THESIS

THERMALLY ENHANCED BIOREMEDIATION OF LNAPL

Submitted by

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ABSTRACT

THERMALLY ENHANCED BIODEGRADATION OF LNAPL

Inadvertent releases of petroleum liquids into the environment have led to widespread soil and groundwater contamination. Petroleum liquids, referred to as Light Non-Aqueous Phase Liquid (LNAPL), pose a threat to the environment and human health. The purpose of the research described herein was to evaluate thermally enhanced bioremediation as a sustainable remediation technology for rapid cleanup of LNAPL zones. Thermally enhanced LNAPL attenuation was investigated via a thermal microcosm study that considered six different temperatures: 4°C, 9°C, 22°C, 30°C, 35°C, and 40°C. Microcosms were run for a period of 188 days using soil, water and LNAPL from a decommissioned refinery in Evansville, WY. The soil microcosms simulated anaerobic subsurface conditions where sulfate reduction and methanogenesis were the pathways for biodegradation. To determine the optimal temperature range for thermal stimulation and provide guidance for design of field-scale application, both contaminant degradation and soil microbiology were monitored.

CH_4 and CO_2 generation occurred in microcosms at 22°C, 30°C, 35°C and 40°C but was not observed at 4°C and 9°C. The total volume of biogas generated after 188 days of incubation was 19 times higher in microcosms at 22°C and 30°C compared to the microcosms at 35°C. When compared to microcosms at 40°C, the total biogas generated was 3 times higher at both 22°C and 30°C. The onset of CH_4 and CO_2 production occurred first within the microcosms held at 30°C beginning after 28 days of incubation, and second within microcosms at 22°C beginning after 58 days of incubation. A delay in CH_4 and CO_2 production was observed within microcosms held at 35°C and 40°C (beginning after 173 and 138 days after incubation)

contributing to lower cumulative biogas generation at these temperatures relative to 22ºC and 30ºC. Microcosms incubated at 4ºC, 22°C, 30ºC, 35 ºC and 40 ºC showed statistically significant biological removal of gasoline range organics (GRO) at the α=0.05 level over the course of the 188-day incubation period. Biotic removal of GRO was significantly higher at 22ºC and 30ºC when compared to 4ºC and 9ºC. The observed removals at 22 ºC, 30 ºC, 35 ºC and 40ºC were not statistically different from each other. The biological removal of DRO compounds was found to be statistically significant at 22ºC, 30 ºC, 35 ºC and 40 ºC and was significantly higher at 22ºC when compared to 4ºC and 9ºC. The percent biological removal of DRO compounds at these temperatures was not statistically different from each other and ranged from 18-22%. Statistically significant biological degradation of all the BTEX compounds only occurred in microcosms at 22°C and 30°C. Benzene, toluene, and xylene biodegradation was observed to be statistically significant in microcosms at 35°C.

Phylogenetic analysis of the microbial communities present at 22°C via a 16S rRNA gene archaeal clone library and pyrosequencing revealed the presence of both hydrogenotrophic and acetoclastic methanogens. Pyrosequencing data from microcosms at 4°C, 22°C and 35°C revealed that temperature impacted both archaeal and bacterial community structure. A potential requirement for changes in microbial community structure at the higher temperatures (35°C and 40°C) could explain the observed delay in biogas production.

The results of the thermal microcosm study led to the development of a pilot study to investigate the efficacy of Sustainable Thermally Enhanced LNAPL Attenuation STELA at the site in Evansville, WY. The thermal pilot study approach and preliminary data is included in this

thesis. The outcome and results of the pilot study will be presented in the masters theses of other students.

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1 INTRODUCTION

Production, storage, transport and refining of petroleum liquids has led to widespread soil and groundwater contamination (Callaghan et al. 2010). Petroleum liquids contain hazardous compounds including benzene, toluene, ethylbenzene, and the xylenes (BTEX), that pose environmental and human health risks (White and Claxton 2004)). Fortunately, natural processes (e.g. biological natural attenuation) often deplete petroleum hydrocarbons in the subsurface, albeit at slow rates (Romantschuk et al. 2000). When natural processes are insufficient relative to cleanup expectations, active remedial measures need to be employed.

Biological natural attenuation of petroleum hydrocarbons within the subsurface is affected by the biogeochemical conditions. In subsurface areas where petroleum liquids, termed light non-aqueous phase liquid (LNAPL), are present, anaerobic conditions often prevail because the O_2 demand exceeds the available O_2 (Bouwer and Zehnder 1993). Under anaerobic conditions, Mn^{4+}, NO_3^-, Fe^{3+}, and SO_4^{-2} can serve as electron acceptors for biological degradation of LNAPL constituents. Once these more thermodynamically favored electron acceptors are depleted, methanogenesis can become a dominant long-term pathway within LNAPL zones (Amos and Mayer 2006). Methanogenesis is a beneficial process because it is not limited by the availability of an electron acceptor. Thus, active remedial technologies must be developed to be viable for application in LNAPL zones under anaerobic conditions, including methanogenic conditions. To support widespread implementation, these technologies should be cost-effective, efficient, and environmentally sustainable.

1.1 RESEARCH MOTIVATION

Both *ex situ* and *in situ* remediation technologies have been developed to treat LNAPL in subsurface environments. Current technologies used to treat and manage petroleum hydrocarbon contaminated sites include hydraulic recovery of LNAPL, soil vapor extraction, hydraulic containment, physical containment, air sparging, bioventing, passive/reactive treatment walls, and natural attenuation (Khan et al. 2004). Physical treatments (e.g. hydraulic recovery of LNAPL and soil vapor extraction) result in only partial depletion of LNAPL. Unfortunately, they are often expensive and can leave significant LNAPL in place. Remediation technologies that rely on biological degradation of contaminants, commonly known as bioremediation, can be an efficient, economical and versatile alternative to physical treatments (Karamalidis et al. 2010) (Couto et al. 2010). A prospective but not widely used biological treatment is thermally enhanced bioremediation (Beyke and Fleming 2005). Thermally enhanced bioremediation is a promising and potentially sustainable remediation technology that has been provisionally termed Sustainable Thermally Enhanced LNAPL Attenuaion (STELA). Specifically, STELA relies on the application of low levels of heating (increases of 10-30 °C) to achieve optimum conditions for biodegradation processes. Although, STELA has the potential to be a cost-effective and efficient treatment, research is needed to determine optimal temperatures for petroleum hydrocarbon biodegradation, to determine its ability to remove contaminants that are regulatory drivers (e.g. benzene), and to verify its broad applicability.

1.2 RESEARCH OBJECTIVES

Herein, temperature impacts on LNAPL source zone biodegradation under anaerobic conditions (sulfate-reducing and methanogenic) were investigated via laboratory microcosm studies. The objectives of the investigations were (1) to study the biological degradation of LNAPL at different temperatures under anaerobic conditions within soils obtained from a former refinery; (2) determine how temperature impacts the indigenous microorganisms; (3) determine which hydrocarbons are degraded; (4) evaluate the potential for the use of thermally enhanced bioremediation for treatment of LNAPL source zones.

1.3 THESIS OVERVIEW

Chapter 2 provides the necessary background information to prepare the reader for the presentation of the research. Chapter 3 presents a thermal laboratory microcosm study investigating STELA. The chapter includes methodology, results, discussion and a conclusion. This chapter is presented in manuscript form. Chapter 4 is dedicated to introducing the concept of STELA at the field scale. Chapter 5 provides thesis conclusions and Chapter 6 presents suggestions for future work. Appendices follow the body of the thesis and contain details on methods and results of a dissolved phase microcosm experiment, statistical analysis of data, complementary raw data, and protocols.

2 BACKGROUND AND LITERATURE REVIEW

2.1 PETROLEUM LNAPL COMPOSITION

Petroleum LNAPL is composed of a large number of hydrocarbon compounds originating from crude oil. Three major categories include: alkanes, alkenes, and aromatics. Crude oil is mainly composed of linear and branched alkanes, cycloalkanes and aromatics. Alkenes are formed during the refining of crude oil and are therefore present in refined petroleum products such as gasoline and diesel (Matar and Hatch 2000). Total petroleum hydrocarbons (TPH) is a common metric used to determine the concentrations of petroleum LNAPL constituents in soil and water samples. TPH is commonly described as gasoline range organics (GRO) and diesel range organics (DRO). GRO includes low boiling point compounds (C_4-C_{10} alkanes, C_4-C_7 alkenes, and aromatics) whereas DRO includes high boiling point compounds (C_{10}-C_{24} alkanes and polynuclear aromatics).

Fate and transport of LNAPL constituents in the environment is a function of the type of hydrocarbons present. For instance, the monoaromatic compounds such as benzene, toluene, ethylbenzene, and xylene (BTEX) are commonly present in gasoline and have relatively high volatility-solubility compared to other petroleum hydrocarbons; therefore BTEX compounds will rapidly form groundwater and vapor plumes. The mobility combined with the toxicity of the BTEX compounds makes them some of the major contaminants of concern at LNAPL contaminated sites.

2.2 LNAPL FATE AND TRANSPORT

When LNAPLs are present in the subsurface, they partition into the gas, aqueous, and sorbed phases. Subsequent advection and diffusion leads to the formation of groundwater and vapor plumes. A schematic of a LNAPL release is shown in Figure 2.1.

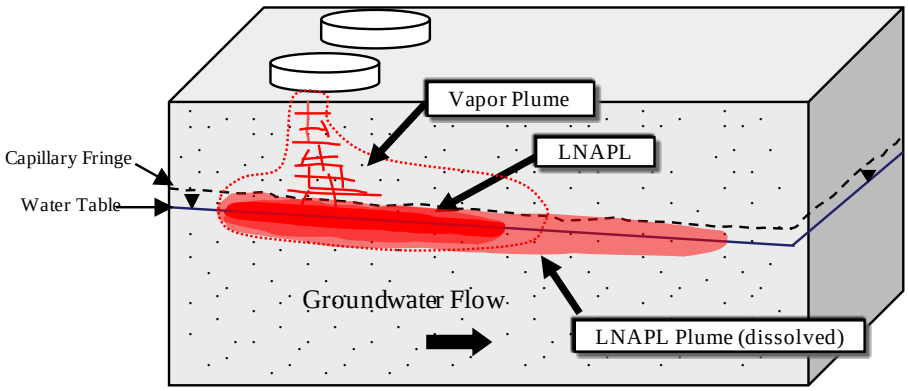

Figure 2.1 Schematic of a subsurface LNAPL release

Released LNAPL migrates down through the subsurface where it eventually reaches groundwater and spreads laterally at the water table surface. Above the water table, volatile components will separate from the LNAPL into soil gas and form vapor plumes. Water soluble compounds present in the LNAPL dissolve into the pore water and migrate away from the LNAPL body via groundwater flow to form groundwater plumes.

Following Ralout's Law (EQ 2.1 and 2.2), the solubility of individual LNAPL constituents is controlled by their pure phase solubility (i.e. gas or aqueous phases) and their mole fraction in the LNAPL.

$$C_a = XC_L \qquad \text{EQ 2.1}$$

Where:

C_a: Concentration of LNAPL constituent in the aqueous phase

X: Mole fraction of compound in LNAPL

C_L: Concentration in equilibrium with pure phase LNAPL

$$C_g = X\left(\frac{P_v}{RT}\right) \qquad \text{EQ 2.2}$$

Where:

C_g: Concentration of LNAPL constituent in the gas phase

X: Mole fraction of compound in LNAPL

P_v: Pure compound vapor pressure

R: Universal gas constant

T: Temperature

Partitioning of LNAPL constituents from the aqueous to the gas phase in the subsurface is governed by Henry's Law (EQ 2.3 and 2.4).

$$C_g = C_a H \qquad \text{EQ 2.3}$$

$$H = \frac{P_p}{C_a} \qquad \text{EQ 2.4}$$

Where:

H: Henry's Constant

P_p: Partial pressure

Over time, the more volatile-soluble compounds (e.g. benzene) move into the gas and aqueous phases to form contaminant plumes. As constituents within the LNAPL partition to gas and aqueous phases their effective volatility-solubility decreases. Correspondingly, there is a tendency, with time, for less soluble-volatile compounds to have increasing effective volatility-solubility.

LNAPL also adsorbs to the solid phase, where adsorption is strongly influenced by soil organic content (fraction of organic carbon present in the porous media). A higher fraction of organic carbon correlates to a higher ability to sorb LNAPL.

Partitioning of LNAPL in the subsurface controls the fate and transport of the contaminants which in turn controls how they are naturally attenuated. When LNAPL constituents become bioavailable, microorganisms within the subsurface will degrade the compounds. Fortunately, many petroleum hydrocarbons associated with LNAPL release are readily attenuated by biological processes that occur in the aqueous phase. Often this leads to LNAPL zones of finite extent (Mahler et al. 2012) and plumes of finite extent (Wiedemeier et al. 1999). Microbiological processes that contribute to the breakdown of LNAPL in the environment are discussed in the following section.

2.3 MICROBIOLOGICAL PROCESSES

Microorganisms have the ability to degrade many organic compounds, including petroleum hydrocarbons present in aqueous phase and vapor phase plumes. Petroleum hydrocarbons can be converted to CO_2 via biological oxidation. The most common electron acceptors for oxidation include O_2, Mn^{4+}, NO_3^-, Fe^{3+}, and SO_4^{-2} (Chapelle 1993). Research has shown that the majority of petroleum hydrocarbons will biodegrade using oxygen as the terminal

electron acceptor (Borden et al. 1995); however, subsurface systems are often anaerobic due to the low aqueous solubility of oxygen and rapid consumption of oxygen by subsurface microorganisms (Wiedemeier et al. 1999). Fortunately, alternative electron acceptors (Mn^{4+}, NO_3^-, Fe^{3+}, and SO_4^{-2}) are often available within the subsurface (Chapelle 1993). Petroleum hydrocarbon degradation has been shown to occur under anaerobic conditions, including via the reduction of sulfate (Rueter et al. 1994; Lovley and Phillips 1994), nitrate (Rabus 1995), or ferric iron (Derek R Lovley et al. 1989). Once these acceptors have been exhausted, further depletion of hydrocarbon can occur via reduction of organic carbon to methane (CH_4) (Chapelle 1999). This process is referred to as methanogenesis. The degradation of petroleum hydrocarbons via methanogenic pathways has been demonstrated by Zengler et al. 1999, Anderson and Lovley 2000, and Li et al. 2012.

Current knowledge suggests that petroleum hydrocarbon degradation by methanogenesis is a multi-step process carried out by strictly anaerobic microorganisms (Mbadinga et al. 2011). Methanogenic end products include CH_4 and CO_2. Microorganisms involved in petroleum hydrocarbon degradation under methanogenic conditions include both *Bacteria* and *Archaea*. Research to date suggests that *Bacteria* are involved in the initial steps of converting petroleum hydrocarbons into methanogenic precursors (formate, acetate, and H_2), and *Archaea* (i.e. methanogens) convert precursors to CH_4 and CO_2 (Prescott et al. 2005; Li et al. 2012). Methanogens utilize a limited number of substrates. The known methanogenic pathways are acetoclastic (acetate), carboxydotrophic (CO), hydrogenotrophic (H_2 and CO_2), methylotrophic (methanol, methylamines, methylsulfides), and methyl respiration (methylated compounds + H_2). The most common substrates are acetate and H_2 and CO_2 (Zinder 1993). Both hydrogenotrophic methanogens and acetoclastic methanogens have been shown to be involved in the anaerobic

conversion of petroleum hydrocarbons to methane (Jones et al. 2008). A schematic of the hypothetical methanogenic degradation of petroleum hydrocarbons is shown in Figure 2.2.

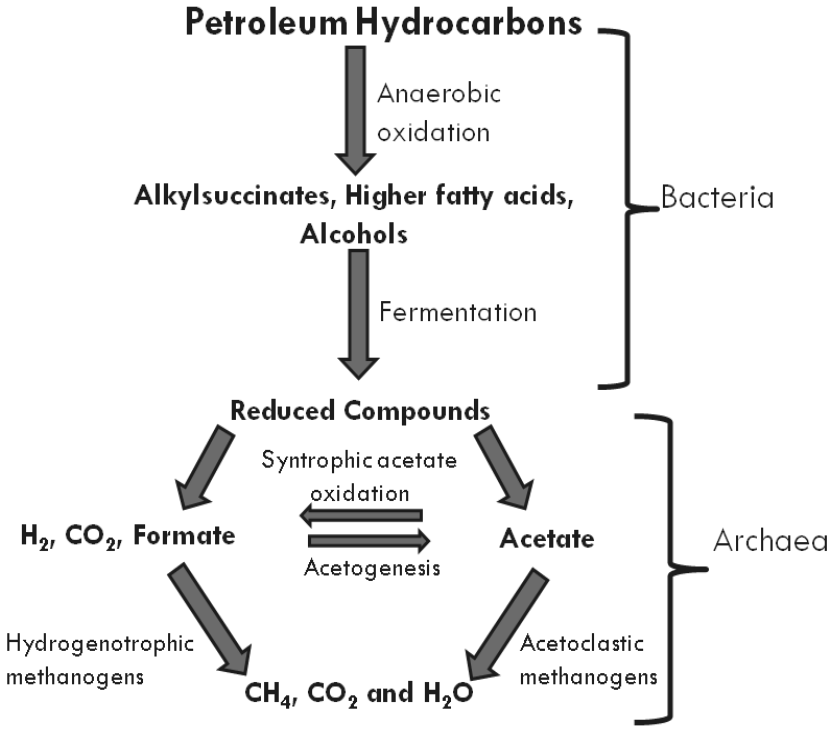

Figure 2.2: Hypothetical schematic of methanogenic degradation of petroleum hydrocarbons (modified from Mbadinga et al. 2011)

2.4 MICROORGANISMS INVOLVED IN ANAEROBIC LNAPL DEGRADATION

Specific types of microorganisms have the ability to transform and degrade petroleum hydrocarbons under anaerobic conditions. Some of these microorganisms have been isolated and identified. Microorganisms that have been isolated from anaerobic hydrocarbon-metabolizing communities include *Azoarcus* spp., *Dechloromonas* spp., *Pseudomonas* spp., *Thauera* spp., *Vibrio* spp., *Geobacter* spp., *Desulfobacula* spp., and *Desulfobacterium* spp. (Ward et al. 2009). *Azoarcus* spp., *Dechloromonas* sp. JJ and *Dechloromonas aromatic* RCB have been shown to

degrade toluene and benzene as well as alkylbenzenes under nitrate-reducing conditions (Burland and Elizabeth A Edwards 1999; J D Coates et al. 2001; Ulrich and Edwards 2003). The oxidation of long-chain n-alkanes ($C_{14} - C_{20}$) under nitrate-reducing conditions is carried out by other members of the γ-*Proteobacteria* class (Ehrenreich et al. 2000), such as strain HdN1, *Marinobacter* sp. BC36, and *Pseudomonas balearica* strain BerOc6 (Bonin et al. 2004; Grossi et al. 2008). *Geobacter* spp. have been isolated from both pristine and petroleum-contaminated anaerobic sediments in which Fe(III) reduction is a dominating pathway (Coates et al. 1996; Zwolinski et al. 2000). More specifically, *Geobacter* spp. have been shown to have the capability to degrade specific petroleum hydrocarbons compounds. For example, an iron-reducing *Geobacter metallireducens* strain GS-15 has been shown to completely oxidize toluene to CO_2 (Lovley et al. 1989).

Sulfate reduction occurs after nitrate and ferric iron have been consumed. Sulfate-reducing conditions are often observed in subsurface environments containing LNAPL. To date, the majority of sulfate-reducing bacteria isolated from environments containing petroleum hydrocarbons are associated with the family *Desulfobacteraceae*. Several mesophilic alkane-degrading sulfate-reducers have been isolated including *Desulfococcus oleovorans* Hxd3 from an oil-water separator (Frank Aeckersberg et al. 1991), strain Pnd3 from petroleum-contaminated marine sediments (Frank Aeckersberg et al. 1998), *Desulfatibacillum alkenivorans* AK-01 from petroleum-contaminated estuarine sediments (So and Young 1999) and *Desulfatibacillum aliphaticivorans* CV2803 from hydrocarbon polluted marine sediments (Cristiana Cravo-Laureau et al. 2004). A thermophilic alkane-degrading sulfate-reducer, *Desulfothermus naphthae* TD3 (member of genus *Desulfotomaculum*), capable of oxidizing n-alkanes at 60°C was isolated from

Guaymas basin (a site in the Gulf of California which has hydrothermal activity) (Rueter et al. 1994).

Once sulfate has been consumed in a subsurface environment, methanogenic pathways take over. Research to date suggests that petroleum hydrocarbon degradation under methanogenic conditions requires the activity of syntrophic microbial communities, and so far only a few studies have provided the phylogeny of microorganisms potentially involved in this syntrophic community (Zengler et al. 1999; Jones et al. 2008; Gieg et al. 2008; Zhang et al. 2012; Li et al. 2012). Zengler et al. (1999) found that an enrichment from anoxic ditch sediments was capable of degrading hexadecane under methanogenic conditions and the development of both archaeal and bacterial 16S rRNA gene (marker used to determine phylogenetic identity of microorganisms) clone libraries showed that the enrichment was comprised of acetogenic bacteria (*Syntrophus* spp.), acetoclastic methanogens (*Methanosaeta* spp.), and hydrogenotrophic methanogens (*Metanospirillum* spp. and *Methanoculleus* spp.). Geig et al. (2008) developed both archaeal and bacterial 16S rRNA gene clone libraries for a methanogenic petroleum oil-degrading enrichment culture from contaminated subsurface sediments and found that archaeal sequences were predominantly associated with acetoclastic *Methanosaeta* spp., but that the bacterial clone library was more diverse. The majority of bacterial sequences were from the phylum *Firmicutes* and included bacteria related to sulfate-reducing, hydrocarbon-degrading *Pelotomaculum* spp. and a syntrophic bacteria *Desulfotomaculum* cluster I. The bacterial community also contained members of the δ-*Proteobacteria* (*Desulfobulbus*, *Desulfovibrio* and *Smithella*) and fermentative bacteria affiliated with *Chloroflexi*, *Bacteroidetes* and *Actinobacteria*.

The data obtained from both of the methanogenic enrichment cultures described by Zengler et al. (1999) and Gieg et al. (2008) suggest that acetoclastic methanogenesis may be the primary biochemical pathway for the conversion of petroleum hydrocarbons to methane due to their dominance within the communities. Other studies have suggested hydrogenotrophic methanogenesis to be the dominant pathway within LNAPL contaminated subsurface environments (Jones et al. 2008; Grabowski et al. 2005). For example, Jones et al. (2008) found that a methanogenic alkane-degrading enrichment derived from estuarine sediments was dominated by *Syntrophus* spp. and hydrogenotrophic methanogens. Furthermore, an archaeal community from a thermophilic enrichment derived from oilfield production fluids with the capability to convert alkanes into methane was found to contain only hydrogen oxidizers within the family *Methanobacteriaceae* suggesting that methane formation was due to hydrogenotrophic methanogenesis. The bacterial community from this same enrichment was dominated by fermentative and syntrophic microorganisms affiliated with the orders *Thermotogales*, *Synergistales*, *Deferribaterales* (Gieg et al. 2010).

Methanogenic microbial communities capable of degrading specific petroleum hydrocarbons, such as the BTEX aromatics, also have been studied. For instance, benzene-degrading cultures have been developed by initially enriching on sulfate and then switching to methanogenic conditions, and these cultures have been shown to contain phylotypes affiliated with *Desulfobacterium* and *Desulfosporosinus* as well as acetoclastic methanogens (Ulrich and Edwards 2003; Da Silva et al. 2007; Mancini et al. 2008). Additionally, Sakai et al. (2009) constructed an archaeal clone library from a benzene-degrading methanogenic enrichment culture derived from non-contaminated soil, and found that the community was dominated by

phylotypes belonging to the *Methanosarcinales* and *Methanomicrobiales*. It was concluded from this study that benzene was degraded by a consortium of fermenters, aceticlastic methanogens and hydrogenotrophic methanogens (Sakai et al. 2009). Furthermore, a toluene-degrading methanogenic consortium enriched from creosote-contaminated aquifer sediments was dominated by archaeal species related to the genera *Methanosaeta* and *Methanospirillum* and a bacterial species related to the genus *Desulfotomaculum* (Ficker et al. 1999).

2.5 BIOREMEDIATION OF PETROLEUM HYDROCARBONS

Bioremediation is a treatment technology that takes advantage of microorganisms' ability to degrade contaminants. Microbial degradation processes can be affected by many factors including the type of microorganisms present, available nutrients, pH, soil moisture content, oxidation-reduction potential, temperature, and contaminant concentration (Pala et al. 2006). In practice, bioremediation involves three general strategies: bioaugmentation, biological natural attenuation, and biostimulation. Bioaugmentation is the addition of microorganisms known to degrade target contaminants and this approach is only necessary when the required types of microorganisms (e.g., benzene-degrading bacteria) are absent at a specific site or are not present in sufficient quantities. Biological natural attenuation involves monitoring the existing biodegradation processes rather than relying on engineered interventions (Khan et al. 2004; Sarkar et al. 2005). Biostimulation involves the addition of elements that are essential to, or enhance the activity of, indigenous degraders; this approach can involve the addition of nutrients (e.g., nitrogen and phosphorus), electron acceptors (e.g., oxygen), or biosurfactants (Tyagi et al. 2011). Environmental conditions such as pH, moisture content, and temperature can also be adjusted as a form of biostimulation (Atlas 1981). The use of temperature as a biostimulation

treatment has the potential to be an effective treatment for LNAPL zones to enhance biological natural attenuation is already occurring.

2.6 BIOLOGICAL NATURAL ATTENUATION

It has been known since the early 1970s that groundwater systems contain microorganisms capable of degrading petroleum hydrocarbons in the aqueous and vapor phases (Litchfield and Clark 1973). Biological natural attenuation has shown to be effective at removing petroleum hydrocarbons at both the laboratory (Lovley et al. 1989; Hutchins et al. 1991; Edwards et al. 1992) and the field scale (Wiedemeier et al. 1999; Rice et al. 1995).

Laboratory studies on the microbial activity within shallow aquifer systems and the ability of those microorganisms to degrade petroleum hydrocarbons in the aqueous phase became an important topic of research throughout the 1980s (Wilson et al. 1986; Heitkamp and Cerniglia 1988). During this same time, natural attenuation of petroleum hydrocarbon groundwater plumes was also being studied and observed at the field scale. Studies that monitored petroleum hydrocarbon concentrations at field sites demonstrated that groundwater plumes eventually cease to expand and reach steady-state (i.e., are characterized by a stable plume boundary) (Baedecker et al. 1988; Cozzarelli et al. 1989; Baedecker et al. 1993). More specifically, studies have shown that 85% to 90% of the petroleum hydrocarbon groundwater plumes in the United States have reached steady-state or are receding (Wiedemeier et al., 1999). The California Leaking Underground Fuel Tank (LUFT) Historical Case Analysis demonstrated that over 90% of groundwater plumes at 271 petroleum hydrocarbon sites in California were either stable or shrinking (59% stable, 33% shrinking) (Rice et al., 1995). Rice et al. (1995) concluded that plume stability or recession was due to natural attenuation processes including biodegradation.

In general, groundwater scientists recognize that the dominant mechanism limiting the spreading of these plumes is aerobic and anaerobic microbial metabolism (biodegradation) (Lovley et al. 1989; Cozzarelli et al. 1994; Baedecker et al. 1993; Rice et al. 1995; Wiedemeier et al. 1999).

Hydrocarbon vapor plumes have also been shown to be naturally attenuated via biodegradation. Because there can be more available O_2 within the vadose zone, vapor plumes are typically removed by aerobic biodegradation. Concentrations of oxygen reaching over 4% in the vadose zone have been shown to be adequate for biodegradation of BTEX constituents (Devaull et al. 2002; DeVaull 2007). Specifically, it has been shown that the genera *Pseudomonas* and *Arthrobacter* have the ability to degrade gasoline and other petroleum vapors within the vadose zone (EPA, 1987). Field sampling studies conducted at three petroleum hydrocarbon-contaminated sites in California concluded that biodegradation was primarily responsible for the observed attenuation of benzene vapors within the vadose zone (Ririe and Sweeny 1996; Ririe et al. 2002). Similarly, another more recent study at a petroleum release site in Stafford, New Jersey indicated that biodegradation was responsible for attenuating benzene vapor concentrations (Sanders and Hers 2006).

