the polar lipids. Solvent was removed from the polar lipids warmed to 37°C with a stream of dry nitrogen. The data for this thesis were derived from the polar lipid extract.

Mild alkaline methanolysis (Guckert et al., 1985) was applied to the polar lipids to transesterify them into fatty acid methyl esters. The dried polar lipid extract was dissolved in chloroform/methanol 1:1 and 1 ml of methanolic KOH and then heated for 30 minutes at 60°C. Two ml of hexane and 2 ml of water to split the phase were added. The organic phase containing the fatty acid methyl esters (FAMES) was retained.

Capillary gas chromatography with flame ionization detection (GC-FID) was used for the analysis of the fatty acid methyl esters on a Hewlett-Packard 5890 Series 2 chromatograph with a non-polar column. The injector temperature was kept at 270°C and the detector at 290°C. The column temperature program began at 60°C for 2 minutes, then escalated at 10°C per minute to 150°C, and then further rose to 312°C at 3°C per minute. The FAME's were identified by comparison to a mixture of known standard fatty acid retention times. The peaks were labeled more precisely through the use of a mass spectrometer (Hewlett-Packard 5971 mass-selective detector) coupled to the gas chromatograph. Mass spectra were determined by electron impact at 70 eV and using the same temperature program as described for the gas chromatograph. Select representative samples were analyzed this way and then applied to the remaining samples. The internal standard added to all samples was 21:0 and each PLFA was measured as equivalent peak response to the internal standard.

2.5.2 Lipid Nomenclature

Lipid nomenclature designates normal straight chain fatty acid methyl esters by two numbers with a colon in between (x:y). The first number is the total number of carbons in the backbone of the fatty acid methyl ester. The methyl carbon of the methoxy group that forms the methyl ester is not included in this number. The second number (y) designates the number of double bonds or sites of unsaturation in the molecule. The number of carbon atoms from the methyl

end of the molecule to the first unsaturation is designated ω z. Different prefixes used include: “i”=iso-branched, “a”=anteiso-branched, “10Me”=methyl branch on the tenth carbon from the carboxylate end, “br”=branched at an unknown location, and “cy”=cyclopropyl. The *cis* and *trans* isomers of the double bond unsaturation are designated with *c* and *t*. Lower case letters were used in the case when the same designation was used for different fatty acids.

2.5.3 Statistical analysis of PLFA profiles

The relative proportion (percentage mole fraction) measured in (pmol/bead) of PLFA was used to determine the # of cells/bead by multiplying by the conversion factor 2.0×10^4 (White et al., 1997). An analysis of variance (ANOVA) using the General Linear Model SAS procedure (SAS Institute Cary, NC) using pmol/bead results was done. Hierarchal Cluster Analysis was performed on the PLFA profiles with Statistica software (StatSoft, Tulsa, OK).

2.6 Nucleic Acid Analysis

2.6.1 DNA Extraction

DNA extraction was performed using (MO BIO Laboratories Inc., Solano Beach, CA) Ultra Clean Soil DNA Isolation Kit. Twenty beads were added to the extraction tubes. Sixty μ L of solution 1, which contains SDS, was added. SDS is a detergent that aids in cell lysis. It breaks down fatty acids and lipids of the cell membranes of many organisms. Two hundred μ L of IRS (inhibitor removal

solution) was added. The IRS is designed to precipitate many PCR inhibitors that may be present in the sample. A modification to the kit included the addition of 10uL of EDTA, which helps to chelate divalent cations, such as heavy metal contaminants, that inhibit PCR. The tubes were then vortexed for ten minutes. Tubes were centrifuged at 10,000 rpm for 2 minutes to pellet cell debris and particulates. The supernatant containing the DNA was transferred to a clean microcentrifuge tube, and 250 uL of solution 2 was added, this solution contained a protein precipitation reagent. The tubes were centrifuged again for 2 minutes, and the supernatant was transferred to a clean microcentrifuge tube. Nine hundred uL of solution 3, a DNA binding salt solution, was added. DNA binds to silica in the presence of high salt concentrations. This mixture was then loaded onto a silica membrane spin filter, centrifuged, and the flow through was discarded. This was repeated until all supernatant passed through the filter. Three hundred uL of solution 4 was added for an ethanol-based wash of the bound DNA. After centrifugation, the flow through was discarded. Tubes were recentrifuged to remove any traces of ethanol. The spin filter was transferred to a clean microcentrifuge tube and the DNA was eluted with 50 uL of 1/10 Tris EDTA (TE) buffer. This buffer was used to stabilize the DNA for storage at 4°C.

2.6.2 PCR Amplification

PCR for DGGE analysis was performed according to Muyzer et al. (1993). The thermocycling program was set at 34 cycles of 94°C for 30s, 60°C for 45s

and 72°C for 45s, using 0.5 units of *Taq* DNA Polymerase (Clonetech, Palo Alto, CA) and 12.5 pmol each of the primers per reaction in a total volume of 25 uL. The forward primer contained the 40bp GC clamp required for DGGE (Muyzer et al., 1993). The final concentration for deoxyribonucleotides (dNTP) was 4 umol and 2 mmol for MgCl₂ per reaction. Thermocycling was performed with a 'Robocycler' PCR block (Stratagene, Lajolla, CA). The primers used targeted the 16S rDNA corresponding to positions 341-519 of *Escherichia coli* bacteria. Five uL of the PCR product were tested through agarose gel electrophoresis (1% agarose, 1x TAE buffer) to determine if amplification occurred. The gels were viewed and captured with software and instrumentation by ALPHA IMAGER (Indiganabar, Bangalore).

2.6.3 DGGE Analysis

DGGE was performed according to Muyzer et al., (1993). A D-code 16/16 cm gel system with 1.5 mm gel width (Bio-Rad, Hercules, CA) was used for DGGE analysis. The PCR amplified product mixed with 5 uL of loading dye for a total of 25 uL was loaded onto the gel and maintained at a temperature of 60°C in 6 L of 0.5x TEA buffer (20 mM Tris acetate, 0.5 mM EDTA). The gradient of the gel was formed between 30% and 65% denaturant with 100% defined as 10 M urea 40% (v/v) formamide. An 8% to 10% acrylamide gradient was used. The gels were run at 55 V for 16 hours. They were then stained in purified water (Millipore, Bedford, Mass.) with ethidium bromide (0.5 mg/L). The gels were

viewed and captured with ALPHA IMAGER (Indiganabar, Bangalore) software and instrumentation.

2.6.4 Extraction of DNA from Acrylamide Gels

Dominant DGGE bands were excised from the gel by using a razor blade to cut the central 1 mm part of the band. The bands were placed into clean microcentrifuge tubes with 50 μ L of purified water (Millipore, Bedford, Mass.) and then frozen for two hours. Freezing releases the DNA from the gel matrix. Two μ L was used as the template in a PCR reaction as described above. The products were analyzed through agarose gel electrophoresis as described above. The Ultraclean PCR Clean-up Kit (MO BIO Laboratories Inc., Solano Beach, CA) was used to prepare the products for sequencing. As described from the kit protocol, 5 volumes of SpinBind (100 μ L) was added to the PCR reaction and transferred to a silica spin filter unit. The DNA binds to the silica membrane and unwanted components of the PCR reaction do not. This was centrifuged for 1 minute at 10,000 rpm and the flow-through liquid was discarded. 300 μ L of SpinClean buffer was added as a wash solution. DNA was eluted with elution buffer.

2.6.5 Sequence Analysis

The cleaned PCR amplification product of the excised gel bands were sequenced using the primer 341f with an ABI-prism model 373 automatic

sequencer with dye terminators (Applied Biosystems, Foster City, CA).

Sequences were compared with the GenBank database using the Blast N facility of the National Center for Biotechnology Information and the Ribosomal Database Project. A neighbor-joining dendrogram showing the relationships of the sequences was constructed using ARB software (Munich, Germany).

2.6.6 Nucleotide Accession Numbers

All unique partial rDNA sequences were submitted to GenBank as 3ftl for the 30 day sampling and 5ftl for the 60 day sampling with accession numbers AY559749-AY559836.

2.7 Quantitative PCR Analysis

A portion of DNA was taken for all quantitative analyses from the aforementioned DNA extraction procedure product. Three targets were chosen for quantitative PCR. These included the eubacterial biomass targeting the rDNA of bacteria, the iron-reducing/sulfate-reducing bacteria targeting the rDNA of delta Proteobacteria species, and the methanogen biomass through primers specific to the mcrA gene present in all methanogens.

2.7.1 Total Bacterial Biomass Estimation via Quantitative-PCR

Oligonucleotide primers targeting the 16S rDNA were 5'-ATGGCTGTCGTCAGCT-3' (1055 forward primer) and 5'-

ACGGGCGGTGTGTAC-3' (1392 reverse primer) from Harms et al., (2003). The probe was TaqMan (Clonetech, Palo, Alto, CA) 16STaq11115 5'-(6-FAM)-CAACGAGCGCAACCC-(TAMRA)-3' (Harms, 2003). The probe had 6-carboxy-fluorescein (FAM) as a reporter fluorochrome at the 5' end, and N,N,N',N' – tetramethyl-6-carboxy-rhodamine (TAMRA) as the quencher dye on the 3' end. The primers and probe were synthesized by Sigma Genosys (The Woodlands, TX) and ABI (Applied Biosystems, Foster City, CA), respectively. The total volume of the reaction was 30 μ L, containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems). This contained the DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$. Three μ L of DNA template was used in each reaction; water was used in negative control reaction tubes. The thermocycling parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A spectrofluorimetric thermal cycler (ABI Prism 7000 Sequence Detection System; Applied Biosystems) was used.

Dilutions (10^5 to 10^9) of extracted DNA from *Nitrosomonas europaea* were used to generate calibration curves for eubacterial quantification (Beller et al., 2002). The quantity of DNA in the standards was determined using a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's protocols. Assumptions used in calculating the number of eubacterial gene copies in a sample included: (i) the approximate size of the genome of *Nitrosomonas europaea* was 2.98 Mbp (ii) there was 3.6 eubacterial gene copies per genome or cell (Harms et al., 2003). The calibration curve

consisted of the cycle threshold versus the log of the DNA concentration. The cycle threshold values retrieved for each individual sample allowed for the determination of the number of cells/bead through comparison to the standard curve and then dividing by the number of gene copies.

2.7.2 Iron-Reducing/Sulfate-Reducing Bacterial Biomass Estimation

The primers used were developed by Stults et al., (2001) to target Delta-Proteobacteria. The primers were 5'-AAGCCTGACGCASCAA-3' (361 forward primer) and 5'-ATCTACGGATTTCCTCCTACA-3' (685 reverse primer). The TaqMan probe used was Eub 1 5'-(FAM)-GTATTACCGCGGNTGCTGGC-(TAMRA)-3' (Perkin-Elmer, Foster City, CA). The probe contained the 6-carboxyfluorescein (FAM) fluorescent reporter dye at the 5' end and 6-carboxy tetramethyl rhodamine (TAMRA) quencher dye at the 3' end. The PCR optimization was as follows: 50uL of total reaction mixture volume, 5uL of DNA template, and TaqMan reaction buffer. The thermocycling parameters were 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 45 cycles of 95°C for 15s and 55°C for 60s (Stults et al., 2001). A spectrofluorimetric thermal cycler (ABI Prism 7000 Sequence Detection System; Applied Biosystems) was used. The DNA standard curve for *Desulfovibrio vulgaris* was generated as before described for *Nitrosomonas europaea* except the assumed calculated genome size was 3.6 Mbp and 2.7 gene copies were assumed per genome (www.sanger.ac.uk/Projects/Microbes/).

2.7.3 Methanogen Biomass Estimation

The primers used were developed to target the α -subunit of the MCR gene present in all methanogens (Hales et al., 1995). The primers were 5'-GCMATGCARATHGGWATGTC-3' (forward primer ME1) and 5'-TCATKGCRTAGTTDGGRTAGT-3' (reverse primer ME2). The PCR optimization was as follows: 30 μ L of total reaction mixture volume and 3 μ L of DNA template and in addition to other reagents SYBR green binding dye which binds to double stranded DNA was used. The thermocycling parameters were 1 cycle of 95°C for 10 min, 30 cycles of 60s at 95°C and 60s at 50°C and 120s at 75°C. A spectrofluorimetric thermal cycler (ABI Prism 7000 Sequence Detection System; Applied Biosystems) was used. A DNA standard curve for methanogens was generated as previously described above but using an assumed calculated genome size of 1.66 Mbp and 2 gene copies per genome (www.Sanger.ac.uk/Projects/Microbes/).

3. Results

3.1 PLFA Analysis Results

All 30-day and 60-day Ambersorb® and regular Bio-Sep® traps from all eight wells were analyzed through PLFA analysis.

3.1.1 Viable Biomass

The graph of the viable biomass as determined by PLFA analysis is represented in Figure 3-1. At 30 days, Ambersorb® beads were not significantly different in biomass in any well. At 30 days, regular Bio-Sep® beads in MW5, closest to the plume, and MW9 had significantly more biomass than other wells except for MW13, which had a high standard deviation however (Figure 3-2).

At 60 days, Ambersorb® beads in MW5 were significantly higher in biomass than MW9, which in turn was significantly different from all wells except MW13. Ambersorb® bead traps had a biomass increase of 6-fold in MW5, 5-fold in MW7, and 7-fold in MW9 when compared to the 30-day sampling of these wells. At 60 days regular Bio-Sep® bead traps in MW5 were significantly higher in biomass from all other wells. Regular Bio-Sep® beads at 60 days in MW5 and MW6 had a 3-fold increase from 30-day beads.

Comparing Ambersorb® to regular Bio-Sep® beads showed a 2-fold increase in regular Bio-Sep® beads in MW5 and MW8, and a 5-fold increase in MW9 at

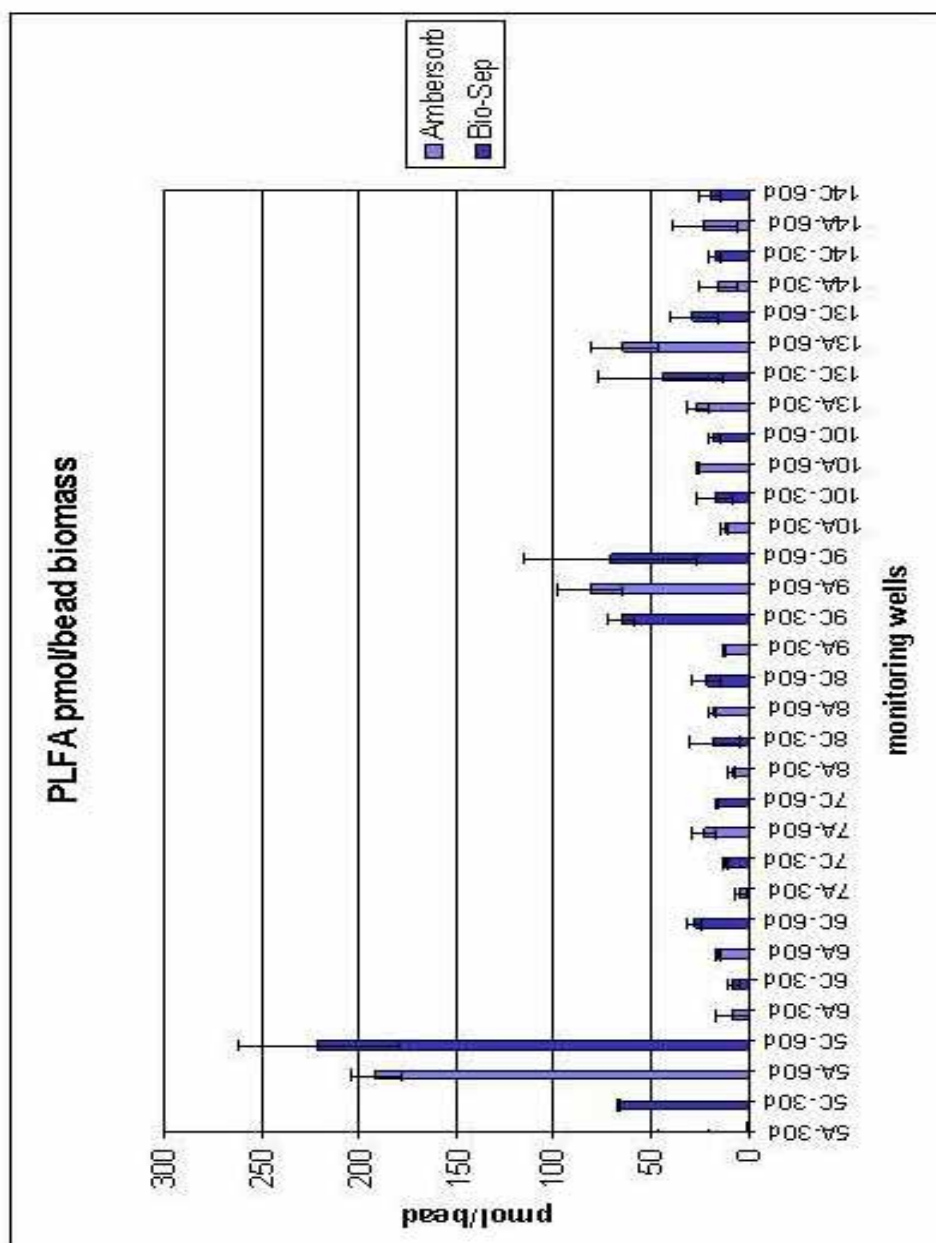


Figure 3-1 PLFA biomass as pmol/bead of Ambersorb® and Bio-Sep® beads. Every four bars represent one well, the first two are from the 30-day sampling and the second two are from the 60-day sampling.

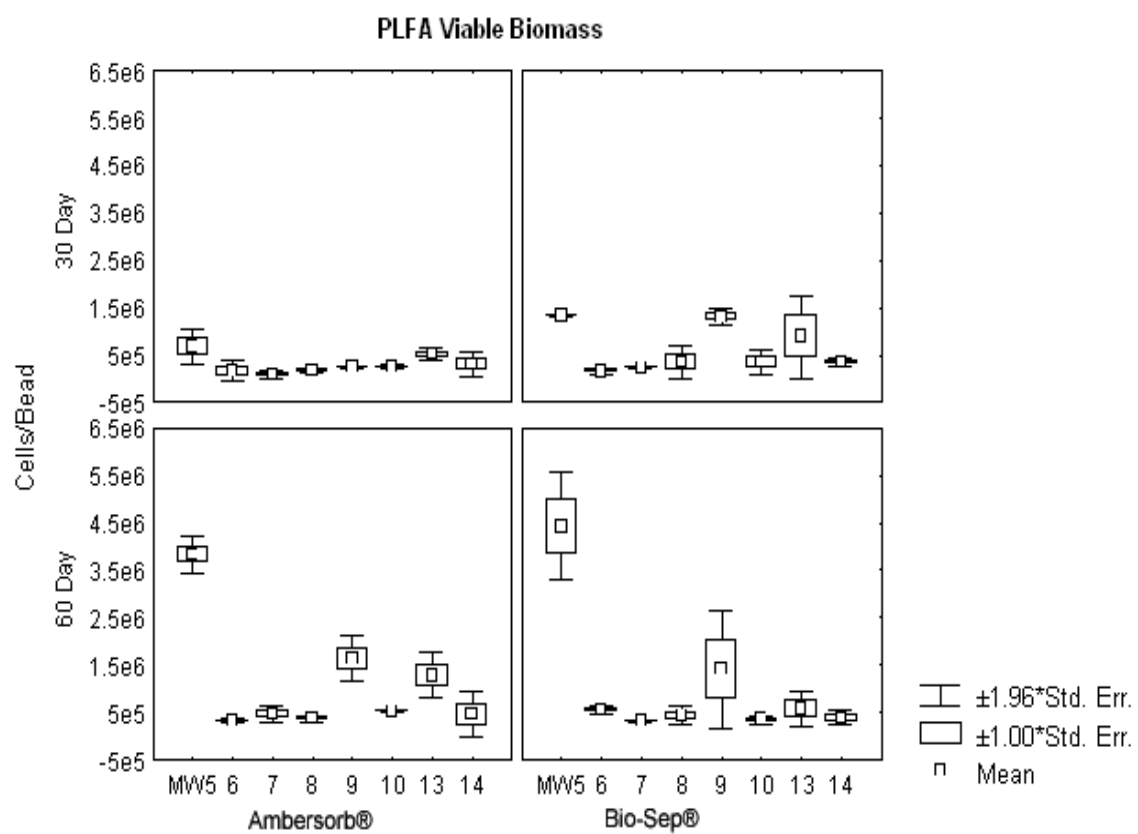


Figure 3-2 PLFA biomass calculated as cells/bead box and whiskers plot.

