Anaerobic Benzene Degradation in the Presence of Salinity

by

Kelvin Kin Hang Chan

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Environmental Engineering

Department of Civil and Environmental Engineering

University of Alberta

Abstract

The environmental presence of benzene is mostly attributed to anthropogenic activities related to the oil and gas sector and industrial production. While intrinsic bioremediation with microbial organisms is an attractive remediation option due to its minimal maintenance, low cost, and non-intrusive nature, biodegradation progress is often stalled upon oxygen depletion in the subsurface. At such instances, the microbial community must shift towards anaerobic conditions and rely on other redox conditions (such as nitrate-, sulfate-reducing, and methanogenesis) without oxygen as a terminal electron acceptor. Furthermore, this situation is often compounded in the oil and gas industry with the release of produced water which contains not only benzene but is also highly saline. In this M.Sc. thesis, the concept in which salt is a co-contaminant to benzene is explored for bioremediation utilizing microbial cultures previously demonstrated to anaerobically biodegrade benzene.

In the first phase of this experiment, the Culture Enrichment period, a benzene biodegrading baseline for the nitrate-, sulfate-reducing, and methanogenic cultures is established. Cultures derived from clay sediments were able to biodegrade benzene at a slightly higher rate (17.7 ± 10.2 µM/d) then the sand counterparts (14.9 ± 10.9 µM/d). In the second phase, the Salinity Experiment, salt contents of 0.0, 0.5, 1.0, and 2.0 g/L NaCl were explored. The nitrate-, sulfate-, and methanogenic treatments demonstrated benzene biodegradation rates of 1.8 ± 0.5, 4.2 ± 5.1, and 2.1 ± 3.4 µM/d respectively. Within a benzene feed concentration between 0.5 and 0.7 mM, biodegradation rates were consistently the highest within the 1.0 g/L NaCl conditions, suggesting this to be the optimal salt content to stimulate benzene biodegradation within this experiment.

Acknowledgements

There is the popular African proverb that “it takes a village to raise a child”. This is very much so the case with the M.Sc. thesis before you. However, I will take it a step further and also mention the other popular village related saying of “every village has its idiot”. I’m fairly sure that is me. There are countless people who contributed through mentorship, guidance, advice, and support which ultimately made this thesis possible. I am beholden to them all.

First and foremost, I would like to thank my supervisor - Dr. Ania Ulrich. She gave me the opportunity to enter graduate studies and join her lab. To those who have ever seen my undergraduate transcript, you’d probably wonder what business I had in attempting further academics and how I had managed to sneak into grad school. Dr. Ulrich was the one who gave me a chance, mentored me, funded me, and believed in me for three years. I will always remember her infectious enthusiasm.

Second, I need to extend my appreciate towards the remaining members of my committee – Dr. Daryl McCartney and Dr. Karen Budwill. They were ready with advice throughout all stages of this thesis and their guidance ensured that my work was continually of good quality.

Next, my lab mates and other graduate students on the sixth floor. Korris Lee has been my mentor from day one. He showed me the ropes in all the analytical techniques and was often the one I called to troubleshoot any instruments. Amy-lynne Balaberda and Luke Gjini showed me that academia is tolerable with a healthy injection of shenanigans and alcohol. Sarah Miles, Lily Yu, Stanley Poon, Chelsea Benally, and Kei Wei consistently purchased lab materials for me and accepted my laziness as I couldn’t be bothered with creating a purchasing account. Petr Kuznetsov is the current lab manager and I cannot thank him more for editing this thesis. Edmund Yu and Heidi Cossey were always ready in their office to hear me rant about whichever frustration that came up for the day. Lastly, David Zhao and Chen Liang took care of all the environmental labs. It was them that troubleshooted the fickle lab instruments I often wanted to hit with a hockey stick.

Apart from the folks at school, I need to thank my mom and dad – Cathy Lai and Edmond Chan. I cannot be the person I am today without their unconditional love and bottomless support. This thesis, and all the other things I aim to do in the future, will hopefully make them proud. Jeffrey Hung, Andrew Wong, and Dimple Ji are my close childhood friends who also went through graduate studies at the same time. They know the frustrations, setbacks, and uncertainties of

research and were always there with advice and words of encouragement. Alison Yeung and Gregory Chu smacked some sense into me when I needed it. Then there is Alyssa Baser, who believes in me, especially in moments when I do not believe in myself.