Rates of natural biodegradation of LNAPL are commonly assumed to be controlled by the rate of partitioning from the non-aqueous to the aqueous phases and the biogeochemical conditions present (Miller et al. 1990). Direct natural biodegradation of the non-aqueous phase at the oil-water interface has also been demonstrated. Several lab studies showed that the rates of biodegradation exceeded the rates of partitioning from non-aqueous and aqueous phases suggesting direct biodegradation of LNAPL under aerobic conditions (Efroymson and Alexander 1991; Ortega-Calvo and Alexander 1994; Ortega-Calvo et al. 1995). Specifically, Efroymson and Alexander, 1991 concluded that the *Arthobacter* species used in their laboratory study was

capable of attaching itself to the solvent-water interface and degrading the target constituent (naphthalene and *n*-hexadecane) directly from the non-aqueous phase. Additionally, Kanaly et al. (2000) found that microbial growth on diesel fuel caused mineralization of the benzo[*a*]pyrene (a polycyclic aromatic hydrocarbon) within the diesel fuel. Studies to date suggest that mineralization of LNAPL constituents at the oil-water interface and growth of microorganisms on the surface of LNAPL requires the presence of entrained water (Kanaly et al. 2000).

2.7 THERMALLY ENHANCED BIOREMEDIATION

Thermal enhancement is a particular type of biostimulation that has the potential to increase LNAPL source zone biodegradation. Microbial metabolism involves enzymatically catalyzed chemical reactions and as temperature is increased, the rates of these reactions tend to increase (Atlas, 1981). Additionally, temperature affects the physical properties of hydrocarbons such as solubility (Whyte et al. 1998), and thus, temperature increases can increase bioavailability (Perfumo et al. 2007). However, it has been also demonstrated that increased LNAPL solubility could potentially lead to aqueous phase concentrations of some constituents reaching levels that are toxic to the microorganisms causing inhibition of degradation (Leahy and Colwell 1990). Furthermore, temperature optima exist for each type of microorganism; beyond these temperatures, microorganisms can die off as enzymes denature and metabolic processes are inhibited (Chapelle, 1993). Microorganisms exhibit different minimum, optimum, and maximum temperatures and can be divided into three categories based on temperature preference: psychrophiles, mesophiles, and thermophiles. Psychrophiles thrive at temperatures between 0°C and 20°C, mesophiles thrive at temperatures between 20°C and 40°C, and

thermophiles prefer temperatures above 45°C (for extremely thermophilic microorganisms [e.g. *Thermococcales* spp.] optimal temperatures lie between 88°C and 100°C). Thus, the temperature optima for thermal stimulation will be site-specific and will depend on the types of microorganism present.

Mulkins-Phillips and Stewart (1974) studied the effect of temperature on the aerobic degradation of Bunker fuel using microbial cultures that were previously enriched on the fuel. After 14 days of incubation, a 52% reduction of benzene-soluble hydrocarbons present in the oil components was observed at 5°C, whereas an 85% reduction was observed after only 7 days of incubation at 15°C (Mulkins-Phillips and Stewart 1975). Temperature effects on the aerobic biotransformation of BTEX compounds were investigated in a batch reactor study using a microbial consortium from a gasoline-contaminated aquifer for a temperature range of 7° to 65°C; the degradation rates were highest at 35°C for all BTEX compounds (Deeb and Alvarez-Cohen 1999). Furthermore, the 35°C-enriched consortium mineralized all the BTEX compounds to CO_2. Perfumo et al. (2007) showed that high temperature (60°C) enhanced the degradation of hexadecane, an alkane hydrocarbon, in an aerobic bench-scale microcosm experiment using soil from an uncontaminated area in Northern Ireland. Microcosms held at 18°C showed a 30% removal by mass of hexadecane while the microcosms held at 60°C experienced up to 56% removal by mass over a period of 40 days. Addition of a rhamnolipid biosurfactant led to a reduction of 71% and 42% after 40 days at 60°C and 18°C, respectively. Furthermore, inoculation with thermophilic *Geobacillus thermoleovorans* in addition to the biosurfactant amendment resulted in 90% removal of the hexadecane from the soil after 40 days (Perfumo et al. 2007). To determine the effects of temperature on the degradation of petroleum hydrocarbons in sub-Arctic soils, Coulon et al. (2005) compared petroleum hydrocarbon degradation at three

different temperatures (4, 10, and 20°C) via aerobic mesocosm studies and found 10°C to be the optimal temperature for this site. After a period of 180 days, the remaining total petroleum hydrocarbons (TPH) level was 15% at 10°C compared to 25% at 4°C (Coulon et al. 2005).

The effect of temperature and nutrient addition on hydrocarbon-degrading microbial communities in temperate estuarine waters was also examined via aerobic microcosms (Coulon et al. 2007). Increasing temperature from 4°C to 20°C had a significant effect ($P<0.001$, statistically significant at 99% confidence) on the TPH biodegradation, where an increase from 4°C to 12°C did not have a significant effect. The maximum petroleum hydrocarbon degradation rate was observed at 20°C; however, the optimal temperature could be 20°C or higher because 20°C was the highest temperature investigated in this experiment (Thames Estuary, UK). Furthermore, after 7 days of incubation, the number of oil-degrading microbes (as measured by plate counts on hydrocarbon-containing media) increased by more than 4 orders of magnitude at 20°C, 3 orders of magnitude at 12°C, and 2 orders of magnitude at 4°C as compared to initial quantities. Additionally, results suggested that temperature, rather than nutrient addition, had the most influence on the microbial community structure. A phylogenetic analysis showed that hydrocarbon-degrading microorganisms were present at both 4°C and 20°C (e.g. *Thalassolituus oleivorans*, *Cycloclastic* sp., *Alcanivorax borkumensis*) demonstrating that thermally versatile microorganisms were present at the site; however, clones related to a pyschrophilic alkane degrader, *Oleispira antarctica*, were dominant within the 4°C community but were not present at 20°C after 7 days of incubation. This suggests that heating may have selected for the growth and activity of a subset of the indigenous microbial community. Rodriguez-Blanco et al. (2010) observed that temperature changes (4°C, 10°C, and 25°C) did not cause a change in the quantity of hydrocarbon-degrading bacteria within seawater microcosms amended with crude oil from the

Mediterranean Sea after 12 weeks of incubation, but a minimal increase in the biodegradation of diesel was observed at 10°C and 25°C compared to 4°C (Rodríguez-Blanco et al. 2010). Thus, research has demonstrated how temperature affects the aerobic biodegradation of petroleum hydrocarbons in site-specific cases; however, many petroleum hydrocarbons contaminated sites are under anaerobic conditions.

One recent study investigated temperature effects on crude oil degradation under sulfate-reducing conditions with enrichment cultures from marine sediments (Higashioka et al. 2011). Sulfide production was tracked as an indicator of the progression of hydrocarbon degradation for cultures held at 8°C and 28°C. Results indicated that after 248 days the sulfide concentration had reached 23.2 mmol/l for the culture held at 28°C, whereas the sulfide concentration of the enrichment incubated at 8°C only increased to 6.1 mmol/l after 301 days. A phylogenetic analysis showed differences in the microbial communities indicating that temperature not only has the ability to influence petroleum hydrocarbon degradation but also the microbial community structure under anaerobic conditions.

Field-scale applications that have taken advantage of temperature include thermal insulation systems (TIS) and heated biopiles in cold climates. Filler et al. (2001) developed and applied TIS to increase temperatures of a soil plume at a leaking underground storage tank site in Fairbanks, AK. The TIS was comprised of an electrical heat mat under a 7.6-cm layer of polystyrene insulation to keep temperatures above freezing. The TIS system resulted in temperatures remaining above freezing (0.5-7.8°C) over two treatment seasons (mid-August to November 1999 and May 2000 to January 2001) at depths ranging from 0.9 to 2.4 m when atmospheric temperatures were as low as -25°C. By monitoring numbers of heterotrophic and diesel-degrading microbes and soil O_2 and CO_2 levels, authors concluded that their system was

successful in extending microbial activity well into the arctic winter (Filler et al. 2001). Reimer et al. (2003) tested a heated biopile system in the Canadian Arctic. In this system, the air was heated prior to being injected in the soil. The soil temperature was maintained at ~15 °C despite daily surface temperatures as low as −42 °C. Soil petroleum hydrocarbon concentrations were reduced by up to ~60% from initial concentrations of ~5000 mg/kg. However, the aerated/heated system caused excessive drying of the soil that may have inhibited microbial activity and likely promoted volatilization of the contaminants (Reimer et al. 2003).

Based on a review of current literature, thermal insulation or active heating to achieve optimal temperatures for microbial activity has not been applied at petroleum-contaminated sites in non-arctic climates. Thermal technologies focusing on delivering low levels of heat could be a potential treatment to enhance microbial degradation or to maintain microbial activity at a maximum through all seasons. This treatment could be particularly successful in LNAPL zones under methanogenic conditions because petroleum hydrocarbon conversion to CH_4 is a slow process compared to other degradation pathways. However, research (laboratory and field pilot studies) investigating the effects of temperature changes on petroleum hydrocarbon degradation under anaerobic conditions (specifically methanogenic conditions) is required to validate the use of thermally enhanced bioremediation for subsurface areas contaminated with LNAPL and to identify temperature optima within moderate climates (e.g. Evansville, WY).

2.8 Molecular Tools to Assess Bioremediation Technologies

Molecular tools are commonly applied in bioremediation to monitor the efficacy of the treatment with respect to promoting an increase in the quantity and activity of required microorganisms as well as to provide a line of evidence that the biological processes are

responsible for the degradation. Furthermore, the application of molecular techniques can provide a more thorough understanding of the microbial community composition and how microorganisms within the community contribute to the major biogeochemical and degradation processes (Mills et al. 2003). Molecular tools are responsible for the discovery of a variety of hydrocarbon-degrading microorganisms (Vogel and Grbìc-galìc 1986; Lovley et al. 1989; Heider et al. 1999; Chakraborty and Coates 2004).

Culture-independent techniques such as cloning and fingerprinting methods have been developed to characterize microbial populations. Fingerprinting techniques (e.g. terminal restriction fragment length polymorphism [TRFLP], and denaturing gradient gel electrophoresis [DGGE]) provide information about the structure or composition of a microbial community based on the sequences of targeted genes. The use of fingerprinting techniques alone does not provide species identification. Cloning and sequencing can be used in conjunction with a fingerprinting technique for species identification. Cloning can also be done independently to identify species within a microbial community. The 16S small subunit (SSU) ribosomal RNA (16S rRNA) gene is a phylogenetic marker that is targeted by a broad range of molecular tools (e.g. TRFLP, DGGE, 16S rRNA gene-targeted polymerase chain reaction (PCR), cloning). Use of this genetic marker has been shown to be a rapid and economical way to assess microbial diversity in environmental samples such as soils and sediments (Torsvik et al. 1996).

Li et al. (2012) developed both bacterial and archaeal 16S rRNA gene clone libraries from enrichment cultures amended with *n*-alkanes established with petroleum reservoir production water. This was done to identify how the microbial community changed under nitrate-reducing, sulfate-reducing and methanogenic conditions. Results of the clone libraries indicated that the microorganisms present within the enrichment cultures changed depending on

the incubation conditions. Scherr et al. (2012) also used DGGE coupled with sequencing to determine changes in bacterial communities from anaerobic digesters during petroleum hydrocarbon degradation. Results suggested that bacterial communities within anaerobic digesters have the ability to change and adapt to degrade LNAPL constituents (Scherr et al. 2012).

3 LNAPL THERMAL MICROCOSM STUDY

3.1 OVERVIEW

A soil microcosm study was completed to determine temperature effects on the anaerobic biological degradation of petroleum hydrocarbons. Light Non-Aqueous Phase Liquid (LNAPL), soils and groundwater were acquired from a former refinery in Evansville, Wyoming for the study. A thermal microcosm study investigated six different temperatures (4ºC, 9ºC, 22ºC, 30ºC, 35ºC and 40ºC) and simulated an anaerobic subsurface where sulfate reduction and methanogenesis were the primary pathways for biodegradation. Complete conversion of some of the petroleum hydrocarbons to CH_4 and CO_2 occurred at 22°C, 30°C, 35°C, and 40°C. No evidence of CH_4 and CO_2 production was observed at 4°C and 9°C. After 188 days of incubation, the highest cumulative total biogas production occurred in microcosms held at 22°C and 30°C suggesting that these temperatures may be optimal for indigenous hydrocarbon-degrading microorganisms. Specifically, average total biogas production in triplicate microcosms at 22°C and 30°C was 19 times higher when compared to microcosms at 35°C and 3 times higher compared to microcosms at 40°C. For the microcosms held at 35°C and 40°C, CH_4 and CO_2 production occurred after a substantial delay (178 days for 35°C and 138 days for 40°C). This delay contributed to the overall lower cumulative biogas production observed during the study period at 35ºC and 40ºC relative to 22ºC and 30ºC. Overall, GRO, DRO and BTEX compounds degraded more readily within microcosms held at temperatures of 22-40°C. Phylogenetic analysis of the microbial communities present at 22°C via a 16S rRNA gene archaeal clone library and pyrosequencing revealed the presence of both hydrogenotrophic and acetoclastic methanogens. Pyrosequencing of 16S rRNA genes found in the microcosms at 4°C,

22°C, and 35°C revealed that temperature impacted both archaeal and bacterial community structure. This result may explain why microcosms at higher temperatures experienced a delay in biogas generation. In general, results indicate that thermally enhanced bioremediation is a promising technology for the treatment of petroleum hydrocarbons within LNAPL source zones, but larger scale studies are needed to verify its feasibility and efficacy.

3.2 INTRODUCTION

The production, transport, refinement and storage of petroleum has led to widespread contamination of soil and groundwater (Callaghan et al., 2010). The United States alone has hundreds of thousands of sites that are impacted by petroleum liquids in the subsurface (Cozzarelli et al., 1990, Kuhad et al. 2009). Petroleum liquids contain a large number of hydrocarbon compounds, some of which are potential or known carcinogens (e.g. benzene, toluene, ethylbenzene, xylene) (White and Claxton 2004). Petroleum liquids present in subsurface environments are referred to as light non-aqueous phase liquid (LNAPL). A continuing drive to cleanup LNAPL releases and the limitations of currently available technologies has resulted in a need for new technologies that are viable, effective and economical.

Biological natural attenuation of petroleum hydrocarbons has been recognized as a removal pathway at LNAPL sites since the early 1970s (Litchfield and Clark 1973). Biological natural attenuation has shown to be effective at removing LNAPL constituents under laboratory conditions (Lovely et al., 1989; Hutchins et al., 1991; Edwards et al., 1992; Sakar et al., 2005; Couto et al, 2010) and field conditions (Wiedemeier et al., 1999; Rice et al. 1995; Newell and Connor 1998; Cozzarelli et al., 2001). Unfortunately, biological processes can be slow relative

to the expectation for timely cleanup (Romantschuk et al., 2000; Rivett and Thornton, 2008). Thus, active remedial technologies must be developed to be viable for application in LNAPL zones under methanogenic conditions.

Biostimulation of the indigenous microbial population via low-level heating (increasing temperatures by 10-20°C) is a promising strategy for enhancing rates of biological natural attenuation of LNAPL zones. Temperature influences hydrocarbon biodegradation, by affecting the metabolic activity and composition of the microbial community as well as the physical properties of LNAPL (Whyte et al. 1998). The rates of microbial metabolism tend to increase as temperature is increased (Atlas, 1981); however, temperature optima exist for the microorganisms involved (Chapelle, 1993).

Several studies have shown that temperature increases can enhance petroleum hydrocarbon biodegradation under aerobic conditions (Mulkins-Phillips and Stewart 1974; Deeb and Alvarez-Cohen 1998; Perfumo et al. 2007). Data suggests that temperature optima are site specific (Coulon et al. 2005; Coulon et al. 2007). Coulon et al. (2005) compared petroleum hydrocarbon degradation within sub-Arctic soils at three different temperatures (4°C, 10°C, and 20°C) via aerobic mesocosm studies and found 10°C to be the optimal temperature for this site. In contrast, 20°C was found to be the optimal temperature for a study examining hydrocarbon biodegradation over the same temperature range in temperate estuarine waters (Thames Estuary, UK) (Coulon et al. 2007). Deeb and Alvarez-Cohen (1998) investigated temperature effects on the aerobic biotransformation of BTEX compounds in a batch reactor study using a microbial consortium from a gasoline-contaminated aquifer for a temperature range of 7° to 65°C. The BTEX degradation rates were highest at 35°C for all BTEX compounds suggesting that

temperature increases up to 35°C increase microbial metabolism for these aquifer sediments but increases beyond 35°C cause a decrease in the biodegradation of BTEX compounds.

Field studies using thermal insulation or temperature treatments to enhance biological metabolism of petroleum hydrocarbons have been conducted in arctic climates (Filler et al. 2001; Reimer et al. 2003). A thermal insulation system (TIS) used in Prudhoe Bay, AK resulted in temperatures remaining above freezing (0.5-7.8°C) over two treatment seasons (mid-August to November 1999 and May 2000 to January 2001) in aerobic petroleum-contaminated soils when atmospheric temperatures were as low as -25°C. By monitoring numbers of diesel-degrading microbes as well as soil O_2 and CO_2 levels, authors concluded that the TIS was successful in extending and enhancing microbial activity well into the arctic winter (Filler et al. 2001).

One recent study investigated temperature effects on crude oil degradation under sulfate-reducing conditions within enrichment cultures from marine sediments (Higashioka et al., 2010). It was observed that an increase in incubation temperature from 8°C to 28°C resulted in an increase in crude oil biodegradation. This suggests that increases in temperatures can also enhance anaerobic biodegradation of petroleum hydrocarbons.

The studies discussed above suggest that temperature optimum is dependent on the location of the contamination, but in general low-level heating (increases of 10-20°C) results in an increase in the metabolism of petroleum hydrocarbons by indigenous microorganisms. Current research has predominantly focused on how temperature affects the aerobic degradation of petroleum hydrocarbons. Conversely, anaerobic conditions (e.g., sulfate-reducing or methanogenic) often prevail at sites containing LNAPL (Amos et al., 2004). Accordingly, research is needed to identify how temperature changes affect LNAPL attenuation under these

anaerobic conditions. Additionally, the concept of thermal insulation or active heating to achieve optimal temperatures for biological metabolism has not been applied to contaminated sites in moderate climates, but could be a potential treatment to aid in keeping microbial activity at a maximum through all seasons. Determining how temperature affects the biodegradation of LNAPL under methanogenic conditions at a non-arctic site will indicate if thermally enhanced bioremediation can effectively increase biodegradation within LNAPL zones at a variety of locations.

The objectives of the research presented herein are to (1) study the impact temperature has on the biological degradation of LNAPL under anaerobic conditions, (2) determine how temperature influences the microbial ecology of the indigenous microorganisms, (3) determine which hydrocarbons are degraded, and (4) evaluate the potential for the use of thermally enhanced bioremediation for treatment of LNAPL source zones. This was accomplished by conducting a thermal soil microcosm study using LNAPL impacted soil and groundwater from a former refinery located in Evansville, WY. Both contaminant degradation and soil microbiology were monitored.

3.3 METHODS

3.3.1 SITE BACKGROUND

LNAPL-impacted soils were collected from a former refinery in Evansville, Wyoming where operations ended at the refinery in 1982. The site is underlain by fluvial sands associated with the North Platte River. Groundwater temperatures at the site change seasonally and will typically be between 8ºC and 15°C.

3.3.2 Soil Collection and Storage

The soils used for the microcosms came from an LNAPL smear zone at the water table approximately 8 feet below ground surface. The soils were obtained using a backhoe. Soils were immediately placed in heavy duty Ziploc™ bags and purged with N_2 gas to displace oxygen from the sample to preserve anaerobic conditions. The soils were placed on ice during transport back to Colorado State University where they were stored at 4 °C for 2 weeks prior to setting up the soil microcosms.

3.3.3 Water Collection and Storage

Groundwater used for the microcosms was collected from a monitoring well at the site (monitoring well RWC) using a peristaltic pump. Four sterile 1-L glass jars were filled up to the top to minimize oxygen exposure. Sulfate concentrations in this water were approximately 1300 mg/L at the time of collection. This concentration is based on an analysis of water samples taken from this same source one month prior to water collection for the microcosm experiment.

3.3.4 Microcosm Set Up

Soils were homogenized and subdivided in an anaerobic chamber. 2-kg subsamples were placed in clean Ziploc™ bags. The soils were then spiked with LNAPL collected from a monitoring well at the former refinery and laboratory grade BTEX. Target concentrations were 5000mg/kg site LNAPL and 100mg/kg BTEX. 100 mL of the site water was added to each 2-kg bag of soil. Saturated soils were homogenized via hand mixing individual bags for approximately 5 minutes in the anaerobic chamber. Abiotic control soils were also prepared in the same way but were amended with sodium azide (ThermoFisher Scientific, Waltham, MA) to arrest microbial activity. Specifically, 20 grams of sodium azide was added per 2 kg bag of soil for a final concentration of 1% sodium azide by weight.

Six sets of triplicate microcosms were set up in an anaerobic chamber. Microcosms consisted of 250-mL serum bottles containing 270 grams of the homogenized soil mixture. Microcosms were sealed using impermeable blue butyl rubber septum stoppers (BellCo Glass, Vineland, NJ). Triplicate killed controls were assembled using the pre-mixed control soil containing sodium azide (Fisher Scientific).

Microcosms were incubated in constant temperature water baths held at different temperatures: 4°C, 9°C, 22°C, 30°C, 35°C, and 40 °C for 188 days. Temperatures of 4°C and 9°C represent colder groundwater temperatures and are not considered to be thermally enhanced. Temperatures of 22°C and above are considered thermally enhanced temperatures for the Evansville site. The bottles were connected to inverted 100-mL glass graduated cylinders to capture gases produced from the microcosms and to limit entry of atmospheric oxygen into the microcosms. In-line glass t-joints were used to pull gas samples for analysis. Figure 3.1 shows the experimental setup for a single microcosm.

In addition, separate 150-mL jars containing 100 g of the soil or abiotic control soil were assembled in the anaerobic chamber for each temperature for characterization and monitoring of microbial communities. Accumulated biogas in these microbial sampling jars was vented periodically using a one way valve.

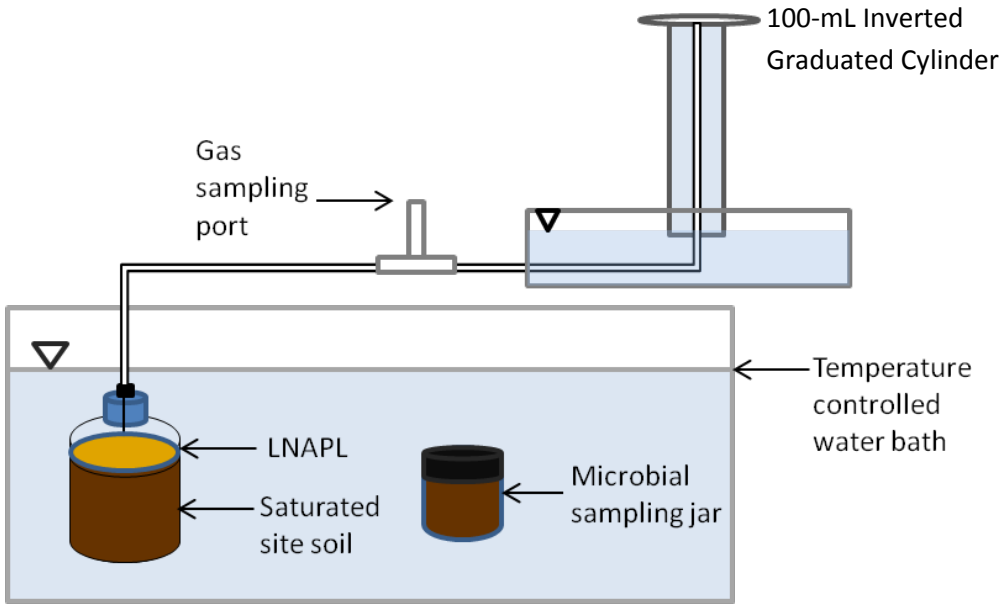

Figure 3.1: Microcosm set up

3.3.5 Analytical Techniques

Data collected during the study included volume of biogas produced, CH_4 and CO_2 concentrations in the biogas, total petroleum hydrocarbons (TPH) in soil as gasoline range organics (GRO) and diesel range organics (DRO), BTEX concentrations in soil, and adenosine tri-phosphate (ATP) concentrations (measure of microbial activity). Sulfate concentrations were not measured over the course of the experiment because this would have required opening and disrupting the microcosms; however, thermodynamics suggest that sulfate initially present was reduced prior to the onset of methanogenesis in the live microcosms at temperatures above 9°C. In addition, a 16S rRNA gene archaeal clone library was constructed for the microcosms held at 22°C after 2 months of incubation to determine the types of methanogens present. Also, bacterial and archaeal 16S rRNA gene targeted 454 pyrosequencing was conducted at the end of the experiment for microcosms at 4°C, 22°C, and 35°C to resolve the effect of temperature on the microbial community structure. The following describes analytical techniques for all of the experimental parameters.

Biogas

The volume of biogas produced per time was determined by monitoring the gas captured in the 100-mL inverted graduated cylinders. The biogas composition (e.g. concentrations of CH_4 and CO_2) was measured by collecting biogas samples from the gas sampling port (Figure 3.1), and manually injecting gas samples into a Hewlett Packard 5890 Series II Gas Chromatograph with a thermal conductivity detector (GC/TCD). The GC column used for the analysis was a Hayesep 8"x 1/8" Q 80/100. 50- µL gas samples were manually injected using a 100- µL gas tight glass syringe. The GC was operated at a constant temperature of 100^0C, and the sample run time was 1.2 minutes. Standards with known concentrations of CH_4 were prepared by diluting commercially available gas containing 80% CH_4 and 20% N_2 with helium. Standards with known concentrations of CO_2 were prepared by diluting pure CO_2 gas with helium. Gas samples were collected from individual microcosms when they produced 5 mL or more of biogas. Thus, the total number of samples from each microcosm was a function of rates of gas production in each of the microcosms.

Hydrocarbons

Soil samples from the microcosms were analyzed for TPH and BTEX at the beginning and end of the experiment. Hydrocarbons were extracted from the soil using high purity methanol (Honeywell Burdick and Jackson ACS/HPLC Methanol). Soil and methanol mixtures (~10g soil, 50-mL methanol) were placed on a multi-tube vortexer (Model 2600, Scientific Manufacturing Industries, Midland, ON, Canada) for 30 minutes and then sonicated using an Aqua Wave 9376 ultrasonic cleaner (Bearnstead/Lab-Line, Waukegan, IL) for 30 minutes. The methanol phase was then analyzed for TPH as GRO and DRO using a Hewlett Packard 5890 Gas

Chromatographer with Mass Spectrometer (GC/MS Hewlett-Packard 5970 Mass Selective Detector). The standards used for this analysis were a GRO mix and a DRO mix (EPA, Restek, Bellefonte, PA). The GC was programmed to start with an initial temperature of 45°C held for 3 minutes followed by a temperature ramp of 12°C per minute until the temperature reached 120°C. The temperature was held at 120°C for 3 minutes followed by another temperature ramp of 25°C per minute until 290°C is reached.

BTEX were also measured in the biogas to account for losses due to volatilization. This was done by manual injection of the gas samples into a HP 5890 Series II GC with a flame ionization detector (GC/FID). The GC column used was a Restek Rxi® -502.2 fused silica column. The run time for each sample was 19.53 minutes. The GC temperature program involved starting at 35^0C with a temperature ramp of 5.8^0C/minute until a temperature of 70^0C was reached. Then the temperature ramp was increased to 20^0C/minute until a final temperature of 240^0C was reached. Standard curves for the BTEX gas phase compounds were developed using a solution containing 100 mg/L of each BTEX compound in de-ionized water.