30 days. There was no significant difference between the biomass found in Ambersorb® and regular Bio-Sep® beads at 60 days (Figure 3-2).

3.1.2 Community Structure

The relative percentages of total PLFA structural groups are represented in Figure 3-3. The monoenoic fatty acids constituted the highest percent of the total PLFA in all wells ranging from 39.5 to 71.8%. Monoenoics are representative of the presence of Gram-negative Proteobacteria, which have an ability to utilize a wide range of carbon sources and to adapt quickly to environmental conditions. At 30 days Ambersorb® bead traps in MW14, control well, had the least amount of monoenoic PLFA at 39.5% (Figure 3-4). MW8 had the highest at 71.8%, but was not significantly different from MW9. MW5, MW6, MW7, MW10, and MW13 were not significantly different from each other. At 30 days regular Bio-Sep® beads in MW14 had the least amount of monoenoic PLFA at 50.2%. MW8 had significantly more monoenoic PLFA than all wells except MW6 and MW9.

At 60 days, Ambersorb® beads in MW8 had the highest monoenoic biomass but were not significantly different from MW13 and MW14 due to their high standard deviations. The next highest in Gram-negative biomass were MW6 and MW10, and they had significantly more biomass at 60 days when compared to 30-day Ambersorb. Regular Bio-Sep® beads in MW7 at 60 days were significantly lower in monoenoic biomass at 50.1%. All other wells were significantly similar to each other ranging from 60-70%. Comparing Ambersorb®

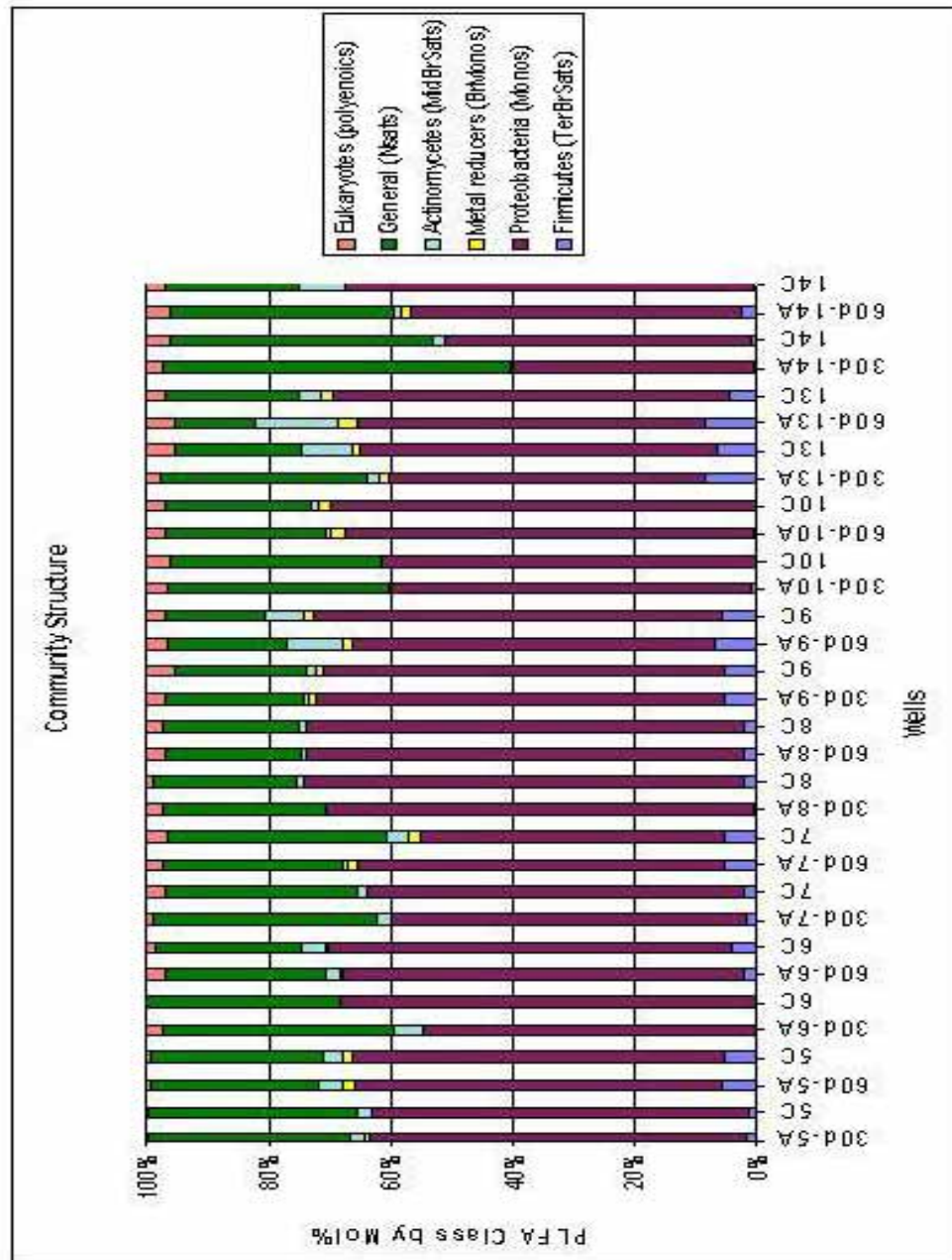


Figure 3-3 PLFA community structure. Every four bars represents one well. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. These fatty acid groups were represented in the samples analyzed. The groups consisted of the monoenoics, terminally branched saturated, branched monoenoic, mid-chain branched saturated, normal saturated, and polyenoics. In addition the monoenoics included cy17:0 and cy19:0.

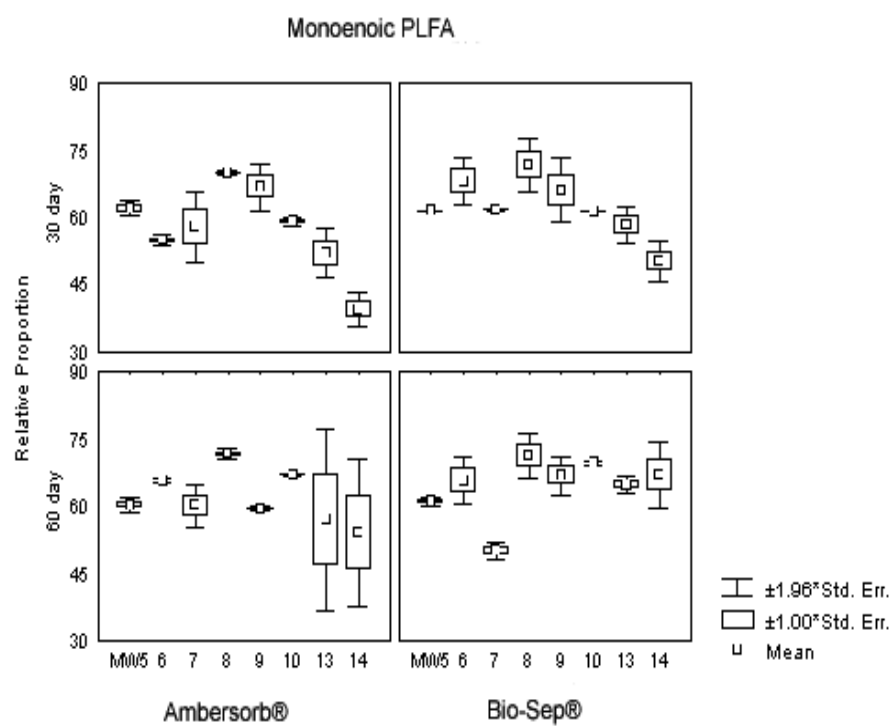


Figure 3-4 Relative proportion of total monoenoic PLFAs including cy17:0 and cy19:0.

to regular Bio-Sep® beads showed a slight increase in biomass in regular Bio-Sep® beads in the majority of the wells with the exception of MW7 at 60 days having a lower biomass.

The normal saturated PLFA were the next abundant group ranging from 13.6 to 57.2%. Normal saturated PLFA are found in all organisms. At 30 days Ambersorb® beads in MW14 had the highest amount at 57.2%. MW14 with regular Bio-Sep® beads also had the highest amount with 42.7%.

At 60 days Ambersorb® beads in MW13 had the least amount of normal saturated with 13.6% but were not significantly different from MW9 and MW14. Regular Bio-Sep® beads at 60 days were relatively similar to each other in normal saturates. MW7 was significantly different with the most biomass. There was no major difference between 60-day and 30-day normal saturates with both types of traps. Comparing Ambersorb® to regular Bio-Sep® beads did not show any major difference in normal saturated biomass. The box and whiskers plot for normal saturated PLFAs can be seen in the appendix.

The terminally branched saturates ranged from 0 to 8.5% of the total PLFA (Figure 3-5). These saturates are representative of Firmicutes (Clostridia-like anaerobes) and some Gram-negative bacteria, such as sulfate-reducers. At 30 days Ambersorb® traps in MW13 had the highest amount at 8.5%, followed by MW9 at 5.3%. The remaining wells were not significantly different and ranged from 0-4%. MW6 had none detected. Regular Bio-Sep® beads at 30 days had the same amount of terminally branched saturates as Ambersorb® beads with

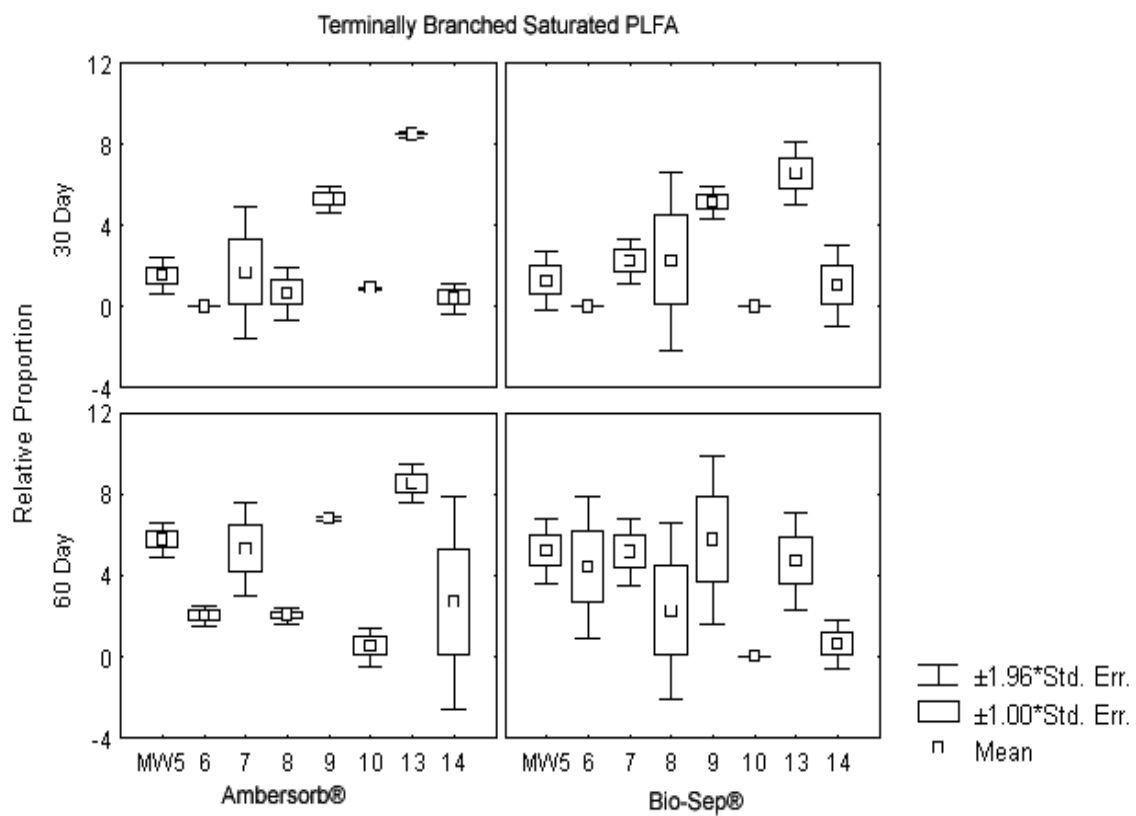


Figure 3-5 Relative proportion of total terminally branched saturated PLFAs.

MW6 and MW10 having a non-detect level. At 60 days Ambersorb® traps in MW6, MW8, and MW9 had a significant increase from 30-day traps. The remaining wells were not significantly different from 30 days. At 60 days regular Bio-Sep® beads in MW5, MW6, and MW7 had a slight increase from the 30-day regular. Regular Bio-Sep® beads in MW13 had 80% less biomass than Ambersorb® MW13 at 60 days.

Mid-chain branched fatty acids are indicative of *Actinomyces*, but also are present in the membranes of some sulfate-and metal-reducing bacteria such as *Desulfobacter*. There was no significant difference between both types of traps and all wells at 30 days. All wells at 60 days with Ambersorb® traps were not significantly different except MW5, which was slightly higher than MW7, MW8 and MW10. There was no significant difference between time-points in Ambersorb® beads. Regular Bio-Sep® beads at 60 days showed a slight significant increase in MW5, MW6 and MW13 from MW8 and MW10. The only significant increase between time-points in regular Bio-Sep® beads was in MW6 from 0 to 4.4% of the total PLFA. There was no significant difference between Ambersorb® and regular Bio-Sep® beads. The box and whiskers plot for mid-chain branched PLFAs can be seen in the appendix.

Branched monoenoic PLFA are indicative of sulfate-and metal-reducing bacteria and *Actinomyces*. Ambersorb® beads at 30 days in MW6, MW7, MW8 and MW14 had 0% branched monoenoics. MW9 and MW13 had the highest percent at 1.2-1.3% but were not significantly different from MW5 and

MW10 at 0.3-0.8%. Regular Bio-Sep® beads at 30 days showed close similarity to Ambersorb® traps. At 60 days Ambersorb® beads in MW5, MW7, and MW10 showed a significant increase from 30-day traps. Regular Bio-Sep® beads at 60 days in MW5, MW7, MW9, and MW10 were significantly higher at 1.4-2.2% from MW8 and MW14 which constituted 0% of the total PLFA, also they showed a significant increase compared to the 30-day traps. Regular Bio-Sep® beads were closely similar to Ambersorb® beads at 60 days.

Polyenoic fatty acids are representative of eukaryotes such as fungi. At 30 days Ambersorb® beads in MW8, MW10, MW13, and MW14 were significantly higher in polyenoics than MW5, closest to the plume. Regular Bio-Sep® beads at 30 days showed MW7, MW10, MW13, and MW14 having more polyenoics than MW5 and MW6, which were closest to the plume. Regular Bio-Sep® beads differed at 30 days from Ambersorb® in that regular Bio-Sep® beads in MW6 showed 0% unsaturates, and regular MW7 had slightly higher polyenoics. At 60 days all Ambersorb® wells had a significantly higher amount of polyenoics than MW5 with the highest contaminants. In addition MW14 had a significantly higher amount than MW6, MW7, and MW10. All 60-day Ambersorb® wells did not differ from 30-day Ambersorb® samples but had smaller standard deviation. Regular Bio-Sep® beads at 60 days showed close similarity to Ambersorb® at 60-days in that all wells were higher in polyenoics than MW5 with a slightly higher standard deviation in some wells. Regular Bio-Sep® beads at 60 days compared to 30

days only showed a slight significant increase in MW6. This plot can be seen in the appendix.

The fatty acid i17:1 ω 7c (Figure 3-6) is a biomarker for sulfate-reducers (Dowling et al., 1986). Ambersorb® beads at 30 days in MW6, MW7, MW8 and MW14 had 0% of this fatty acid. MW9 and MW13 had the highest significant amount from all wells except MW5. In regular Bio-Sep® beads only MW9 showed a statistical difference. At 60 days, Ambersorb® traps in MW5, MW7, MW9, and MW13 were statistically different, while MW6, MW8, MW10, and MW14 were not different. MW7 had a big increase from the 30-day sampling. In regular Bio-Sep® traps at 60 days MW7 had the highest relative amount. MW7 increased significantly compared to its 30-day result and to 60-day Ambersorb® traps. MW5, MW9, and MW13 had significant relative proportions but only MW5 showed an increase in relative proportion from 30-days.

Figure 3-7 shows the hierarchical cluster analysis of the wells based on PLFA. Two main groups are evident labeled I and II. In group II, all MW5 well samples clustered together indicating a unique PLFA profile. The microbial community differed probably due to the high concentration of contaminants in this well.

3.1.3 Metabolic Status

The stress biomarker for the Gram-negative community is assessed by the ratios of cyclopropyl fatty acids to their metabolic precursors. As Gram-negative bacteria change from logarithmic to stationary phase, they increasingly convert

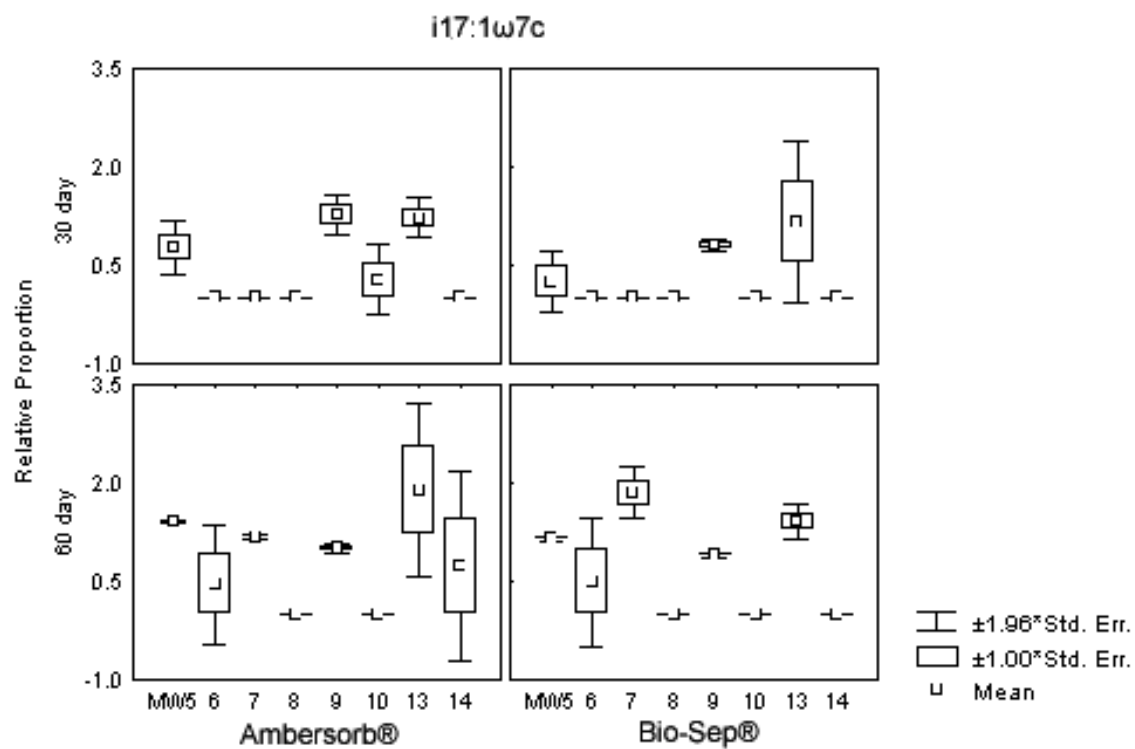


Figure 3-6 Relative proportion of i17:1ω7c as a biomarker for sulfate-reducers.