Last (and most importantly), my cat Jupiter. She taught me the value of self care, specifically the importance in eating (eight meals a day) and sleeping (13 hours a day).

Table of Contents

List of Figures

List of Tables

CHAPTER 1

General Introduction

1.1 - Introduction

Ever since the onset of the industrial revolution, petroleum hydrocarbons (PHCs) have become a staple use in everyday life. Globally, PHCs are used not only in solvents, lubricants, and building materials, but most importantly, they contribute approximately from one-third to one-half of the world's energy supply in the form of fueling transportation and heating buildings (Speight 2014). Soils and groundwater contaminated with PHCs are therefore one of the most frequent forms of contamination in Canada and presents as a multibillion dollar problem due to the plentiful contaminated sites each with differing degrees of contamination, risk of off-site migration, and logistics or technologies required to remediate (CCME 2014).

In Canada, one method of classification is defining PHCs into four fractions based on the number of carbon atoms within the compound's structure. The compounds within in each fraction contain similar physical-chemical properties and toxicological characteristics which allows for general predictions on chemical fate, exposure, and potential risk (CCME 2008). Fraction 1 (F1) encompasses PHCs with six to ten carbon atoms (C_6 to C_{10}). Similarly, Fractions 2, 3 and 4 (F2, F3, and F4) are classified as $C_{>10}$ to C_{16}, $C_{>16}$ to C_{34}, and $C_{>34}$ to C_{50} respectively (CCME 2008).

Within the F1 component of PHCs, there exists a smaller family of aromatic hydrocarbons collectively known as BTEX as consists of benzene, toluene, ethylbenzene, and xylenes (Figure 1 below). These compounds can readily volatize into the air, are soluble into groundwaters, and mobile in subsurface environments. Due to these characteristics, BTEX can be indicators of PHC migration (CCME 2008). As significant constituents of petroleum and industrial products, PHC F1-BTEX are a common soil and groundwater contaminant due to underground storage tank leakages, spills, and improper disposal methods (Mancini et al. 2008).

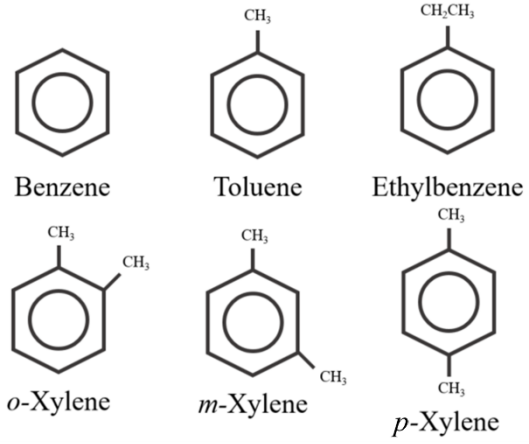

Figure 1-1 – BTEX Compounds Structures (Ulrich 2004)

Within the BTEX family, benzene has the molecular formula C_6H_6 and a structure as seen in Figure 1-1 above. While it may be the structurally simplest member of the six monocyclic aromatic hydrocarbons shown, this organic chemical compound is often problematic on PHC contaminated sites due to its difficulty to remediate, mobility, and carcinogenic nature.

1.2 – General Properties of Benzene

Benzene has a CAS registry number of 71-43-2 and is also known by synonyms including benzol, carbon oil, coal naptha, light oil, phene, and phenyl hydride (CCME 2004). It is a clear, colourless, flammable, and volatile liquid possessing a sweet, aromatic odour at room temperature and has a density that is slightly lighter than water (Becalski 2014; CCME 2004; Knovel 2008; Wilson et al. 2009). When this compound is released into the environment, sorption onto soil particles occurs but remaining fractions will continue to flow downwards until reaching the groundwater where it becomes easily dispersed due to its high solubility (CCME 2014; Shiu et al. 1990; Vallero 2008; Wilson et al. 2009). Lastly, benzene is also miscible in fats and oils (Becalski 2014). The specific physical, chemical, and partitioning properties of benzene which results in the previously mentioned environmental behaviors are discussed in detail within the literature review of Chapter 2.

1.3 - Sources of Benzene Contamination

Benzene contamination results from both anthropogenic releases as well as naturally occurring sources (Becalski 2014). Human related benzene releases are most significantly associated with activities originating in both the energy sector and industrial production. Specifically, atmospheric deposition, above- and under-ground storage tank leakages, seepage from improperly designed waste disposal sites, and spillage of oil and gasoline during storage, transportation, and handling are the common reasons for benzene release within these two industries (CCME 2004). An estimated annual amount of 34 kilotonnes of benzene is released into the atmosphere, 1 kilotonne into the water, and 0.2 kilotonne onto soil within Canada each year (Government of Canada, Environment Canada 1993).