The percent biological degradation of GRO and DRO and BTEX compounds was calculated using the following formula.

$$\%\ Biological\ Degradation = \frac{C_a - C_l}{C_i} \qquad \text{(EQ 3.1)}$$

Where:

C_a: average final concentration in the triplicate abiotic control microcosms

C_l: average final concentration in the triplicate live culture microcosms

C_i: average initial concentration from the soil used for the microcosms

The error for percent biological degradation was determined by propagation of error using standard deviations. The following equation was used.

$$\sigma_{\%\ biological\ degradation} = \sigma_a^2 \left[\frac{1}{C_i}\right]^2 + \sigma_l^2 \left[\frac{1}{C_i}\right]^2 + \sigma_i^2 \left[\frac{C_l - C_a}{C_i^2}\right]^2 \qquad \text{(EQ 3.2)}$$

Where:

σ_a: standard deviation of the final concentrations in the abiotic controls

σ_l: standard deviation of the final concentrations in the live culture microcosms

σ_i: standard deviation of the initial concentration in the soil used for the microcosms

Molecular-ATP

ATP levels were measured periodically throughout the experiment to monitor microbial activity. ATP was measured using BacTiter-Glo™ Microbial Cell Viability Assay (Promega Corporation, Madison, WI). 0.5 g of soil was removed from the microbial sampling jars (Figure 3.1) in the anaerobic chamber for each temperature and mixed with 500 µL of the BacTiter-Glo™ reagent in 1.5-mL sterile tubes. The soil and reagent mixture was vortexed for approximately 10 seconds and then allowed to incubate at room temperature for 4 minutes. Then, the reaction was centrifuged to pellet the soil and 100 µL of the supernatant was then pipetted into a Costar® EIA/RIA 96 well plate. Sample luminescence was then measured using a Synergy HT plate reader with a luminometer (BioTek®, Winooski, VT).

Soil color varied within the microcosms; therefore separate standard curves were made for each temperature at each sampling time until the standard curves no longer changed. A solution was prepared to generate a standard curve that accounted for the change in luminescence

caused by the pigment of the soil. The standards were prepared by autoclaving soil from each temperature and spiking the soil with 4 known amounts of ATP.

Molecular-Clone Library Construction and Pyrosequencing

A 16S rRNA gene archaeal clone library was generated for the 22°C microcosm to identify the types of methanogens present. DNA was extracted from the 22°C soil from the microbial sampling jar after incubating for a period of 2 months. DNA was extracted from soil using the PowerLyzerTM PowerSoil® DNA Isolation Kit and the PowerLyzerTM 24 Homogenizer at 4000rpm for 45 seconds (MoBio Laboratories Inc., Carlsbad, CA). Primers 344F and 915R were used (Benlloch et al. 2002) for amplification of archaeal 16S rRNA gene sequences. Each PCR reaction contained 0.5mM MgCl2, 0.8mM dNTPs, 0.5µM of both forward and reverse primers, 1.25U of DNA Taq polymerase (5 PRIME, Inc. Gaithersburg, MD), and 0.4 mg/L Bovine Serum Albumin (BSA). The PCR program ran for 2 minutes at 98°C with 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 90 seconds with a final elongation at 72°C for 7 minutes. PCR products were cloned with the TOPO TA Cloning® Kit for sequencing version O (Invitrogen Corporation, Carlsbad, CA) using chemical transformation according to the manufacturer's instructions. A total of twenty clones were selected from plates and grown overnight at 37°C in LB broth containing ampicillin (50mg/L). Plasmid DNA was extracted using the FastPlasmid Mini kit (5 PRIME, Inc. Gaithersburg, MD) according to the manufacturer's instructions. The plasmid DNA concentration was quantified using Quant-iT^{TM} Broad-Range DNA Assay Kit (Invitrogen Corporation, Carlsbad, CA). Clone sequencing was performed at the Proteomic and Metabolomics Facility at Colorado State University for sequencing.

At the end of the microcosm experiment, DNA was extracted from microcosm soil at all temperatures as described previously. DNA from the triplicate microcosms at 4°C, 22°C, and 35°C was pooled. The pooled DNA was sent to Research and Testing Laboratories LLC (Lubbock, Texas) for bacterial and archaeal 16S rRNA gene 454 pyrosequencing. Primers 939F and 1492R were chosen for the bacterial 16S rRNA gene targeted assay. Primers 341F and 958R were chosen for the archaeal 16S rRNA gene targeted assay. A Roche Genome Sequencer FLX+ (plus) 454 pyrosequencer was the sequencing platform used. The 454 pyrosequencing data analysis methodology can be found in Appendix C. The analysis returns relative abundance data for phyla, order, family, genus and species for the samples submitted. The relative abundance data meets the following match criteria. DNA sequences with identity scores similar to well characterized 16S rRNA gene sequences that have greater than 97% identity (<3% divergence) are reported at the species level. If the sequence similarity is between 95% and 97% results are reported at the genus level. When similarity is between 90% and 95% results are reported at the family level and if between 85% and 90% it is reported at the order level. Results are reported as class when similarity is between 80% and 85% and finally phyla if similarity is 77% to 80%. Any match below this percent identity is discarded.

3.4 Results and Discussion

3.4.1 Biogas Data

Produced biogas indicates complete degradation of petroleum hydrocarbon species to CH_4 and CO_2 at temperatures 22°C, 30°C, 35°C, and 40°C. No biogas was produced within the killed controls, indicating a successful abiotic control. Also, over the course of the experiment, no biogas was produced from the 4°C or 9°C microcosms. Figure 3.2 shows the total biogas production within the microcosms over 188 days.

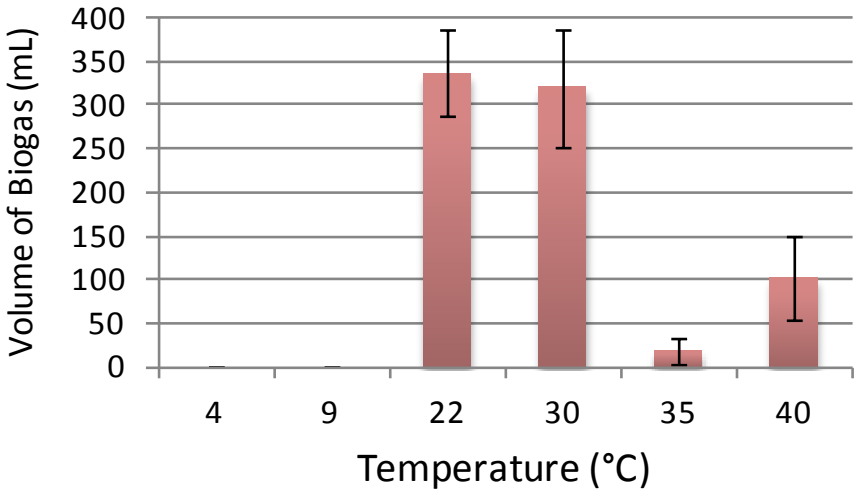

Figure 3.2: Total biogas production as a function of temperature over 188 days. The values shown are averages for triplicate microcosms, and error bars represent the standard deviations for triplicates.

Figure 3.3 presents cumulative CO_2 and CH_4 production in triplicate microcosms as a function of time.

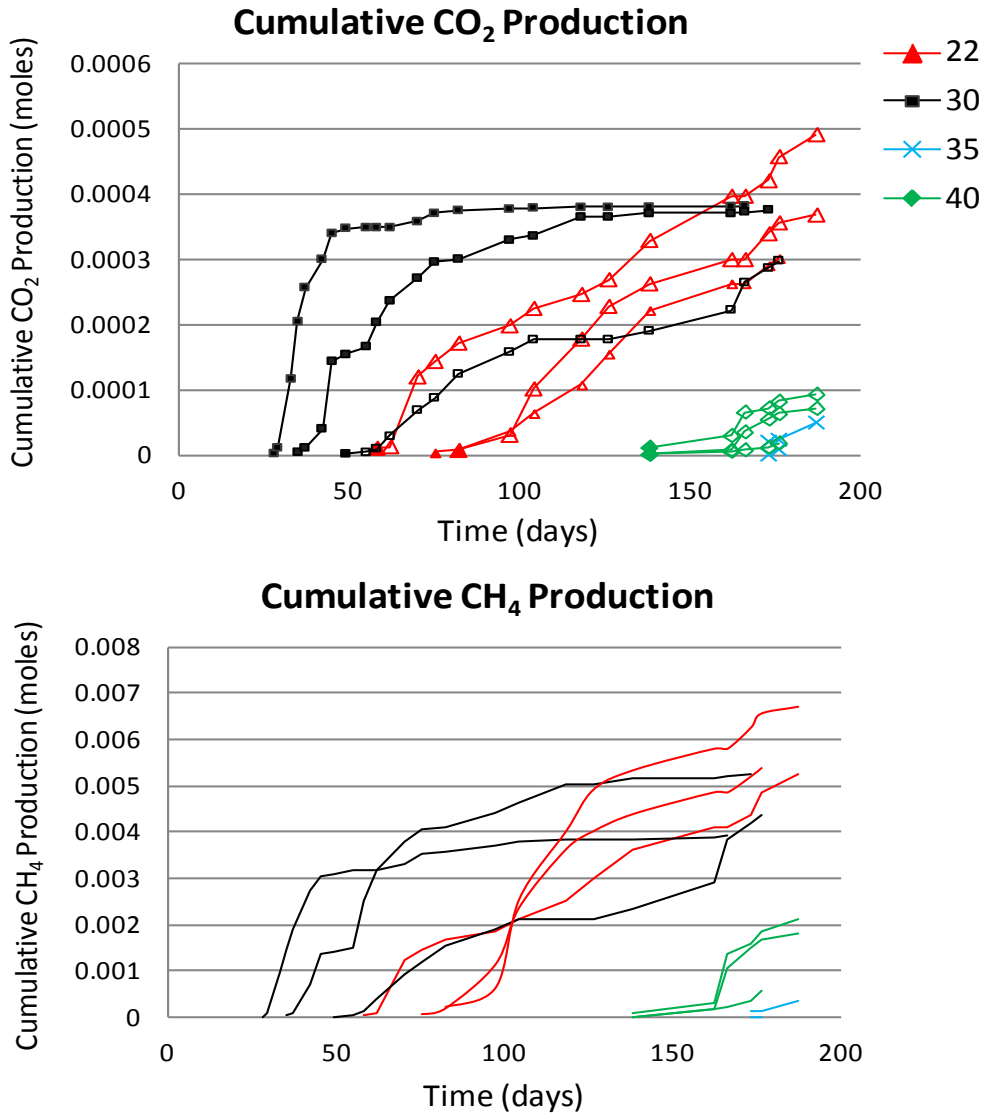

Figure 3.3: Cumulative CO_2 and CH_4 production as a function of time. Three lines shown for each temperature represent the triplicate microcosms. Data is not shown for 4°C and 9°C because no biogas was produced.

The most CH_4 and CO_2 production occurred at 22°C and 30°C during the 188-day incubation period. CH_4 and CO_2 production occurred first within the microcosms held at 30°C beginning after 28 days of incubation followed by production beginning within microcosms at

22°C at 58 days of incubation. Production of biogas continued at 22°C and 30°C throughout the course of the experiment, with the most production occurring within one of the triplicates held at 22°C. A significant delay (138-173 days of incubation) in CH_4 and CO_2 production occurred within microcosms held at 35°C and 40°C. Microcosms held at 40°C began producing after 138 days of incubation followed by microcosms at 35°C at day 173. The delay in biogas production occurred in all three triplicate microcosms at 35°C and 40°C indicating that these results are reproducible.

Groundwater temperatures range seasonally from approximately 8-15°C at the Evansville, WY site where soils were collected. Thus, it is possible that the indigenous microbial communities present in the soils tested required time to acclimate to temperatures above 30°C. Based on the CH_4 and CO_2 generation results, approximately 5-6 months were required to generate a sufficient number of microorganisms capable of thriving at temperatures of 35°C and 40°C to drive consequential degradation. Further research is required to determine if, after an acclimation period, rates of biodegradation at 35°C and 40°C would prove to be comparable to or even higher than rates at 22°C and 30°C.

3.4.2 HYDROCARBON DATA

After the microcosms were incubated for a period of 188 days, chemical analysis was carried out to evaluate losses of petroleum hydrocarbons. GRO and DRO concentrations of the live culture microcosms were compared to the concentrations in the abiotic controls as well as the initial soil hydrocarbon concentrations to determine biological removal. Figure 3.4 shows the GRO results.

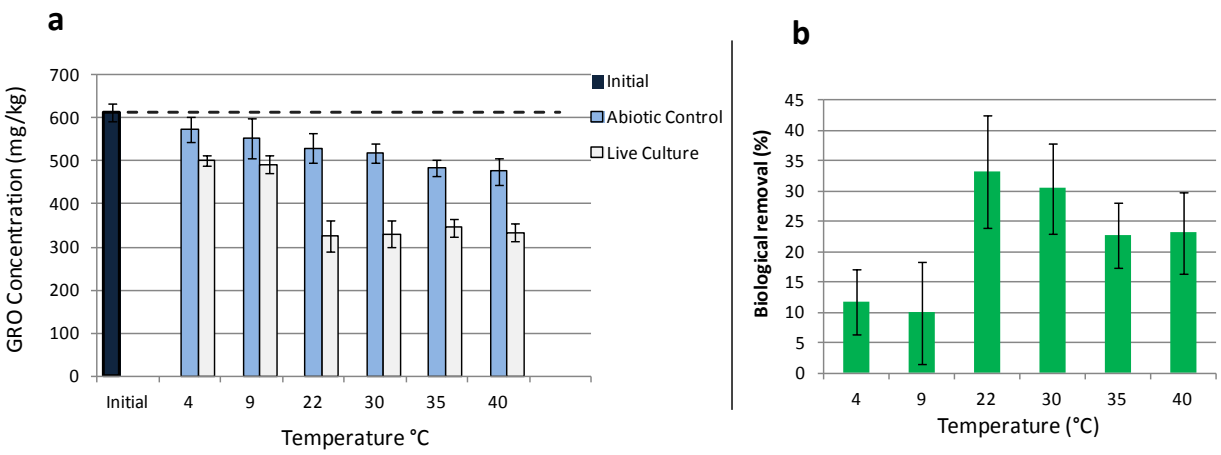

Figure 3.4: GRO degradation with temperature (a) Initial concentration in soil is shown along with the final concentrations within the abiotic controls and live culture microcosms (b) Percent biological degradation is shown. Error is represented by standard deviations from triplicate microcosms

When compared to the abiotic controls in a two sample, two-tailed t-test, microcosms at 4°C, 22°C, 30°C, 35°C and 40°C showed statistically significant biological degradation of GRO at the α=0.05 level over the course of the 188-day incubation period. Biotic removal of GRO was significantly higher at 22ºC and 30ºC when compared to 4ºC and 9ºC. The observed removals at 22 ºC, 30 ºC, 35 ºC and 40ºC were not statistically different from each other and percent removal ranged from 23-33%.

Similar trends in DRO degradation were observed. DRO results are shown in Figure 3.5 below. Using a t-test comparing the concentrations within the abiotic controls to the live culture microcosms, the biological removal of DRO was found to be statistically significant at 22°C, 30°C, 35°C, 40°C. The removal at these temperatures ranged from 18-22%.

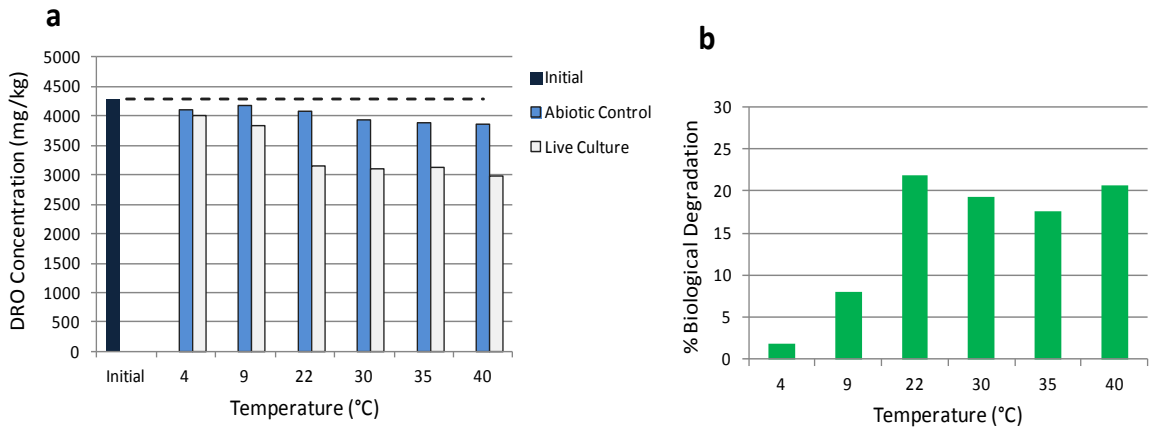

Figure 3.5: DRO degradation with temperature (a) Initial concentration in soil is shown along with the final concentrations within the abiotic controls and live culture microcosms (b) Percent biological degradation is shown. Error is represented by standard deviations from triplicate microcosms.

Although the CH_4 and CO_2 biogas results indicated that degradation of LNAPL constituents occurred to a higher degree within 22°C and 30°C relative to 35°C and 40°C over the course of the experiment, the biological hydrocarbon removal at all of these temperatures was not statistically different at the α=0.05 level for both GRO and DRO (determined by an ANOVA test). This result suggests that the GRO and DRO compounds at 35°C and 40°C were still in the initial steps of the anaerobic or methanogenic breakdown and that there may have been a buildup of intermediates. For example, at the time the experiment was ended, many of the GRO and DRO hydrocarbons in microcosms at 35°C and 40°C may have been converted into pathway intermediates (e.g. alcohols), but had not yet been converted to CH_4 and CO_2 by the methanogens.

Benzene, toluene, ethylbenzene, and xylene degradation also was observed in the live microcosms (Figure 3.6, 3.7, 3.8, 3.9). Statistically significant ($p<0.05$ for two-tailed t-test comparing abiotic microcosms to live culture microcosms) biological removal of benzene was observed at temperatures of 22°C, 30°C, and 35°C and ranged from 19-26% (Fig. 3.6).

Similarly, biological removal of toluene was found to be statistically significant at temperatures of 22°C, 30°C, and 35°C and ranged from 23-34% (Figure 3.7). Biological removal of ethylbenzene was only found to be significant within microcosms held at 22°C and 30°C at 31% and 25% respectively (Figure 3.8). Biological removal of the xylenes was statistically significant at 22°C, 30°C, 35°C, and 40°C and ranged from 14-26% (Figure 3.9). The percentages of contaminant removed due to biological degradation for GRO, DRO, and individual BTEX compounds are summarized in Table 3.1 for comparison.

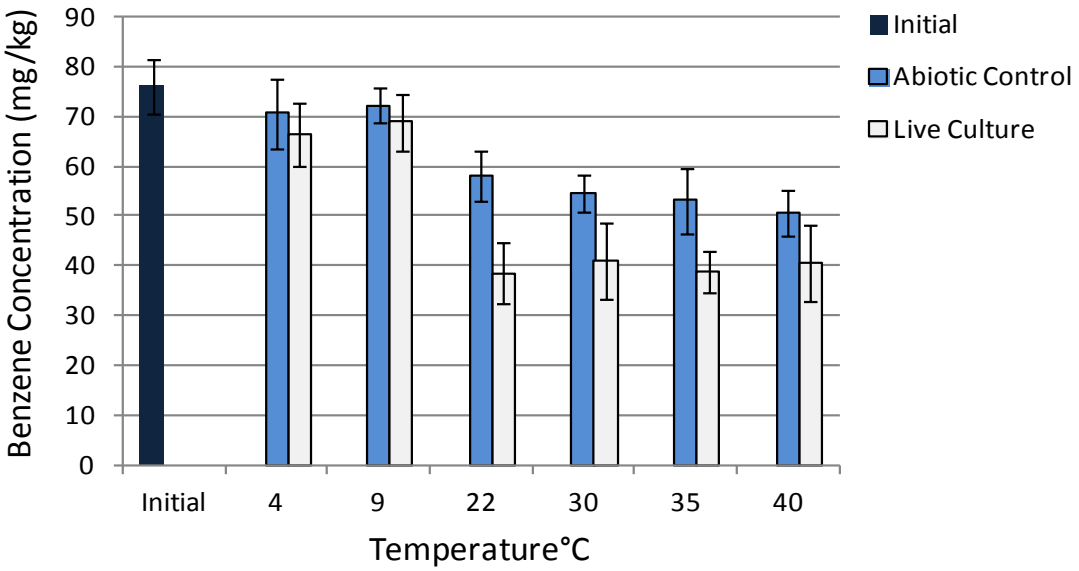

Figure 3.6: Benzene degradation with temperature: Initial concentration in soils is shown along with the final concentrations within the abiotic control and live culture microcosms.

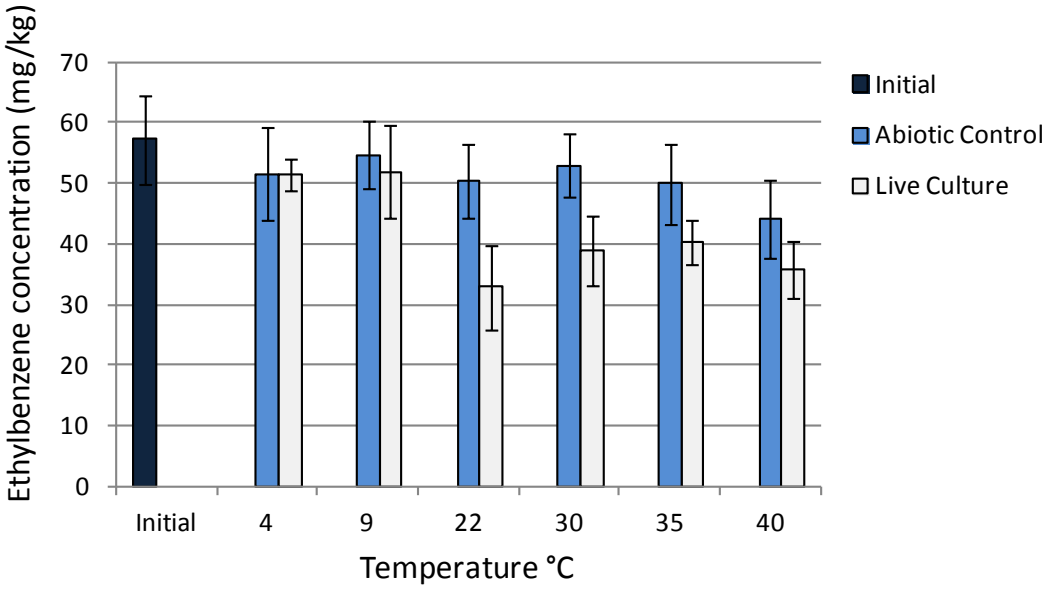

Figure 3.7: Toluene degradation with temperature: Initial concentration in soils is shown along with the final concentrations within the abiotic control and live culture microcosms.

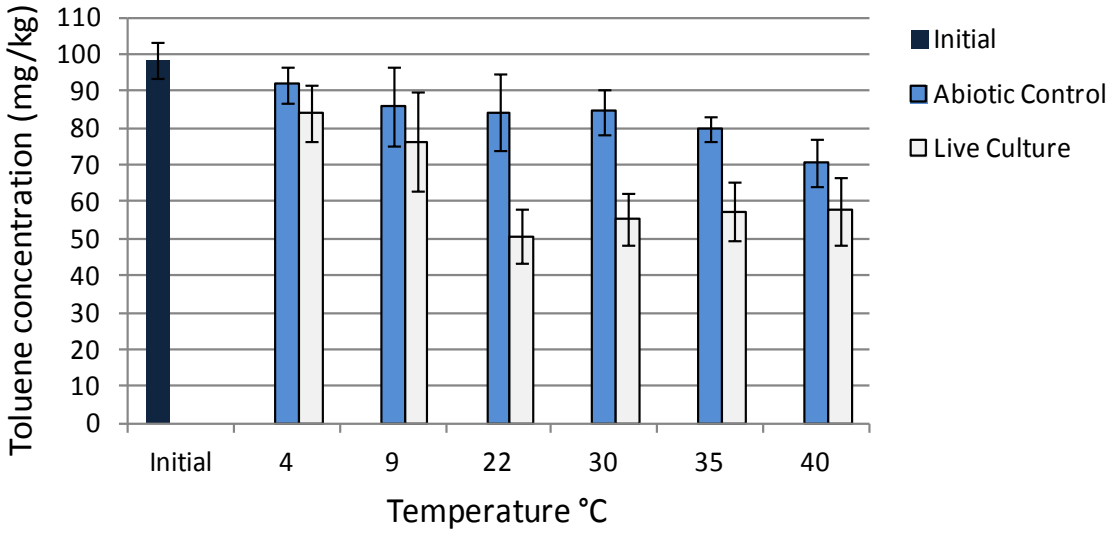

Figure 3.8: Ethylbenzene degradation with temperature: Initial concentration in soil is shown along with the final concentrations within the abiotic control and live culture microcosms.

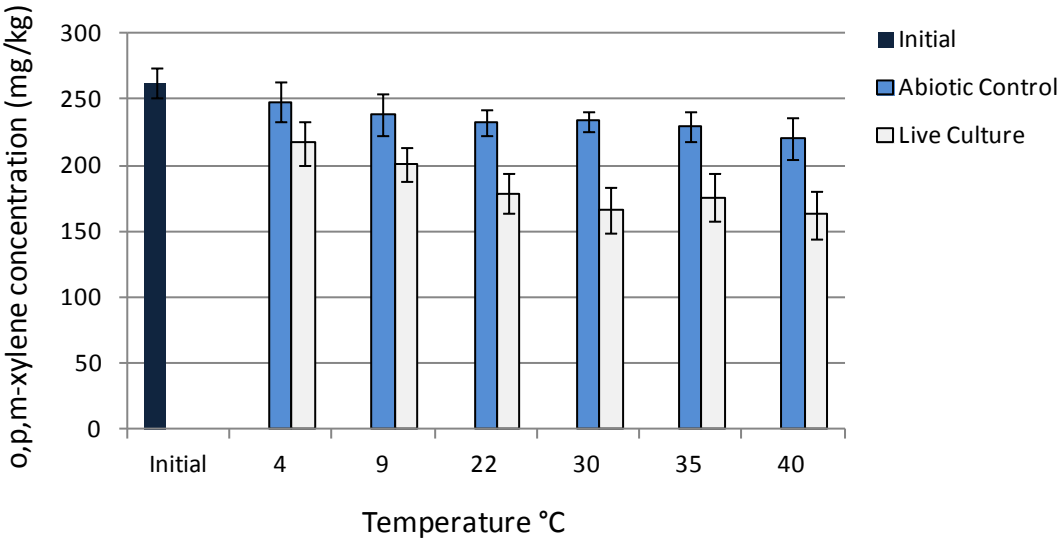

Figure 3.9: Xylene degradation with temperature: Initial concentration in soil is shown along with the final concentrations within the abiotic control and live culture microcosms.

Table 3.1: Percent biological degradation of GRO, DRO, and BTEX compounds

	Percent Biological Degradation					
Temperature	**DRO**	**GRO**	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylenes**
4	**1.8**	**12***	**5.5**	**7.7**	**0.37**	**12**
9	**8.0**	**10**	**4.4**	**9.6**	**4.9**	**14***
22	**22***	**33***	**26***	**34***	**31***	**21***
30	**19***	**31***	**18***	**30***	**25***	**26***
35	**18***	**23***	**19***	**23***	**17**	**21***
40	**21***	**23***	**13**	**13**	**15**	**22***

*** indicates statistically significant biological removal based on a two-tailed t-test**

The hydrocarbon and biogas results presented herein collectively suggest that the optimal temperature for thermal stimulation of biological degradation within these soils is between 22°C and 30°C. Above 30°C, biological degradation still occurs but the conversion to methanogenic end products takes longer to occur. Higher temperatures of 35°C and 40°C also may be suitable for thermal stimulation, but likely require changes to the subsurface microbial communities before significant methanogenic activity can occur. Increases in temperature also resulted in an increase in abiotic losses (e.g. volatilization) particularly for GRO; therefore, over a longer incubation time in which microorganisms can acclimate, higher temperatures of 35°C and 40°C could result in higher overall removal of LNAPL. Further research is required to resolve whether temperatures above 30°C are beneficial or even viable for field applications of STELA.