16:1 ω 7c and 18:1 ω 7c to the cyclopropyl fatty acids cy17:0 and cy19:0, respectively (Guckert et al., 1986). This ratio usually ranges from .05 (log) to 2.5 and greater (stationary) varying with the environment and the microorganism (Mikell et al., 1987; White, 1983). The ratio of cy17:0/16:1 ω 7c in the 30-day Ambersorb® beads had MW8 with a ratio of 0.3 which was significantly greater than MW6, MW7, MW9, and MW13. In the regular Bio-Sep® 30-day traps only MW10 with a ratio of 0.19 was significantly greater than all wells except MW8 and MW14. In 60-day Ambersorb® traps MW10 and MW13 were significantly greater, at a ratio of 0.26 and 0.29, than other wells except MW8 and MW14. A few wells in the 60 day Ambersorb® traps showed an increased ratio from the 30 day sampling. Regular Bio-Sep® traps at 60 days showed a significant difference in MW8 and MW14 with a higher ratio than all other wells except MW13. These include the control wells and MW8, an aerobic well. Comparing regular Bio-Sep® beads between time-points showed no significant difference. Comparing the 60-day samples between traps showed very little difference.

The two different ratios representative of Gram-negative stress may also represent two different Gram-negative communities. The ratio of cy19:0/18:1 ω 7c in the 30-day Ambersorb® traps had MW7 with the highest ratio above all other wells at .61. Due to the higher standard deviation in regular traps at 30 days MW7 was greater than only 2 other wells and no real difference was seen from the Ambersorb® traps. Ambersorb® 60-day traps were similar to 30-day traps, except MW13 had a significant 92% increase. The only significant difference in

regular Bio-Sep® beads at 60 days was the high ratio in MW14 of 2.57. The highest ratio wells were MW8, MW9, MW13, and MW14 suggesting that the Gram-negative community in these wells had environmental nutrient-limiting conditions limiting bacterial growth (Table 3-1).

A secondary metabolic stress may be assessed by observing the ratio of *t/c* fatty acids (Table 3-2). Gram-negative bacteria generate *trans*-fatty acids to maintain membrane integrity as an adaptation to a less favorable environment. Ratios greater than .1 are indicative of adaptation to a toxic or stressful environment (Guckert et al., 1986). The 16:1 ω 7*t/c* ratio was significantly higher in MW8, MW10, and MW13 than MW6 and MW7, where it was non-detected, in Ambersorb® beads at 30 days. Regular Bio-Sep® beads at 30 days showed MW5, MW9, and MW10 with the highest significant ratios. There was no significant difference between trap types at 30 days. Ambersorb® beads at 60 days showed an increase in MW5, closest to the plume, from the 30 day sampling to a ratio of 0.32 (77% increase). The next highest ratios were in MW7, MW8, and MW9, all downgradient, with a range of 0.11-0.14, and were significantly higher from the corresponding 30-day Ambersorb® sampling. Regular Bio-Sep® traps at 60 days also had MW5 as the highest significant ratio with a ratio of 0.47, which was significantly higher from the regular Bio-Sep® 30-day traps as well as the Ambersorb® traps. MW9 still remained at second highest with a ratio of 0.14. Regular Bio-Sep® MW6 and MW7 increased from being non-detected, while MW10 decreased slightly when compared to the 30

Table 3-1 Physiological stress cy/monoenoic PLFA ratio.

Sample	Cy17:0/16:1 ω 7c	Cy19:0/18:1 ω 7c	Std.Dev.(Cy17)	Std.Dev.(Cy19)
30d 5A ^a	0.04	0.04	0.04 ^b	0.06
30d 5C	0.06	0.06	0.01	0.04
60d 5A	0.05	0.04	0.01	0.02
60d 5C	0.04	0.08	0.00	0.01
30d 6A	0.00	0.18	0.00	0.06
30d 6C	0.00	0.21	0.00	0.12
60d 6A	0.08	0.18	0.02	0.15
60d 6C	0.05	0.06	0.00	0.09
30d 7A	0.00	0.61	0.00	0.04
30d 7C	0.00	0.80	0.00	0.33
60d 7A	0.06	0.31	0.00	0.21
60d 7C	0.05	0.57	0.00	0.45
30d 8A	0.33	0.13	0.06	0.19
30d 8C	0.17	1.47	0.14	1.12
60d 8A	0.14	0.14	0.15	0.00
60d 8C	0.25	0.16	0.04	0.09
30d 9A	0.06	0.06	0.00	0.01
30d 9C	0.07	0.07	0.00	0.01
60d 9A	0.08	1.17	0.02	1.51
60d 9C	0.07	0.42	0.04	0.48

Table 3-1 Continued

Sample	Cy17:0/16:1 ω 7c	Cy19:0/18:1 ω 7c	Std.Dev.(Cy17)	Std.Dev.(Cy19)
30d 10A	0.15	0.20	0.17	0.14
30d 10C	0.19	0.40	0.01	0.23
60d 10A	0.26	0.07	0.03	0.05
60d 10C	0.16	0.11	0.02	0.03
30d 13A	0.05	0.25	0.00	0.02
30d 13C	0.05	1.65	0.01	1.83
60d 13A	0.29	0.48	0.01	0.10
60d 13C	0.23	0.1.	0.24	0.07
30d 14A	0.15	0.32	0.13	0.06
30d 14C	0.25	0.45	0.29	0.10
60d 14A	0.17	0.37	0.15	0.05
60d 14C	0.43	2.57	0.02	0.09

^a A represents Ambersorb® beads, C represents Bio-Sep® beads

^b Standard deviations generally ranged from 0.00-0.19, except for a few exceptions

Table 3-2 Physiological stress *trans/cis* PLFA ratio.

Sample	16:1 ω 7t/c	18:1 ω 7t/c	Std.Dev.(16t/c)	Std.Dev.(18t/c)
30d 5A ^a	0.18	0.21	0.01 ^b	0.30
30d 5C	0.15	0.00	0.00	0.00
60d 5A	0.32	0.04	0.01	0.00
60d 5C	0.47	0.04	0.03	0.00
30d 6A	0.00	0.00	0.00	0.00
30d 6C	0.00	0.00	0.00	0.00
60d 6A	0.07	0.00	0.01	0.00
60d 6C	0.06	0.00	0.00	0.00
30d 7A	0.00	0.00	0.00	0.00
30d 7C	0.00	0.00	0.00	0.00
60d 7A	0.12	0.00	0.00	0.00
60d 7C	0.07	0.01	0.00	0.00
30d 8A	0.05	0.00	0.07	0.00
30d 8C	0.03	0.00	0.04	0.00
60d 8A	0.11	0.00	0.01	0.00
60d 8C	0.09	0.00	0.00	0.00
30d 9A	0.10	0.00	0.01	0.00
30d 9C	0.11	0.08	0.00	0.00
60d 9A	0.14	0.11	0.01	0.15
60d 9C	0.14	0.05	0.00	0.01

Table 3-2 Continued

Sample	16:1 ω 7t/c	18:1 ω 7t/c	Std.Dev.(16t/c)	Std.Dev.(18t/c)
30d 10A	0.09	0.04	0.00	0.06
30d 10C	0.12	0.00	0.02	0.00
60d 10A	0.06	0.03	0.00	0.04
60d 10C	0.07	0.00	0.00	0.00
30d 13A	0.07	0.03	0.01	0.04
30d 13C	0.06	0.05	0.00	0.07
60d 13A	0.08	0.09	0.01	0.13
60d 13C	0.06	0.00	0.00	0.00
30d 14A	0.04	0.00	0.05	0.00
30d 14C	0.03	0.00	0.05	0.00
60d 14A	0.03	0.00	0.05	0.00
60d 14C	0.09	0.00	0.00	0.00

^a A represents Ambersorb® beads, C represents Bio-Sep® beads

^b Standard deviations generally ranged from 0.00-0.05, except for a few exceptions

day sampling.

The 18:1ω7t/c ratio was not significantly different in any Ambersorb® well at 30 days. In regular Bio-Sep® beads at 30 days MW9 (0.08) was significantly greater than all wells except MW13. There was no significant difference between trap types at 30 days. At 60 days no well had a ratio of 0.1 or higher and was not significantly different from 30-day sampling or Ambersorb®.

3.2 Nucleic Acid Analysis Results

Nucleic acid analysis was performed on all samples at both time-points. It included DGGE analysis and quantitative PCR.

3.2.1 Phylogenetic Association

Figure 3-8 and 3-9 are DGGE gel images of the 30-day time point Bio-Sep® samples showing the 55 excised and sequenced DGGE bands labeled A-BE. Of the 55 excised bands, 11 did not produce a readable sequence. Figure 3-10 and 3-11 are DGGE gel images of the 60-day time point Bio-Sep® samples showing the 64 excised and sequenced DGGE bands labeled A-BL. Of the 64 excised bands, 11 did not produce a readable sequence. The sequence data for the 30 and 60-day excised DGGE bands showing the closest genus match, phylogenetic affiliation, and the similarity index as compared to the Ribosomal Database Project (RDP) are listed in the appendix. Neighbor-joining dendograms, constructed with the sequence data through the ARB alignment

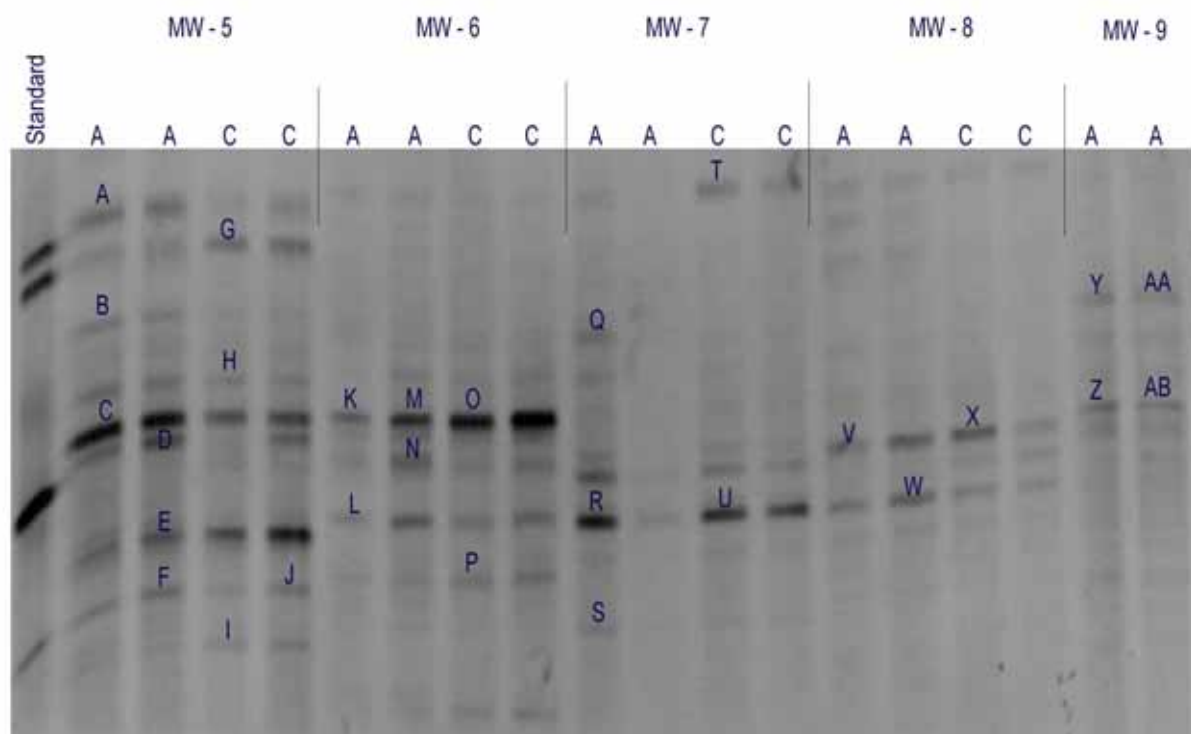


Figure 3-8 Thirty day DGGE profile of amplified DNA from a portion of the 16s rRNA gene (1st gel). The first two columns labeled A represent the Ambersorb® duplicates. The next two columns labeled C are the Bio-Sep® duplicates. Every four columns represent one well. The lettered bands were excised and sequenced.

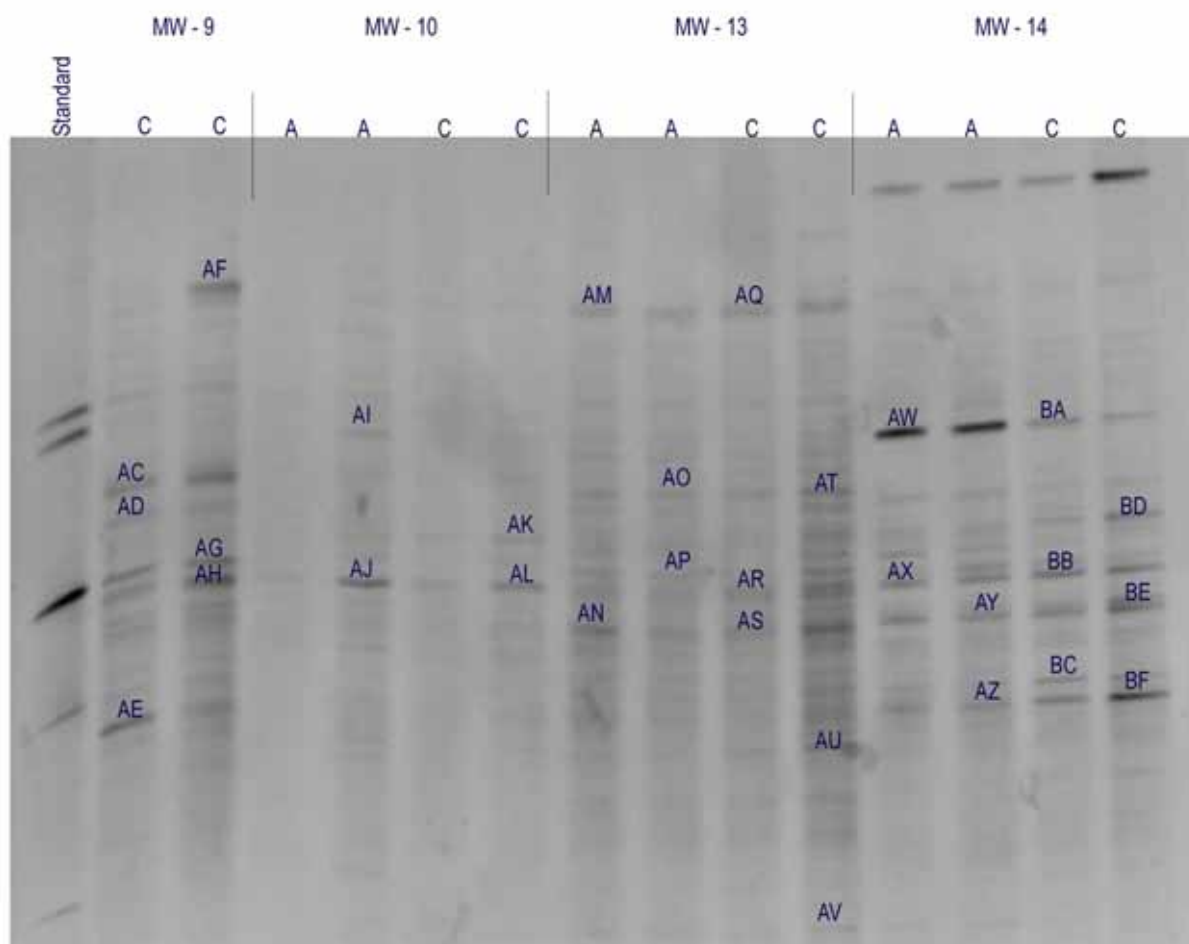


Figure 3-9 Thirty day DGGE profile of amplified DNA from a portion of the 16s rRNA gene (2nd gel). The first two columns labeled A represent the Ambersorb® duplicates. The next two columns labeled C are the Bio-Sep® duplicates. Every four columns represent one well. The lettered bands were excised and sequenced.

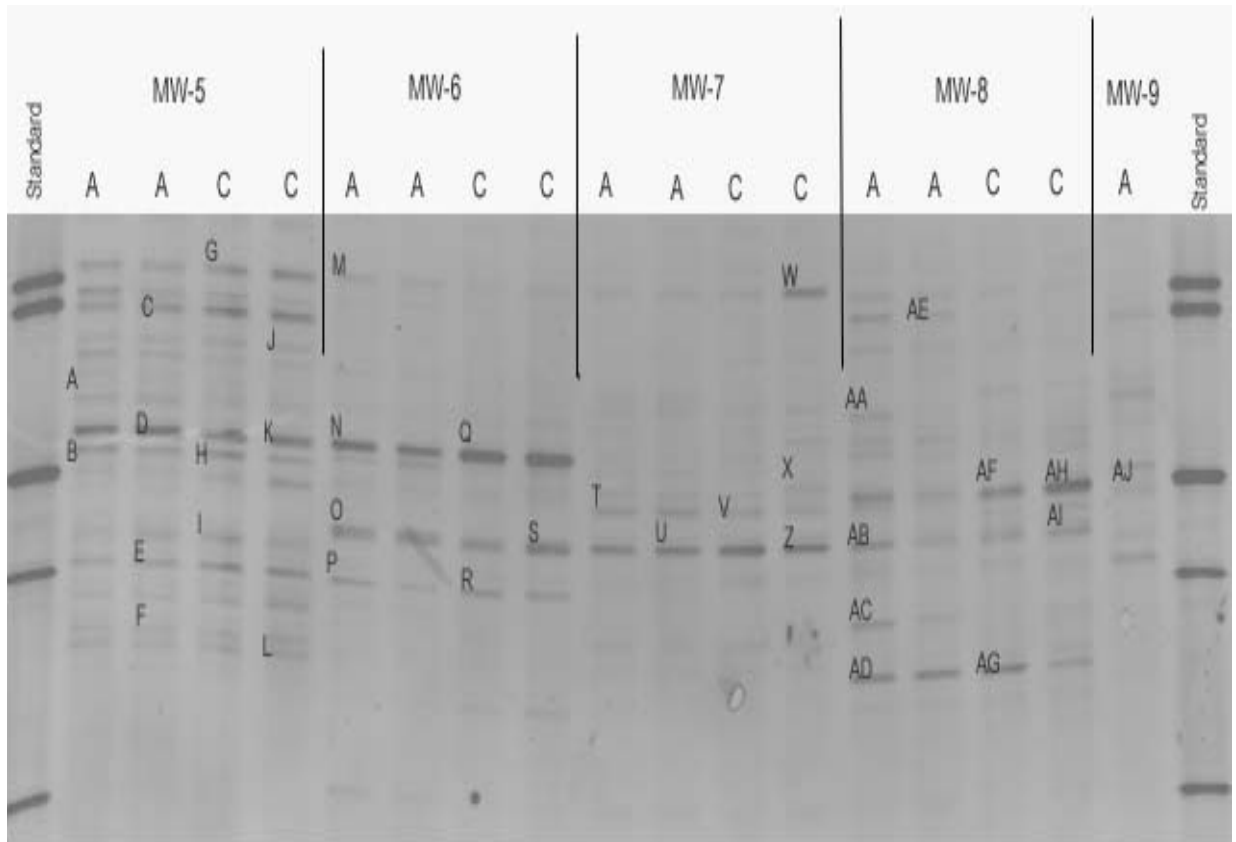


Figure 3-10 Sixty day DGGE profile of amplified DNA from a portion of the 16s rRNA gene (1st gel). The first two columns labeled A represent the Ambersorb® duplicates. The next two columns labeled C are the Bio-Sep® duplicates. Every four columns represent one well. The lettered bands were excised and sequenced.