In regards to the energy sector, petroleum refining and processing of oil and gas activities contribute mostly to anthropogenic benzene contamination since the chemical is a natural constituent of petroleum: making up between 1 to 4% of gasoline (Barker et al. 1989; Kirk et al. 1991) and up to 3% of crude oil (Becalski 2014). A secondary and lesser source within the energy

sector involves the extraction of natural gas condensate and slow distillation of coal (Jacques 1990).

In terms of the industrial sector, benzene is associated as a precursor or intermediate product in the production of plastics, synthetic rubbers, dyes, solvents, paints, and other chemicals and pharmaceuticals (Becalski 2014; CCME 2004). These other chemicals include, but are not limited to, styrene, phenol, cyclohexane, ethylbenzene, cumene, and maleic acid anhydride (Fishbein 1985; Jacques 1990; Soares et al. 2010).

In 2013, USEPA reports 8% of the superfund sites undergoing remediation efforts were impacted by benzene contaminant (U. S. Environmental Protection Agency (EPA) 2000). Due to the recalcitrant and mobile nature of benzene, even trace amounts possess the potential to contaminate large volumes of both soil and groundwater, and often to concentrations levels exceeding the limits defined by local regulations (Soares et al. 2010).

Aside from anthropogenic origins, benzene can also be a natural occurring contaminant. These natural sources include discharges from petroleum seeps, forest fires, volcanic eruptions, and vegetation (CCME 2004; Westberg et al. 1981). CCME (2004) reported three case studies in Canada in which the benzene content of non-impacted soils was analyzed. The data in these respective studies reported low to non-detect concentrations, suggesting that natural occurrences of benzene to be relatively minor in comparison to those of anthropogenic origin in Canada (CCME 2004).

1.4 - Effects of Benzene Exposure

Acute and low level exposure effects to benzene include drowsiness, dizziness, and unconsciousness (Wilson et al. 2009) and is often rapidly metabolized by the liver and excreted as urinary metabolites (Becalski 2014). In higher levels of exposure, benzene is distributed throughout the body and has an affinity to remain within fatty tissues (Becalski 2014). The International Agency for Research on Cancer has deemed benzene as a carcinogen, linking countless cases of long-term and high level exposure towards the onset of acute myelogenous leukemia (Becalski 2014; Mancini et al. 2008).

Insufficient data is available regarding the effect of benzene exposure on wild animals, thus relying on the extrapolation of mammalian studies in a laboratory setting. In such studies, benzene is not only classified as a highly acute toxic compound via inhalation and ingestion exposure but

is reported to absorb readily into the digestive tract (Becalski 2014; Government of Canada (GOC) 2015).

Plants grown with benzene content in soils have been reported to transform benzene into metabolites, suggesting possibilities for phytoremediation. Conversely, plants sprayed with higher concentrations of benzene display symptoms of cellular damage (CCME 2004).

1.5 - Benzene Contamination Plumes and Fate

The process in which benzene is released, either in pure solution or as part of fuel, and infiltrating the environment is similar to that of other organic contaminants (Mackay et al. 1985). The spilled liquid containing benzene will flow downwards in the unsaturated subsurface at a rate governed by the liquid's hydraulic conductivity and fill the pores of the soils directly below and adjacent to the spill point. A small residual amount is left within the soil pores of the slug path as the downward and gravity driven movement continues. This process proceeds until either one of two eventualities occur: firstly, this downward migration ceases when the volume of the spilled liquid is equivalent to the volume of liquid retained in the pore spaces of the unsaturated zone as described above; or secondly, the downward migration arrives at the water saturated zone or some impermeable layer and upon which advection and dispersion processes dominate (ATSDR 2007; Domenico and Schwartz 1998; Government of Canada (GOC) 2015; Mackay et al. 1985). Throughout such occurrences, there exists four mechanisms that determine the fate of the released benzene and are as follows.

Benzene is a readily volatile compound; thus the first fate is volatilization into the air-filled pore space from contaminated soils or groundwater. GOC (2015) reported a 4.8 hour half-life of benzene in contaminated waters as a result of volatilization. However, volatilization and subsequent diffusion of benzene in the unsaturated zone can also increase the area of contamination as the contaminated gas diffuses throughout the soil's pore spaces (Domenico and Schwartz 1998).