3.4.3 Molecular Data

Microbial activity, monitored via ATP testing, was measured throughout the experiment for all temperatures. Results of ATP concentrations for all temperatures are shown in Figure 3.10 as a function of time.

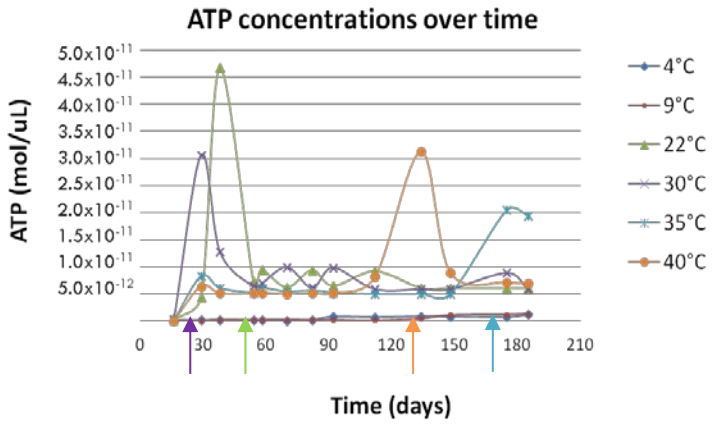

Figure 3.10: ATP concentrations: Individual lines on the graph represent the different temperatures. The arrows on the x-axis indicate onset of CH_4 and CO_2 production at the different temperatures.

Throughout the course of the experiment, ATP concentrations remained at least an order of magnitude lower within the soil held at 4°C and 9°C compared to ATP concentrations measured from soil at 22°C, 30°C, 35°C and 40°C. The elevated ATP concentrations observed at temperatures 22-40°C suggest that increases in temperature result in an increase in microbial activity. It was also observed that ATP concentrations within soils held at the temperatures of 22-40°C peaked just prior to the onset of CH_4 and CO_2 production within the microcosms held at these temperatures. The increases in ATP at the onset of biogas production may indicate a microbial shift in the anaerobic breakdown of the LNAPL constituents. The microcosms were initially operating under sulfate-reducing conditions, which results in higher energy yields as compared to methanogenesis. Additionally, the initial steps of converting petroleum hydrocarbons into methanogenic precursors (e.g. formate, acetate, H_2) generate more energy than the conversion to CH_4 and CO_2 (Mbadinga et al. 2011; Li et al. 2012). The peak in ATP observed at the onset of CH_4 and CO_2 production could correlate with sulfate-reducing metabolism.

The archaeal clone library derived from soil held at 22°C after 2 months of incubation indicated the presence of both hydrogenotrophic methanogens and acetoclastic methanogens (Table 3.2).

Table 3.2: Archaeal Clone Library Results

Clone ID	Blast Result	% Match	Identities
Arch 1	*Methanosaeta concilii* strain	98%	433/441
Arch 2	*Methanosaeta concilii* strain	100%	514/514
Arch 3	*Methanobacterium bryantii* strain	99%	508/514
Arch 4	*Methanosaeta concilii* strain	98%	433/441
Arch 5	*Methanobacterium bryantii* strain	99%	509/514
Arch 6	*Methanocella paludicola* SANAE	99%	510/514
Arch 7	*Methanobacterium bryantii* strain	99%	509/514
Arch 11	*Methanobacterium bryantii* strain	99%	509/514
Arch 12	*Methanobacterium bryantii* strain	99%	509/514
Arch 13	*Methanobacterium bryantii* strain	99%	509/514
Arch 14	*Methanobacterium bryantii* strain	99%	509/515
Arch 15	*Methanobacterium bryantii* strain	99%	509/516
Arch 16	*Methanobacterium bryantii* strain	99%	511/514
Arch 17	*Methanobacterium bryantii* strain	99%	508/515
Arch 18	*Methanobacterium bryantii* strain	99%	508/516
Arch 19	***Methanocella paludicola* SANAE**	**89%**	**461/516**
Arch 20	*Methanobacterium bryantii* strain	99%	509/514
Arch 22	*Methanocella paludicola* SANAE	99%	510/514
Arch 25	*Methanobacterium bryantii* strain	99%	509/514
Arch 26	*Methanobacterium bryantii* strain	99%	509/515

The results indicated that the methanogenic community within cultures at 22°C was predominantly comprised of *Methanobacterium bryantii* strains after 2 months of incubation. The library also contained *Methanosaeta concilli* strains and *Methanocella paludicola* SANAE. Both *Methanobacterium bryantii* strains and *Methanosaeta concilli* strains are acetoclastic methanogens whereas *Methanocella paludicola* is a hydrogenotrophic methanogen. Nineteen of the 20 Archaeal 16S rRNA gene clones showed >97% sequence similarity to 16S rRNA gene sequences in the public database, suggesting these have been previously described, but one of the clones, Arch 19, had only 89% sequence similarity to the closest relatives in the public database suggesting that it may represent a novel archaeal species.

The archaeal 16S rRNA gene 454 pyrosequencing data represents the composition of the archaeal community at the end of the experiment. Cultures held at 4°C, 22°C, and 35°C were

chosen for this analysis in order to investigate how the community may have changed due to different incubation temperatures. These results are presented in Figure 3.11.

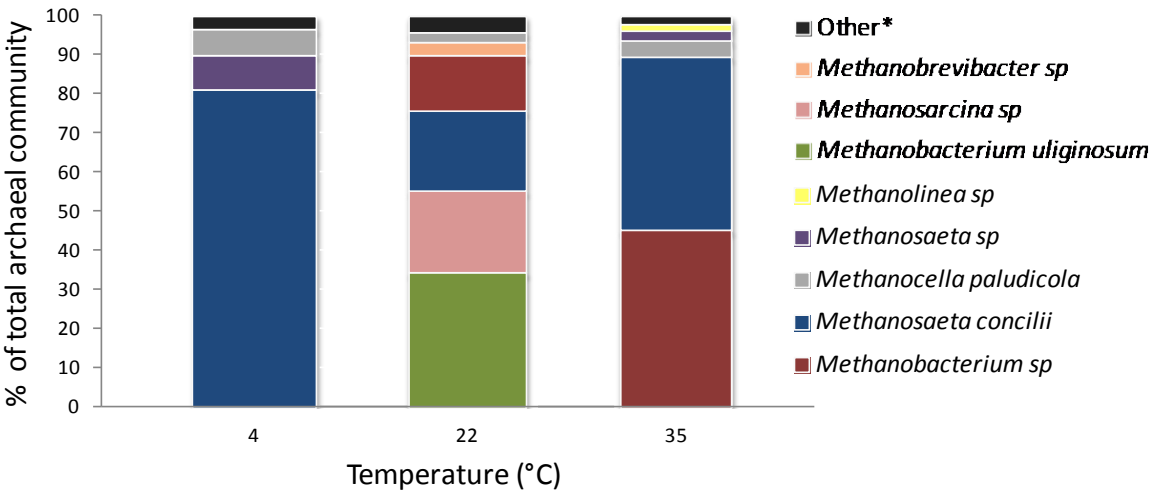

Figure 3.11: Relative abundance of methanogenic phylotypes based on archaeal pyrosequencing. *Microorganisms that make up less than 3% of total community. Additional information is presented in Appendix C.

The results indicate that the archaeal community contained the orders *Methanobacteriales*, *Methanocellales*, *Methanomicrobiales*, and *Methanosarcinales* at the three temperatures investigated (4°C, 22°C, and 35°C). Some species within these orders were present at all three temperatures and others were only observed at one or two temperatures. Hydrogenotrophic methanogens, *Methanobrevibacter* sp., *Methanocella paludicola*, *Methanobacterium* sp., and *Methanolinea* sp. were present at all three temperatures. Two species from the genera *Methanosarcina* (*Methanosarcina barker* and *Methanosarcina* sp.) were only present within the microcosms held at 22°C. *Methanosarcina* species possess the capability to utilize all of the methanogenic substrates (e.g. methanol, acetate and H_2-CO_2), making them the most diverse methanogens (Zinder 1993). The presence of this particular type of methanogen within the 22°C culture could have contributed to the increased CH_4 and CO_2 production observed within microcosms held at this temperature. Because *Methanosarcina* was

not seen at the other temperatures (4°C and 35°C) it is possible that it prefers mesophilic temperatures.

The 16S rRNA bacterial 454 pyrosequencing data indicated that the bacterial community was dominated by the phyla *Firmicutes* and *Proteobacteria* at the three temperatures investigated (4°C, 22°C, and 35°C). The community from the 22°C culture was much higher in *Proteobacteria* compared to 4°C and 35°C. For example, 78.5% of the bacterial sequences determined by pyrosequencing within 22°C were within the phylum *Proteobacteria* whereas the bacterial communities from 4°C and 35°C only had 37.9% and 46.5 % of sequences representing *Proteobacteria* respectively (Table 3.3).

Table 3.3: Phylum data from bacterial community at 4°C, 22°C, and 35°C.

Phylum	4°C	22°C	35°C
Firmicutes	53.4	10.7	35.1
Proteobacteria	37.9	78.5	46.5
Chloroflexi	0.6	0.8	8.0
Bacteroidetes	7.3	7.3	4.9
Spirochaetes	0.1	1.1	0.6
Actinobacteria	0.2	0.4	3.0
Acidobacteria	0.1	0.1	0.6
TM7	0.2	ND	0.2
Gemmatimonadetes	0.1	ND	ND
Caldiserica	0.1	ND	0.1
Thermodesulfobacteria	0.1	0.2	0.1
Verrucomicrobia	ND	0.5	0.1
Synergistetes	ND	0.3	ND
Tenericutes	ND	0.1	ND
OP11	ND	ND	0.4
Chlamydiae	ND	ND	0.1
Chlorobi	ND	ND	0.1

Genera and species data from the bacterial community based on the pyrosequencing results is presented for each temperature in Figure 3.12.

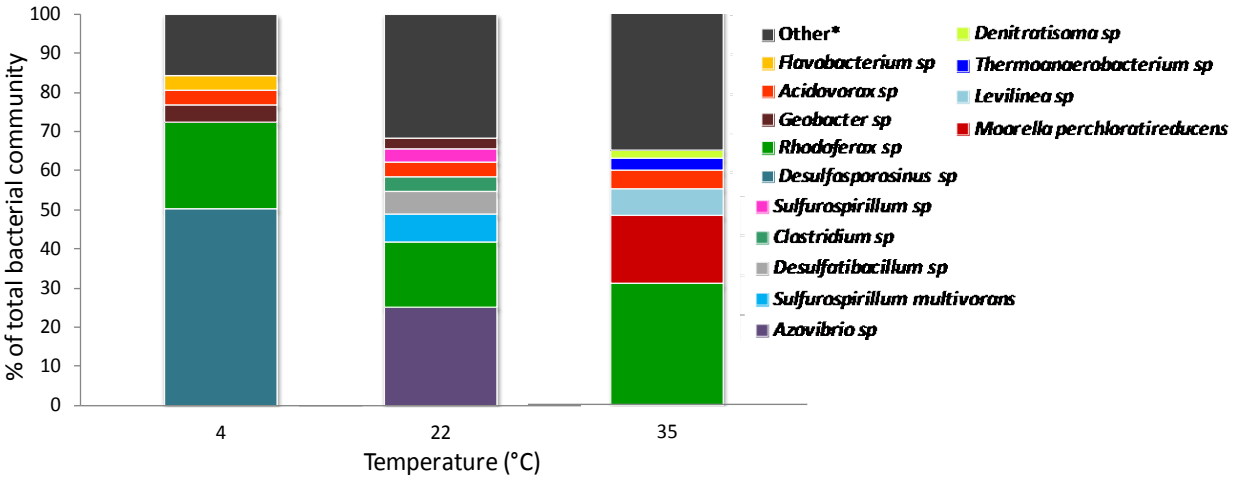

Figure 3.12: Relative abundance of anaerobic bacteria phylotypes based on bacterial pyrosequencing. *Microorganisms that make up less than 3% of total community. Additional information is presented in Appendix C.

The bacterial community within microcosms held at 4°C for 188 days was dominated by species relating to the genera *Desulfosporosinus* and *Rhodoferax*. Other organisms present at this temperature included *Geobacter* sp., *Acidovorax* sp., and *Flavobacterium* sp. *Desulfosporosinus* sp. represented 50% of the sequences within the 4°C culture and was detected at 22°C but did not represent a large portion of the community (0.39%). The same strain was not present at 35°C indicating that it may not be capable of thriving at temperatures higher than 22°C. A benzene-degrading culture that was initially enriched on sulfate and then switched to methanogenic conditions also contained phylotypes affiliated with *Desulfosporosinus* (Ulrich and Edwards 2003). The dominance of *Desulfosporosinus* sp. at 4°C could indicate that sulfate-reducing conditions were still present at this temperature at the end of the experiment. The generation of methanogenic biogas within microcosms held at 4°C was not observed further supporting that complete consumption of sulfate did not occur at this temperature. Based on the

ATP results, microbial activity within the microcosms held at 4°C was an order of magnitude lower than that of 22°C and 35°C. We hypothesize that the decreased microbial activity observed in microcosms at 4°C slowed down the consumption of sulfate resulting in a system that did not reach methanogenic conditions.

Species from the genera *Rhodoferax* (95-97% similarity) were detected at all three temperatures investigated. *Rhodoferax* sp. represented 22% of the community at 4°C, 17% at 22°C, and 31% at 35°C. This genera contains microorganisms capable of anaerobic fermentation (e.g. *Rhodoferax fermentans*). The dominance of *Rhodoferax* sp. within the microbial communities from microcosms held at 4°C, 22°C, and 35°C suggests that it is a versatile microorganism capable of living at a range of temperatures. The presence of this microorganism within the soil microcosms suggests that it contributes to a community involved in the anaerobic breakdown of petroleum hydrocarbons. Additional research is needed to investigate species within the genera *Rhodoferax* and how they are involved in the breakdown of petroleum hydrocarbons under sulfate-reducing and methanogenic conditions.

The bacterial community within cultures held at 22°C was dominated by *Azovibrio* sp. (25% of the community) and *Rhodoferax* sp. Other organisms detected included *Sulfurospirillum multivorans* and *Desulfatibacillum* sp. The genera *Azovibrio* is closely related to *Azoarcus*, *Azospira* and *Azonexus*. These genera contain mostly nitrogen-fixing Betaproteobacteria. The only described species in the genera *Azovibrio* is *A. restrictus* (Reinhold-Hurek and Hurek 2000). *Azovibrio restrictus* is capable of nitrogen fixation but is not known to be fermentative. The dominance of an *Azovibrio* sp. within methanogenic cultures at

22°C could indicate that some microorganisms within the genera *Azovibrio* also are capable of fermentation.

A species that made up 7% of the community in the cultures at 22°C was *Sulfurospirillum multivorans*. This microorganism is strictly anaerobic and is known for reductive dehalogenation of chlorinated ethenes (e.g. tetrachloroethene [PCE] and trichloroethene [TCE]) (Schmitz and Diekert 2004; Ye et al. 2010). Another species from the genera *Sulfurospirillum*, *Sulfurospirillum cavolie*, was isolated from a petroleum contaminated aquifer (Kodama et al. 2007). The presence of *Sulfurospirillum multivorans* in the hydrocarbon-degrading microcosms held at 22°C and the isolation of *Sulfurospirillum cavolie* from a petroleum contaminated aquifer suggest that species within this genus might be capable of petroleum hydrocarbon degradation. Additionally, a species within the genera *Desulfatibacillum* was detected at all three temperatures but was more dominant at 22°C (6%). Isolates from the genera *Desulfatibacillum* capable of degrading alkanes have been isolated previously; *Desulfatibacillum alkenivorans* AK-01 from petroleum-contaminated estuarine sediments (So and Young 1999) and *Desulfatibacillum aliphaticivorans* CV2803 from hydrocarbon polluted marine sediments (Cravo-Laureau et al. 2004).

The community at 35°C was dominated by a species from the genera *Rhodoferax* (31%) and *Moorella perchloratireducens* (17%). *Moorella perchloratireducens* is a thermophillic microorganism that has been previously shown to be capable of reducing perchlorate and nitrate under anaerobic conditions. It was not detected in the cultures held at 4°C or 22°C. This species was recently isolated from the produced water from underground gas storage in Russia (Balk et al. 2008). The dominance of this microorganism in the microcosms held at 35°C indicates that it

might contribute to petroleum hydrocarbon degradation within environments at elevated temperatures.

Shannon weaver index values revealed that the bacterial community was more diverse than the archaeal community at all temperatures (Figure 3.13).

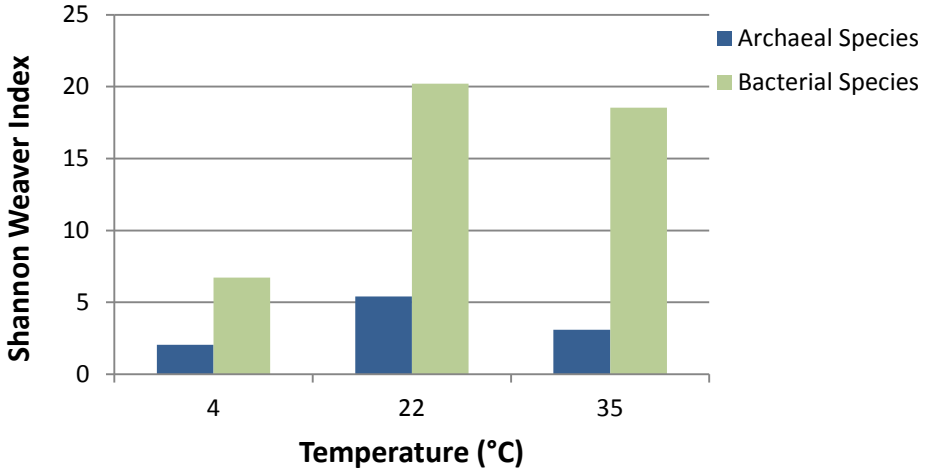

Figure 3.13: Archaeal and Bacterial Community Shannon Weaver Index Values with Temperature

With respect to different incubation temperatures, the most diverse archaeal and bacterial communities were present at 22ºC. The more diverse archaeal community at 22ºC might be related to the higher biogas generation within microcosms held at 22°C relative to microcosms at 4°C and 35°C. Microcosms at 4°C did not see sufficient biological degradation of LNAPL constituents and also had the lowest archaeal and bacterial community diversity. Temperature increases from 4ºC to 22ºC and 35ºC not only increased the biological degradation of GRO and DRO but also increased the diversity of the microbial community. This result suggests that the biological degradation of petroleum hydrocarbons under methanogenic conditions is more

efficient with a diverse bacterial and archaeal community which results from temperature elevations.

Overall, the pyrosequencing results showed that both the archaeal and bacterial communities were impacted by the incubation temperature. The difference in the community structure at 4ºC, 22ºC and 35ºC suggests that different temperatures selected for different microorganisms. Furthermore, the results support the hypothesis that the observed lag in methanogenesis at 35°C and 40°C may have been due to required shifts in both the archaeal and bacterial communities.

3.5 CONCLUSION

Results indicate that biodegradation of petroleum hydrocarbons within LNAPL zones under anaerobic conditions can be accelerated by maintaining temperatures at ~20-40°C. However, per work by others, the optimal temperature depends on the indigenous microbial community present. Based on the results of this microcosm experiment, the optimal temperature for biodegradation within the subsurface at Evansville, WY is likely between 20-30°C. If it is desirable to increase volatilization as well as microbial activity, a higher temperature (e.g. 40°C) could be used, but results indicate that there may be a delay in the onset of methanogenesis and further research is required to determine impact of longer incubations at higher temperatures. Overall, GRO, DRO and BTEX compounds degraded more readily within microcosms held at temperatures of 22-40°C suggesting that the microbial community present was capable of degrading a broad range of petroleum hydrocarbons at elevated temperatures.

Both the archaeal clone library and 16S archaeal pyrosequencing data showed that both acetoclastic and hydrogenotrophic methanogens were present within the archaeal community.

Furthermore, the methanogens *Methanosarcina barkeri* and *Methanosarcina* sp. were present at 22°C and not at 4°C and 35°C. The absence of this microorganism could explain why CH_4 and CO_2 production was lower in 35°C and did not occur at 4°C. 16S rRNA gene bacterial pyrosequencing data for microcosms held at temperatures of 4°C, 22°C and 35°C indicated that the structure of the bacterial community varied based on incubation temperature. The bacterial community was much more diverse than the archaeal community for all three temperatures and was dominated by phyla *Firmicutes* and *Proteobacteria*. Apparent shifts were observed in both the bacterial and archaeal community with temperature with community diversity varying with temperature. Based on the pyrosequencing results, the most diverse archaeal and bacterial community was present at 22ºC.

Overall, the results of this study indicate that the use of thermally enhanced bioremediation for LNAPL zones under sulfate-reducing and methanogenic conditions has the capability to increase the rate and extent of petroleum hydrocarbon biodegradation as well as have an impact on microbial ecology. Additional research is warranted to validate the efficacy of this technique at a field scale.

Acknowledgements

This research was supported by Chevron and the University Consortium for Field-Focused Groundwater Contamination Research. I would also like to express my gratitude to Julio Zimbron, Mitchell Olson, Emilie Lefèvre and Gary Dick for the help and support that they provided throughout the course of this work.

4 SUSTAINABLE THERMALLY ENHANCED LNAPL ATTENUATION PILOT STUDY

4.1 INTRODUCTION

In order to study the efficacy of a thermal treatment on the biological degradation of LNAPL, a pilot study will be implemented at the decommissioned refinery site in Evansville, WY. This pilot study builds on the microcosm study presented in Chapter 3, seasonal CO_2 flux data from the site collected by Kevin McCoy (McCoy Thesis, 2012) and an ongoing (unpublished) Engineered Attenuation Zones field study being conducted at the Evansville site. Based on the results of the microcosm study presented in Chapter 3, the target temperature for the pilot is between 20 and 30°C.

The purpose of the pilot study is to evaluate the cost, feasibility and performance of STELA. There are several specific objectives associated with the STELA pilot. The first objective is to determine the amount of energy needed to increase subsurface temperatures to temperatures within the range of 20-30°C. The second objective is to investigate heat transport through an aquifer by monitoring temperature laterally and with depth. Another objective is to determine how the performance of a STELA system could be impacted by seasonal temperature changes. Furthermore, the STELA pilot will allow us to determine the amount of time it takes for a thermal treatment to achieve plausible cleanup levels in an LNAPL zone.

The STELA pilot is in the process of being implemented in an LNAPL source zone area at the Evansville site. Multiple level sampling systems (MLS) have been installed for monitoring purposes at 17 locations. The MLS allow for depth discrete temperature, water and

gas sampling. Furthermore, at grade CO_2 traps are being used in addition to the MLS to monitor CO_2 flux. By monitoring the CO_2 flux LNAPL losses can be determined (See Figure 4.1).

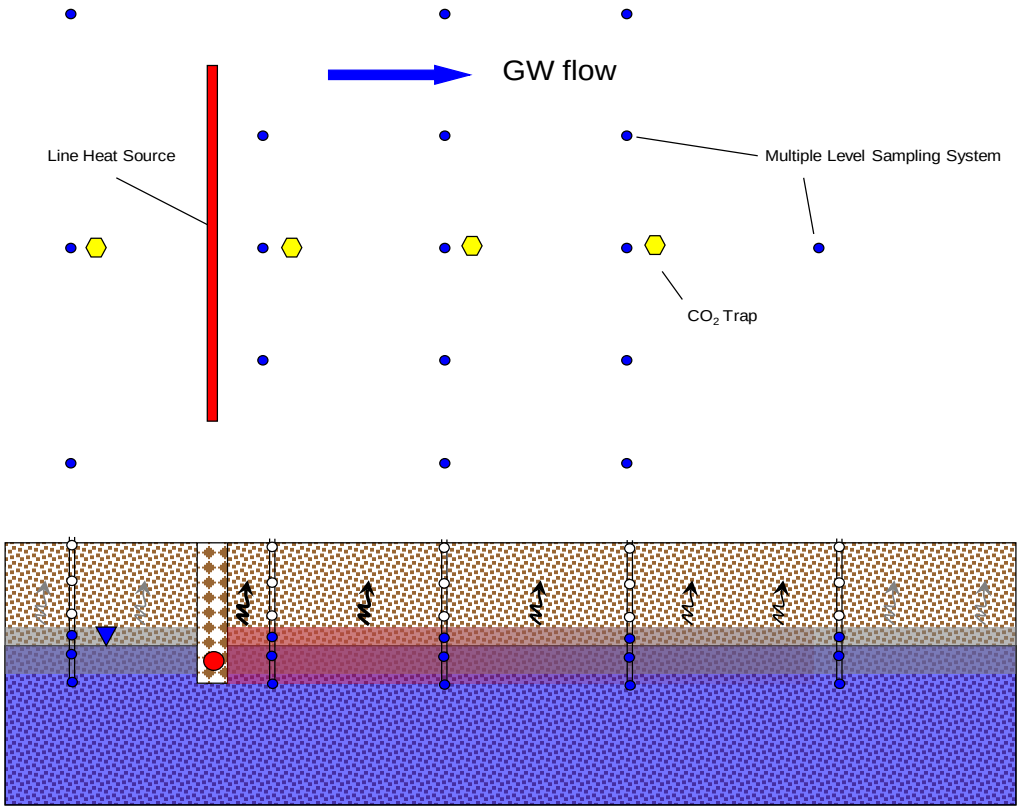

Figure 4.1: STELA pilot study layout: plan view and side view

The rest of this chapter will present the work that has been done to date on the STELA pilot. This includes collection of soil cores, design and installation of MLS, basic heating element design, and presentation of preliminary data.

4.2 SOIL CORE COLLECTION

Direct push drilling was used during the soil core collection. There is no rotation involved in direct push drilling and this method allowed for the recovery of soil sample cores sealed inside a tube so that no direct handling of the sample took place. Approximately 14 ft of material was extracted at each of the 17 MLS locations. Soil cores were collected in a series of 5 ft long acetate sleeves. Soil cores were flash frozen using dry ice and transported back to Colorado State University for analysis of hydrocarbon content, microbial ecology, and mineralogy to establish baseline data for the area.

4.3 MULTILEVEL SAMPLING SYSTEM DESIGN AND INSTALLATION

Multilevel Sampling System Design

The MLS allows for depth discrete gas (CO_2 and CH_4) and water sampling with a total of six ports; three gas sampling ports in the vadose zone and three groundwater sampling ports. Additionally, at each sample port is a thermocouple which allows for temperature data collection. Multilevel samplers were made at the Center for Contaminant Hydrology at Colorado State University by Gary Dick.

Each MLS consists of 1/2 inch inner diameter (ID) schedule 40 PVC pipe (RNR supply, Fort Collins, CO). 1/8 inch ID Fluorinated Ethylene Propylene (FEP) tubing is secured at different intervals along the PVC pipe for gas and water sample ports (US Plastic). Six individual pieces of FEP tubing is spaced at 2-ft intervals to allow for 3 gas sample ports and 3

water sample ports. The ends of the tubing are covered with nytex screen (153µm) to filter out silt and sand particles (Wildlife Supply Co, Yulee, FL).

The thermocouples are attached next to each of the sample ports. The thermocouples are made with Type K parallel construction thermocouple wire (24 AWG, Teflon coated) and Type K miniature thermocouple connectors. The sensing end was spot welded to create the Type K thermocouple (TC Direct, United Kingdom). In order to protect the thermocouple, a glass casing was fabricated from 4mm OD soft glass tubing. The fabricated glass cup was filled with epoxy (Tra-Bond Bipax) and the spot welded end of the thermocouple wire was inserted into the cup.