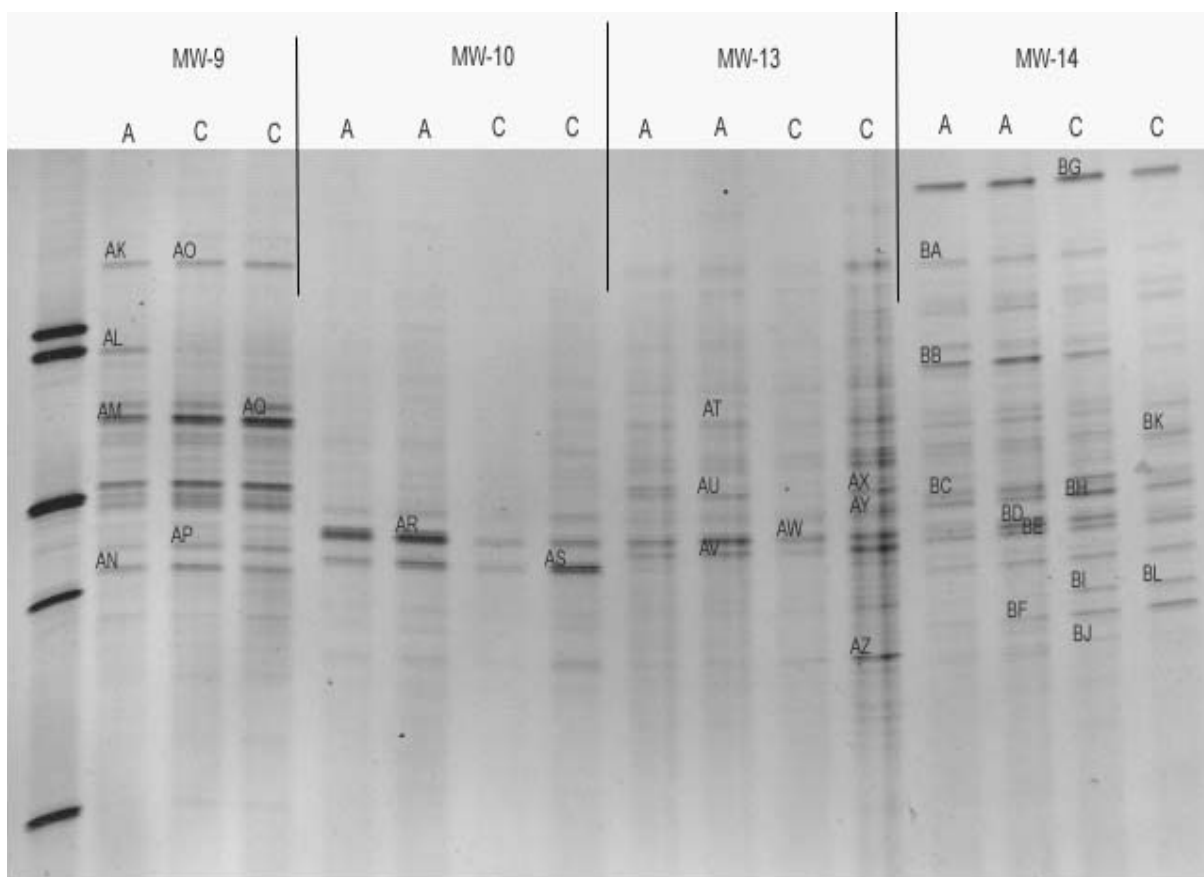


Figure 3-11 Sixty day DGGE profile of amplified DNA from a portion of the 16s rRNA gene (2nd gel). The first two columns labeled A represent the Ambersorb® duplicates. The next two columns labeled C are the Bio-Sep® duplicates. Every four columns represent one well. The lettered bands were excised and sequenced.

program, were organized into four trees based on the observed lineages (Figures 3-12, 3-13, 3-14, 3-15). In the delta Proteobacteria tree (Figure 3-12) organisms closely related to delta sulfate reducing bacteria were present in all wells except MW5 and MW6. *Desulfobulbus* was associated with a band in MW6 (N) at 30 days (Figure 3-8) and at 60 days only had a band with a low association (.664) when blasted against the Ribosomal Database. Especially dominant bands of delta sulfate reducing bacteria were seen on the DGGE gel Figure 3-8(R,U) and 3-10(U,Z) corresponding to MW7 at both time points. At 60 days the DGGE profile of MW10 no longer detected delta sulfate reducing bacteria while for MW13 they were detected only at 60 days. *Geobacter/Pelobacter* species were present in all wells downgradient of the plume (MW5, MW6, MW7, MW8, MW9, MW10). The *Geobacter/Pelobacter* related bands in MW5, Figure 3-8(C), 3-10(D, K, H) and MW6 Figure 3-8(L, O, K, M), 3-10 (O, Q, N, P, R) at both time points were much brighter and dominant than these same related bands in other wells. At 60 days the DGGE profile of MW9 and MW10 no longer detected *Geobacter/Pelobacter* species. Figure 3-13 shows band sequences of organisms closely related to *Campylobacter /Sulfurospirillum*, *Nitrospira*, *Spirochaete*, *Clostridia* and Gram-positive organisms. Sequences closely related to *Campylobacter/Sulfurospirillum* species were found in MW9 at both time-points, and MW5 at 30 days only. Sequences closely related to Gram-positive organisms were found in MW5 at 30 days only, MW14 at 60 days, and MW8 and MW13 at both times. MW8 was the only well whose band was medium in



Figure 3-12 Delta Proteobacteria tree.

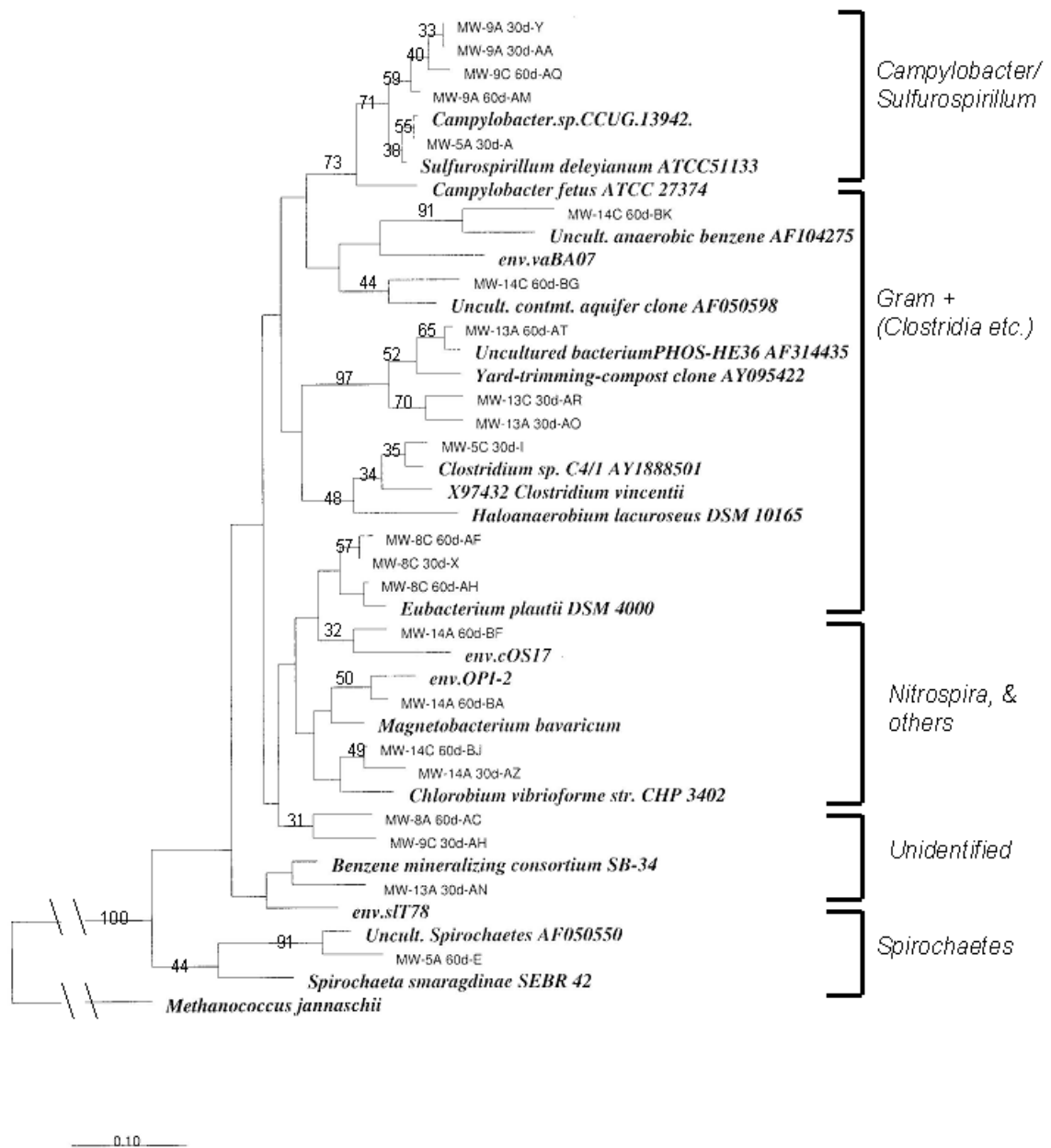


Figure 3-13 Epsilon, Gram-positive, and others tree.

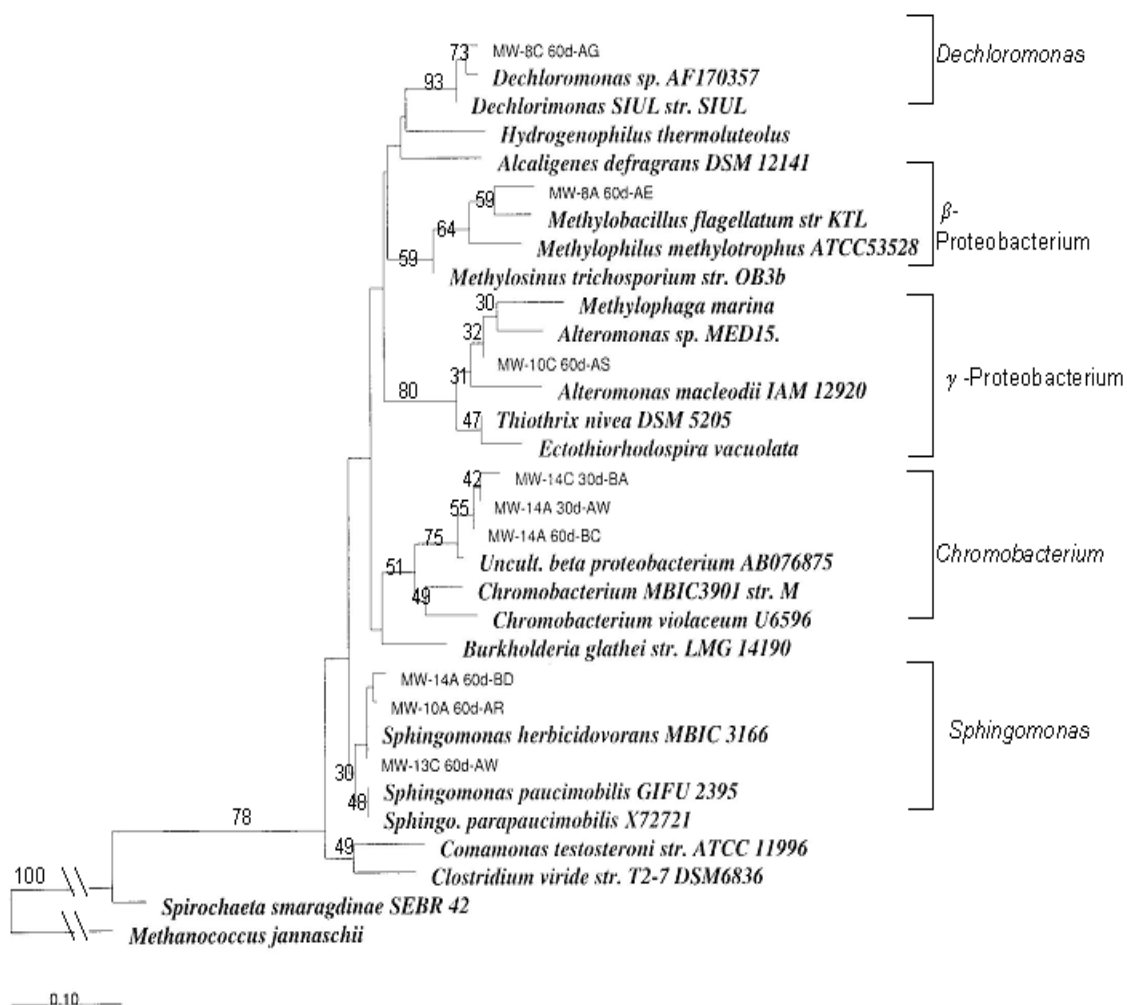


Figure 3-14 α, β, γ--Proteobacteria tree.

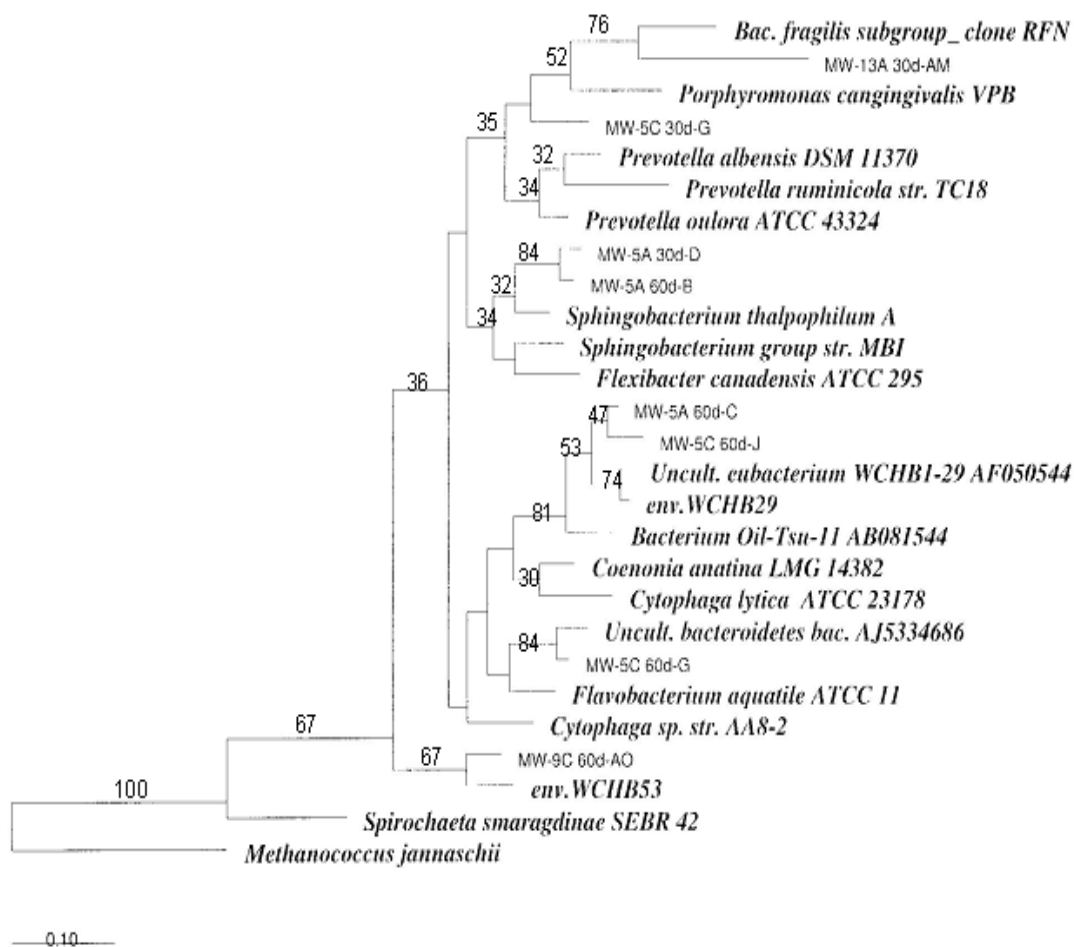


Figure 3-15 *Bacteroides* , *Flexibacter*, *Cytophaga* tree.

intensity (Figure 3-8(X), 3-10(AF), the rest were very light. All of the sequences in the tree related to Gram-positive organisms, when blasted through the Ribosomal Database Project showed association with Clostridia/Firmicutes. Bands AZ in Figure 3-9 and BF,BJ in Figure 3-11 of MW14 were found to be closely related to *Nitrospira*. Related organisms to *Spirochaetes* were detected in MW5 at 60 days (Figure 3-10 E). MW6 had sequenced bands closely related to an environmental clone F2 associated with petroleum contaminated iron-reducing conditions (Holmes et al., 2003), Geo-83 associated with anaerobic benzene degradation in petroleum-contaminated aquifer (Rooney et al., 1999) and a benzene-contaminated groundwater clone 2212. A band in MW9 was related to an uncultured delta Proteobacteria clone HYD 89-51 associated with sulfate-reducers and other bacteria in sediments above gas hydrate (Knittel et al., 2003).

The α , β , γ -Proteobacteria tree had sequences associated with it from MW8, MW10, MW13, and MW14. The DGGE profile of MW8 at 60 days had sequenced bands associated with *Dechloromonas* (AG) and other β -Proteobacteria (AE) (Figure 3-10). MW10 at 60 days had a band (AS) associated with γ -Proteobacteria (Figure 3-11). Sequenced bands from MW14 at both time-points showed relation to *Chromobacterium*. MW10, MW13, and MW14 all had bands in their DGGE profile at 60 days with close similarity to *Sphingomonas* species. This *Sphingomonas* band in MW10 (AR) was brighter in intensity when compared to MW13 (AW) and MW14 (BD) (Figure 3-11).

The Gram-negative *Flexibacter-Cytophaga-Bacteroides* phylum included sequenced bands from primarily MW5 and MW9 (Figure 3-15) with the exception of a faint band (AM) in MW13 at 30 days that had an association with *Bacteroides* (Figure 3-9). *Sphingobacterium* was associated with MW5 bands D (Figure 3-8) and B (Figure 3-10). MW9 had a band (AO) at 60 days (Figure 3-11) closely associated with environmental clone WCHB53 that was isolated from a hydrocarbon and chlorinated solvent-contaminated aquifer undergoing intrinsic bioremediation (Dojka et al., 1998). MW5 had 3 bands at 60 days (G,C,J in Figure 3-10) that were associated with various environmental clones .

Certain bands were much more intense or only present in Ambersorb® samples when compared to the control samples. These included bands associated to *Chromobacterium* in MW14 (AW) at 30 days, *Sphingomonas* in MW10 (AR) at 60 days, β -Proteobacteria in MW8 (AE) at 60 days, *Campylobacter* in MW5 (A) at 30 days, and one band (AA) of the delta sulfate reducing bacteria in MW8 at 60 days. Other bands were more intense or only present in control samples when compared to Ambersorb® samples. These included bands associated to Gram-positive organisms in MW5 (I) at 30 days, and uncultured delta Proteobacteria in MW9 (AE) at 30 days.