Sorption onto solids within the soil is the second process governing the environmental fate of benzene. Soil organic matter content acts as a partitioning medium and largely possess the fraction retained within the soil media (Chiou 1989; Rebhun et al. 1992). Sorption to the mineral and clay portions of the soil will also occur, but to a lesser extent in comparison to the organic matter (Chiou 1989).

The third removal mechanism of benzene in contaminated soils is the mere leaching away and consequent dilution by infiltrating precipitation. This process is limited by obvious factors

such as annual rainfall or rate of groundwater recharge but this simply transport process often result in the off-site migration of benzene via underlying aquifers (CCME 2004).

Biodegradation of vapor-phase, sorbed, and dissolved benzene by indigenous microbial organisms is the fourth and last removal mechanism (CCME 2004). Most hydrocarbon degrading microorganisms are ubiquitous in most contaminated sites and rely on oxygen as an electron acceptor during their metabolic processes to degrade benzene (CCME 2004; English and Loehr 1991). However, due to the high oxygen demand imposed by the benzene load, sites often become oxygen deficient or anaerobic (Song et al. 1990). Upon oxygen depletion, microorganisms must rely on the next readily available electron acceptor to metabolize benzene. In most cases, such electron acceptors exists in the form of nitrate, manganese (IV), iron (III), sulfate, and carbon dioxide. (U. S. Environmental Protection Agency (EPA) 2000). This transition between aerobic and anaerobic biodegradation often tends to be the stalling point in many contaminated sites due to the absence of a microbial culture capable of utilizing these secondary electron acceptors in anaerobic conditions or simply the insufficient amount of these electron acceptors (Vogt et al. 2011). Figure 1-2 below depicts a benzene plume in which the leading edge undergoes aerobic biodegradation due to the availability of oxygen but becomes anaerobic and relies upon remaining electron acceptors in proximity to the benzene source.

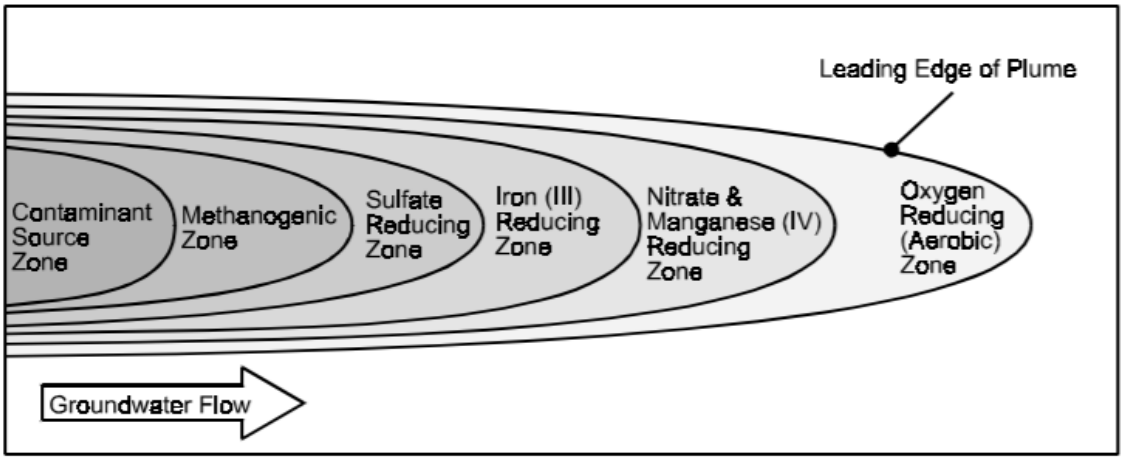

Figure 1-2 - Redox Zones of a Typical Petroleum Plume in an Aerobic Aquifer (Aerial View) (U. S. Environmental Protection Agency (EPA) 2000)

1.6 – Current Benzene Remediation Technologies

Numerous remediation strategies have been applied to target benzene contaminated soils and groundwaters. The tradition and most direct method involve the excavation and disposal of

impacted soils coupled with a pump and treat approach for the groundwater. Benefits of this technique include the operational simplicity and time efficiency at the price of greater economics as well as the higher degree of site intrusion.