Multilevel Sampling System Installation

After soil core collection, multilevel samplers (MLS) were installed at the locations where soil core was taken. Direct push drilling was followed up by closed stem agar drilling to increase the diameter of the excavation. Once drilling had reached 14 ft below ground surface, three inch ID slotted PVC was lowered to the bottom of the bore hole. The MLS was then placed inside the slotted PVC down the bottom of the bore hole (~14 feet below ground surface). The area between the MLS and slotted PVC was backfilled with sand to act as a filter pack. Figure 4.2 is a schematic of an installed MLS.

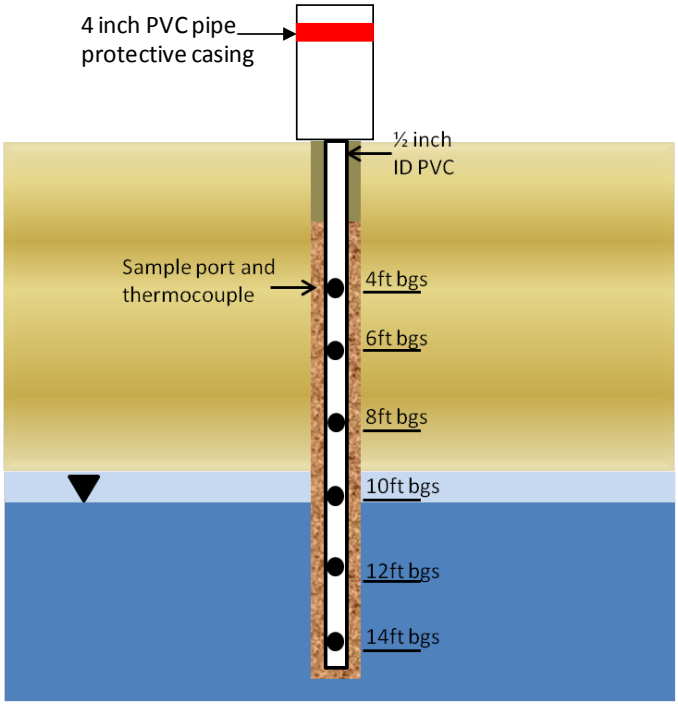

Figure 4.2: Multilevel Sampler schematic

4.4 HEATING ELEMENT

The heating element will be installed once the baseline data has been collected and analyzed. Heat will be delivered through the formation using submersible heat trace wire. A line of 10 wells will be drilled between the A and B monitoring wells and submersible heat trace helixed around PVC pipe will be lowered into each of the wells. Figure 4.3 depicts how heat will be delivered through the formation. Line power will be used initially to allow for an accurate understanding of heat transport through the formation.

Eventually, power could be delivered by a 2000 Watt solar panel system to increase the sustainability of the remediation treatment. The use of a sustainable power source is part of the development of STELA.

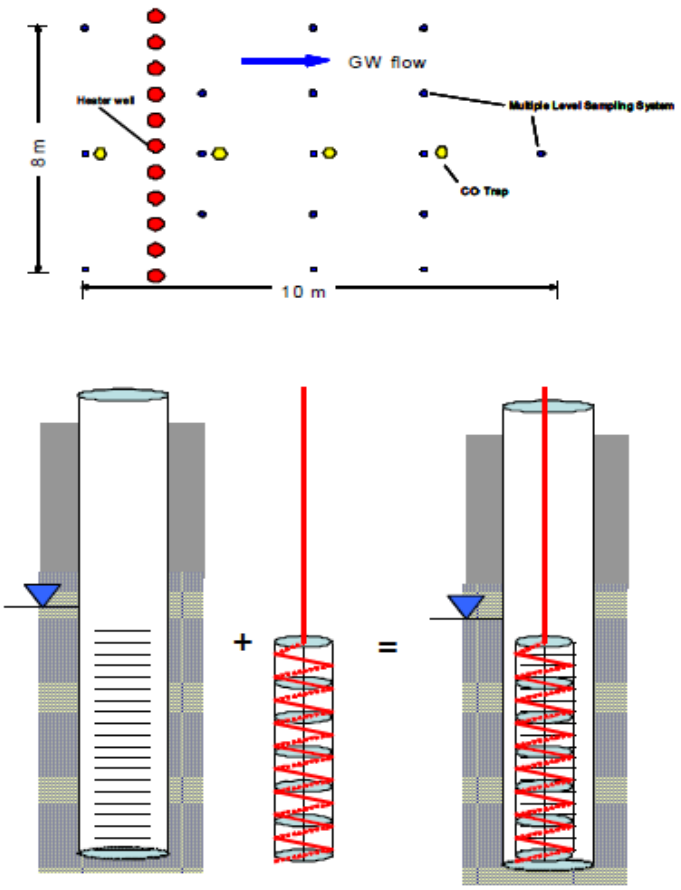

Figure 4.3: Heating Element Design

4.5 PRELIMINARY BASELINE DATA

Thus far, gas samples, water samples, and soil samples have been collected for each of the MLS locations. Soil cores were collected during MLS installation on November 8th, 2011. Temperature data within the formation has also been collected for each location with depth. Temperature data was collected in December of 2011, and February, March and May of 2012.

A full sampling event occurred on March 28th, 2012. During this sampling event three water samples were collected from each of the aqueous MLS ports for anion, cation, and TPH analysis. pH and ORP measurements for groundwater samples were also taken in the field. Gas

samples from the vadose zone were analyzed for CH_4, CO_2 and O_2 at all of the vapor sampling ports on the MLS systems. Methods used in the field can be found in Appendix C.

The available data that will be presented here includes soil hydrocarbon data from the soil cores (diesel range organics DRO, gasoline range organics GRO), CH_4 and CO_2 concentrations in the vadose zone, temperature data, and sulfate data from water samples. The data presented in the following graphs will show data with depth from the wells along the center transect (Figure 4.4).

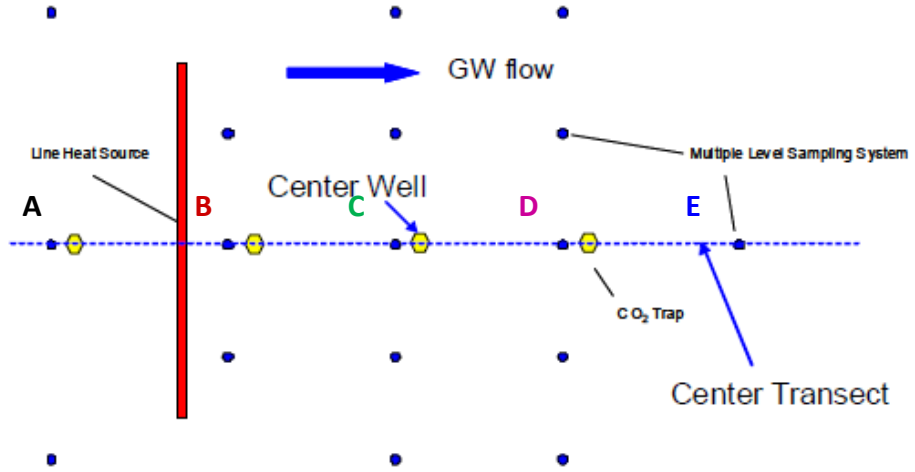

Figure 4.4: Center Transect Wells

Biogas Data

A Landtec GEM™2000 gas analyzer was used in the field to measure CH_4, CO_2, and O_2 within the vadose zone for all MLS locations. The baseline gas data along the center transect wells is shown in Figures 4.5, 4.6 and 4.7. CH_4 concentrations increase with depth for all of the center transect wells with the exception of Well A. The concentration in A increases from 4 to 6 feet below ground surface (bgs) and then decreases from 6 to 8 feet bgs. The presence of CH_4 within the vadose zone indicates that methanogenesis is occurring within this LNAPL zone. The

CO_2 concentration shows an increase from 4 to 6 ft bgs and then levels out from 6 to 8 ft and the O_2 concentrations decrease with depth with the exception of Well A. The outlier data point for CH_4, CO_2 and O_2 in Well A needs to be verified.

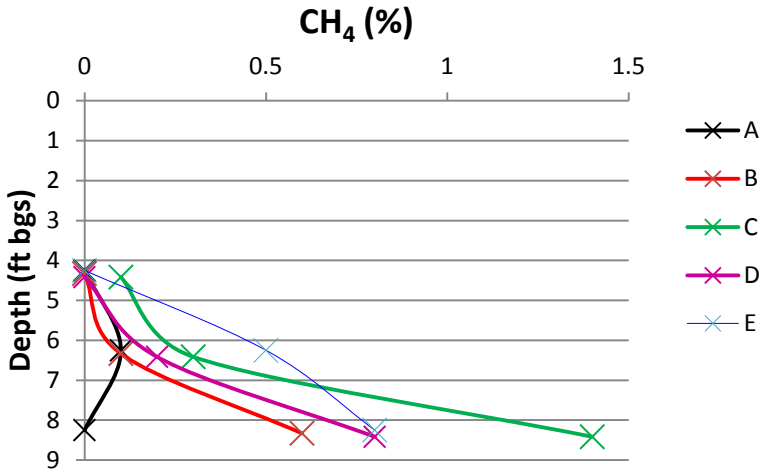

Figure 4.5: CH_4 data from MLS points along center transect

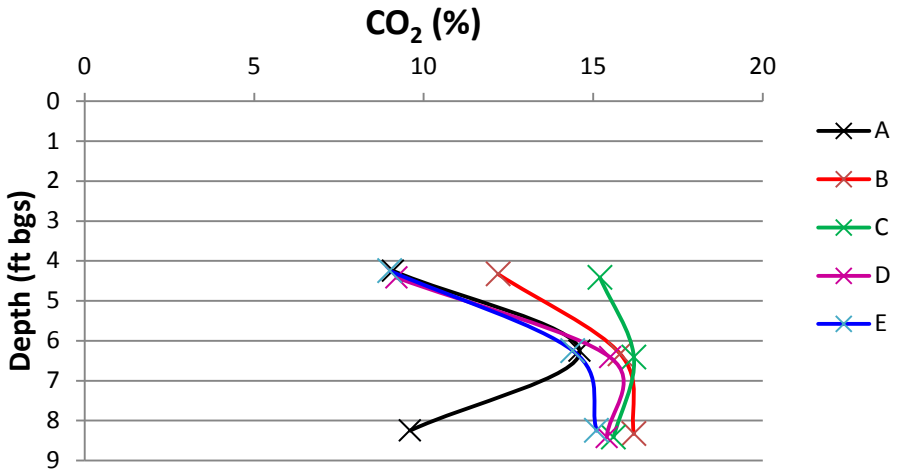

Figure 4.6: CO_2 data from MLS points along center transect

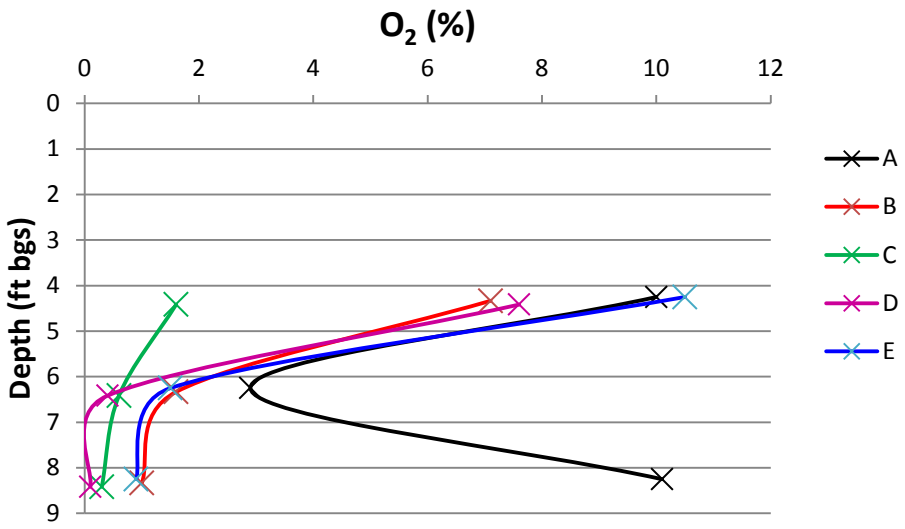

Figure 4.7: O_2 data from MLS points along center transect

Soil Hydrocarbon Data

Soil cores were sub-sampled for hydrocarbon analysis with depth by taking slices of the soil cores and placing the soil into high purity methanol (Honeywell Burdick and Jackson ACS/HPLC Methanol) for extraction. The soil hydrocarbon analysis was performed by Maria Irianni Renno and the plots were put together by Daria Akhbari. Results are shown in Figure 4.8 for DRO and in Figure 4.9 for GRO. The results show higher concentrations showing up near the water table which sits between 8 and 9 feet bgs with DRO and GRO concentrations peaking in well A at 25,226 mg/kg and 6,467 mg/kg respectively. The concentrations reveal and confirm that the area chosen for the pilot is a highly contaminated LNAPL zone. Soil cores will be taken at the end of the study at locations directly next to the MLS locations to determine if soil concentrations decreased. Water samples are also being collected to track hydrocarbon concentrations in the aqueous phase.

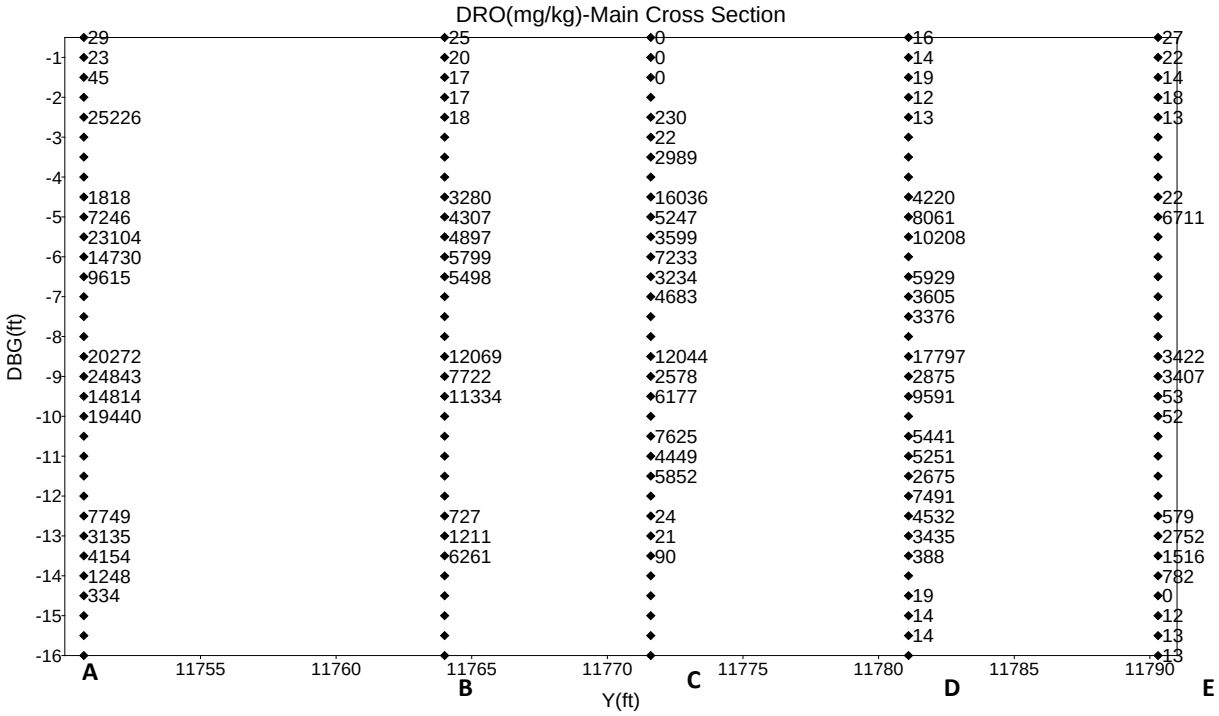

Figure 4.8: DRO results from soil cores along the center transect wells with depth

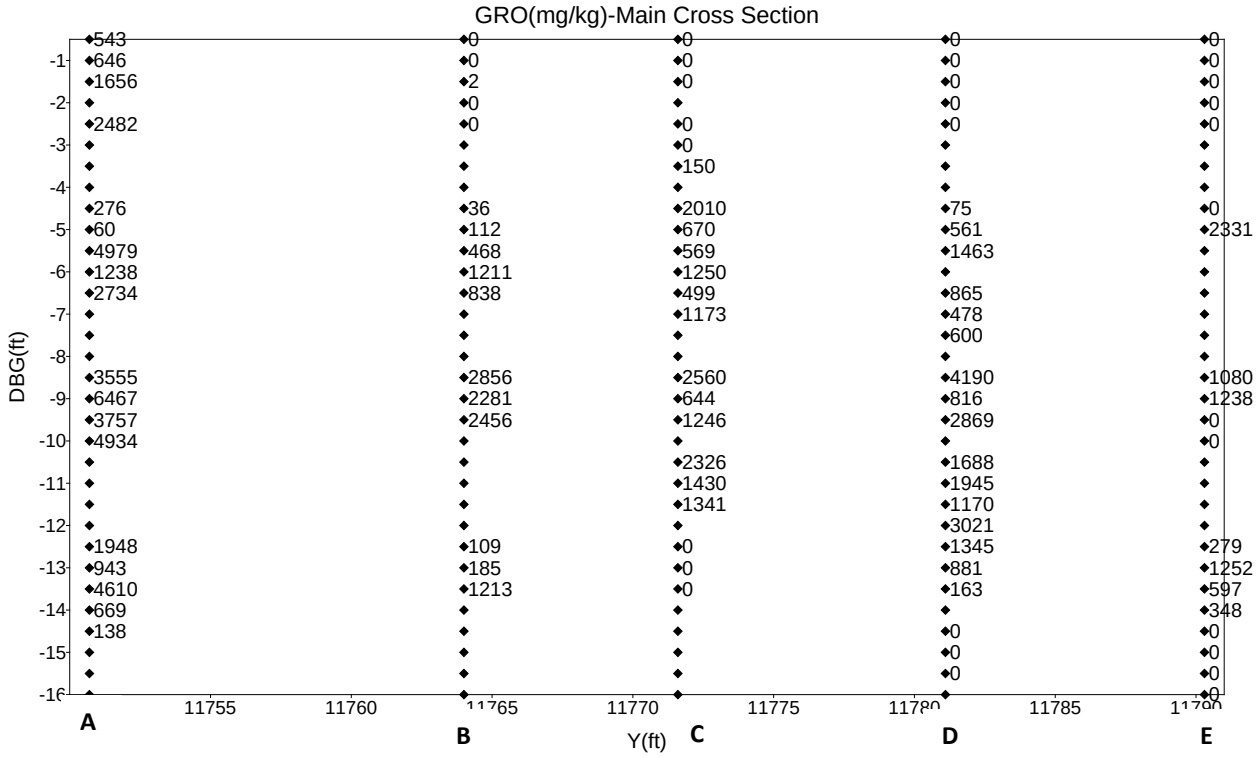

Figure 4.9: GRO results from soil cores along the center transect wells with depth

Temperature Data

The temperature data is shown in Figure 4.10. The figure was put together by Daria Akhbari. This data demonstrates that temperatures fluctuate in the subsurface with changing seasons. During the colder months of December and February, temperature increases proportionally with depth. In May, temperature tends to decrease with depth. Summer temperatures are not presented here but will likely reveal an increase in temperatures at all depths. The target temperature for the design is between 20-30°C per the laboratory LNAPL microcosm study results. Some subsurface temperatures measured were at 16°C during the month of December. This reveals that only a small amount temperature increase will be necessary in order to reach optimal temperatures during some months. Natural temperature fluctuations in the subsurface present challenges that will need to be addressed during the design and implementation of the heating system.

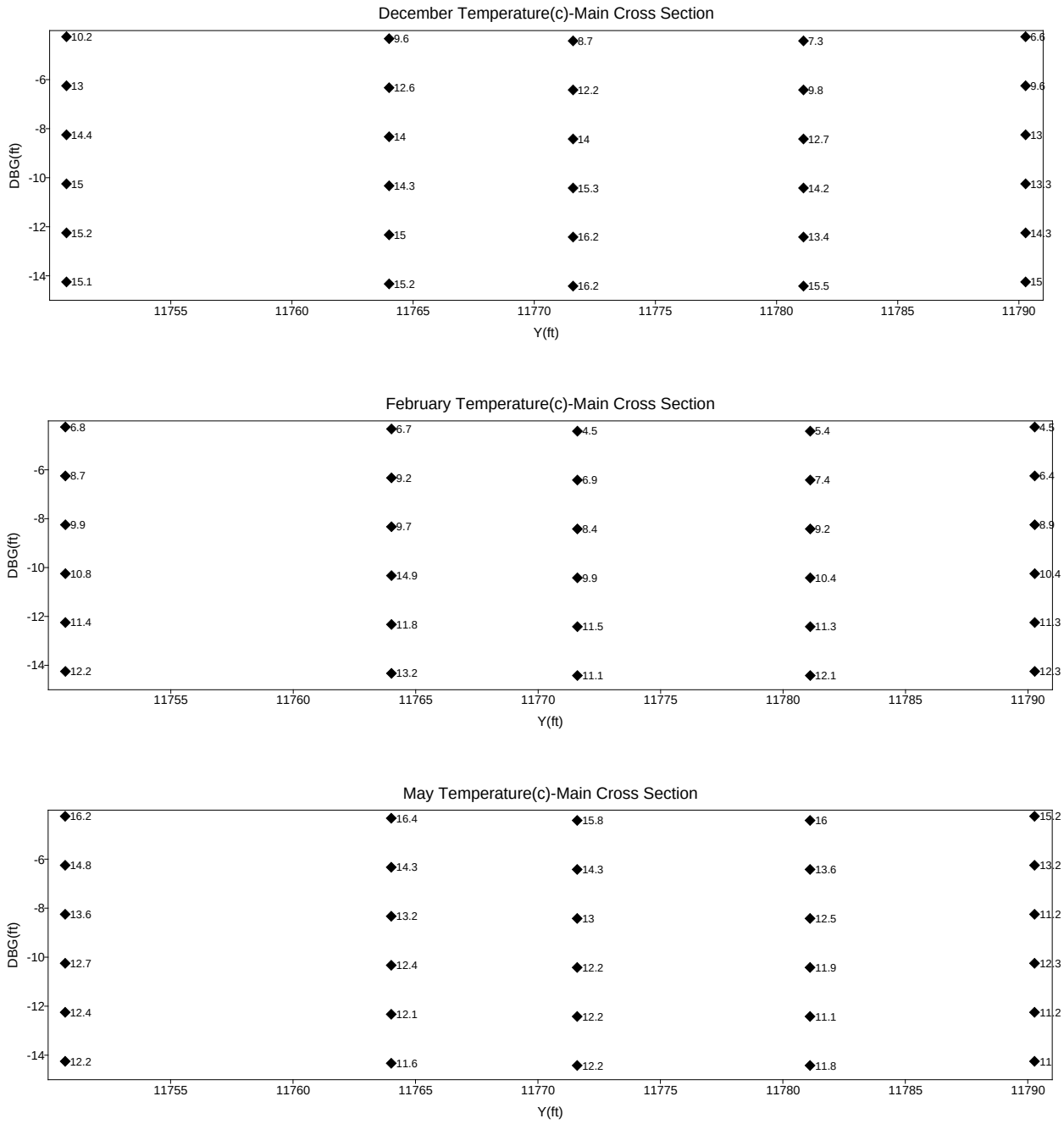

Figure 4.10: Field temperature data for wells along center transect for the months of December, February and May.

Sulfate Data

Water samples collected from the MLS during the sampling event on March 28th, 2012 were analyzed for sulfate (SO_2^{-4}) concentrations. This analysis was done at the Center for Contaminant Hydrology at Colorado State University by Ellen Daugherty using an Ion Chromatograph. This data is shown in Figure 4.11.

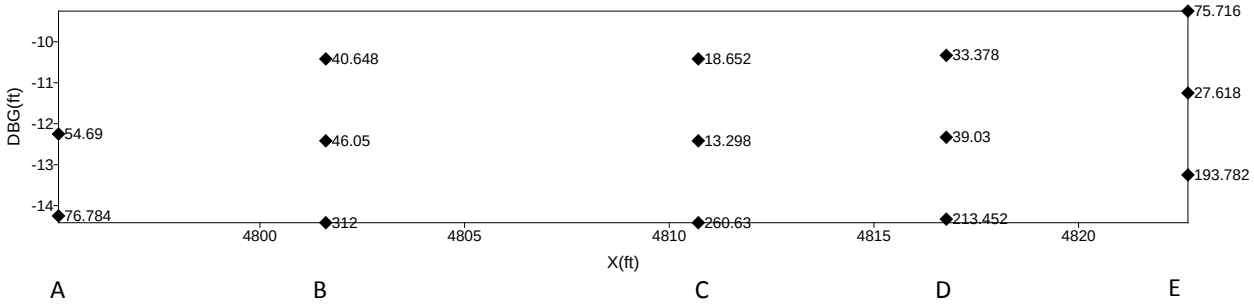

Figure 4.11: Sulfate concentrations in water samples collected from center transect MLS

The presence of SO_2^{-4} within the groundwater indicates that sulfate reduction is a likely pathway that could contribute to the biological degradation of LNAPL constituents in the aqueous phase. Because CH_4 was detected in the vadose zone the primary degradation pathways present at the site of the thermal pilot are sulfate reduction and methanogenesis.

4.4 CONCLUSION

The thermal pilot in Evansville, WY will be the first application of low levels of heat *in situ* with the intent to increase the anaerobic biodegradation rates of LNAPL. Target temperatures are between 20 and 30°C and are based on the results of the LNAPL thermal microcosm study presented in section 3 of this thesis. Temperature results indicate that temperatures will need to be elevated from about 5 to 15°C depending on the time of year and depth bgs. Gas data as well as SO_2^{-4} data indicate that anaerobic conditions are present in the thermal pilot area with sulfate reduction and methanogenesis being the dominant pathways for LNAPL biodegradation. Petroleum hydrocarbon levels measured in the soil cores were higher in DRO than GRO and had overall TPH of up to 31,310 mg/kg at 9 ft in well A. The conditions at the thermal pilot site are consistent with the conditions of the LNAPL microcosm study. Thus, it is anticipated that if temperatures are kept between 20-30°C, petroleum hydrocarbon degradation will be increased significantly.

4.5 ACKNOWLEDGEMENTS

I would like to thank my co-workers at both Colorado State University and Trihydro for their contribution to this project. Specifically, I would like to thank Maria Irianni Renno and Daria Akhbari for continuing the work on the thermal pilot study and helping to put together the preliminary baseline data. Also, I would like to thank Ellen Daugherty for her anion analysis of the water samples. Finally, I would like to thank Chevron and the University Consortium for Field-Focused Groundwater Contaminations Research for the financial support of this project.

5 THESIS CONCLUSIONS

The focus of this thesis was to investigate thermally enhanced bioremediation of LNAPL. This remedial strategy focuses on managing temperature to optimize conditions for microbial depletion of petroleum hydrocarbons in LNAPL zones. This work focused specifically on the anaerobic breakdown of LNAPL at a former refinery located in Evansville, WY.

A laboratory thermal microcosm study using soils, groundwater, and LNAPL from the site indicated an optimal temperature within the range of 22-30°C. The anaerobic microbial breakdown of petroleum hydrocarbons to CH_4 and CO_2 at temperatures of 22°C and 30°C occurred faster and to a higher degree when compared to the other temperatures investigated during the duration of the microcosm experiment. An investigation into the microbial ecology within cultures held at 4°C, 22°C, and 35°C revealed that a thermal treatment impacts the microorganisms present. The bacterial and archaeal communities changed depending on the incubation temperature. Interestingly, microcosms held at 22°C contained species from the genera *Methanosarcina* that was not present at the other two temperatures analyzed for microbial ecology. Species within this genera of methanogens possess the capability to utilize all three of the major methanogenic substrates (methanol, acetate and H_2/CO_2), making them the most diverse methanogens. The microcosm study showed that a thermal treatment could enhance the anaerobic biodegradation of petroleum hydrocarbons and select for different types of microorganisms.