3.3 Quantitative Nucleic Acid Analysis Results

3.3.1 Eubacterial Biomass Results

The quantitative eubacterial biomass box and whiskers plot is pictured in Figure 3-16. The major significant difference in both traps at 30 days was the higher biomass in MW14 with regular beads. MW5 with Ambersorb® beads and MW5 and MW6 with regular Bio-Sep® beads had significantly more biomass than most other wells within each trap type. At 60 days Ambersorb® in MW14 had the highest biomass and increased greatly from 30 days. MW5, MW9, and MW13 were significantly similar and the next highest in biomass. These wells increased from the 30-day time point as well. Regular Bio-Sep® traps at 60 days showed MW13 with the highest significant biomass, but it was not significantly different from MW14 due to its high standard deviation. MW5 and MW9 were the next highest significant wells, followed by MW6, MW8, and MW10 grouping together. Many wells were significantly higher in biomass from the 30-day sampling as well as the Ambersorb® beads.

3.3.2 Iron-Reducing Bacteria/ Sulfate-Reducing Bacteria Results

All samples from the 30-day and 60-day time-points were tested for iron-reducing /sulfate-reducing (IRB/SRB) bacteria (Figure 3-17). At 30 days there was no significant difference between any wells and both types of traps. At 60 days MW14 was significantly greater than all other Ambersorb® traps in other wells. Ambersorb® MW14 was not significantly greater than regular Bio-

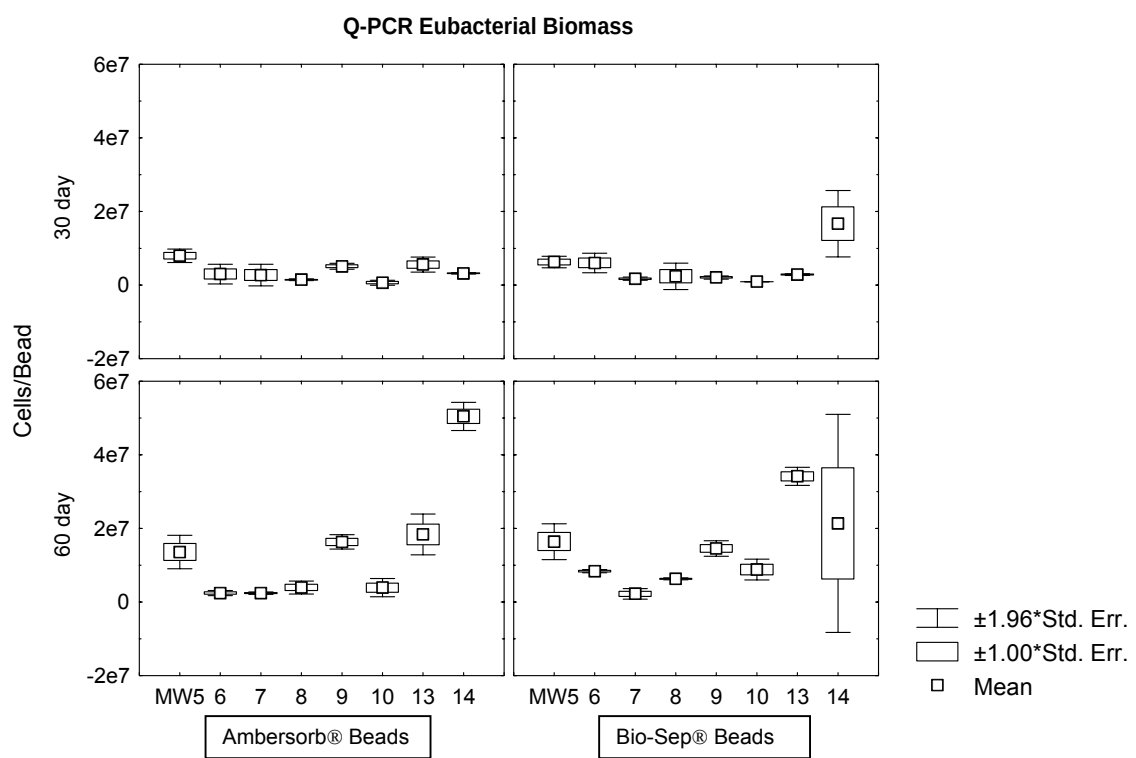


Figure 3-16 Quantitative eubacterial biomass.

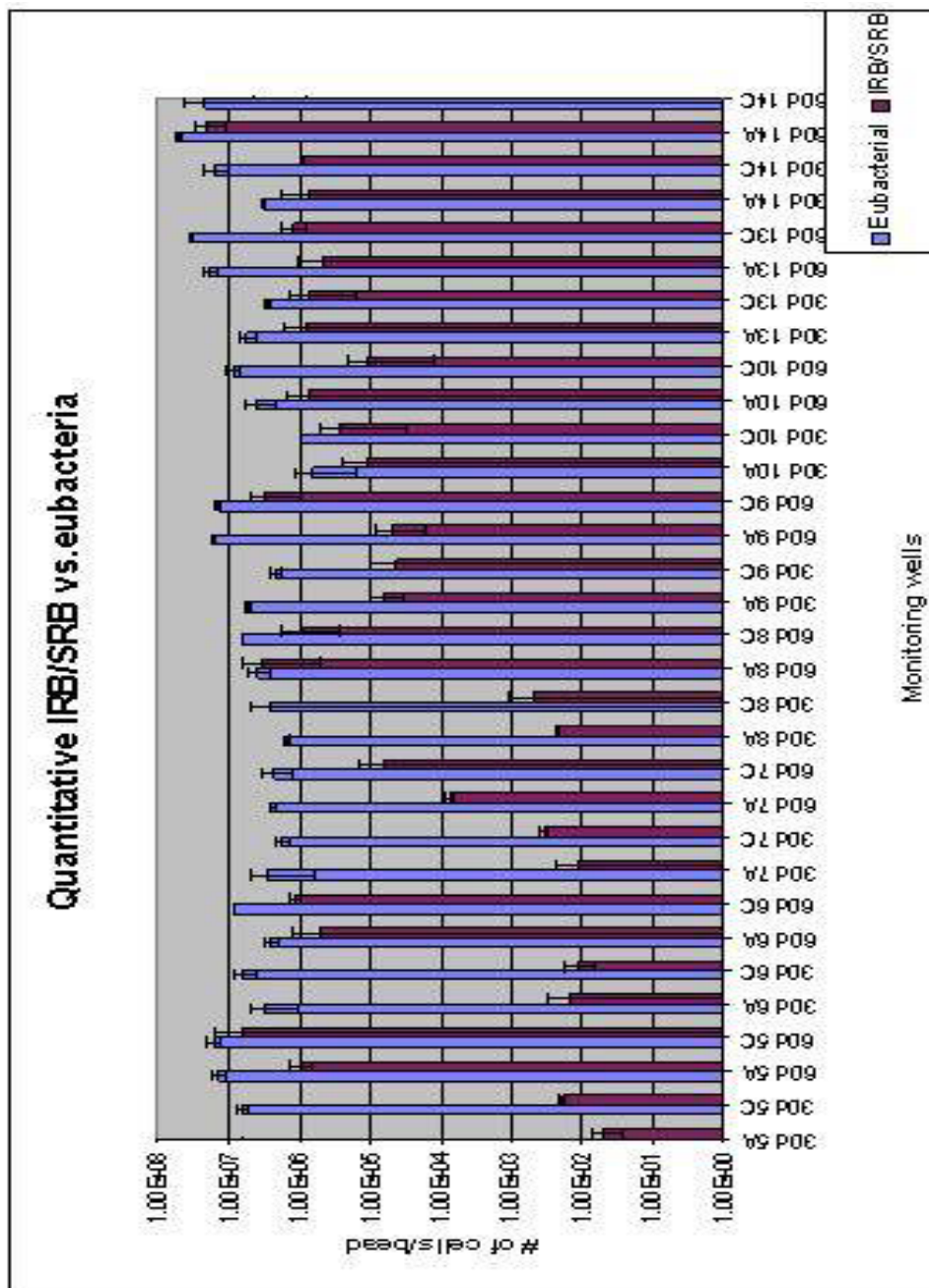


Figure 3-17 Iron-and sulfate-reducing quantitative PCR biomass vs. quantitative PCR eubacterial biomass. The A represents Ambersorb® beads and the C represents Bio-Sep® beads.

Sep® MW5. All other wells with regular Bio-Sep® traps showed no significant difference from each other or all Ambersorb® trap wells except MW14.

3.3.3 Methanogen Results

Quantitative methanogen PCR was tested only on 60-day time-point samples. The range of methanogen cells/bead was 10^3 to 10^5 . In Ambersorb® traps, MW9 had the highest significant biomass (Figure 3-18). Next highest were MW6, MW8, MW10, and MW13. MW7 was significantly lower in methanogen biomass followed by MW14. MW5 was close to MW9 but its high standard deviation did not make it significantly different from the 4 wells that grouped together in the Ambersorb® traps. Regular Bio-Sep® bead traps in MW5 and MW9 were both significantly higher in methanogen biomass than all other wells with this trap type. MW10 was the next highest in biomass, followed by MW6, MW8, and MW13, which all grouped together. MW6 was not significantly different from MW14, which was lower than the group. Comparing between traps showed that overall regular Bio-Sep® bead traps showed higher biomass. Figure 3-18 shows the comparison between methanogen biomass and eubacterial biomass as obtained from quantitative PCR.

3.4 Geochemical Parameter Results

The results to the geochemical parameters tested are listed in Table 3-1. They were performed on the day of the first sampling at 30 days. Geochemical

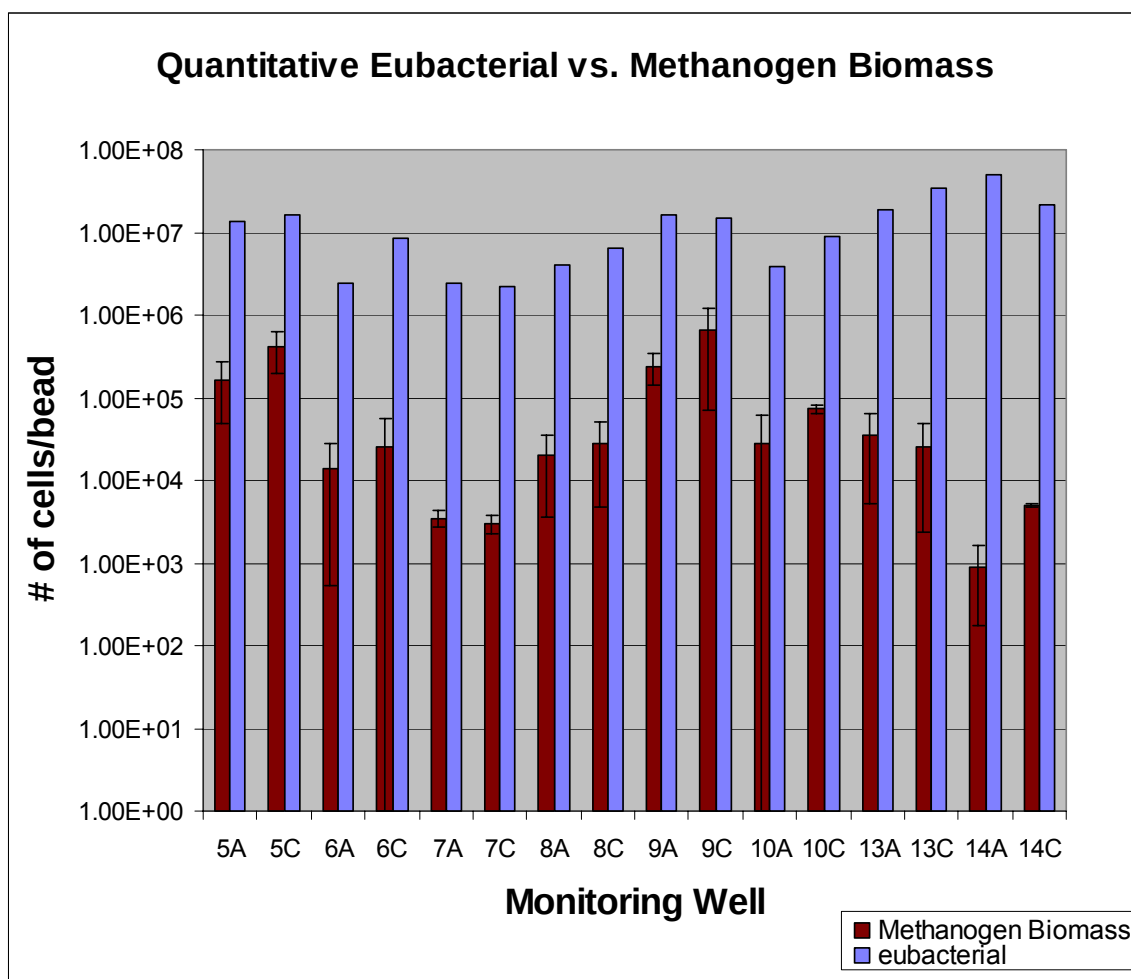


Figure 3-18 Methanogen biomass 60-day time-point as determined through quantitative PCR. The A represents Ambersorb® beads and the C represents Bio-Sep® beads.

Table 3-3 Geochemical parameters.

Reporting Laboratory: Microseeps LaPalma, CA			
Sample Description:	Analyte(s)	Result	Units
MW-5	Hydrogen	2.6	nM
MW-6		5.4	nM
MW-4		4.6	nM
MW-8		1.9	nM
MW-5	Oxygen	1.1	mg/L
MW-6		1.5	mg/L
MW-7		0.42	mg/L
MW-4		2.0	mg/L
MW-10		0.85	mg/L
MW-8		6.7	mg/L
FB		7.0	mg/L
MW-5	Carbon dioxide	190	mg/L
MW-6		140	mg/L
MW-7		310	mg/L
MW-4		17	mg/L
MW-10		59	mg/L
MW-8		17	mg/L
FB		0.63	mg/L
MW-5	Alkalinity as CaCO ₃	430	mg/L
MW-6		220	mg/L
MW-7		480	mg/L
MW-4		160	mg/L
MW-10		260	mg/L
MW-8		270	mg/L
MW-8 MS		380	mg/L
MW-8 MSD		390	mg/L
FB		15	mg/L
MW-5	Ferric Iron	< 1.0	mg/L
MW-6		1.2	mg/L
MW-7		< 1.0	mg/L
MW-4		< 1.0	mg/L
MW-10		< 1.0	mg/L
MW-8		< 1.0	mg/L
MW-8 MS		12	mg/L
MW-8 MSD		12	mg/L
FB		< 1.0	mg/L

Table 3-3 Continued

Reporting Laboratory:

Microseeps LaPalma, CA

MW-5	Ferrous Iron	5.2	mg/L
MW-6		18	mg/L
MW-7		17	mg/L
MW-4		< 1.0	mg/L
MW-10		< 1.0	mg/L
MW-8		< 1.0	mg/L
MW-8 MS		10	mg/L
MW-8 MSD		10	mg/L
FB		< 1.0	mg/L
MW-5	Divalent Manganese	1.8	mg/L
MW-6		1.9	mg/L
MW-7		2.3	mg/L
MW-4		< 1.0	mg/L
MW-10		< 1.0	mg/L
MW-8		< 1.0	mg/L
MW-8 MS		10	mg/L
MW-8 MSD		10	mg/L
FB		< 1.0	mg/L
MW-5	Methane	9500	ug/L
MW-6		7900	ug/L
MW-7		7400	ug/L
MW-4		2000	ug/L
MW-10		210	ug/L
MW-8		100	ug/L
FB		17	ug/L
MW-5	Nitrate	< 0.50	mg/L
MW-6		< 0.50	mg/L
MW-7		< 0.50	mg/L
MW-4		< 0.50	mg/L
MW-10		< 0.50	mg/L
MW-8		2.4	mg/L
MW-8 MS		11	mg/L
FB		< 0.50	mg/L
MW-5	Nitrogen	7.5	mg/L
MW-6		9.3	mg/L
MW-7		5.6	mg/L
MW-4		15	mg/L
MW-10		16	mg/L
MW-8		15	mg/L
FB		11	mg/L

Table 3-3 Continued

Reporting Laboratory:

Microseeps LaPalma, CA

MW-5	Sulfate	< 1.0	mg/L
MW-6		4.5	mg/L
MW-7		7.6	mg/L
MW-4		14	mg/L
MW-10		13	mg/L
MW-8		23	mg/L
MW-8 MS		27	mg/L
FB		< 1.0	mg/L
MW-5	Sulfide	2.9	mg/L
MW-6		< 2.0	mg/L
MW-7		< 2.0	mg/L
MW-4		< 2.0	mg/L
MW-10		< 2.0	mg/L
MW-8		< 2.0	mg/L
MW-8 MS		18	mg/L
MW-8 MSD		18	mg/L
FB		< 2.0	mg/L
MW-5	Ethane	5600	ng/L
MW-6		290	ng/L
MW-7		120	ng/L
MW-4		16	ng/L
MW-10		31	ng/L
MW-8		< 5.0	ng/L
FB		21	ng/L
MW-5	Ethene	< 5.0	ng/L
MW-6		< 5.0	ng/L
MW-7		51	ng/L
MW-4		< 5.0	ng/L
MW-10		17000	ng/L
MW-8		< 5.0	ng/L
FB		13	ng/L

parameter tests were performed only on monitoring wells 5, 6, 7, 8, and 10 and not on monitoring wells 9, 13, and 14. The dissolved hydrogen concentration of greater than 5 nM in MW6 is indicative of methanogenesis. High methane concentrations were evident in MW5, MW6, and MW7. Only MW8 had a high dissolved oxygen measurement (6.7mg/L). Carbon dioxide was highest in MW7 followed by MW5 and then MW6. MW10 had approximately half the amount of carbon dioxide as MW6. Fe (II) was high in MW6 and MW7 and moderate in MW5. Sulfate was highest in MW8 followed by MW10 at about half the amount. MW6 and MW7 had moderate sulfate levels. Sulfate was lowest in MW5 at below the level of detection. Sulfide levels were below the level of detection in all wells tested except MW5 (2.9 mg/L). Nitrate was below the level of detection in all wells except MW8 (2.4 mg/L).

Contaminants BTEX, MTBE, and TBA were tested on all wells and reported in parts per billion. These results are listed by well in the appendix. The most recent monitoring in 2003 showed non-detect levels of contaminants in MW13 and MW14. MW5 had the highest amount of BTEX, MTBE, and TBA in 2003 followed by MW6 with much lower amounts in comparison. MW6 was last tested in 2002. MW7 had low MTBE (5.7ppb) and high TBA (183 ppb). MW8 was last sampled in 1996 and had 59 ppb of MTBE. MW9 had non-detect levels of all contaminants as of 2002. MW10 had MTBE (10.2 ppb) and TBA (95.3 ppb) when last sampled in 2002.

4. Discussion

Figures 4-1 and 4-2 show a summary of the microorganisms found in each well after 30 days (4-1) and after 60 days (4-2) according to the Ribosomal Database Project and are very similar to the phylogenetic trees. There were more microbes and biomass detected after the 60-day sampling and thus was mainly used over the 30-day sampling. There was no great difference detected between the Ambersorb® and regular Bio-Sep® beads. Overall regular Bio-Sep® beads collected more biomass, however both types of beads varied in biomass.