Soil vapor extraction (SVE) is an in-situ remediation strategy demonstrated to be extremely efficient at targeting benzene and other volatile organic compounds within the soil's unsaturated zone (Soares et al. 2010). SVE requires the installation of a series of extraction wells on site which induces a vacuum in the soil matrix and creates an airflow towards the surface. This technology takes advantage of the high volatility and low vapour pressure of benzene to encourage the mass transfer of desorption from soils and dissolution from the aqueous phase towards volatilization into the gas phase. However, limitations include inability to target residual or trace amounts of contaminant once the bulk and accessible portions of the plume has been treated (Soares et al. 2010).

Intrinsic biodegradation of benzene (as mentioned in Section 1.5) by indigenous microbial communities is another widely used remediation technique. While this method is far more time consuming and operates at a lower removal rate in comparison to other benzene targeting remediation technologies, is it an applicable option for sites with less restrictive scheduling demands and is also an attractive option due to its minimal operating costs, lack on on-site operation, non-intrusive nature, and ability to treat residual contamination that traditional means of remediation cannot access. Most pitfalls associated with this method involve a stall in degradation progress due to the lack of an appropriate benzene degrading microbial community, the depletion of a usable electron acceptor critical for the metabolism of these microorganisms, low nutrient conditions, and colder climates (Ulrich et al. 2009).

1.7 – Salinity Co-contamination

In addition to PHC contamination, many sites in the petroleum industry are also plagued by elevated salt content due to incidental releases of hypersaline produced waters which simultaneously contain both benzene and salt (Ulrich et al. 2009). This occurrence of salt existing as a co-contaminant aside benzene is a common situation in many upstream sites (such as flare pits and drilling sites) or downstream processing facilities within the oil and gas industry (Ulrich et al. 2009). It is worth noting that salinity impacted soils in this context is defined as soils which possess a salt content over 0.45% and need not be as extensive as some hypersaline marine environments (Qin et al. 2012). Currently, not enough is known in the scientific community

regarding halophilic and halotolerant terrestrial microbes' ability to biodegrade PHC in the presence of salt (Sei and Fathepure 2009; Ulrich et al. 2009). Seldom do existing and comparable studies analyze the effect of salt specifically against the aerobic biodegradation of benzene. To date, no research has been identified to examine the biodegradation of benzene under both **saline and anaerobic conditions** in the preparation of this thesis. Literature suggests that the presence of salinity can vary from disrupting tertiary protein structures and denatured enzymes of cell dehydration of microbial communities (Ulrich et al. 2009). While different species react differently in the presence of salt, it is suspected that salinity deceases the accessibility of soil organic matter to the microbial community (Qin et al. 2012). Understanding the implications of elevated salt content against the anaerobic biodegradation of benzene has valuable applications towards the petroleum industry, specifically the reclamation of oil and brine impacted soils, remediation of oil polluted hypersaline lakes and oceans, treatment of oily hypersaline wastewater, and hydrocarbon degradation process in hypersaline petroleum reservoirs (Martins and Peixoto 2012). The real-world implications of saline benzene contamination are undeniable and the complexity of this subject demand further investigation in the scientific community. This thesis and its experiment are but a drop in the crashing waterfall that is PHC research, but it aims to be a part of the trickle that brings upon the river of progress and knowledge. It is filled with the hope that benzene contamination would one day be a trivial matter in the petroleum and industrial industry.

1.8 – Objective, Research Questions, Hypothesis, and Overall Thesis Organization

The objective of this thesis is *to investigate the effect of salinity on anaerobic benzene biodegradation.* From this goal, two research questions were developed to guide the research:

I. "*What differences in anaerobic benzene biodegradation capabilities exist between treatments based on nitrate-reducing, sulfate-reducing, and methanogenic conditions? Furthermore, are these biodegradation capabilities affected by whether the treatments were derived from clay or sand sediments originating from the same source?" and,*

II. "*For the same treatments in Research Question I, is anaerobic benzene biodegradation also possible under varying salinity conditions? And if it is possible, what is the optimal salinity concentration which yields the greatest degradation rate?"*

A literature review on anaerobic benzene biodegradation and aerobic benzene biodegradation with the influence of salinity has been accomplished in Chapter 2. This chapter not

only discusses the theoretical aspects of benzene biodegradation but also the results from publications of past experiments in this field. In these studies, degrees of degradation were not demonstrated equally across all salinity ranges. Conversely, certain intervals of salt concentrations resulted in greater degradation rates. These conclusions in conjunction with the previously mentioned research questions ultimately led to the hypothesis that *trace amounts of salinity will not impede (but may even stimulate) benzene biodegradation. However, further addition in salinity past an optimum range will result in inhibitory effect towards benzene biodegradability*.