Building on the results of the thermal microcosm study, a pilot study is underway at the Evansville site. This pilot study will reveal if thermal enhancement of an LNAPL zone can increase the anaerobic biodegradation of the contaminant at a larger scale. Sustainability has

been incorporated into the design of the pilot study by using solar power to deliver heat to the subsurface. Conclusions and outcomes of the pilot study will be summarized in the masters theses of other students.

Thermal enhanced bioremediation of petroleum hydrocarbons has the potential to increase biodegradation rates at other LNAPL impacted sites. Based on results from this thesis, thermal treatments could be particularly successful at sites in which there is seasonal variability similar to that of Evansville, WY. Determining the optimal temperature and keeping that temperature constant through all seasons could prevent a lag that occurs when seasonal temperatures decrease.

6 SUGGESTIONS FOR FUTURE RESEARCH

A mixed LNAPL was the contaminant investigated in the research presented in this thesis. An understanding of how temperature impacts the biodegradation of individual compounds within LNAPL bodies is an important step in developing thermal bioremediation. For example, microorganisms involved in benzene degradation might have a different optimal temperature than microorganisms involved in naphthalene degradation. Thermal microcosm studies looking at individual compounds and individual microorganisms involved in their removal is warranted.

Furthermore, thermally enhanced bioremediation could be applied to environmental contaminants other than petroleum liquids. Determining optimal temperatures for biodegradation of a variety of environmental contaminants (e.g. chlorinated solvents, heavy metals) could increase the applicability and versatility of thermally enhanced bioremediation.

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APPENDIX A: SUPPLEMENTAL DATA FOR THERMAL MICROCOSM STUDY

16S Bacterial Pyrosequencing Data: Kingdom; Phylum; Class; Order; Family; Genus; Species	Temperature °C		
	4	22	35
Classification	Relative Abundance (%)		
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Rhodoferax ; Rhodoferax sp	22	17	31
Bacteria ; Firmicutes ; Clostridia ; Thermoanaerobacterales ; Thermoanaerobacteraceae ; Moorella ; Moorella perchloratireducens	0	0	17
Bacteria ; Chloroflexi ; Anaerolineae ; Anaerolineales ; Anaerolineaceae ; Levilinea ; Levilinea sp	0.2	0.6	7.1
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Acidovorax ; Acidovorax sp	3.9	3.8	4.5
Bacteria ; Firmicutes ; Clostridia ; Thermoanaerobacterales ; Thermoanaerobacteraceae unclassified ; Thermoanaerobacterium ; Thermoanaerobacterium sp	0	0.17	3.18
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Coriobacteriales ; Coriobacteriaceae ; Eggerthella ; Eggerthella sp	0	0.13	2.07
Bacteria ; Proteobacteria ; Betaproteobacteria ; Rhodocyclales ; Rhodocyclaceae ; Denitratisoma ; Denitratisoma sp	1.02	0.95	1.99
Bacteria ; Bacteroidetes ; Bacteroidia ; Bacteroidales ; Bacteroidaceae ; Bacteroides ; Bacteroides sp	1.02	1.99	1.77
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfotomaculum ; Desulfotomaculum sp	0.05	0.04	1.74
Bacteria ; Firmicutes ; Clostridia ; Thermoanaerobacterales ; Thermoanaerobacteraceae ; Thermacetogenium ; Thermacetogenium phaeum	0	0	1.74
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfotomaculum ; Desulfotomaculum arcticum	0	0	1.74
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Clostridiaceae ; Clostridium ; Clostridium sp	1.38	3.93	1.59
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfotomaculum ; Desulfotomaculum alcoholivorax	0	0	1.48
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Pelotomaculum ; Pelotomaculum sp	0	0.04	1.14
Bacteria ; Bacteroidetes ; Flavobacteria ; Flavobacteriales ; Flavobacteriaceae ; Flavobacterium ; Flavobacterium sp	3.70	1.68	1.11
Bacteria ; Bacteroidetes ; Flavobacteria ; Flavobacteriales ; Flavobacteriaceae ; Lutibacter ; Lutibacter maritimus	1.64	1.68	1.11
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Xanthomonadales ; Xanthomonadaceae ; Lysobacter ; Lysobacter sp	0.63	0.43	1.03
Bacteria ; Firmicutes ; Clostridia ; Thermoanaerobacterales ; Thermoanaerobacteraceae ; Moorella ; Moorella glycerini	0	0.0	0.96
Bacteria ; Chloroflexi ; Anaerolineae ; Anaerolineales ; Anaerolineaceae ; Longilinea ; Longilinea sp	0	0.13	0.81
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Bacillus ; Bacillus sp	0	0.09	0.74
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfobacterales ; Desulfobacteraceae ; Desulfatibacillum ; Desulfatibacillum sp	0.10	5.79	0.70
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Geobacillus ; Geobacillus sp	0	0	0.70
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Xanthomonadales ; Xanthomonadaceae ; Xanthomonas ; Xanthomonas translucens	0.50	0.39	0.66
Bacteria ; Proteobacteria ; Betaproteobacteria ; Gallionellales ; Gallionellaceae ; Sideroxydans ; Sideroxydans sp	0.10	2.37	0.63
Bacteria ; Spirochaetes ; Spirochaetia ; Spirochaetales ; Spirochaetaceae ; Treponema ; Treponema sp	0.03	0.47	0.55
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Vulcanibacillus ; Vulcanibacillus modesticaldus	0	0	0.55
Bacteria ; Proteobacteria ; Betaproteobacteria ; Rhodocyclales ; Rhodocyclaceae ; Azovibrio ; Azovibrio sp	0.18	25.13	0.52
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Clostridiaceae unclassified ; Sedimentibacter ; Sedimentibacter sp	1.28	1.60	0.48
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Xanthomonadales ; Xanthomonadaceae ; Rhodanobacter ; Rhodanobacter sp	0.36	0.09	0.48
Bacteria ; OP11 ; OP11 (class) ; OP11 (order) ; OP11 (family) ; OP11 (genus) ; OP11 uncultured	0	0	0.44

16S Bacterial Pyrosequencing Data: Kingdom; Phylum; Class; Order; Family; Genus; Species	Temperature °C		
	4	22	35
Classification	Relative Abundance (%)		
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Chromatiales ; Chromatiaceae ; Thioflavicoccus ; Thioflavicoccus mobilis	0	0	0.18
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Solirubrobacterales ; Conexibacteraceae ; Conexibacter ; Conexibacter sp	0	0	0.18
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Syntrophobacterales ; Syntrophaceae ; Smithella ; Smithella sp	0.16	1.04	0.15
Bacteria ; Verrucomicrobia ; Verrucomicrobiae ; Verrucomicrobiales ; Verrucomicrobiaceae ; Verrucomicrobium ; Verrucomicrobium sp	0	0.52	0.15
Bacteria ; Bacteroidetes ; Cytophagia ; Cytophagales ; Cytophagaceae ; Cytophaga ; Cytophaga sp	0.03	0.22	0.15
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfuromonadales ; Geobacteraceae ; Geobacter ; Geobacter psychrophilus	0.03	0.22	0.15
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Hydrogenophaga ; Hydrogenophaga sp	0.18	0.04	0.15
Bacteria ; Caldiserica ; Caldisericia ; Caldisericales ; Caldisericaceae ; Caldisericum ; Caldisericum exile	0.05	0.04	0.15
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinomycetales ; Cellulomonadaceae ; Cellulomonas ; Cellulomonas bogoriensis	0	0	0.15
Bacteria ; Acidobacteria ; Solibacteres ; Solibacterales ; Solibacteraceae ; Solibacter ; Solibacter sp	0	0	0.15
Bacteria ; Acidobacteria ; Holophagae ; Holophagales ; Holophagaceae ; Geothrix ; Geothrix sp	0	0	0.15
Bacteria ; Chlorobi ; Ignavibacteria ; Ignavibacteriales ; Ignavibacteriaceae ; Ignavibacterium ; Ignavibacterium album	0	0	0.15
Bacteria ; Thermodesulfobacteria ; Thermodesulfobacteria (class) ; Thermodesulfobacteriales ; Thermodesulfobacteriaceae ; Thermodesulfobacterium ; Thermodesulfobacterium sp	0.05	0.17	0.11
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinomycetales ; Streptomycetaceae ; Streptomyces ; Streptomyces sp	0	0.13	0.11
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Alteromonadales ; Alteromonadaceae ; Microbulbifer ; Microbulbifer sp	0	0.13	0.11
Bacteria ; Chloroflexi ; Caldilineae ; Caldilineales ; Caldilineaceae ; Caldilinea ; Caldilinea sp	0.36	0	0.11
Bacteria ; Proteobacteria ; Betaproteobacteria ; Hydrogenophilales ; Hydrogenophilaceae ; Thiobacillus ; Thiobacillus sp	0	0	0.11
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Enterobacteriales ; Enterobacteriaceae ; Serratia ; Serratia proteamaculans	0	0	0.11
Bacteria ; Chlamydiae ; Chlamydiia ; Chlamydiales ; Parachlamydiaceae ; Parachlamydia ; Parachlamydia sp	0	0	0.11
Bacteria ; Proteobacteria ; Betaproteobacteria ; Hydrogenophilales ; Hydrogenophilaceae ; Hydrogenophilus ; Hydrogenophilus sp	0	0	0.11
Bacteria ; Spirochaetes ; Spirochaetia ; Spirochaetales ; Spirochaetaceae ; Spirochaeta ; Spirochaeta sp	0.03	0.52	0.07
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Brachymonas ; Brachymonas petroleovorans	0.34	0.47	0.07
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfovibrionales ; Desulfovibrionaceae ; Desulfovibrio ; Desulfovibrio sp	0	0.43	0.07
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Polaromonas ; Polaromonas sp	0.05	0	0.07
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Chromatiales ; Halothiobacillaceae ; Halothiobacillus ; Halothiobacillus sp	0.03	0	0.07
Bacteria ; Proteobacteria ; Betaproteobacteria ; Methylophilales ; Methylophilaceae ; Methylovorus ; Methylovorus sp	0	0	0.07
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Alcaligenaceae ; Pigmentiphaga ; Pigmentiphaga daeguensis	0	0	0.07
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfosporosinus ; Desulfosporosinus lacus	0	0	0.07
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Chromatiales ; Chromatiaceae ; Thiococcus ; Thiococcus sp	0	0	0.07
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinomycetales ; Microbacteriaceae ; Microbacterium ; Microbacterium sp	0	0	0.07

	4	22	33
Classification	Relative Abundance (%)		
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Solirubrobacterales ; Conexibacteraceae ; Conexibacter ; Conexibacter woesei	0	0	0.07
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Paenibacillaceae ; Paenibacillus ; Paenibacillus sp	0	0	0.07
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfotomaculum ; Desulfotomaculum acetoxidans	0	0	0.07
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinomycetales ; Nocardiaceae ; Nocardia ; Nocardia sp	0	0	0.07
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Myxococcales ; Polyangiaceae ; Polyangium ; Polyangium sp	0	0	0.07
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Solirubrobacterales ; Solirubrobacteraceae ; Solirubrobacter ; Solirubrobacter pauli	0	0	0.07
Bacteria ; Proteobacteria ; Epsilonproteobacteria ; Campylobacterales ; Campylobacteraceae ; Sulfurospirillum ; Sulfurospirillum multivorans	0.57	6.82	0
Bacteria ; Proteobacteria ; Epsilonproteobacteria ; Campylobacterales ; Campylobacteraceae ; Sulfurospirillum ; Sulfurospirillum sp	0.44	3.24	0
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfuromonadales ; Geobacteraceae ; Trichlorobacter ; Trichlorobacter sp	0.08	2.55	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Eubacteriaceae ; Eubacterium ; Eubacterium sp	0	1.04	0
Bacteria ; Proteobacteria ; Epsilonproteobacteria ; Campylobacterales ; Campylobacteraceae ; Campylobacter ; Campylobacter sp	0.03	0.99	0
Bacteria ; Bacteroidetes ; Cytophagia ; Cytophagales ; Cytophagaceae ; Meniscus ; Meniscus glaucopis	0	0.69	0
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Paenibacillaceae ; Paenibacillus ; Paenibacillus turicensis	0.05	0.60	0
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Virgibacillus ; Virgibacillus sp	0.03	0.60	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Rhodocyclales ; Rhodocyclaceae ; Azoarcus ; Azoarcus sp	0	0.52	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Acidovorax ; Acidovorax delafieldii	0.05	0.47	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Desulfosporosinus ; Desulfosporosinus sp	50.21	0.39	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Rhodocyclales ; Rhodocyclaceae ; Azospira ; Azospira sp	0	0.35	0
Bacteria ; Synergistetes ; Synergistia ; Synergistales ; Synergistaceae ; Synergistes ; Synergistes sp	0	0.26	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Neisseriales ; Neisseriaceae ; Eikenella ; Eikenella corrodens	0	0.26	0
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Oceanobacillus ; Oceanobacillus kapialis	0	0.26	0
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinobacteria (order) ; Actinobacteria (family) ; Nostocoida (Actinobacteria) ; Nostocoida limicola actinobacteria	0.10	0.17	0
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Thiotrichales ; Piscirickettsiaceae ; Methylophaga ; Methylophaga sp	0.03	0.17	0
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Oceanobacillus ; Oceanobacillus sp	0	0.13	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Rhodoferax ; Rhodoferax antarcticus	0.08	0.09	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Clostridiaceae unclassified ; Tissierella ; Tissierella creatinini	0.05	0.09	0
Bacteria ; Spirochaetes ; Spirochaetes (class) ; Spirochaetales ; Spirochaetaceae ; Spirochaeta ; Spirochaeta caldaria	0	0.09	0
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Bdellovibrionales ; Bacteriovoracaceae ; Bacteriovorax ; Bacteriovorax sp	0	0.09	0
Bacteria ; Firmicutes ; Bacilli ; Bacillales ; Bacillaceae ; Halobacillus ; Halobacillus sp	0	0.09	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Syntrophomonadaceae ; Pelospora ; Pelospora sp	0	0.09	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Graciibacteraceae ; Gracilibacter ; Gracilibacter sp	0	0.09	0
Bacteria ; Firmicutes ; Bacilli ; Lactobacillales ; Lactobacillaceae ; Lactobacillus ; Lactobacillus sp	0	0.09	0
Bacteria ; Tenericutes ; Mollicutes ; Acholeplasmatales ; Acholeplasmataceae ; Phytoplasma ; Phytoplasma mali	0	0.09	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Alcaligenaceae ; Achromobacter ; Achromobacter sp	0	0.09	0
Bacteria ; Chloroflexi ; Anaerolineae ; Anaerolineales ; Anaerolineaceae ; Anaerolinea ; Anaerolinea sp	0	0.09	0

16S Bacterial Pyrosequencing Data: Kingdom; Phylum; Class; Order; Family; Genus; Species	Temperature °C		
	4	22	35
Classification	Relative Abundance (%)		
Bacteria ; Firmicutes ; Bacilli ; Lactobacillales ; Lactobacillaceae ; Lactobacillus ; Lactobacillus sp	0	0.09	0
Bacteria ; Tenericutes ; Mollicutes ; Acholeplasmatales ; Acholeplasmataceae ; Phytoplasma ; Phytoplasma mali	0	0.09	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Alcaligenaceae ; Achromobacter ; Achromobacter sp	0	0.09	0
Bacteria ; Chloroflexi ; Anaerolineae ; Anaerolineales ; Anaerolineaceae ; Anaerolinea ; Anaerolinea sp	0	0.09	0
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfobacterales ; Desulfobulbaceae ; Desulfocapsa ; Desulfocapsa sp	0	0.09	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Peptococcaceae ; Dehalobacter ; Dehalobacter sp	0	0.09	0
Bacteria ; Firmicutes ; Clostridia ; Clostridiales ; Clostridiaceae ; Clostridium ; Clostridium ragsdalei	0	0.09	0
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Pseudomonadales ; Pseudomonadaceae ; Pseudomonas ; Pseudomonas sp	0.31	0.04	0
Bacteria ; Proteobacteria ; Alphaproteobacteria ; Rhizobiales ; Xanthobacteraceae ; Ancylobacter ; Ancylobacter dichloromethanicum	0.03	0.04	0
Bacteria ; Proteobacteria ; Alphaproteobacteria ; Rhizobiales ; Rhizobiaceae ; Sinorhizobium ; Sinorhizobium sp	0	0.04	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Ramlibacter ; Ramlibacter tataouinensis	0	0.04	0
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfobacterales ; Desulfobulbaceae ; Desulfocapsa ; Desulfocapsa thiozymogenes	0	0.04	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Comamonas ; Comamonas sp	0.10	0	0
Bacteria ; Gemmatimonadetes ; Gemmatimonadetes (class) ; Gemmatimonadales ; Gemmatimonadaceae ; Gemmatimonas ; Gemmatimonas sp	0.10	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Alcaligenaceae ; Castellaniella ; Castellaniella defragrans	0.08	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Hydrogenophilales ; Hydrogenophilaceae ; Thiobacillus ; Thiobacillus denitrificans	0.08	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Rhodocyclales ; Rhodocyclaceae ; Rhodocyclus ; Rhodocyclus sp	0.08	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Oxalobacteraceae ; Herbaspirillum ; Herbaspirillum hiltneri	0.05	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Comamonadaceae ; Giesbergeria ; Giesbergeria sinuosa	0.05	0	0
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Rubrobacterales ; Rubrobacteraceae ; Rubrobacter ; Rubrobacter sp	0.05	0	0
Bacteria ; Actinobacteria ; Actinobacteria (class) ; Actinomycetales ; Microbacteriaceae ; Leifsonia ; Leifsonia sp	0.05	0	0
Bacteria ; Proteobacteria ; Alphaproteobacteria ; Rhizobiales ; Bradyrhizobiaceae ; Bradyrhizobium ; Bradyrhizobium canariense	0.05	0	0
Bacteria ; Chloroflexi ; Dehalococcoidetes ; Dehalococcoideales ; Dehalococcoideaceae ; Dehalococcoides ; Dehalococcoides sp	0.05	0	0
Bacteria ; Proteobacteria ; Betaproteobacteria ; Burkholderiales ; Alcaligenaceae ; Alcaligenes ; Alcaligenes sp	0.05	0	0
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Xanthomonadales ; Xanthomonadaceae ; Arenimonas ; Arenimonas sp	0.05	0	0
Bacteria ; Proteobacteria ; Gammaproteobacteria ; Xanthomonadales ; Xanthomonadaceae ; Xanthomonas ; Xanthomonas hortorum	0.05	0	0
Bacteria ; Proteobacteria ; Deltaproteobacteria ; Desulfobacterales ; Desulfobacteraceae ; Desulfobacterium ; Desulfobacterium sp	0.03	0	0

16S Archaeal Pyrosequencing Data: Kingdom, Phylum, Class, Order, Family, Genus, Species	Temperature (°C)		
	4	22	35
Name	Relative Abundance (%)		
Archaea ; Euryarchaeota ; Methanobacteria ; Methanobacteriales ; Methanobacteriaceae ; Methanobrevibacter ; Methanobrevibacter sp	0.72	3.49	0.99
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanocellales ; Methanocellaceae ; Methanocella ; Methanocella paludicola	6.40	2.35	4.21
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanomicrobiales ; Methanomicrobiaceae ; Methanoculleus ; Methanoculleus receptaculi	0	0	0.15
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanomicrobiales ; Methanomicrobiaceae ; Methanoculleus ; Methanoculleus sp	0.05	0	0.17
Archaea ; Euryarchaeota ; Methanobacteria ; Methanobacteriales ; Methanobacteriaceae ; Methanobacterium ; Methanobacterium sp	0.02	13.99	45.02
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanomicrobiales ; Methanomicrobiales (family) ; Methanolinea ; Methanolinea sp	2.66	0.76	1.57
Archaea ; Euryarchaeota ; Methanobacteria ; Methanobacteriales ; Methanobacteriaceae ; Methanobacterium ; Methanobacterium beijingense	0	0	0.97
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanosarcinales ; Methanosaetaceae ; Methanosaeta ; Methanosaeta concilii	80.96	20.51	44.29
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanosarcinales ; Methanosaetaceae ; Methanosaeta ; Methanosaeta sp	8.98	2.22	2.64
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanosarcinales ; Methanosarcinaceae ; Methanosarcina ; Methanosarcina sp	0	20.81	0
Archaea ; Euryarchaeota ; Methanobacteria ; Methanobacteriales ; Methanobacteriaceae ; Methanobacterium ; Methanobacterium uliginosum	0	34.29	0
Archaea ; Euryarchaeota ; Methanomicrobia ; Methanosarcinales ; Methanosarcinaceae ; Methanosarcina ; Methanosarcina barkeri	0	1.57	0
Archaea ; Thaumarchaeota ; Thaumarchaeota (class) ; Nitrosopumilales ; Nitrosopumilaceae ; Candidatus Nitrosopumilus ; Candidatus Nitrosopumilus sp	0.09	0	0
Archaea ; Euryarchaeota ; Methanobacteria ; Methanobacteriales ; Methanobacteriaceae ; Methanobacterium ; Methanobacterium formicicum	0.03	0	0
Archaea ; Crenarchaeota ; Thermoprotei ; Desulfurococcales ; Desulfurococcaceae ; Staphylothermus ; Staphylothermus hellenicus	0.03	0	0
Archaea ; Crenarchaeota ; Thermoprotei ; Desulfurococcales ; Desulfurococcaceae ; Desulfurococcus ; Desulfurococcus mobilis	0.05	0	0

APPENDIX B: DISSOLVED PHASE THERMAL MICROCOSM EXPERIMENT

B.1 BACKGROUND AND METHODS

Thermal microcosm studies were conducted to determine thermal effects on the biodegradation of petroleum hydrocarbons. Microcosms consisted of 250 mL serum bottles containing petroleum hydrocarbon contaminated soil and water. The bottles were connected to 60-mL plastic syringes to capture the biogas produced. In-line valves were used to pull gas samples for analysis. The study was conducted using soils collected from an LNAPL smear zone at the Evansville site. Site water was used to saturate the soils in each microcosm. The microcosms were prepared in an anaerobic chamber to simulate an oxygen-free subsurface environment. Triplicate microcosms were placed in water baths at seven different temperatures: 4, 10, 22, 30, 35, 40, and 60 ^{0}C. The experiment was conducted for 55 days. Figure 1 shows the experimental set up.

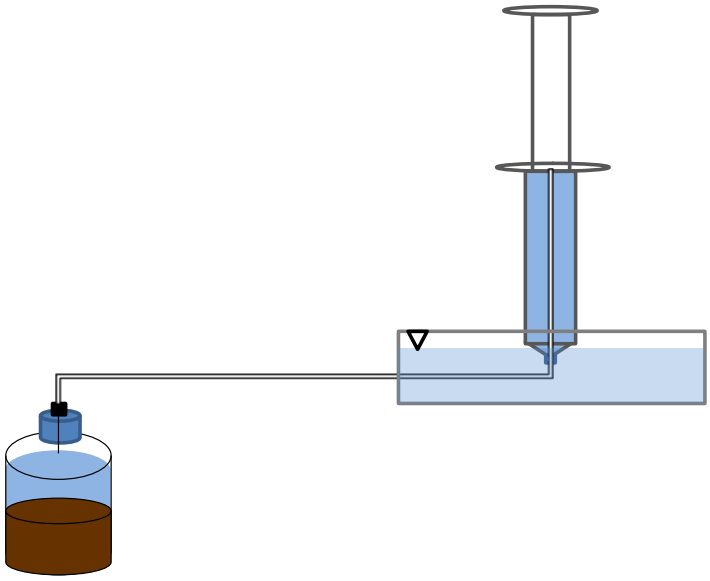


Figure B.1: Microcosm set up

To compare biodegradation potential at different temperatures, data collected included volume of biogas production, CH_4 and CO_2 concentrations, total petroleum hydrocarbons (TPH), adenosine triphosphate (ATP), and total extractable DNA. The volume and composition of biogas was measured by monitoring the gas captured by the 60-mL syringes. The gas was then analyzed for CH_4 and CO_2 by taking a gas sample from the in-line valve and manually injecting into a Gas Chromatograph and using a Thermal Conductivity Detector (GC/TCD). Total petroleum hydrocarbon measurements were taken at the beginning and end of the experiment and were measured using a Gas Chromatograph and Flame Ionization Detector (GC/FID). ATP levels were measured throughout the experiment to provide information on microbial activity. Higher levels of ATP indicate increased microbial activity within a system. ATP was measured using BacTiter-Glo™ Microbial Cell Viability Assay (Promega Corporation). Additionally, DNA was extracted from all microcosms at the end of the study using MoBio Powerlyzer Power Soil DNA Extraction kit. DNA yield was enhanced by making modifications to the kit protocol. DNA was quantified using Quant-iT™ Broad-Range DNA Assay Kit from Invitrogen.

B.2 RESULTS

CH_4 and CO_2 production results are shown in Figures 2 and 3.

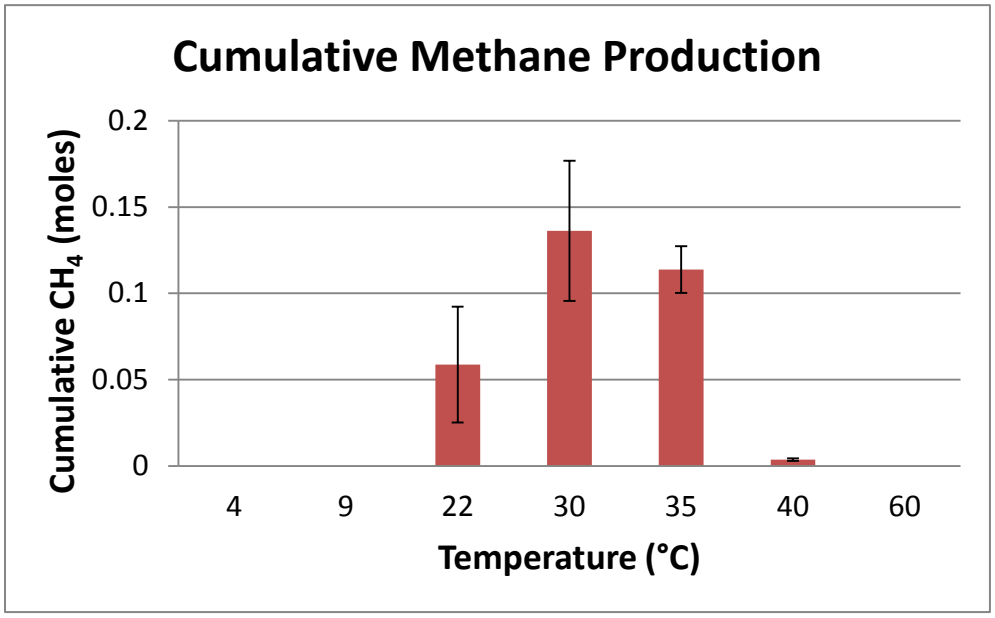


Figure B.2: Cumulative methane production through 55 days

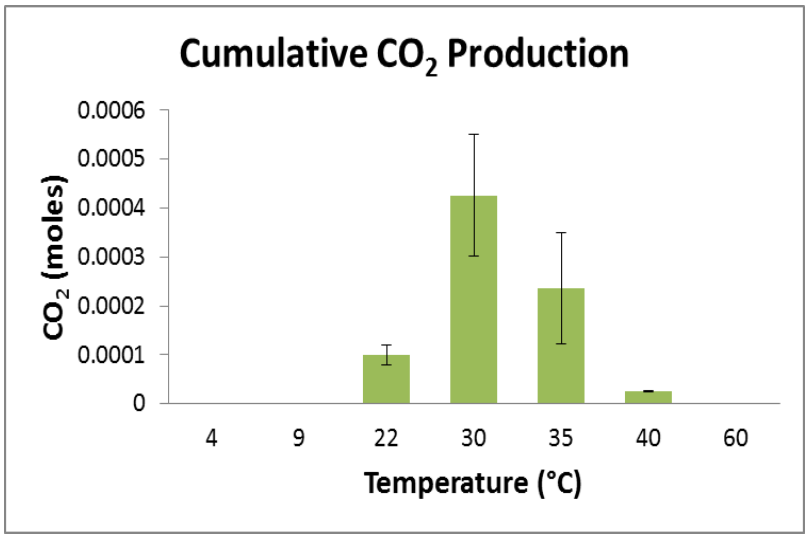


Figure B.3: Cumulative CO2 production through 55 days

Total Petroleum Hydrocarbons (TPH) was measured in the soil and water phases at the beginning and end of the study. Results for total TPH, or the sum of water-phase and soil-phase TPH, are shown in Figure 4. Results indicate that TPH had a maximum reduction of 6.7%.