MW13 and MW14 were located distal to the plume yet distant (~ 60-90ft.) (Figure 2-1). Unfortunately geochemical parameters were not tested on MW13 and MW14. MW13 showed non-detect levels of contaminants since 2000, while MW14 showed non-detect levels in 2003 but just 3 months prior showed the presence of MTBE and TBA. The microbial community in MW13 and MW14 consisted of delta sulfate-reducing bacteria, Gram-positive organisms (Firmicutes), *Nitrospira* (MW14 only), *Chromobacterium* (MW14 only) and *Sphingomonas*. The DGGE profile of MW14 showed a more diverse microbial community. PLFA analysis showed that MW13 had the highest amount of terminally branched saturates which are representative of Firmicutes and some Gram-negative bacteria, such as sulfate-reducers, both of which were

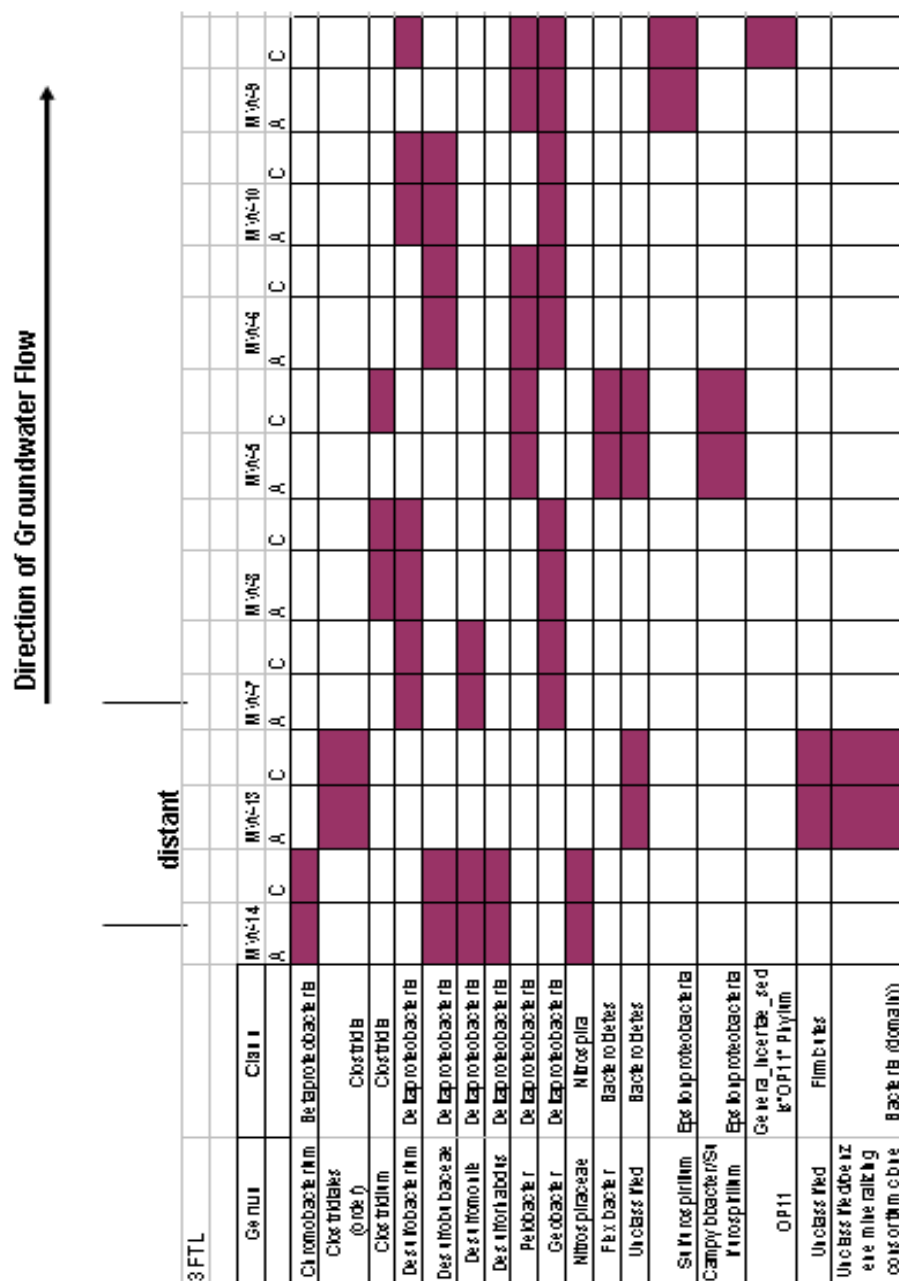


Figure 4-1 Summary of microorganisms found at 30-day time-point. Distant wells were MW13 and MW14, which were considered controls. The A represents Ambersorb® beads and the C represents Bio-Sep® beads.

represented in the nucleic acid analysis. PLFA analysis also showed a high amount of i17:1 ω 7c, and branched monoenoics, both biomarkers for sulfate-reducers in MW13 but not MW14. But quantitative PCR showed one of the highest IRB/SRB biomass estimates (10^6 - 10^7 cells/bead) for MW14. The stress biomarkers (cy/cis ratio) for the Gram-negative community were high for both MW13 and MW14 (1.7, 3.0 respectively) indicating the Gram-negative community did not have a sufficient source of carbon or other nutrients (i.e. phosphate, trace minerals) and were in stationary phase. Since contaminants can serve as a carbon source, the high starvation/stress biomarker may indicate the lack of contaminants in MW13 and MW14. Due to the position and contaminant levels of these wells, they may be considered controls to show which organisms were present indigenously without the presence of any contaminants. MW13 can be considered the overall control due to the detection of MTBE and TBA for a short period of time in MW14. Surprisingly MW14 also contained two different bands associated to two Gram-positive uncultured clones involved in anaerobic benzene degradation (Rooney-Varga et al., 1999) and hydrocarbon remediation (Dojka et al., 1998). The high presence of sulfate-reducers in the background wells may indicate that the presence of sulfate-reducers in the plume may not necessarily be responsible for the observed contaminant degradation. However contaminant degradation has been shown to occur through sulfate reduction (Somsamak et al., 2001), but usually the presence of contaminants enriches a certain community of microorganisms that

are present in background control wells in low amounts and usually not enriched. Monoenoic PLFA levels, indicative of Gram-negative bacteria, were relatively high and similar in all wells including the controls.

There was a shift in the bacterial community within the plume from the community distal and downgradient from the plume (MW13 and MW14). MW5 is located closest to the source of the plume and had the highest concentration of MTBE and BTEX, more than any other well since the first monitoring of the site in 1993 (Figure 2-1). PLFA showed that this well had the highest viable biomass probably due to the high amount of contaminants that can serve as a carbon source. Gram-negative communities in the plume showed more evidence of adapting to their stressful environment (.5 *trans/cis*) to maintain membrane integrity. The low *cy/cis* ratio indicated the Gram-negative community was in log growth phase. Geochemical parameters suggested that there was a moderate level of Fe(II) accumulation (5.2 mg/L) indicative of iron reduction but also showed Fe(III) depleted to below the level of detection. This indicates that iron reduction was occurring but may have already been exhausted. The *Pelobacter* genus was dominant only in MW5 at 60 days. *Pelobacter* are strict anaerobes that can use Fe(III) or sulfur as electron acceptors. The presence of *Pelobacter* is probably the result of the indigenous microbial response to MTBE/BTEX contamination due to selection pressure. Quantitative PCR supports IRB/SRB biomass, especially after 60 days. PLFA analysis also showed that MW5 was moderate in mid-chain branched saturates which are indicative of Gram-positive

bacteria and some metal reducing bacteria and moderate in terminally branched saturates found in *Bacteroides* and some Gram-negative bacteria (especially anaerobes). MW5 was moderate in branched monoenoics indicative of iron- and sulfate-reducers. Also unlike the control wells, MW5 had low eukaryotic PLFA (as determined through the polyenoic PLFA) indicating an anaerobic environment. MW5 had association to the *Sphingobacterium* genus, which is capable of aerobic biodegradation of fluorobenzene (Carvalho et al., 2002) and has been isolated from the rhizosphere of plants growing on oil-contaminated soil (Boronin et al., 2003). Also MW5 had association to environmental clones from the *Flexibacter-Cytophaga-Bacteroides* phylum including one associated with a hydrocarbon and chlorinated solvent-contaminated aquifer undergoing intrinsic bioremediation (Dojka et al., 1998). *Flavobacteria-Cytophaga* have been identified as part of degrading cultures that were shown to biodegrade MTBE and BTEX in a laboratory reactor (Sedran et al., 2002). Hierarchical cluster analysis showed that this well contained unique grouping of fatty acids probably due to *Pelobacter*, and *Flavobacteria-Cytophaga* being present only in this well. There was no detection of sulfate reducers in the DGGE profile, but due to the depletion of sulfate and sulfide concentration (2.9mg/L), this may have been a responsible process for contaminant biodegradation prior to sampling. In addition high methane concentrations (9500 ug/L) and high methanogen biomass estimation through quantitative PCR indicate that methanogenesis is a dominant process and that methanogens may be responsible for the reported decrease in

contaminant concentrations. Carbon dioxide, indicative of biodegradation of contaminants, was second highest in this well (190 mg/L) and also may provide an electron acceptor for methanogens. Methanogenesis has been found to be responsible for MTBE degradation in more and more studies. Kolhatkar et al. found a positive correlation between MTBE biodegradation at 6 sites and methanogenic conditions (2001).

MW6 had the second highest amount of BTEX originally and currently. It also had a high amount of MTBE and showed a gradual decrease of MTBE over the years of monitoring (Figure 2-1). MW6 did not show high level of biomarkers indicative of metabolic response to environmental stress and its Gram-negative community exhibited no lack of limiting nutrients. Geochemical monitoring suggested that there was a high level (18 mg/L) of reduced Fe(II) accumulation as well as a moderate level of sulfate (4.5 mg/L) indicating possible iron and sulfate reduction. Carbon dioxide (140 mg/L) was third highest in this well and indicative of contaminant biodegradation as well as possible methanogenesis. MW6 was mainly dominated by the *Geobacter* genus. *Geobacter* are anaerobes able to oxidize organic matter to carbon dioxide with Fe(III) as the electron acceptor (Coates and Lovley, 2001), and are most readily isolated from hydrocarbon-contaminated environments (Coates and Anderson, 2000). The prevalence of *Geobacter* in the monitoring wells closest to the plume is probably the result of the indigenous microbial community response to MTBE/BTEX contamination. MW6 had association with only one sulfate-reducing organism,

Desulfobulbus. Also MW6 had bands associated to environmental delta Proteobacteria clones that were associated with anaerobic benzene degradation and one clone F2 associated with petroleum contaminated iron-reducing conditions (Holmes et al., 2003). PLFA analysis showed moderate levels of mid-chain branched saturates and terminally branched saturates supporting anaerobes and some metal reducing bacteria. There is also evidence for methanogenesis occurring in this well through the methane concentration (7900 ug/L) and the methanogen PCR results.

Monitoring wells 7 and 8 had a somewhat similar DGGE banding profile. MW7 was located east of the plume and about 30 feet farther away than MW8 (Figure 2-1). This well had a lower initial concentration of MTBE but after a few years received a migration of more MTBE into the well. MW8 was closer and west of the plume with a higher initial MTBE concentration. BTEX was not detected in MW7 and MW8. The starvation/stress of Gram-negative bacteria was relatively greater than most wells with MW7 at .6 and MW8 at ~1.7 indicating a possible lack of limiting nutrients and stationary phase. The microbial community as determined through nucleic acid analysis consisted of sulfate-reducing delta Proteobacteria, this trend increased at 60 days. The sulfate-reducing bands associated with other sulfate-reducing bands in MW13 and MW14 in the phylogenetic tree but according to the Ribosomal Database Project MW7 had greater variety in the different types of sulfate-reducing genus (Figure 4-2). The bands associated with delta sulfate-reducing bacteria were especially

dominant in MW7 and both wells had very faint *Geobacter* bands. PLFA analysis in MW7 showed moderate levels of terminally branched saturates and branched monoenoics supporting anaerobic organisms and sulfate-reducing bacteria. Also MW7 showed a high level of i17:1 ω 7c biomarker for sulfate-reducers. Sulfate-reducing conditions have been associated with contaminant degradation (Somsamak et al., 2001). MW8 was not monitored since 1997 so it is not evident as to the concentration of contaminants in this well to date. In addition MW8 showed Gram-positive association, *Dechloromonas*, and β -Proteobacteria species. One band in MW8 belonging to the β -Proteobacteria had close association to *Methylosinus trichosporium* OB3b, which when grown on methane under copper-limited conditions oxidized diethyl ether, propane, and propylene with a low level of MTBE degradation (Hyman and O'Reilly, 1999). *Methylosinus trichosporium* OB3b is a methanotroph that uses oxygen as an electron acceptor and methane as an electron donor, both of which were present in MW8. Geochemical parameters showed that MW8 had a higher amount of sulfate (23 mg/L) detected than did MW7 (7.6 mg/L). Due to the higher intensity of the sulfate-reducing band on the DGGE gel and the lower sulfate concentration in this well, MW7 is probably experiencing more sulfate reduction, although quantitative PCR shows MW8 having a higher biomass of IRB/SRB bacteria than MW7. MW8 showed no geochemical signs of iron reduction and little support for methanogenesis (100 ug/L methane). But the lower methane concentration in MW8 may be due to its usage by *Methylosinus trichosporium* OB3b. Quantitative

PCR supports methanogens in both MW7 and MW8. On the other hand MW7 had high amounts of reduced Fe(II) accumulation (17 mg/L) and methane production (7400 ug/L). It appears that the microbial community in MW7 already depleted Fe(III) as an electron acceptor and may currently be undergoing sulfate-reduction and methanogenesis. Carbon dioxide (310mg/L) was the highest in MW7 indicating the biodegradation of contaminants and methanogenesis but also the high level may be explained by possible migration of carbon dioxide from upstream. A high dissolved oxygen measurement (6.7 mg/L) in MW8 supports the evidence that this is aerobic groundwater. Also MW8 had no detection of branched monoenoics, which are representative of obligate anaerobes, such as iron and sulfate-reducers, but had a high level of monoenoics. Other anaerobic indicators, terminally branched saturates and mid-chain branched saturates, were observed at a lower level from all other wells. The last time MW8 was sampled in 1996 it had an MTBE concentration of 59ppb. It is unknown what occurred in MW8 since then and what it contains presently.

MW10 had a high concentration of MTBE originally and it showed some fluctuation of MTBE over the years with a concentration of 10.2ppb in 2002. This well contained no BTEX and was further downgradient of the plume than MW7 and MW8 (Figure 2-1). The microbial community in MW10 changed between time-points from *Geobacter* and delta sulfate-reducers to *Sphingomonas* and γ -Proteobacteria closely associated to the *Alteromonas* genus, a bacterium that in the absence of oxygen can reduce Fe(III). The metabolic stress (t/c) ratio

decreased to below .1 after 60 days. MW10 had a lot of monoenoic PLFA and branched monoenoics but almost no mid-chain branched and terminally branched saturates supporting the presence of Gram-negative, sulfate-and iron-reducing bacteria in MW10. Geochemical parameters at 30 days showed a moderate amount of sulfate (13 mg/L) indicating the possibility of sulfate reduction and dissolved oxygen concentration (.85mg/L) was indicative of a primarily anaerobic community. Quantitative PCR supports iron-and sulfate-reducers at 60 days, although the DGGE profile does not. In addition Fe(III) and Fe(II) were below the limit of detection. Other parameters were very low including methane production (210 ug/L) at 30 days. Despite the reported concentration for methane, quantitative PCR methanogen biomass was moderate (10^4 cells/bead) and may support methanogenesis at 60 days. MW10 may be experiencing sulfate reduction and methanogenesis.

MW9 is positioned between MW5 and MW6 but further away from the source and general direction of groundwater flow (Figure 2-1). MW9 early on had trace evidence of BTEX, but as of 2000 was not detected. MW9 had a relatively high amount of MTBE originally but it decreased greatly after one year probably due to migration. Since 1994 the concentration of MTBE was low but varied and was not detected in 2002 when the well was last sampled. Surprisingly this well had the second highest amount of biomass with about half of that of MW5. The *cy/cis* stress biomarker was 0.5-1.25, indicating possible stationary phase. The community changed in between sampling dates where

Geobacter was no longer present at 60 days. One band was closely related to delta sulfate-reducing bacteria and another to *Campylobacter/Sulfurospirillum*, which usually reduce sulfur. This well had an association to a delta Proteobacteria clone HYD 89-51 associated with sulfate-reducers and other bacteria in sediments above gas hydrate (Knittel et al., 2003). Also MW9 had association to another environmental clone WCHB53 that was isolated from a hydrocarbon and chlorinated solvent-contaminated aquifer undergoing intrinsic bioremediation (Dojka et al., 1998). Quantitative IRB/SRB PCR and the amount of i17:1ω7c both support sulfate-reducing bacteria. Terminally branched saturates, mid-chain saturates, and branched monoenoics were relatively high indicative of an anaerobic environment. A high level of methanogens were detected through quantitative PCR. It is uncertain whether the primers for detection of methanogens may also be detecting other archaeal bacteria, or if methanogenesis is a naturally occurring process at this site, considering background wells also had a level of detection of methanogens. Even if methanogenesis is naturally occurring without the presence of contaminants, it still may play a large role in the degradation of MTBE and BTEX. Unfortunately geochemical parameters were not tested on this well. Due to the information gathered from the microbial analysis, this well needs to be monitored again to see if there was any migration of contaminants.

5. Conclusions

There appears to be no significantly important difference between Ambersorb® and Bio-Sep® beads. The 60-day time-point for sampling collected more biomass and more variety of microorganisms and thus was preferred over the 30-day time-point.

Methanogenesis seemed to be prevalent in all wells according to quantitative PCR analysis. Geochemical parameter testing supported high methane concentrations especially in MW5, MW6, and MW7. Methanogenesis could be naturally occurring at the site and may not simply be the response to the presence of MTBE and BTEX. It is also very likely that methanogens and methanogenesis are the signature organisms and process responsible for the biodegradation of MTBE and BTEX at this site.

The samples from the plume showed a difference from the control samples. Gram-negative communities in the samples closest to the plume (MW5 and MW6) did not show a lack of limiting nutrients (i.e. carbon) as did other wells further from the plume and were in log growth phase. Also the Gram-negative community in MW5 showed the highest adaptation to the environmentally stressful conditions through decreased membrane permeability.

Production of iron (II) and methane as end products of iron-reduction and methanogenesis was evident in MW5. *Pelobacter* was only present in MW5 at 60-days and may be responsible for the degradation of both BTEX and MTBE. There is strong microbial evidence for iron-reducers and methanogens in MW5

as well. Further studies are needed to conclude whether iron-reducers or methanogens, or both, were responsible for the MTBE and BTEX biodegradation. Also environmental clones associated to the *Flexibacter-Cytophaga-Bacteroides* phylum associated with hydrocarbon intrinsic bioremediation may play a role in contaminant biodegradation in MW5.

MW6 showed strong evidence for sulfate-reduction, iron-reduction, and methanogenesis in both the geochemistry and microbial community. *Geobacter* was dominant in MW6 and may have been part of the signature response to MTBE and BTEX contamination.

MW7 and MW8 only had MTBE and TBA contamination. MW7 had strong geochemical and microbial evidence for iron-reduction, sulfate-reduction, and methanogenesis. It appears that Fe(III) has already been depleted in MW7 and currently sulfate-reduction and methanogenesis may be responsible for the biodegradation of MTBE. *Geobacter*, delta sulfate-reducing bacteria, and methanogens may have been the organisms responsible for MTBE biodegradation. MW8 may be primarily aerobic with possible sulfate-reduction and methanogenesis. *Methylosinus trichosporium OB3b* may have been responsible for MTBE biodegradation in MW8, especially since MW8 appears to have oxygen and methane to support this methanotroph.

The shifting contaminant levels observed in some wells may be an indication that the groundwater is fluctuating back and forth and not flowing exactly in the

proposed direction of groundwater flow. This may explain the temporary MTBE and TBA concentrations in MW14 and the unusual characteristics of MW9.

MW9 had the second highest biomass, showed a high level of methanogens, a high level of sulfate-reducing bacteria, and had environmental clones associated with hydrocarbon remediation. It is uncertain as to the activity in this well considering in 2002 it had non-detect levels of contaminants. MW9 should be put under observation again to determine whether the detected results are a result of an influx of contaminants.

There was evidence for sulfate-reducing bacteria in all wells. MW7, which only had MTBE and TBA contamination, contained a greater variety of sulfate-reducing genus when compared to other wells, including the controls. Sulfate-reducing organisms may have been responsible for MTBE and BTEX biodegradation.