Chapter 3 details the materials, experimental set up, and analytical procedures used within the laboratory and summarizes the calculation methods for the metrics of analysis used in the following chapters. Chapter 4 reports benzene biodegradation with treatments within nitrate-, sulfate-reducing, and methanogenic conditions. It also explores differences between treatments originating from clay and sand sediments. This chapter serves to provide a baseline on the biodegradation capabilities of the microbial cultures studied. Chapter 5 is the backbone of this thesis as it introduces a salinity factor and discusses differences in benzene biodegradation in varying salt concentrations. Lastly, Chapter 6 offers a conclusion for the work undertaken and suggests future directions in hopes of further developing the knowledge of the scientific community on this topic.

1.9 – Chapter 1 References

ATSDR. (2007). "Toxicological Profile for Benzene." *Agency for Toxic Substances and Disease Registry (ATSDR)*, (August), 438.

Barker, J. F., Sudicky, E. A., Mayfield, C. I., and Gillham, R. W. (1989). *Petroleum Hydricarbon Contaminantion of Groundwater. Natural Fate and In Situ Remediation, a Summary Report. PACE Report No. 89-5.* Ottawa, Canada.

Becalski, A. (2014). "Chapter 20 - Processing Contaminants: Benzene." *Encyclopedia of Food Safety*, 376–380.

CCME. (2004). *Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health: Benzene*.

CCME. (2008). *Canada-Wide Standard for Petroleum Hydrocarbons (PHC) in Soil: Scientific Rationale Supporting Technical Document*.

CCME. (2014). "Canada-wide standards for petroleum hydrocarbons (PHC) in soil." *Ccme*, (January), 1–8.

Chiou, C. T. (1989). "Theoretical COnsiderations of the Partition Uptake of Nonionic Organic COmpounds by Soil Organic Matter." *Reactions and Movement of Organic Chemicals in Soils, SSSA Special Publication 22*, Soil Science Society of America and American Society of Agronomy.

Domenico, P. A., and Schwartz, F. W. (1998). "Chapter 19 - Multiphase Fluid Systems." *Physical and Chemical Hydrogeology (2nd Edition)*, 405–416.

English, C. W., and Loehr, R. C. (1991). "Degradation of organic vapors in unsaturated soils." *Journal of Hazardous Materials*, 28(1–2), 55–64.

Fishbein, L. (1985). "An overview of environmental and toxicological aspects of aromatic hydrocarbons IV. Ethylbenzene." *Science of the Total Environment, The*, 44(3), 269–287.

Government of Canada, Environment Canada, H. and W. C. (1993). *Benzene - Priority Substances List Assessment Report*.

Government of Canada (GOC). (2015). *Canadian Environmental Protection Act, 1999*.

Jacques, A. P. (1990). *National Inventory of Sources and Emissions of Benzene (1985). Report EPS 5/AP/1.* Ottawa.

Kirk, R. E., Othmer, D. F., Grayson, M., and Eckroth, D. (1991). *Kirk-Othmer Encylopedia of Chemical Technology (4th Edition)*. John Wiley, New York.

Knovel. (2008). “Basic Physical Properties of Chemical Compounds.” *Knovel Critical Tables (2nd Edition)*.

Mackay, D. M., Roberts, P. V, and Cherry, J. a. (1985). “Transport of organic contaminants in groundwater - Distribution and fate of chemicals in sand and gravel aquifers.” *Environmental science & technology*, 19(5), 384–92.

Mancini, S. A., Lacrampe-Couloume, G., and Lollar, B. S. (2008). “Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses.” *Environmental Forensics*, 9(2–3), 177–186.

Martins, L. F., and Peixoto, R. S. (2012). “Biodegradation of petroleum hydrocarbons in hypersaline environments.” *Brazilian Journal of Microbiology*, 43(3), 865–872.

Qin, X., Tang, J. C., Li, D. S., and Zhang, Q. M. (2012). “Effect of salinity on the bioremediation of petroleum hydrocarbons in a saline-alkaline soil.” *Letters in Applied Microbiology*, 55(3), 210–217.

Rebhun, M., Kalabo, R., Grossman, L., Manka, J., and Rav-Acha, C. (1992). “Sorption of organics on clay and synthetic humic-clay complexes simulating aquifer processes.” *Water Research*, 26(1), 79–84.