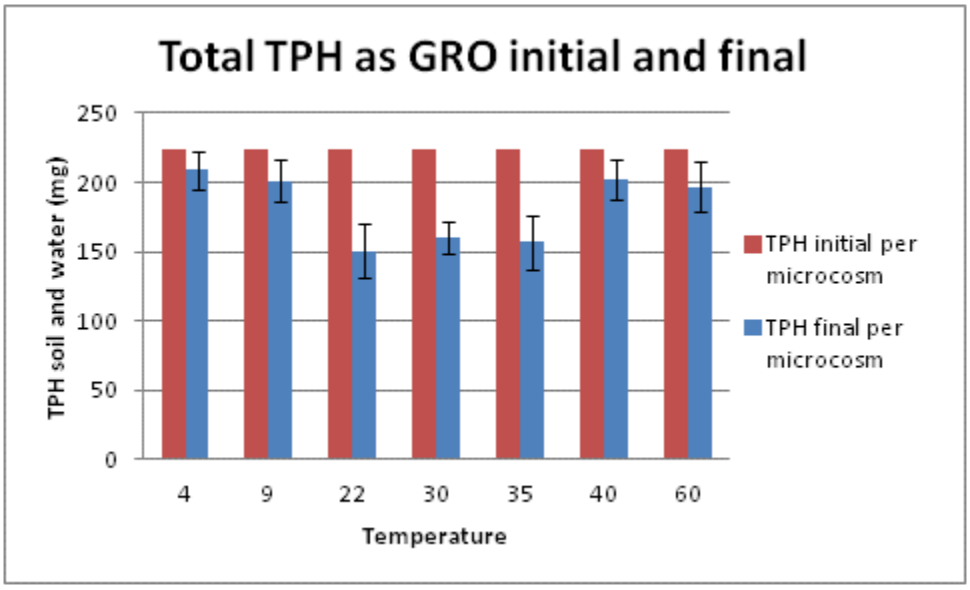


Figure B.4: TPH data

DNA extraction yields are shown below in Figure 7 for each temperature. The DNA in ng/uL represents the amount of DNA extracted from 0.6 grams of soil from each microcosm. Increases in DNA extracted could indicate microbial growth at certain temperatures.

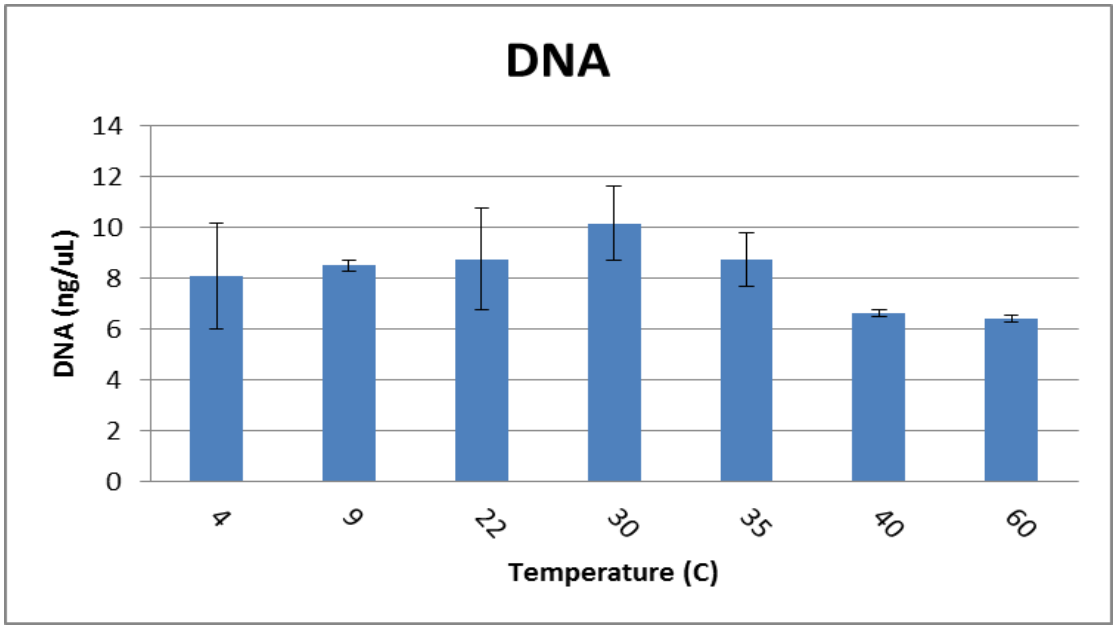


Figure B.5: DNA extraction yield

DNA can be used to evaluate differences among the microbial communities at different temperatures. This portion of the study has not been completed, but additional method development is scheduled to be completed in 2012. The extracted DNA is currently being stored at -80°C.

B.3 CONCLUSIONS

Results indicate that temperature does have an impact on the biodegradation of hydrocarbons within the microcosms. Results showed that microcosms at 30^0C and 35^0C had the highest production levels of biogas, CH_4, and CO_2. Similar reductions in TPH content were noted in microcosms at 22^0C, 30^0C and 35°C. There was a correlation between biogas production and ATP content at different temperatures. When biogas production was observed at 30^0C, the soil contained elevated levels of ATP. A similar trend was seen when biogas production was dominated by the 35^0C system.

APPENDIX C: STATISTICAL DATA

C.1 DRO ABIOTIC VS. BIOTIC 2 TAILED T-TEST STATISTICAL DATA

Abiotic vs. Biotic DRO 4 deg C

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	947.4	947.4	0.08	0.7900
Error	4	46748.5	11687.1		

testing homogeneity of variance

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.999987	**Pr < W**	0.9932
Kolmogorov-Smirnov	**D**	0.175171	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.027909	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.189502	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.97536	**Pr < W**	0.6990
Kolmogorov-Smirnov	**D**	0.238583	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.033968	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.217117	**Pr > A-Sq**	>0.2500

control

testing normality

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-0.47	0.6626
Satterthwaite	Unequal	3.7621	-0.47	0.6640

ttest - pval>=alpha *not statistically different*

Level of trt	N	amount	
		Mean	Std Dev
biotic	3	4011.43333	224.166406
control	3	4088.39333	173.364199

mean & std. dev.

Abiotic vs. Biotic DRO 9deg C

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-2.17	0.0958
Satterthwaite	Unequal	3.4636	-2.17	0.1063

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.981092	**Pr < W**	0.7365
Kolmogorov-Smirnov	**D**	0.229746	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.032558	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.21066	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.991107	**Pr < W**	0.8196
Kolmogorov-Smirnov	**D**	0.210023	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.030094	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.199421	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	4916.5	4916.5	0.54	0.5037
Error	4	36508.1	9127.0		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
biotic	3	3829.03333	227.652534
control	3	4170.78667	150.179272

Abiotic vs. Biotic DRO 22 deg C

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-6.02	0.0038
Satterthwaite	Unequal	3.999	-6.02	0.0038

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.974101	**Pr < W**	0.6913
Kolmogorov-Smirnov	**D**	0.240376	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.034277	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.218537	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.875173	**Pr < W**	0.3103
Kolmogorov-Smirnov	**D**	0.325017	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.058705	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.333149	**Pr > A-Sq**	0.2173

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	43.3807	43.3807	0.01	0.9390
Error	4	26149.8	6537.5		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	3137.98333	192.509217
Control	3	4077.20333	189.517591

Abiotic vs. Biotic DRO 30 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-4.32	0.0125
Satterthwaite	Unequal	2.5587	-4.32	0.0314

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.941591	**Pr < W**	0.5338
Kolmogorov-Smirnov	**D**	0.27657	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.042278	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.255543	**Pr > A-Sq**	>0.2500

Biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.9567	**Pr < W**	0.5997
Kolmogorov-Smirnov	**D**	0.261609	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.038558	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.238268	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	30817.6	30817.6	3.12	0.1522
Error	4	39527.7	9881.9		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
biotic	3	3102.14667	309.231855
control	3	3926.00000	116.740379

Abiotic vs. Biotic DRO 35 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-4.21	0.0136
Satterthwaite	Unequal	3.9349	-4.21	0.0141

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.882072	**Pr < W**	0.3305
Kolmogorov-Smirnov	**D**	0.32081	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.05699	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.324957	**Pr > A-Sq**	0.2308

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.877074	**Pr < W**	0.3158
Kolmogorov-Smirnov	**D**	0.323872	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.058232	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.330889	**Pr > A-Sq**	0.2211

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	741.6	741.6	0.10	0.7643
Error	4	28782.7	7195.7		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
biotic	3	3111.99000	243.939789

Level of trt	N	amount	
		Mean	Std Dev
control	3	3900.63667	214.335581

Abiotic vs. Biotic DRO 40 deg C

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-5.10	0.0070
Satterthwaite	Unequal	2.9251	-5.10	0.0155

Tests for Normality				
Test	Statistic		p Value	
Shapiro-Wilk	W	0.994576	Pr < W	0.8592
Kolmogorov-Smirnov	D	0.200552	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.029241	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.195541	Pr > A-Sq	>0.2500

biotic

Tests for Normality				
Test	Statistic		p Value	
Shapiro-Wilk	W	0.925934	Pr < W	0.4736
Kolmogorov-Smirnov	D	0.290002	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.046137	Pr > W-Sq	>0.2500

Tests for Normality				
Test	**Statistic**		**p Value**	
Anderson-Darling	**A-Sq**	0.273588	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	11352.9	11352.9	0.98	0.3775
Error	4	46172.4	11543.1		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	2969.17667	268.774892
Control	3	3852.47333	133.088458

C.2 DRO STATISTICAL DATA FROM ANOVA TEST

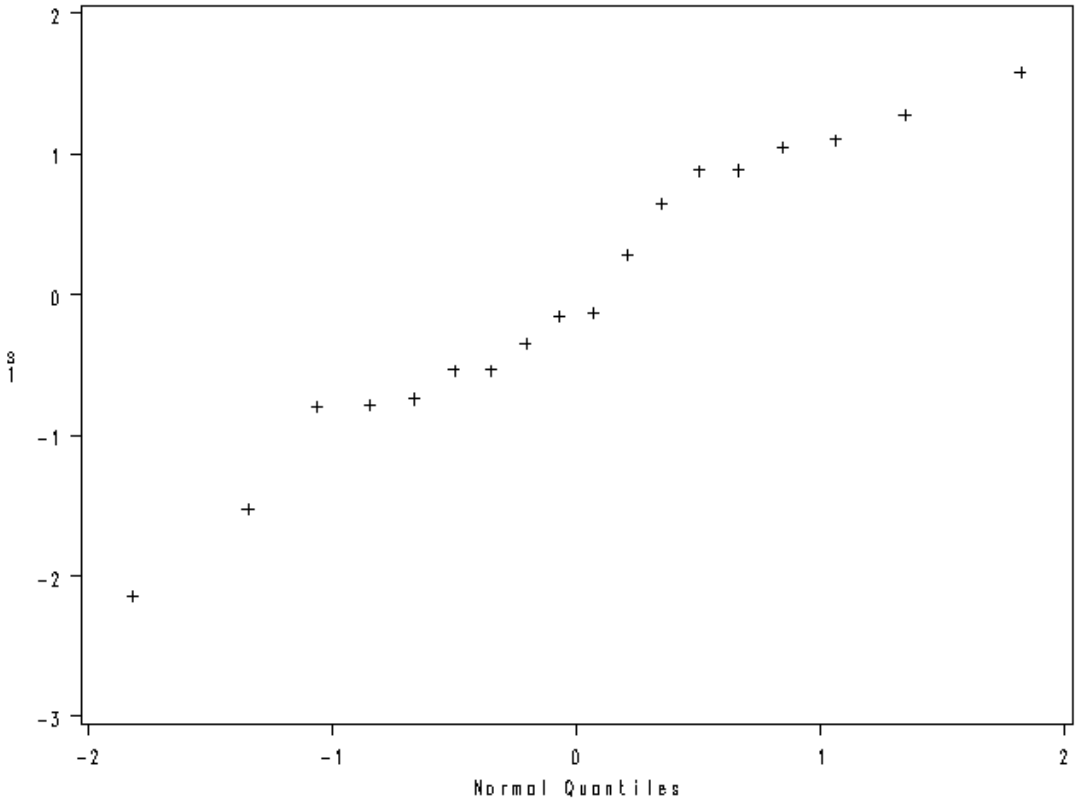
Comparisons significant at the 0.05 level are indicated by *.**				
temperature Comparison	**Difference Between Means**	**95% Confidence Limits**		
22 - 40	15.10	-127.82	158.01	
22 - 30	31.15	-111.77	174.06	
22 - 35	40.65	-102.26	183.57	
22 - 9	161.32	18.40	304.23	***
22 - 4	232.81	89.89	375.73	***
40 - 22	-15.10	-158.01	127.82	
40 - 30	16.05	-126.87	158.97	
40 - 35	25.56	-117.36	168.47	
40 - 9	146.22	3.30	289.13	***
40 - 4	217.71	74.80	360.63	***
30 - 22	-31.15	-174.06	111.77	
30 - 40	-16.05	-158.97	126.87	
30 - 35	9.51	-133.41	152.42	
30 - 9	130.17	-12.75	273.08	
30 - 4	201.66	58.75	344.58	***
35 - 22	-40.65	-183.57	102.26	
35 - 40	-25.56	-168.47	117.36	
35 - 30	-9.51	-152.42	133.41	
35 - 9	120.66	-22.25	263.58	

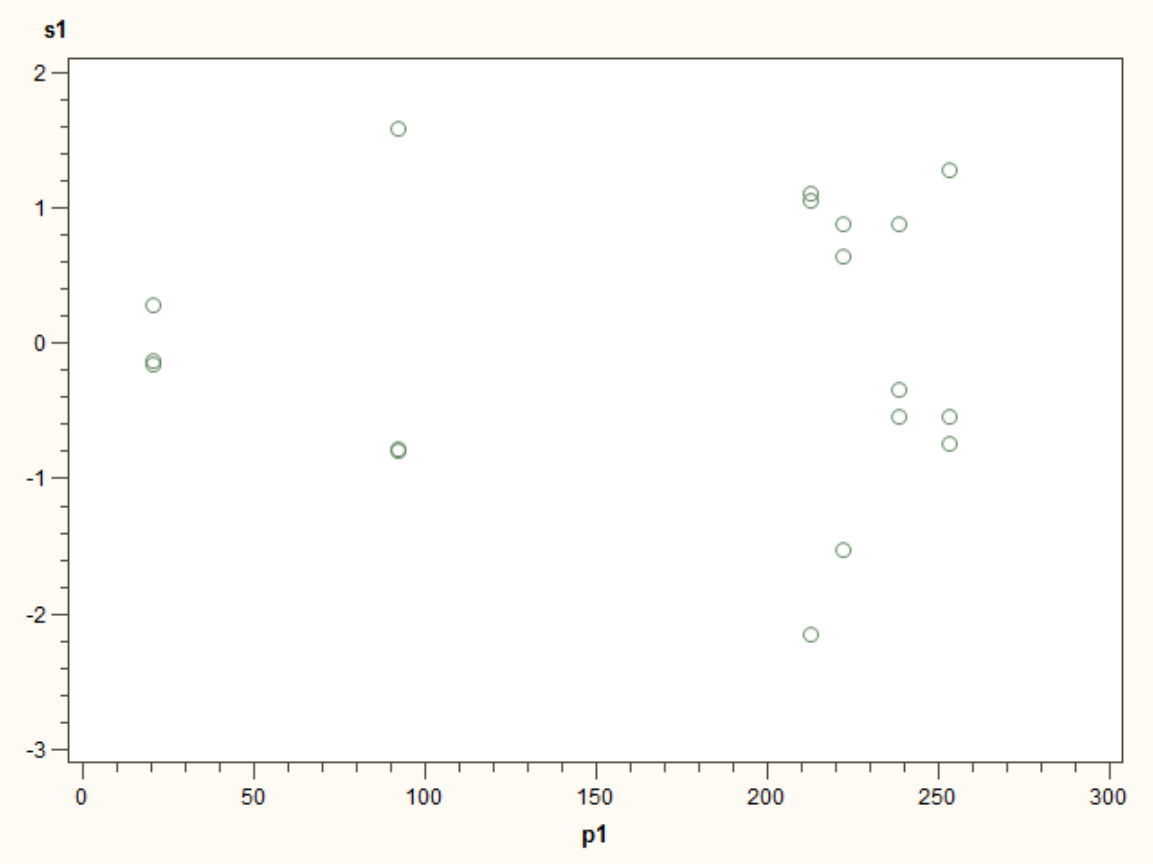
Comparisons significant at the 0.05 level are indicated by ***.				
temperature Comparison	**Difference Between Means**	**95% Confidence Limits**		
35 – 4	192.16	49.24	335.07	***
9 – 22	-161.32	-304.23	-18.40	***
9 – 40	-146.22	-289.13	-3.30	***
9 – 30	-130.17	-273.08	12.75	
9 – 35	-120.66	-263.58	22.25	
9 – 4	71.49	-71.42	214.41	
4 – 22	-232.81	-375.73	-89.89	***
4 – 40	-217.71	-360.63	-74.80	***
4 – 30	-201.66	-344.58	-58.75	***
4 – 35	-192.16	-335.07	-49.24	***
4 – 9	-71.49	-214.41	71.42	

Level of temperature	**N**	**amount**	
		Mean	**Std Dev**
4	3	20.779200	16.173224
9	3	92.273400	89.999955
22	3	253.589400	72.969697
30	3	222.440400	87.160714
35	3	212.934600	121.974468
40	3	238.490100	50.610232

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
temperature	5	11780.1	2356.0	3.19	0.0464
Error	12	8868.6	739.1		

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.95746	**Pr < W**	0.5534
Kolmogorov-Smirnov	**D**	0.137912	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.053874	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.334715	**Pr > A-Sq**	>0.2500




s1
2
1
0
-1
-2
-3
0
50
100
150
200
250
300
p1

C.3 GRO ABIOTIC VS. BIOTIC STATISTICAL DATA FROM 2 TAILED T-TEST

abiotic vs. biotic 4 deg C

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-4.09	0.0150
Satterthwaite	Unequal	2.6994	-4.09	0.0322

2 sample two tailed ttest

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.994075	**Pr < W**	0.8528
Kolmogorov-Smirnov	**D**	0.202079	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.029364	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.196101	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.960944	**Pr < W**	0.6201
Kolmogorov-Smirnov	**D**	0.256921	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.037514	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.23344	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	226.2	226.2	2.25	0.2078
Error	4	401.8	100.4		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	500.473033	12.0079846
Control	3	572.981062	28.2597351

abiotic vs. biotic 9 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-2.11	0.1022
Satterthwaite	Unequal	2.7096	-2.11	0.1347

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.997191	**Pr < W**	0.8987
Kolmogorov-Smirnov	**D**	0.191069	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.028597	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.192621	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.992873	**Pr < W**	0.8386
Kolmogorov-Smirnov	**D**	0.205494	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.02966	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.197445	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	515.3	515.3	1.50	0.2884
Error	4	1377.6	344.4		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
biotic	3	491.665771	19.6947420
control	3	552.692022	45.9930624

abiotic vs. biotic 22 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-6.94	0.0023
Satterthwaite	Unequal	3.9842	-6.94	0.0023

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.943903	**Pr < W**	0.5433
Kolmogorov-Smirnov	**D**	0.274427	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.041708	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.25289	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.80757	**Pr < W**	0.1327
Kolmogorov-Smirnov	**D**	0.360425	**Pr > D**	0.1244
Cramer-von Mises	**W-Sq**	0.075671	**Pr > W-Sq**	0.1660
Anderson-Darling	**A-Sq**	0.415185	**Pr > A-Sq**	0.1059

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	0.6494	0.6494	0.00	0.9566
Error	4	774.2	193.6		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	326.356023	36.9941636
control	3	529.599573	34.7363870

abiotic vs. biotic 30 deg C

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	4	-8.16	0.0012
Satterthwaite	Unequal	3.6169	-8.16	0.0019

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.770987	**Pr < W**	0.0470
Kolmogorov-Smirnov	**D**	0.376393	**Pr > D**	0.0909
Cramer-von Mises	**W-Sq**	0.085017	**Pr > W-Sq**	0.1185
Anderson-Darling	**A-Sq**	0.460997	**Pr > A-Sq**	0.0750

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.994962	**Pr < W**	0.8643
Kolmogorov-Smirnov	**D**	0.199327	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.029146	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.19511	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	118.7	118.7	0.90	0.3976
Error	4	530.5	132.6		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	330.682545	32.2714573
Control	3	517.395076	23.0216965

abiotic vs. biotic 35 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-8.40	0.0011
Satterthwaite	Unequal	3.9878	-8.40	0.0011

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.851639	**Pr < W**	0.2448
Kolmogorov-Smirnov	**D**	0.338421	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.064574	**Pr > W-Sq**	0.2421
Anderson-Darling	**A-Sq**	0.36134	**Pr > A-Sq**	0.1709

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.953959	**Pr < W**	0.5870
Kolmogorov-Smirnov	**D**	0.264509	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.039233	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.241392	**Pr > A-Sq**	>0.2500

control

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	3.0882	3.0882	0.05	0.8369
Error	4	256.1	64.0161		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
biotic	3	345.260517	20.7291062
control	3	483.724382	19.6141901

abiotic vs. biotic 40 deg C

Method	**Variances**	**DF**	**t Value**	**Pr > |t|**
Pooled	Equal	4	-7.11	0.0021
Satterthwaite	Unequal	3.0304	-7.11	0.0055

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.98778	**Pr < W**	0.7884
Kolmogorov-Smirnov	**D**	0.217453	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.030913	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.203149	**Pr > A-Sq**	>0.2500

biotic

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.85833	**Pr < W**	0.2630
Kolmogorov-Smirnov	**D**	0.334747	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.062902	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.353287	**Pr > A-Sq**	0.1841

Levene's Test for Homogeneity of amount Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
trt	1	208.8	208.8	2.31	0.2029
Error	4	361.0	90.2419		

Level of trt	**N**	**amount**	
		Mean	**Std Dev**
Biotic	3	336.025938	15.8868803
Control	3	475.910143	30.1631519

C.4 GRO STATISTICAL DATA FROM ANOVA TEST

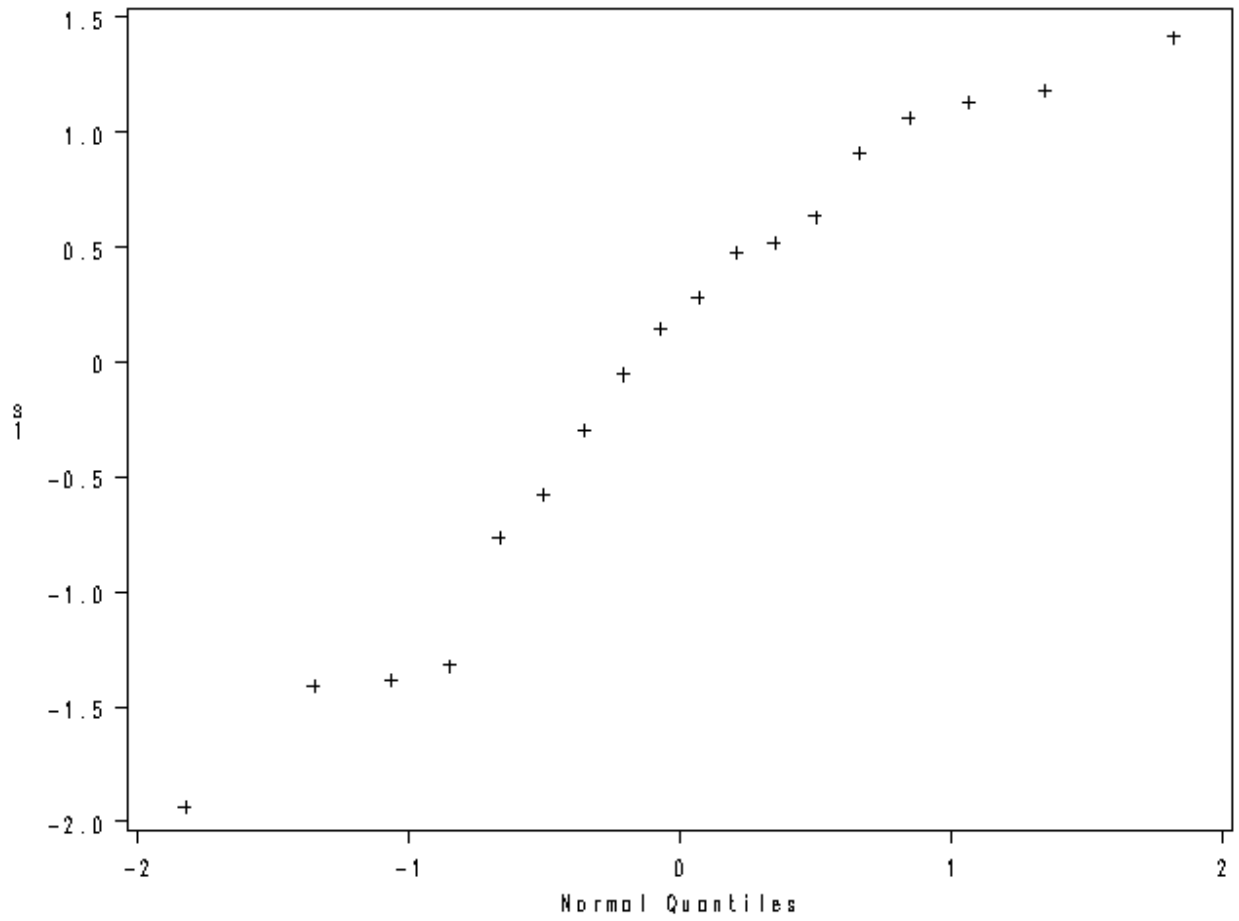
ANOVA assumptions:

Normally distributed data, equality of variance, independent observations,

Normal data:

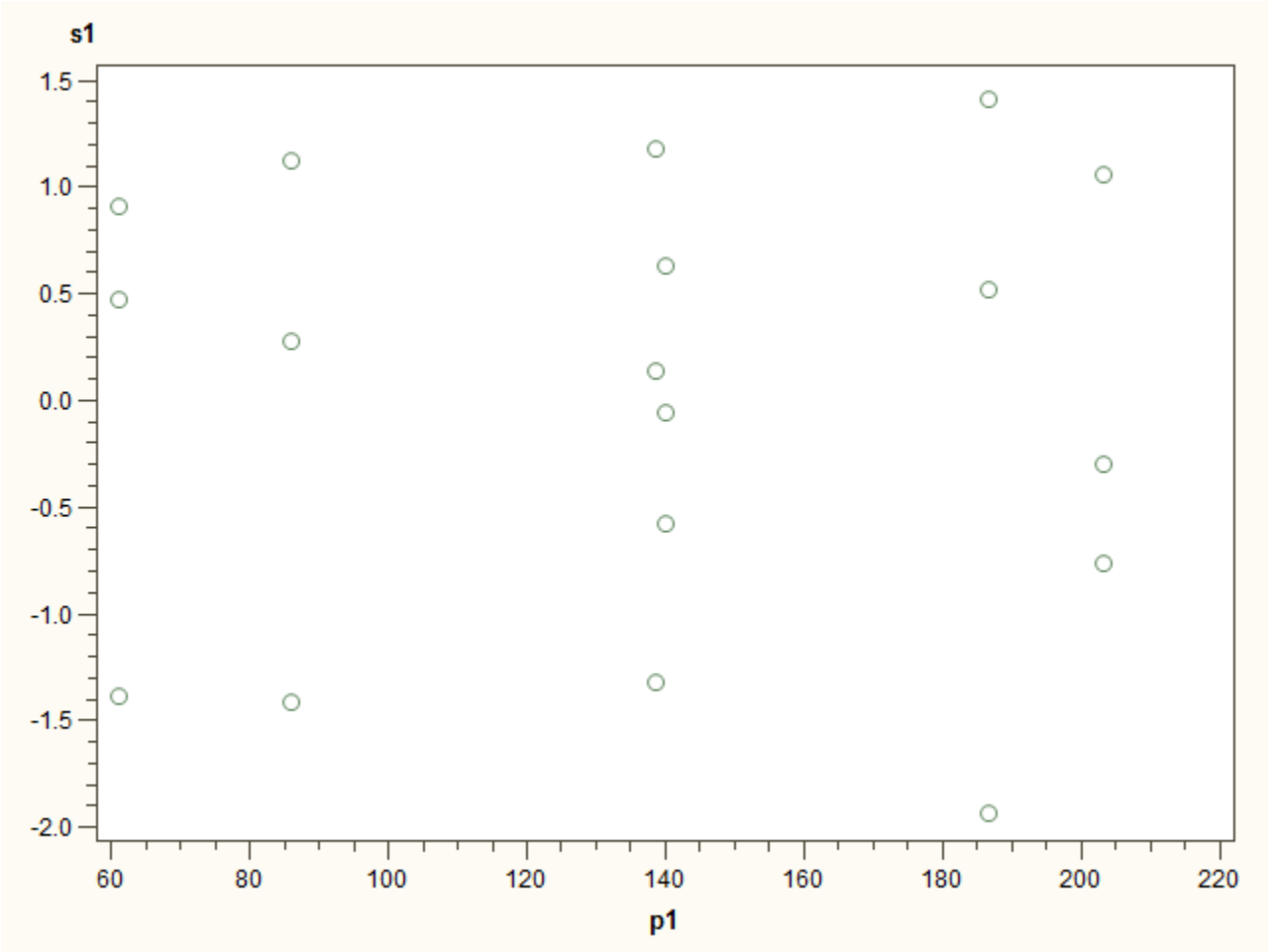
Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.93762	**Pr < W**	0.2637
Kolmogorov-Smirnov	**D**	0.122559	**Pr > D**	>0.1500
Cramer-von Mises	**W-Sq**	0.058552	**Pr > W-Sq**	>0.2500
Anderson-Darling	**A-Sq**	0.400928	**Pr > A-Sq**	>0.2500

All pvalues > 0.1 -indicates normal data



Studentized residuals vs. predicted residuals plot

Equality of Variance:



Studentized residuals vs. predicted residuals

Levene's Test for Homogeneity of concentration Variance ANOVA of Absolute Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
temperature	5	1157.1	231.4	0.86	0.5364
Error	12	3240.1	270.0		

Pvalue>=alpha - indicates equal variance

alpha=0.05

Comparisons significant at the 0.05 level are indicated by ***.				
temperature Comparison	**Difference Between Means**	**95% Confidence Limits**		
22 - 30	16.53	-50.23	83.30	
22 - 40	63.36	-3.41	130.12	
22 - 35	64.78	-1.99	131.54	
22 – 4	117.40	50.64	184.17	***
22 – 9	142.22	75.45	208.98	***
30 - 22	-16.53	-83.30	50.23	
30 - 40	46.83	-19.94	113.59	
30 - 35	48.25	-18.52	115.01	
30 – 4	100.87	34.11	167.64	***
30 – 9	125.69	58.92	192.45	***
40 - 22	-63.36	-130.12	3.41	
40 - 30	-46.83	-113.59	19.94	
40 - 35	1.42	-65.34	68.19	
40 – 4	54.04	-12.72	120.81	
40 – 9	78.86	12.09	145.62	***
35 - 22	-64.78	-131.54	1.99	
35 - 30	-48.25	-115.01	18.52	
35 - 40	-1.42	-68.19	65.34	
35 – 4	52.62	-14.14	119.39	
35 – 9	77.44	10.67	144.20	***
4 – 22	-117.40	-184.17	-50.64	***

Comparisons significant at the 0.05 level are indicated by ***.				
temperature Comparison	**Difference Between Means**	**95% Confidence Limits**		
4 - 30	-100.87	-167.64	-34.11	***
4 - 40	-54.04	-120.81	12.72	
4 - 35	-52.62	-119.39	14.14	
4 - 9	24.82	-41.95	91.58	
9 - 22	-142.22	-208.98	-75.45	***
9 - 30	-125.69	-192.45	-58.92	***
9 - 40	-78.86	-145.62	-12.09	***
9 - 35	-77.44	-144.20	-10.67	***
9 - 4	-24.82	-91.58	41.95	

Means with the same letter are not significantly different.				
t Grouping		**Mean**	**N**	**temperature**
	A	203.24	3	22
	A			
	A	186.71	3	30
	A			
B	A	139.88	3	40
B	A			
B	A	138.46	3	35
B				
B	C	85.84	3	4

Means with the same letter are not significantly different.				
t Grouping		**Mean**	**N**	**temperature**
	C			
	C	61.03	3	9

Level of temperature	**N**	**concentration**	
		Mean	**Std Dev**
4	3	85.841362	39.5868598
9	3	61.026251	37.2941384
22	3	203.243549	29.0667007
30	3	186.712531	53.1260790
35	3	138.463947	38.4726516
40	3	139.884204	18.5853711

APPENDIX D: PROTOCOLS

D.1 BTEX ANALYSIS FROM SOIL

1. Weigh out 2 grams of soil and add to 60mL vial containing 50mL of Methanol
2. Add 1mL of internal standard solution
    a. 9986uL of Methanol, 14uL of 1,2 Dibromoethylene (4986uL+5000uL MeOH)
3. Place vial containing soil, MeOH and standard solution on the shaker for 30 minutes for extraction
4. Place vial in sonication bath for 30 minutes for further extraction
5. Remove vials and allow to settle (at this point samples can be refrigerated for up to two weeks)
6. Dilute extraction in Purge and Trap glass vials with DI water for a 1:50 dilution
    a. 0.1mL of extract, 4.9mL DI water (move quickly to prevent volitalization losses)
    b. Add the 0.1mL (100uL) of the extract into the glass vial with a syringe next to machine
7. Place samples in the autosampler and tighten with wrench
8. Program the Purge and Trap selecting lines you will be using to inject the samples
    a. →ALS select the start and stop position (F2)
    b. →EXIT to confirm choice
    c. Use Method 4 for BTEX
9. Open all upper valves on GC for FID
10. Push button next to valves to ignite FID (if detector reads 0-0.1 it is not lit, should be anywhere from 5-25 and varying)
11. Set oven temperature to 250 to bake off excess for 10-20min then set back to 35
12. At computer, open GC-Inst, Method→Load→FID-GRO→Sequence→Use quick sequence generator
13. Enter Data file name ending with a number
14. At the sample log table cut out Keyword entries
15. Enter sample name (be specific entering name study and dilution)
16. When you Run Sequence make sure FULL METHOD is marked
17. Change the data file directory (use sequence name)
18. Run Sequence
19. Press start on Purge and Trap Auto Sampler
20. Watch for bubbles in samples to verify that it is working

D.2 Diesel Range Organics and Gasoline Range Organics Analysis from Soil

Sample Preparation

- 5-10 gm of soil extracted in 50 ml of MeOH (based on 8015b method and Chi Wan Jeon, 2007)
- Measured against GRO & DRO standards (Restek catalog # DRO 31064 & GRO #30065)

Sample Run

- GC/MS at the Central Instrument Facility at the Chemistry Department Colorado State University
- Method: JZGRODRO
- DB5 MS column
- Inject 1/10 std dilution in MeOH
- Injection volume 1 uL

Program

- Duration 18 minutes
- Initial temp Cº45 hold for 3 minutes
- Temperature ramp 1: 12 Cº/minute till 120 Cº hold for 3 minutes at temperature
- Temperature ramp 2: 25 Cº/minute till 290 Cº hold for 3 minutes

D.3 ARCHAEAL 16SRRNA PCR PROTOCOL

Archaeal
PCR

Primers: Use primers 340F and 1000R

MASTER MIX: Use Sigma or Takara Taq Polymerase

Reagent	Per Rxn (uL)	Total for 5 Rxns	Final Conc.
PCR Water	29.25	146.25	
10XPCR Buffer	5	25	1X
MgCl2 (50mM)	0.5	2.5	0.5mM
dNTP (10mM)	4	20	0.8mM
F Primer (10uM)	2.5	12.5	0.5uM
R Primer (10uM)	2.5	12.5	0.5uM
5 prime	0.25	1.25	1.25U
BSA (10mg/L)	2	10	0.4mg/L
DNA	4		
Total (uL)	50	230	

Run on Thermocycler program (ARCNZ)
2 minutes 98C
30 cycles: 95C for 30sec, 57C for 30 sec, 72C for 90 sec

Final elongation: 72C for 7 minutes

The PCR product should be 560bp

D.4 Protocol for Performing the BacTiter-Glo™ Assay

Materials to Be Supplied by the User

• opaque-walled multiwell plates

• multichannel pipette or automated pipetting station for delivering reagent

• plate shaker or other device for mixing contents of multiwell plates

• luminometer (e.g., GloMax® 96 Microplate Luminometer [Cat.# E6501] or GloMax® 20/20 Luminometer [Cat.# E5311]), or CCD camera capable of reading multiwell plates

• optional: ATP for generating a standard curve

Caution: Skin contains ATP. Because this assay is so sensitive, we recommend wearing gloves to avoid contamination.

Reagent Preparation

1. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature before use. For convenience the BacTiter-Glo™ Buffer may be thawed and stored at room temperature for up to 48 hours before use.

2. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature.

3. Transfer the appropriate volume (10ml for Cat.# G8230, G8231 or 100ml for Cat.# G8232, G8233) of BacTiter-Glo™ Buffer into the amber bottle containing BacTiter-Glo™ Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the BacTiter-Glo™ Reagent.

4. Mix by gently vortexing, swirling or by inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo™ Substrate should go into solution easily, in less than one minute.

5. Equilibrate Reagent at room temperature for at least 15 minutes before use. To achieve maximum sensitivity, additional equilibration time may be required.

D.5 454 PYROSEQUENCING DATA ANALYSIS

Term Definitions

Terms used within this guide are defined as follows:

Tag
o The term tag refers to the 8-10 bp sequence at the 5' end of the sequence read.
o The tag is also known as the barcode in some programs.
Identity Percentage
o Identity percentage for a read is defined as the length of the HSP Identity divided by the length of the hit HSP.
o BLAST is run to return 5 hits and 1 HSP per hit.
HSP – High-Score Pair
o The highest scoring local alignment between a sequence read and the database sequence such that the score cannot be improved by extension or trimming of the alignment.
HSP coverage
o The HSP length divided by the query length, giving the percentage of the query sequence covered by the HSP.

Data Analysis Methodology

Overview of the Data Analysis Process

Once sequencing has completed, the data analysis pipeline will begin processing the data. The data analysis process consists of two major stages, the quality checking and reads denoising stage and the diversity analysis stage. During the read quality checking and denoising stage, denoising and chimera checking is performed on all the reads for each region of data. Then each remaining read is quality scanned to remove poor reads from each sample. The primary output of this stage is a quality checked and denoised FASTA formatted sequence, quality, and mapping file. This stage is performed for all customers whose data we know the encoded tags for. During the diversity analysis stage, each sample is run through our analysis pipeline to determine the taxanomic information for each read and then analyzed to provide the sample's microbial diversity. The output for this stage is a set of files detailing the taxanomic information for each read as well as the number and percentage of each species found within each sample. This stage is performed for all customers whose data is sequenced using primers based within the 16S, 18S, 23S, ITS and SSU regions.

The data analysis pipeline is broken down into the following steps, each of which is discussed more thoroughly in the sections below:

□□ Quality Checking and Denoising
1. Denoising and Chimera Checking
2. SFF File Generation
3. Quality Checking and FASTA Formatted Sequence/Quality File Generation

- Microbial Diversity Analysis

1. Taxanomic Identification
2. Data Analysis

Denoising and Chimera Checking

Denoising

The process of denoising is used to correct errors in reads from next-generation sequencing technologies including the Roche 454 technologies. According to the paper “Accuracy and quality of massively parallel DNA pyrosequencing” by Susan Huse, et al. and “Removing noise from pyrosequenced amplicons” by Christopher Quince, et al. the per base error rates from 454 pyrosequencing attain an accuracy rate of 99.5% [1] [2]. However, the large read numbers that the machine can generate mean that the total number of noisy reads can be substantial. In order to determine true diversity it becomes critical to determine which reads are good and which reads contain noise introduced by the experimental procedure. The Research and Testing Laboratory analysis pipeline attempts to correct this issue by denoising entire regions of data prior to performing any other steps of the pipeline.

The Research and Testing analysis pipeline performs denoising by performing the following steps on each region:

- Reads within the data set are sorted from longest read to shortest read.
- Using USEARCH [3], reads are then dereplicated meaning they are clustered together into groups such that each sequence is an exact match to portion of the seed sequence for the cluster. Each cluster is marked with the total number of member sequences.

- The seed sequence from each cluster is then sorted by abundance, largest cluster to smallest cluster. Keep in mind that no minimum size restrictions exist on the clusters, thus single member clusters will exist.
- Clustering at a 1% divergence using the USEARCH [3] application is performed on the seed sequences in order to determine similar clusters. The result of this stage is the consensus sequence from each new cluster, each tagged to show their total number of member sequences (dereplicated + clustered).

- The consensus sequences are re-sorted based upon their abundance, largest cluster to smallest cluster. However at this point a minimum size restriction if put into place and any cluster that does not contain at least two member sequences is removed from consideration.
- Clustering at a 5% divergence is once again performed using USEARCH [3] on the consensus sequences in order to determine if the consensus sequences have created additional clusters. The result of this stage is the seed sequence from each new cluster,

Once this process has completed we have removed all reads that failed to have a similar or exact match elsewhere on the region and through the use of consensus sequences we have helped correct base pair errors generated during sequencing.

Chimera Checking

As discussed in the paper “Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons” by Brian Haas, et al. the formation of chimeric sequences occurs when an aborted sequence extension is misidentified as a primer and is extended upon incorrectly in subsequent PCR cycles.

Because amplification produces chimeric sequences that stem from the combination of two or more original sequences [4], we will perform chimera detection using the *de novo* method built into UCHIIME.

The Research and Testing analysis pipeline performs chimera detection and removal by executing UCHIIME in *de novo* mode on the clustered data that was output by our denoising methods. By using this method we can determine chimeras across entire region of data even after accounting for noise and removing low quality sequences.

SFF File Generation

SFF files are a binary file containing many data about a read in a single file. For each read, the sff contains a flowgram, quality score and sequence with defined lengths from QC measures performed by the machine. The sff represents the raw data and includes many reads that may have been excluded due to length or chimera detection or any other filter requested for custom processing. Since the files are binary, they cannot be opened with standard text editors. Special programs like Mothur [5] or BioPython [6] are able to load their data into human readable formats and output fasta, qual, flowgram or text (sff.txt) versions. Sff files or their derivatives can then be used for further processing of the data. Sff files provided may be of two forms. In the case of an entire region containing a single investigator's samples, the entire region plus mapping file is provided. In cases where multiple investigators had samples on a single region, each sample is demultiplexed from the sff file using the Roche sffinfo tool by providing its barcode, effectively eliminating it from any read extracted. The split sff can then be used for raw data or submitted directly to archives like the NCBI's SRA. In cases where a single sff for all samples is desired but an entire quadrant is not used, an investigator may request a single sff for a nominal charge. Alternatively, it is possible to use the provided split sff files for denoising/chimera removal by modifying the mapping files. Additional instructions are available if you wish to do so.

Quality Checking and FASTA Formatted Sequence/Quality File Generation

The denoised and chimera checked reads generated during sequencing are condensed into a single FASTA formatted file such that each read contains a one line descriptor and one to many lines of sequence/quality scores. The Research and Testing Laboratory analysis pipeline takes the FASTA formatted sequence and quality files and removes all sequences that meet the following quality control requirements:

1. Failed sequence reads,
2. Sequences that have low quality tags, primers, or ends and
3. Sequences that fail to be at least 250 bp in length.

Sequences that pass the quality control screening are condensed into a single FASTA formatted sequence and quality file such that each read has a one line descriptor followed by a single line of sequence/quality data. The descriptor line in both files has been altered to contain the samples name followed by the original descriptor line, separated with a unique delimiter (::).

This stage of the pipeline creates the FASTA reads archive which contains the following files:

1. The sequence reads from all samples concatenated into a single sequence file. The original tags have been removed from each sequence and an “artificial tag” has been added in its place. The title of the file will be <name>.fas.
2. The quality scores from all samples concatenated into a single quality file. The scores are labeled with the corresponding sample name and will have a matching line in the .fas file. Since the original tags were removed from the sequence and an “artificial tag” was put into its place, the quality scores have been similarly altered such that the original scores for the tag have been removed and an “artificial quality tag” has been added in its place. The artificial quality tag consists of Q30s for the length of the tag. This file will be labeled <name>.qual.
3. A mapping file consisting of sample names included in the analysis. This file contains the information for each sample such that each line has the sample name, tag and primer used for the sample. This file will be labeled as: <name>.txt

Taxonomic Identification

In order to determine the identity of each remaining sequence, the sequences will first be sorted such that the FASTA formatted file will contain reads from longest to shortest. These sequences are then clustered into OTU clusters with 100% identity (0% divergence) using USEARCH [3]. For each cluster the seed sequence will be put into a FASTA formatted sequence file. This file is

then queried against a database of high quality sequences derived from NCBI using a distributed .NET algorithm that utilizes BLASTN+ (KrakenBLAST www.krakenblast.com). Using a .NET and C# analysis pipeline the resulting BLASTN+ outputs were compiled and data reduction analysis performed as described previously [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29].

Based upon the above BLASTn+ derived sequence identity percentage the sequences were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level , 80 and 85% at the class and 77% to 80% at phyla. Any match below this percent identity is discarded. In addition, the HSP must be at least 75% of the query sequence or it will be discarded, regardless of identity.

After resolving based upon these parameters, the percentage of each organism will be individually analyzed for each sample providing relative abundance information within and among the individual samples based upon relative numbers of reads within each. Evaluations presented at each taxonomic level, including percentage compilations represent all sequences resolved to their primary identification or their closest relative [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29].

Analysis description

These folders contain the actual result files from analysis. The folder contains the results for blasting the seed reads against the appropriate Research and Testing Laboratory database. It also contains a seqs_otu_table.txt file showing the predicted OTUS (defined by unique species) with the confidence interval used to give the level of classification for it. Also present is the seqs_otus.txt file which gives the clusters formed by USEARCH. The seed read used for a cluster is the first listed and is the longest sequence of the cluster.

The .csv files are in a comma separated format and can be opened with Excel or another text editor program. Each file can be dragged and dropped into Excel or you may choose to right click on the file name, select “Open With” and choose Excel as the program. Each file contains information about all the samples. Sample names span the first row with the bacterial/fungal designations at each respective taxonomic level are listed in the first column. Counts (or

Percentages- if looking at the …Percent.csv file) of each of the respective taxonomic levels found within the sample are listed below the sample name.

Generally, the most relevant files are the Percent composition files, although the PercentTraceback can be used when confidence intervals are desired. The files include composition information forced to the top blast hit at a specific taxonomic level. For example, in the species files, the nearest well described species for each sequence is listed; similarly, the genus files contain the genus of the species as catalogued in the database, and so on for each taxonomic level. Research and Testing Laboratory uses 7 taxonomic levels for each organism: Kingdom, Phylum, Class, Order, Family, Genus, Species.

The information is organized by taxonomic level (each file specifies for which taxonomic level the information included is for). Files names include:

<name>Kingdom

<name>BelowMinimum

<name>Excluded

<name><taxa level>Counts

<name><taxa level>Percent

<name>SpeciesOptions

<name>SpeciesFullTaxa

<name>SpeciesTraceback

<name>SpeciesPercentTraceback

File descriptions:

<name>Kingdom.csv with the following columns followed by samples:

1. Query sequence name (with sample label) and its cluster information

2. The hit name with “-I” followed by the identity percentage and “Q” followed by the query length,

3. Identity column, indicating the identity percentage of the query to the hit sequence along the HSP (High Scoring Pair) region.

4. The count for the hit, based on the number of cluster members. If clustering is not performed, this is always 1.

<name>BelowMinimum.csv

Contains all hits from the blast results that fell below 77% identity OR had HSP coverage below 70%. These are given a classification at the closest species, but do not appear in any other file. These hits did not have sufficient similarity to any reference sample to have confidence in assigning to an organism. If a sample had all its reads fall into this file, all values in the Counts file will show as 0 and all the values in the Percent files will show as NaN for the sample as a result of division by zero.

<name>Excluded.csv

Contains hits against organisms that have been requested to be excluded from results by the sender. By default, this is always empty. Some items may be dropped from the blastout and will not appear in the excluded file. This currently includes plastid and mitochondrial sequences that may be present in the database.

Additionally, some reads generated may be present in none of the above files if they were not sufficiently similar to any of the reference sequences in the database. This may occur due to a spurious amplification or poor quality read. These reads are considered to be further noise in the data.

Each of the taxa levels contains 2 files

<name><taxa level>Counts.csv

Counts are merged on the organism term, with separation given to those with top hits among different species, condensing all identity scores and query lengths. A set of hits by a read against multiple organisms but with identical similarity have each organism listed, separated by "::". A read may have up to 5 hits, but only the best are used for determination. If a non-specific species is found as the top hit, a similar quality hit on a full species is set as the top hit for the Percent computations. Each unique set of terms has a single line with summed counts for all of its samples.

<name><taxa level>Percent.csv

Converts the previous file of raw read counts into percentages of composition per sample per organism, for the top hit only. Multiple organism hits are converted to top hit only for merging with other reads with similar top hits, except those with generic species as the top hit, which are changed to the next hit with a specific name. Some entries here may appear as NaN, indicating

that the count was zero as a result of discarding all hits for the sample due to percent identity or filtering.

Additional species files

<name>SpeciesOptions.csv

Shows the percent file without discarding any top hits and calculating percents based on this. This shows what percent of the reads had only a single similar species based on sequence or multiple similarities.
<name>SpeciesFullTaxa.csv

Shows the Percent file with the full taxa of every organism listed. This way, multiple files do not need to be examined to determine the catalogued taxonomy used in the groupings
Due to incomplete taxonomic data for some organisms, the top hits may have unusual naming conventions. Some entries have no specific name, so they are abbreviated <Genus> sp. If a sample from the database had missing taxonomic data at the class level e.g. *Cyanobacteria*, then the class of the organism would be *Cyanobacteria (class)*. Others may be named with an "unclassified" e.g. *Clostridiaceae unclassified.* This occurs in many places, but the naming convention remains the same for any taxonomic level. Those without at least a defined class are not added to the database for bacteria and fungi. Also seen are species like *Pseudomonas sp Rhizobiaceae* or a genus like *Pseudomonas (Rhizobaceae).* This is a generic Pseudomas species belonging to the Rhizobiaceae family. Since there are multiple entries for Pseudomonas sp in the NCBI Taxonomy, the suffix has been added to distinguish at each level where there may be confusion over which species it is, if only the usual nomenclature had been used.

Also included in the **Percent** folder is a "traceback" file, *SpeciesPercentTraceback*. This file reflects taxonomic information for organisms found in the sample based on the goodness of the alignment against reference sequences. This file is similar to the previously described percent file, however, the nearest neighbor isn't forced, but instead based on the percent identity of the query sequence to the reference sequence; the "most certain" taxonomic level is listed. A

summary table of the level which is used for identification based on identity score is provided below.

Identity to reference sequence	**Traceback Designation**
I > 97%	
97% ≥ I > 95%	(unk species)
95% ≥ I > 90%	(unk genus)
90% ≥ I > 85%	(unk family)
85% ≥ I > 80%	(unk order)
80% ≥ I > 77%	(unk class)

For example, if one of the sequences was identified as a *Staphylococcus aureus* with an identity of 98%, it would still be labeled as *Staphylococcus aureus* in the Traceback file. However, if the identity was 88%, it would be grouped with other sequences with identities between 85-89% belonging to the Bacillales order and labeled as *Bacillales (unk family)*. The traceback changes are made to all entries, and then additional merging is done to combine now repeated terms. This may also split hits against an organism into several levels of confidence giving a *Staphylococcus aureus, Staphylococcus (unk species), Staphylococcaceae (unk genus)* from the *S. aureus* hits. Since taxonomic database entries are used, there may be entries similar to *Bacillales(family)(unk genus)* which is a hit against an organism with incomplete taxa (only up to order) and an identity above 89% but below 94%. Any entry with a full species name and no parenthetical notation means the hit was above 96% identity.

Due to the changes made between the *SpeciesPercent* file and the *SpeciesTracebackPercent* file, the two are not directly comparable. The Percent file shows only the top hit result while the Traceback further divides this into confidence measures based on percent identity.

D.6 FIELD PROTOCOL THERMALLY ENHANCED PILOT STUDY

Water Sampling

1. Attach sample system to DI water source and purge.
2. Hook up water sample tubing from MLS to a peristaltic pump and connect a three way valve on the outlet (or "Christmas tree" valve system)
3. Connect flow cell and flush line until pH and ORP stabilize.
    a. Insert pH and ORP probes into flow cell by first removing the top of the flow cell and inserting probes through the holes in the top portion.
    b. Then put O rings on each probe, then insert probes into bottom of flow cell.
    c. Adjust electrode position to where you want, then tighten the hex screws. Test o-ring seals by flushing cell with DI water.
    d. In order to fill the cell without getting air bubbles, turn the flow cell upside down while pumping water through, being careful to cover any holes on the barrels of the electrodes to prevent leakage of fill solution. Chase small bubbles that form in the cell out by rotating and tapping the cell.
4. Switch flow to sample port.
5. *Attach an in-line 0.45µm filter and fill
    a. 10-mL serum vial† for anion analysis (Be careful not to fill to top, this will build up pressure and may pop the cap off.) Fill via injection with a needle.
    b. 10-mL crimp-top vial for cation analysis. Immediately add 40 µL of nitric acid via syringe.
6. Switch flow to 2nd sample port.
7. Fill a 10-mL crimp-top vial for TPH analysis (no air).
8. Fill a 10-mL crimp-top vial for methane analysis (no air).
9. Store samples on ice for shipment back to CSU, and then at 4 ^{0}C until analysis or sample preparation.

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10. Collect 1 purge blank in every 7 ports (immediately after pH ORP measurement)
11. Collect triplicates for every 10 ports
12. Make 2 shipping blanks for every 100 samples

*Pre-label sample vials with Well ID, port ID, sample type, date, sampler. Record this info on the field form spreadsheet along with sampling time.

†Prepare the serum vials according to the following procedure:
- Insert gray chlorobutyl serum stoppers (VWR) and crimp down with aluminum cap.
- Use a microspatula to remove the inner seal of the aluminum cap
- Insert a needle attached to a 2-way valve for about 10 vials.
- Leaving valves in the open position, transfer vials to the anaerobic chamber.
- Move the vials into the chamber and close valves. Transfer them to outside the chamber.
- Use the vacuum pump to evacuate the vials, one at a time, bringing the pressure to just past 25 on the wall gauge. Then remove the needle.

Gas/Vapor sampling

Rent a Landtec GEM2000 from either TRS Environmental or Equipco Rental Services for measurements in the field for CH_4 and CO_2.
http://www.equipcoservices.com/rentals/gas_detection/landfill_gas_analyzers.html

Cost: ~ $145/day

A carbon filter is recommended to be used when known or suspected levels of petroleum hydrocarbons are present. These can be bought from TRS Environmental for $15. For more information on the carbon filters refer to Lantec GEM 2000 FAQs document.

Temperature sampling

Temperature will be measured at all six points on each well by connecting a digital thermometer to the thermocouples in place on the multi-level samplers.

Temperature will also be logged continuously at the center point using a data logger.