Further studies are needed to determine exactly which of the aforementioned processes are responsible for the observed degradation of MTBE and BTEX. One proposition is to investigate the anaerobic biodegradation of ^{14}C -MTBE in bench-scale microcosms that may use aquifer sediments from this site with various electron acceptors. For comparison to field samples Bio-Sep® bead “bug traps” may also be placed into the microcosms and analyzed using the same molecular techniques and may help in identifying microorganisms responsible for any observed MTBE biodegradation.

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Appendix

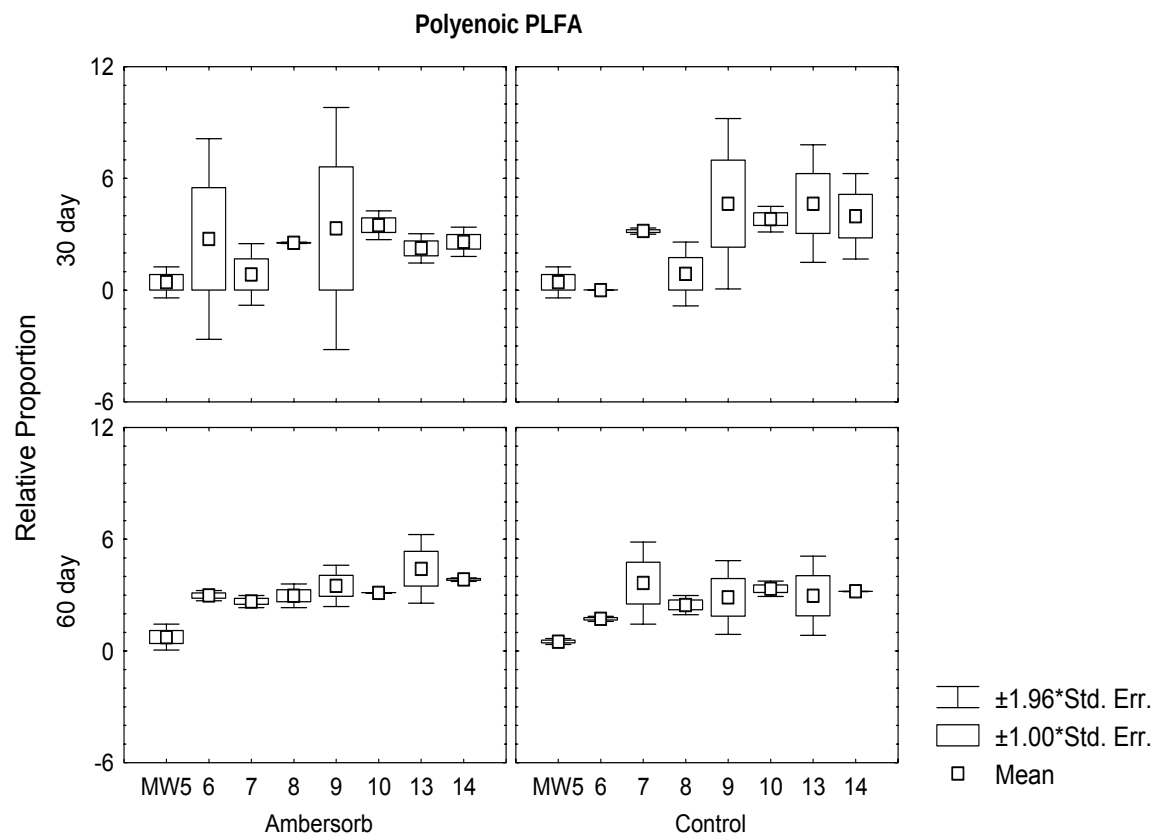


Figure A1 Relative proportion of total polyenoic PLFAs. Control indicates Bio-Sep® beads.

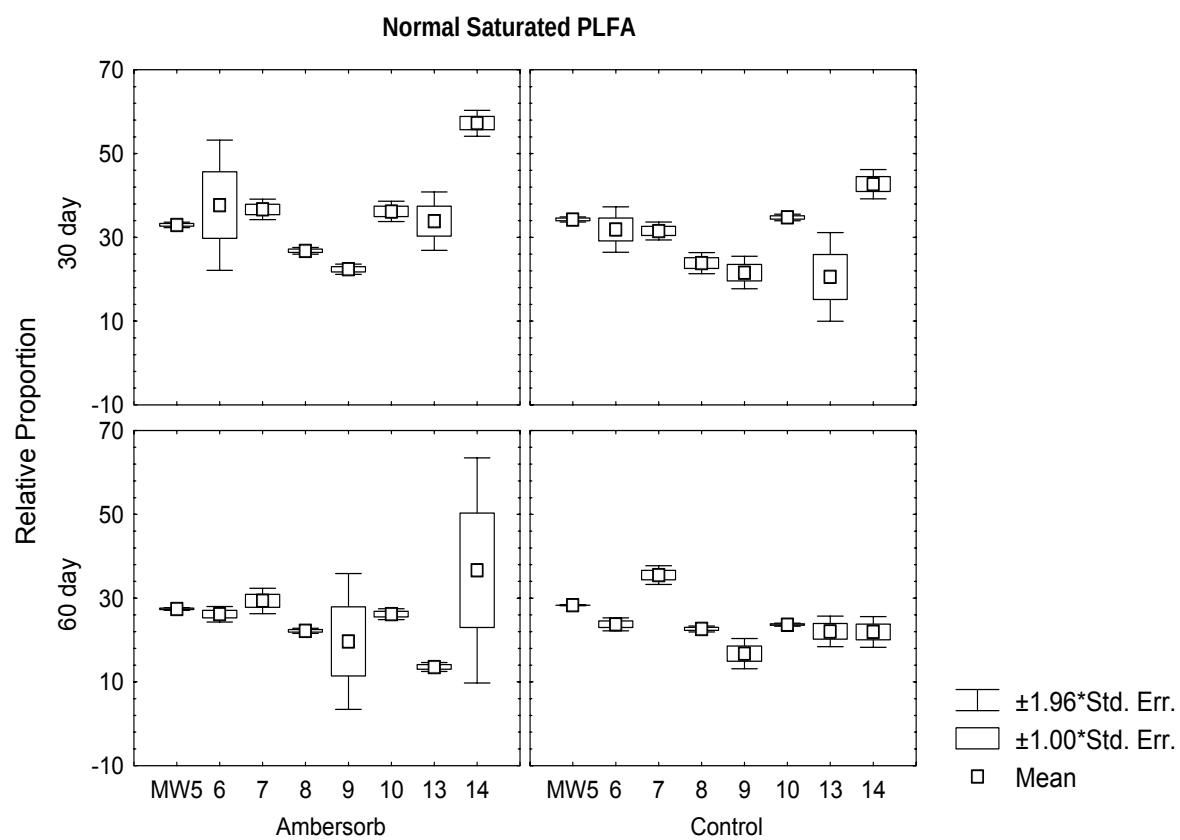


Figure A2 Relative proportion of total normal saturated PLFAs. Control indicates Bio-Sep® beads.

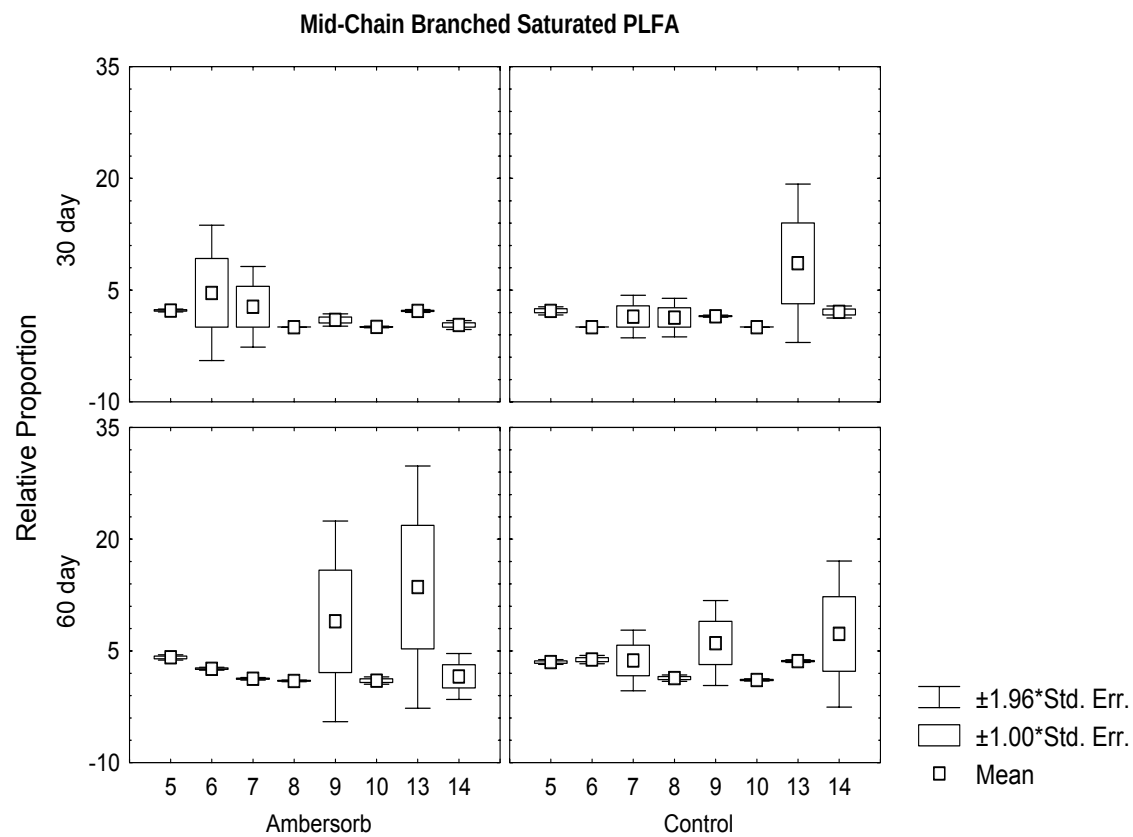


Figure A3 Relative proportion of total mid-chain branched saturated PLFAs. Control indicates Bio-Sep® beads.

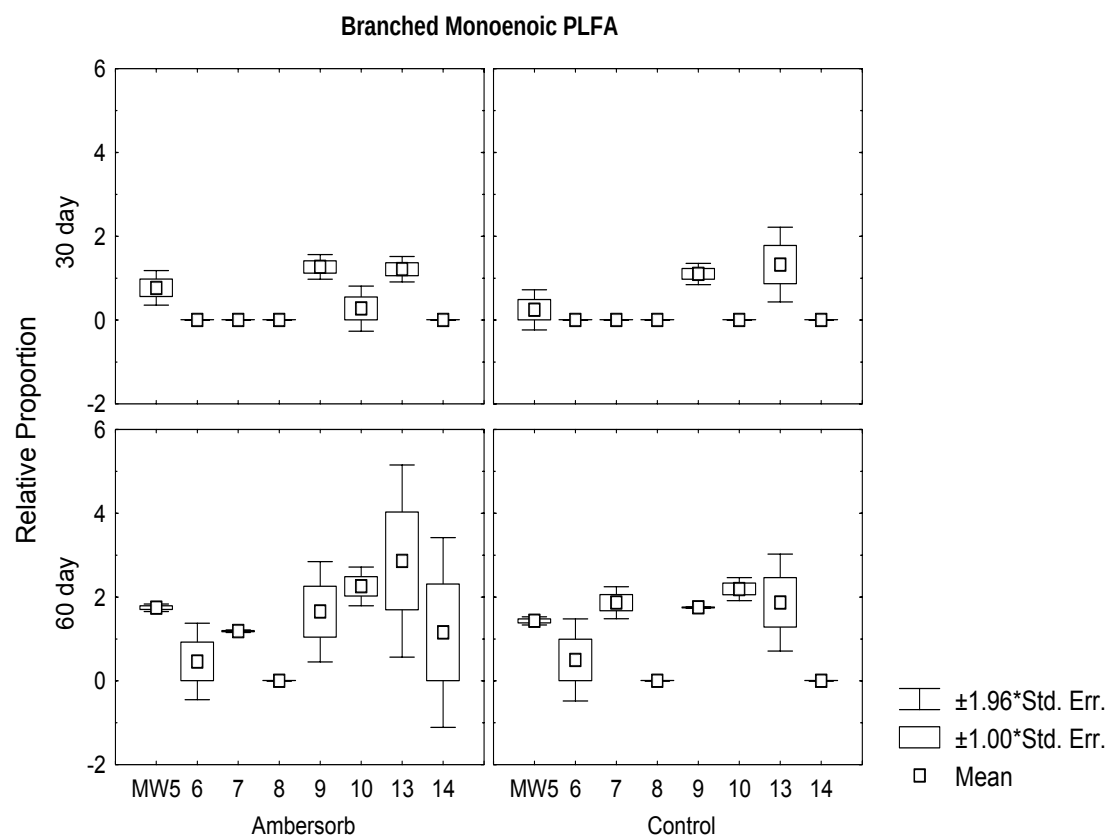


Figure A4 Relative proportion of total branched monoenoic PLFAs. Control indicates Bio-Sep® beads.

Table A1 Sequence results from bands excised from 30-day DGGE gel. Identifications are based on DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above .900 are considered excellent, .700-.800 are good, and below .600 are considered to be unique sequences. Phylogenic affiliations are presented as phylum followed by family unless otherwise noted.

Band	Closest Match	Similarity Index	Phylogenetic Affiliation
A	Campylobacter/Sulfurospirillum	.992	EpsilonProteobacteria
B	Failed		
C	Pelobacter	.915	DeltaProteobacteria
D	Flexibacter	0.623	Bacteroidetes
E	failed		
F	Failed		
G	Unclassified	.885	Bacteroidetes
H	Failed		
I	Clostridium	.926	Clostridia
J	Failed		
K	Geobacter	.882	DeltaProteobacteria
L	Pelobacter	.813	DeltaProteobacteria
M	Geobacter	0.814	Geobacter
N	Desulfobulbus	0.724	DeltaProteobacteria
O	Geobacter	.879	DeltaProteobacteria
P	Geobacter	0.852	DeltaProteobacteria
Q	Desulfobacterales (order)	.732	DeltaProteobacteria
R	Desulfobacterium	.737	DeltaProteobacteria
S	Desulfomonile	0.671	DeltaProteobacteria
T	Geobacter	0.865	DeltaProteobacteria
U	Unclassified	0.744	DeltaProteobacteria
V	novel		
W	Desulfobacterium/Desulfomonile	0.689	DeltaProteobacteria
X	Clostridium	.658	Clostridia
Y	Sulfurospirillum	1.00	EpsilonProteobacteria
Z	Geobacter	.778	DeltaProteobacteria
AA	Sulfurospirillum	1.00	EpsilonProteobacteria

Table A1 Continued

Band	Closest Match		Phylogenetic Affiliation
AC	Sphingobacteriales (order)	.780	Sphingobacteria
AD	Pelobacter	.735	DeltaProteobacteria
AE	Desulfobacterium	.756	DeltaProteobacteria
AF	OP11	.901	Genera_incertae_sedis"OP11" Phylum
AG	Thermodesulforhabdus	.651	Deltaproteobacteria
AH	Failed		
AI	NCBI		
AJ	Geobacter	.776	DeltaProteobacteria
AK	Geobacter	.649	DeltaProteobacteria
AL	Desulfobacterium	.737	DeltaProteobacteria
AM	Tannerella	.403	Bacteroidetes
AN	Unclassified/benzene mineralizing consortium clone SB-34	.627	Bacteria (domain))
AO	Clostridiales (order)	.785	Clostridia
AP	Failed		
AQ	Failed		
AR	Unclassified	.885	Firmicutes
AS	Failed		
AV	Failed		
AW	Chromobacterium	.845	BetaProteobacteria
AX	Desulfomonile	.746	DeltaProteobacteria
AY	Desulforhabdus	.761	DeltaProteobacteria
AZ	Nitrospiraceae	.649	Nitrospira
BA	Chromobacterium	.780	BetaProteobacteria
BB	Desulfomonile	.711	DeltaProteobacteria
BC	Desulfobulbus/Desulfocapsa/Desulforhopalus	.557	DeltaProteobacteria
BD	Failed		
BE	Desulfogustis	.696	DeltaProteobacteria

Table A2 Sequence results from bands excised from the 60-day DGGE gel. Identifications are based on DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above .900 are considered excellent, .700-.800 are good, and below .600 are considered to be unique sequences. Phylogenetic affiliations are presented as phylum followed by family unless otherwise noted.

Band	Closest Match	Similarity Index	Phylogenetic Affiliation
A	Failed	.	
B	Flavobacteriaceae	.708	Flavobacteria
C	Rickenella	.948	Bacteroidetes
D	Pelobacter	.954	DeltaProteobacteria
E	unclassified-Spirochaetaceae (family)	.719	Spirochaetes
F	Failed		
G	Capnocytophaga/Coenonia	.725	Flavobacteria
H	Pelobacter sp.	.650	DeltaProteobacteria
I	NCBI		Bacteroidetes
J	Rikenella	.800	Bacteroidetes
K	Pelobacter	.898	DeltaProteobacteria
L	Failed		
M	Failed		
N	Geobacter	.837	DeltaProteobacteria
O	unclassified-Desulfobulbaceae (family)	.664	DeltaProteobacteria
P	Geobacter	.860	DeltaProteobacteria
Q	Geobacter/NCBI	.836	DeltaProteobacteria
R	Trichlorobacter	.862	DeltaProteobacteria
S	Failed		
T	Syntrophobacter	.752	DeltaProteobacteria
U	Unclassified	.742	DeltaProteobacteria
V	Desulfomonile/NCBI	.767	DeltaProteobacteria
W	Geobacter	.817	DeltaProteobacteria
X	Desulforegula	.588	DeltaProteobacteria
Y	Desulfarculus	.779	DeltaProteobacteria
Z	Desulfobacterium/NCBI	.772	DeltaProteobacteria
AA	Desulfonema/Unclassified	.530	DeltaProteobacteria
AB	Desulfomonile	.765	DeltaProteobacteria
AC	unclassified/NCBI	.675	

Table A2 Continued

Band	Closest Match	Similarity Index	Phylogenetic Affiliation
AE	Unclassified	.828	BetaProteobacteria
AF	Clostridiaceae (family)	.695	Clostridia
AG	Dechloromonas	.856	BetaProteobacteria
AH	Clostridium/Unclassified	.632	Clostridia
AI	Syntrophobacter/Desulfarcus	.719	DeltaProteobacteria
AJ	Desulfocapsa/Desulfarcus	.711	DeltaProteobacteria
AK	Unclassified-Bacteroidales(order)/NCBI	.644	Bacteroidetes
AL	Failed		
AM	Sulfurospirillum	.863	EpsilonProteobacteria
AN	Failed		
AO	Bacteroidales(order)/NCBI	.594	Bacteroidetes
AP	NCBI		
AQ	unclassified/(Campylobacterales (order)	.963	EpsilonProteobacteria
AR	Sphingomonas/Porphyrobacter/Sphingobium	.873	AlphaProteobacteria
AS	uclassified/Oceanospiralles (order)	1.00	GammaProteobacteria
AT	Unclassified /NCBI	.964	Proteobacteria (phylum)
AU	Desulfobacterium/NCBI	.673	DeltaProteobacteria
AV	NCBI/uncultured		
AW	Sphingomonas	.969	AlphaProteobacteria
AX	Failed		
AY	Failed		
AZ	NCBI		
BA	Unclassified	.878	EpsilonProteobacteria
BB	Failed		
BC	Chromobacterium	.894	BetaProteobacteria
BD	Porphyrobacter	.952	AlphaProteobacteria
BE	Desulfomonile	.708	DeltaProteobacteria
BF	Unclassified/NCBI	.678	Nitrospira
BG	Unclassified	.733	Firmicutes (phylum)
BH	Unclassified-Syntrophobacteraceae(family)	.664	DeltaProteobacteria
BI	Desulfomonile/NCBI	.743	DeltaProteobacteria
BJ	Unclassified/NCBI	.780	Nitrospira (phylum)
BK	Unclassified/NCBI	.815	Clostridia
BL	Desulfomonile	.735	DeltaProteobacteria

Well ID	Date	Monitoring Data				Constituents of Concern					
		TOC	DTW	LPH Thickness	CGW/E	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
MW-13	9/2/2000	208.40	2.35	---	206.05	ND	ND	ND	ND	ND	ND
	12/2/2000	208.40	1.4	---	207	Well Not Sampled					
	3/16/2001	208.40	---	---	---	Well Not Sampled					
	6/27/2001	208.40	1.4	---	207	ND	ND	ND	ND	1.6	ND
	9/7/2001	208.40	3.25	---	205.15	ND	ND	ND	ND	ND	ND
	12/14/2001	208.40	2.17	---	206.23	ND	ND	ND	ND	ND	ND
	3/15/2002	208.40	1.5	---	206.9	ND	ND	ND	ND	6.1	ND
	7/12/2002	208.40	3.87	---	204.73	ND	ND	ND	ND	1.5	ND
	9/27/2002	208.40	0.82	---	207.58	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	ND (0.24)	ND (6.4)
	12/23/2002	208.40	1.76	---	206.64	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	ND (0.24)	ND (6.4)
	3/13/2003*	NS	0.8	---	---	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	ND (0.24)	ND (6.4)
	6/9/2003	---	1.11	---	---	ND (0.96)	ND (1.2)	ND (3.2)	ND (0.28)	ND (3.9)	ND (29)
MW-14 1-10	9/27/2002	Not Surveyed	1.17	---	Not Surveyed	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	ND (0.24)	ND (6.4)
	12/23/2002	Not Surveyed	2.51	---	Not Surveyed	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	12.7	182
	3/13/2003*	99.37	1.81	---	97.56	1.8	ND (0.15)	ND (0.12)	ND (0.11)	21.6	190
	6/9/2003	99.37	1.11	---	98.26	ND (0.19)	ND (0.24)	ND (0.65)	ND (0.56)	ND (0.77)	ND (5.9)
Notes:											
All concentrations are in parts per billion (ppb)											
TOC = Top of casing											
DTW = Depth to water											
LPH = Liquid phase hydrocarbons											
CGW/E = Corrected ground water elevation											
* = First event after well network survey on 1-28-03											

Figure A5 Contaminant levels in MW13 and MW14.