Sei, A., and Fathepure, B. Z. (2009). “Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake.” *Journal of Applied Microbiology*, 107(6), 2001–2008.

Shiu, W. Y., Ma, K. C., Mackay, D., Seiber, J. N., and Wauchope, R. D. (1990). “Solubilities of pesticide chemicals in water. Part I: Environmental physical chemistry.” *Reviews of environmental contamination and toxicology*, 116, 1–13.

Soares, A. A., Albergaria, J. T., Domingues, V. F., Alvim-Ferraz, M. da C. M., and Delerue-Matos, C. (2010). “Remediation of soils combining soil vapor extraction and bioremediation: Benzene.” *Chemosphere*, 80(8), 823–828.

Song, H. G., Wang, X., and Bartha, R. (1990). “Bioremediation potential of terrestrial fuel spills.” *Applied and Environmental Microbiology*, 56(3), 652–656.

Speight, J. G. (2014). *The Chemistry and Technology of Petroleum (5th Edition)*. CRC Press.

U. S. Environmental Protection Agency (EPA). (2000). “Engineered approaches to in situ bioremediation of chlorinated solvents: Fundamentals and field applications.” (EPA 542-R-00-008), 1–144.

Ulrich, A. C. (2004). “Characterization of Anaerobic Benzene-Degrading Cultures.” University of Toronto.

Ulrich, A. C., Guigard, S. E., Foght, J. M., Semple, K. M., Pooley, K., Armstrong, J. E., and Biggar, K. W. (2009). “Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater.” *Biodegradation*, 20(1), 27–38.

Vallero, D. (2008). “Chapter 6 - Air Pollution Systems and Processes.” *Fundamentals of Air Pollution (4th Edition)*, 154–198.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). “Anaerobic benzene degradation by bacteria.” *Microbial Biotechnology*, 4(6), 710–724.

Westberg, H., Sexton, K., and Flyckt, D. (1981). “Hydrocarbon production and photochemical ozone formation in forest burn plumes.” *Journal of the Air Pollution Control Association*, 31(6), 661–664.

Wilson, S., Card, G., and Haines, S. (2009). “Table 3.3 - Properties of Some Commonly Occurring Vapours.” *Ground Gas Handbook*, Whittles Publishing.

CHAPTER 2

Literature Review

2.1 – Introduction

This chapter is a literature review for the topic of anaerobic benzene biodegradation in the presence of salinity. The first portion of this chapter sets up the context of viewing benzene as an environmental contaminant and the intricacies involved with the bioremediation of this substrate. In Section 2.2, the physical, chemical, and partitioning properties of benzene are listed to describe its behavior upon release in the subsurface. Next, the shift between aerobic biodegradation towards other redox conditions and the thermodynamic challenges which arise when utilizing these other terminal electron acceptors are discussed in Section 2.3. Three degradation pathways for anaerobic benzene biodegradation are commonly proposed in literature. Section 2.4 explores these three initial activation mechanisms (benzene methylation, hydroxylation, and carboxylation) and provides evidence in support and opposition for each pathway. The complexity and challenges of anaerobic or saline benzene biodegradation studies are discussed in Section 2.5 while Section 2.6 follows with a literature review of past anaerobic degradation experiments. Each of the three redox conditions pertinent to this thesis (nitrate-, sulfate-reducing, and methanogenic) are explored via both a general overview as well as a detailed discussion involving each individual study. Lastly, Section 2.7 provides a literature review on past publications which involved a salinity factor within benzene degradation experiments.

2.2 – Physical, Chemical, and Partitioning Properties of Benzene

Benzene is a clear, colourless, flammable, volatile liquid possessing a sweet, aromatic odour at room temperature (CCME 2004; Wilson et al. 2009). With a chemical formula of C_6H_6, benzene has a molecular weight of 78.11 $g \cdot mol^{-1}$ (CCME 2004). It has a melting point of 5.5°C (Knovel 2008) and a boiling point of 80.09°C (Becalski 2014; Yaws 2014), thus allowing for a liquid state at room temperature. With a density of 0.877 $g \cdot cm^{-3}$, benzene is slightly lighter than water (Becalski 2014; Knovel 2008). Furthermore, it is readily soluble in water with a reported solubility between 1,761 (Lyons et al. 2016) and 1,780 $mg \cdot L^{-1}$ at 25°C (Shiu et al. 1990). In terms of volatility, the vapor pressure at 20°C is 12.7 kPa (Vallero 2008). The Henry's Law (K_H) partitioning coefficient at 25°C is 5.5385 x 10^{-3} $atm \cdot m^3 \cdot mol^{-1}$ (Yaws 2014) or dimensionless at 0.2289 (Wilson et al. 2009). Moreover, benzene has a relatively low log octanol-water partitioning (log K_{ow}) coefficient of 2.13 and is readily miscible with fats, oils, alcohol, chloroform, ether, carbon disulphide, carbon tetrachloride, glacial acetic acid, and acetone (Becalski 2014; CCME 2004). Lastly, benzene contains sorption to organic carbon partitioning coefficient log K_{oc} value of 1.77 or K_{oc} value of 59 (Vallero 2008; Wilson et al. 2009).