Well ID	Date	Monitoring Data				Constituents of Concern					
		TOC	DTW	LPH Thickness	CGM/E	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
MW-5	3/12/1993	210.42	184	---	208.78	8200	14000	1700	11000	1500	ND
	5/11/1993	210.42	161	---	208.81	NS	NS	NS	NS	NS	NS
	9/17/1993	210.42	332	---	207.10	10000	21000	2300	16000	1900	NS
	9/23/1994	210.42	263	---	207.79	10000	19000	2700	17000	1800	ND
	5/17/1996	210.42	140	---	208.02	7500	11000	1800	8900	1300	1300
	8/10/1996	210.42	206	---	208.36	4000	5500	1100	4600	980	ND
	11/7/1996	210.42	235	---	208.07	6200	8700	1700	10800	620	ND
	12/6/1997	210.42	254	---	207.88	5220	6400	2240	18320	500	ND
	3/27/1998	210.42	128	---	209.14	3990	9310	1810	10000	635	337
	7/23/1998	210.42	283	---	207.59	6130	8030	2530	11200	470	ND
2-15	9/18/1998	210.42	345	---	206.97	5590	5950	2490	129	1210	ND
	12/16/1998	210.42	312	---	207.30	3760	4000	2030	7130	379	491
	3/1/1999	210.42	122	---	209.20	5110	5490	1620	6180	700	1040
	6/21/1999	210.42	227	---	208.15	4510	5040	2280	10900	574	581
	9/7/1999	210.42	240	---	208.02	4450	5670	2490	12000	1050	ND
	12/30/1999	210.42	208	---	208.34	5040	6400	2070	9480	525	ND
	3/20/2000	210.42	129	---	209.13	5360	9690	1660	6970	501	ND
	6/22/2000	210.42	122	---	209.20	7800	13600	1680	7090	537	2280
	9/2/2000	210.42	203	---	208.39	3160	2860	920	1630	313	1210
	12/2/2000	210.42	195	---	208.47	3990	3300	1540	4940	3640	ND
	3/16/2001	210.42	130	---	209.12	5250	9750	1450	6040	386	ND
	6/27/2001	210.42	120	---	209.22	4500	7370	932	3290	314	ND
	9/7/2001	210.42	268	---	207.74	2720	1090	1920	7890	734	ND
	12/14/2001	210.42	291	---	207.51	4210	1360	2150	7330	733	ND
	3/15/2002	210.42	194	---	208.48	3910	5060	1300	5260	349	546
	7/12/2002	210.42	249	---	207.93	2810	593	1580	5610	251	300
	9/27/2002	210.42	159	---	208.83	4470	8160	984	3860	234	560
	12/23/2002	210.42	156	---	208.86	3230	5200	1420	5630	208	130
	3/13/2003*	100.71	107	---	99.84	4550	8690	1610	7290	301	ND (160)
	6/9/2003	100.71	111	---	99.60	2740	6740	748	3300	120	403

Figure A6 Contaminant levels in MW5.

Well ID	Date	Monitoring Data			Constituents of Concern						
		TOC	DTW	LPH Thickness	CGWE	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
M006	3/12/1993	210.37	1.90	---	208.47	490	1,200	290	1300	140	140
	5/11/1993	210.37	2.03	---	208.34			Well Not Sampled			
	9/17/1993	210.37	3.63	---	206.74	980	2,400	520	2600	270	NS
	9/23/1994	210.37	2.53	---	207.84	880	2,500	750	3200	200	130
	5/17/1996	210.37	1.67	---	208.70	1000	3600	630	2500	120	280
	8/10/1996	210.37	2.35	---	208.02	550	1,400	290	930	120	ND
	11/6/1996	210.37	2.59	---	207.78	340	600	140	584	66	ND
	12/8/1997	210.37	2.54	---	207.83			Well Not Sampled			
	3/27/1998	210.37	1.69	---	208.68	218	666	148	497	71.2	ND
	7/23/1998	210.37	3.14	---	207.23			Well Not Sampled			
	9/18/1998	210.37	3.71	---	206.66	330	92.2	30.2	129	44	ND
	12/16/1998	210.37	3.14	---	207.23			Well Not Sampled			
	3/1/1999	210.37	1.37	---	209.00	239	852	183	632	42.2	ND
	6/21/1999	210.37	2.45	---	207.92			Well Not Sampled			
	2-15	9/7/1999	210.37	2.51	---	207.86	609	2830	544	2140	61.8
12/30/1999		210.37	3.01	---	207.36			Well Not Sampled			
3/20/2000		210.37	1.58	---	208.79	176	502	99.2	284	36	140
6/22/2000		210.37	1.52	---	208.85			Well Not Sampled			
9/2/2000		210.37	2.25	---	208.12	254	418	103	281	35.6	229
12/2/2000		210.37	2.14	---	208.23			Well Not Sampled			
3/16/2001		210.37	1.59	---	208.78	135	362	107	293	50.7	235
6/27/2001		210.37	1.54	---	208.83			Well Not Sampled			
9/7/2001		210.37	2.94	---	207.43	184	155	43.5	200	34.7	138
12/14/2001		210.37	2.99	---	207.38			Well Not Sampled			
3/15/2002		210.37	2.15	---	208.22	181	487	113	363	27.4	124
7/12/2002		210.37	2.84	---	207.53			Well Not Sampled			
9/27/2002		210.37	2.01	---	208.36			Well Not Sampled			
12/23/2002		210.37	1.89	---	208.48			Well Not Sampled			
3/13/2003*		100.69	1.31	---	99.38			Well Not Sampled			
6/9/2003	100.69	1.36	---	99.33							

Figure A7 Contaminant levels in MW6.

Well ID	Date	Monitoring Data				Constituents of Concern					
		TOC	DTW	LPH Thickness	CGME	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
MW7 2-15	3/12/1993	209.85	1.60	---	208.25	ND	ND	ND	ND	19	67
	5/11/1993	209.85	1.90	---	207.95	---	---	Well Not Sampled	---	---	---
	9/17/1993	209.85	3.09	---	206.76	ND	ND	ND	0.57	30	NS
	9/23/1994	209.85	2.21	---	207.64	4.8	ND	ND	ND	150	110
	5/17/1996	209.85	1.55	---	208.30	3.8	ND	ND	ND	100	410
	8/10/1996	209.85	2.31	---	207.54	ND	ND	ND	ND	20	ND
	11/07/1996	209.85	2.32	---	207.53	ND	ND	ND	ND	17	60
	12/8/1997	209.85	2.26	---	207.59	---	---	Well Not Sampled	---	---	---
	3/27/1998	209.85	1.44	---	208.41	---	---	Well Not Sampled	---	---	---
	7/23/1998	209.85	2.78	---	207.07	---	---	Well Not Sampled	---	---	---
	9/18/1998	209.85	3.21	---	206.64	---	---	Well Not Sampled	---	---	---
	12/16/1998	209.85	2.74	---	207.11	---	---	Well Not Sampled	---	---	---
	3/1/1999	209.85	1.22	---	208.63	---	---	Well Not Sampled	---	---	---
	6/21/1999	209.85	1.92	---	207.93	---	---	Well Not Sampled	---	---	---
	9/7/1999	209.85	2.01	---	207.84	---	---	Well Not Sampled	---	---	---
	12/30/1999	209.85	2.48	---	207.37	---	---	Well Not Sampled	---	---	---
	3/20/2000	209.85	1.41	---	208.44	---	---	Well Not Sampled	---	---	---
	6/22/2000	209.85	1.43	---	208.42	---	---	Well Not Sampled	---	---	---
	9/2/2000	209.85	2.08	---	207.77	---	---	Well Not Sampled	---	---	---
	12/2/2000	209.85	1.94	---	207.91	---	---	Well Not Sampled	---	---	---
	3/16/2001	209.85	1.34	---	208.51	---	---	Well Not Sampled	---	---	---
	6/27/2001	209.85	---	---	---	---	---	Well Not Sampled	---	---	---
	9/7/2001	209.85	2.74	---	207.11	---	---	Well Not Sampled	---	---	---
	12/14/2001	209.85	1.47	---	208.38	---	---	Well Not Sampled	---	---	---
	3/15/2002	209.85	1.88	---	207.97	---	---	Well Not Sampled	---	---	---
	7/12/2002	209.85	2.81	---	207.04	---	---	Well Not Sampled	---	---	---
	9/27/2002	209.85	1.42	---	208.43	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	1.3	28
	12/23/2002	209.85	1.95	---	207.9	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	5.9	358
	3/13/2003*	100.11	1.19	---	98.92	ND (0.23)	ND (0.15)	ND (0.12)	ND (0.11)	5.7	183
	6/9/2003	100.11	1.56	---	98.55	---	---	---	---	---	---

Figure A8 Contaminant levels in MW7.

Well ID	Date	Monitoring Data				Constituents of Concern					
		TOC	DTW	LPH Thickness	CGWE	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
MW8	5/11/1993	210.50	1.98	---	208.52	ND	ND	ND	ND	73	26
	9/17/1993	210.50	3.47	---	207.03	0.65	ND	ND	1.6	67	NS
	9/23/1994	210.50	2.51	---	207.99	3.6	ND	ND	ND	70	12
	5/17/1996	210.50	1.58	---	208.92	ND	ND	ND	ND	84	38
	8/10/1996	210.50	2.29	---	208.21	ND	ND	ND	ND	48	ND
	11/7/1996	210.50	2.43	---	208.07	ND	1.2	ND	ND	59	ND
	12/6/1997	210.50	2.57	---	207.93	Well Not Sampled					
	3/27/1998	210.50	1.55	---	208.95	Well Not Sampled					
	7/23/1998	210.50	2.94	---	207.56	Well Not Sampled					
	9/18/1998	210.50	3.52	---	206.98	Well Not Sampled					
	12/16/1998	210.50	3.45	---	207.05	Well Not Sampled					
	3/1/1999	210.50	1.61	---	208.89	Well Not Sampled					
	6/21/1999	210.50	2.49	---	208.01	Well Not Sampled					
	9/7/1999	210.50	2.70	---	207.80	Well Not Sampled					
	12/30/1999	210.50	3.14	---	207.36	Well Not Sampled					
	3/20/2000	210.50	1.62	---	208.88	Well Not Sampled					
	6/22/2000	210.50	1.56	---	208.94	Well Not Sampled					
	9/2/2000	210.50	2.28	---	208.22	Well Not Sampled					
	12/2/2000	210.50	2.08	---	208.42	Well Not Sampled					
	3/16/2001	210.50	1.60	---	208.90	Well Not Sampled					
	6/27/2001	210.50	1.50	---	209.00	Well Not Sampled					
	9/7/2001	210.50	3.00	---	207.50	Well Not Sampled					
	12/14/2001	210.50	3.06	---	207.44	Well Not Sampled					
	3/15/2002	210.50	2.29	---	208.21	Well Not Sampled					
	7/12/2002	210.50	2.69	---	207.81	Well Not Sampled					
	9/27/2002	210.50	2.40	---	208.10	Well Not Sampled					
	12/23/2002	210.50	1.75	---	208.75	Well Not Sampled					
	3/13/2003*	100.82	1.32	---	99.50	Well Not Sampled					
	6/9/2003	100.82	1.62	---	99.20	Well Not Sampled					

Figure A9 Contaminant levels in MW8.

Well ID	Date	Monitoring Data				Constituents of Concern					
		TOC	DTW	LPH Thickness	CGWE	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA
MW9	5/11/1993	211.49	2.96	---	208.53	ND	ND	7.7	7.9	140	94
	9/17/1993	211.49	4.45	---	207.04	11	ND	ND	12	140	NS
	9/23/1994	211.49	3.25	---	208.24	13	ND	ND	9.7	14	NS
	5/17/1996	211.49	2.54	---	208.95	2.6	ND	ND	ND	ND	ND
	8/10/1996	211.49	3.36	---	208.13	4.1	ND	ND	2	24	ND
	11/7/1996	211.49	3.52	---	207.97	1.7	4.3	1.5	ND	12	ND
	12/6/1997	211.49	3.63	---	207.86	---	---	Well Not Sampled	---	---	---
	3/27/1998	211.49	2.65	---	208.84	ND	ND	ND	ND	4.4	4
	7/23/1998	211.49	4.12	---	207.37	---	---	Well Not Sampled	---	---	---
	9/18/1998	211.49	4.71	---	206.78	ND	ND	ND	ND	7.68	23.7
	12/16/1998	211.49	4.34	---	207.15	---	---	Well Not Sampled	---	---	---
	3/1/1999	211.49	2.39	---	209.10	ND	ND	ND	ND	1.6	ND
	6/21/1999	211.49	3.73	---	207.76	---	---	Well Not Sampled	---	---	---
	9/7/1999	211.49	3.92	---	207.57	10.2	3.1	ND	1.4	12.5	ND
	12/30/1999	211.49	2.07	---	209.42	---	---	Well Not Sampled	---	---	---
	3/20/2000	211.49	2.71	---	208.78	ND	ND	ND	ND	2	11.3
	6/22/2000	211.49	2.96	---	208.53	---	---	Well Not Sampled	---	---	---
	9/2/2000	211.49	3.59	---	207.90	ND	ND	ND	ND	2.8	79.1
	12/2/2000	211.49	3.39	---	208.10	---	---	Well Not Sampled	---	---	---
	3/16/2001	211.49	2.74	---	208.75	ND	ND	ND	ND	1.9	ND
	6/27/2001	211.49	2.08	---	209.41	---	---	Well Not Sampled	---	---	---
	9/7/2001	211.49	4.20	---	207.29	ND	1.8	ND	ND	4.9	24.3
	12/14/2001	211.49	4.67	---	206.82	---	---	Well Not Sampled	---	---	---
	3/15/2002	211.49	3.43	---	208.06	ND	ND	ND	ND	2.7	ND
	7/12/2002	211.49	4.00	---	207.49	ND	ND	ND	ND	ND	ND
	9/27/2002	211.49	2.87	---	208.62	---	---	Well Not Sampled	---	---	---
	12/23/2002	211.49	2.96	---	208.53	---	---	Well Not Sampled	---	---	---
	3/13/2003*	102.00	2.23	---	99.77	---	---	Well Not Sampled	---	---	---
	6/9/2003	102.00	1.39	---	100.61	---	---	---	---	---	---

Figure A10 Contaminant levels in MW9

Well ID	Date	Monitoring Data			Constituents of Concern							
		TOC	DTW	LPH Thickness	CGW/E	Benzene	Toluene	Ethylbenzene	Xylenes	MTBE	TBA	
MW-10	5/11/1993	210.39	2.30	---	208.09	ND	ND	ND	27	290	72	
	9/17/1993	210.39	3.93	---	206.46	12	ND	3.5	12	180	NS	
	9/23/1994	210.39	3.23	---	207.16	ND	ND	ND	ND	5.3	ND	
	5/17/1996	210.39	1.73	---	208.66	ND	ND	ND	ND	5.3	19	
	8/10/1996	210.39	2.79	---	207.60	ND	ND	ND	ND	23	120	
	11/7/1996	210.39	2.92	---	207.47	ND	0.75	1.3	1	13	ND	
	12/8/1997	210.39	2.96	---	207.43	Well Not Sampled						
	3/27/1998	210.39	2.09	---	208.30	ND	ND	ND	ND	3.4	3.7	
	7/23/1998	210.39	3.69	---	206.70	Well Not Sampled						
	9/18/1998	210.39	4.24	---	206.15	ND	ND	ND	ND	ND	ND	
	12/16/1998	210.39	3.51	---	206.88	Well Not Sampled						
	3/1/1999	210.39	1.14	---	209.25	ND	ND	ND	ND	4.41	ND	
	6/21/1999	210.39	3.03	---	207.36	Well Not Sampled						
	9/7/1999	210.39	3.03	---	207.36	ND	ND	ND	ND	18.6	269	
	12/30/1999	210.39	3.01	---	207.38	Well Not Sampled						
	3/20/2000	210.39	2.07	---	208.32	ND	ND	ND	ND	6.4	50	
	6/22/2000	210.39	1.85	---	208.54	Well Not Sampled						
	9/2/2000	210.39	2.85	---	207.54	ND	1.1	ND	ND	18.9	152	
	12/22/2000	210.39	2.85	---	207.74	Well Not Sampled						
	3/16/2001	210.39	2.06	---	208.33	ND	ND	ND	ND	4	44.1	
	6/27/2001	210.39	1.98	---	208.41	Well Not Sampled						
	9/7/2001	210.39	3.60	---	206.79	ND	ND	ND	ND	12.8	160	
	12/14/2001	210.39	3.50	---	206.89	Well Not Sampled						
	3/15/2002	210.39	2.70	---	207.69	ND	ND	ND	ND	10.2	95.3	
	7/12/2002	210.39	3.66	---	206.83	Well Not Sampled						
	9/27/2002	210.39	1.05	---	209.34	Well Not Sampled						
	12/23/2002	210.39	2.43	---	207.96	Well Not Sampled						
	3/13/2003*	101.02	1.55	---	99.47	Well Not Sampled						
	6/9/2003	101.02	1.89	---	99.13	Well Not Sampled						

Figure A11 Contaminant levels in MW10

Vita

Anita Eva Biernacki was born June 29, 1979 in Warsaw, Poland. She graduated from Seminole High School in Seminole, FL in 1997. She received her undergraduate degree in biology from the University of South Florida in Tampa, FL in 2001. A desire to continue studies at the graduate level resulted in enrollment at the University of Tennessee, Knoxville. She entered the Microbiology department and worked in the laboratory of Dr. David White, completing the Master of Science degree in 2004.