2.3 – Redox Conditions and Electron Acceptors of Anaerobic Benzene Biodegradation

The biodegradation of most aromatic compounds often begin with the oxidation or reduction of substituent groups, carbon-carbon cleavage of substituents from the ring, decarboxylations, removal of O-methyl, sulfur, nitrogen, or halogens from the ring (Coates et al. 2002). Unfortunately, these initial biodegradation strategies to cleave the aromatic ring are not applicable for benzene as it lacks a function group such as carboxyl or hydroxyl and is further strengthened by a delocalized cloud of π-electrons which stabilizes its carbon-to-carbon bonds. Ultimately, these factors lead to a thermodynamic stability which resists degrading under chemical reactions and the enzymatic attacks during biological degradation, allowing for its persistence in the natural environment (Coates et al. 2002; Foght 2008; Langenhoff et al. 1996; Phelps et al. 1996).

For aerobic microorganisms, this resonance stability to resist ring cleavage can be overcome by the insertion of an oxygen molecule. Specifically, Coates et al. attributed this process in a 2002 literature review as Rieske non-heme iron dioxygenase, during which "dioxygen is cleaved and both atoms of oxygen are inserted across the double bond of the inactivated aromatic ring to yield a *cis*-dihydrodial." This aerobic biodegradation of benzene is a topic that is well studied and demonstrated throughout literature. However, these processes are not possible under anaerobic circumstances in which oxygen is not available. As a result, anaerobic biodegradation must rely on another terminal electron acceptor as discussed in the following paragraphs and utilize other metabolic pathways (discussed in the following section 2.4).

One basis to access and utilize chemical energy during the metabolism of microbial bacteria involves reduction-oxidation (or redox) reactions. The oxidation process involves the removal of an electron(s) from a substance while the reduction component describes the addition the electron(s) to another substance. Coupled together, these redox reactions defines the situation when an electron(s) is taken from the electron donor and given to the electron acceptor (Ulrich 2004). In benzene biodegradation, the goal involves microbial organisms utilizing benzene in such redox reactions and converting it into less harmful or even benign end-products (Johnson et al. 2003).

During aerobic benzene biodegradation, the most well studied form of benzene biodegradation, oxygen is used as the electron acceptor during the degradation process. However, the subsurface and aquifer environment quickly become anaerobic as the available oxygen content

becomes depleted by microbial activity in the contaminated plume. It is in this circumstance which results in a sudden shift away from the ability for microbial organisms to rely on oxygen as a terminal electron acceptor in benzene biodegradation and towards anaerobic biodegradation. Due to the slow degradation rates under anaerobic conditions, biodegradation of benzene was not thought to be possible until the beginning of the 1980's (Atlas 1981). However, it should be noted that anaerobic benzene bioremediation does have its advantages such as a generally lower cost and management requirement than aerobic biodegradation (Sakai et al. 2009). In the last 39 years of research, a handful of laboratories around the world had published findings demonstrating the possibility of anaerobic benzene biodegradation under several other redox conditions but are often associated with lengthy enrichment periods, considerable lag phases, and generally lower degradation rates (Foght 2008; Vogt et al. 2011). Such redox conditions commonly include nitrate-, sulfate-, chlorate-, perchlorate-, manganese-, and iron- (or ferric-) reducing conditions as well as methanogenic conditions in which methane (CH_4) is formed (Foght 2008). The experiment in this thesis focuses on nitrate-, sulfate- and methanogenic conditions of anaerobic benzene biodegradation.

In terms of thermodynamics, spontaneous reactions are ones which occur without the addition of external energy. It should be noted that this is simply the definition of a spontaneous reaction and reactions of this nature can occur both quickly or slowly, but is not related to either kinetics or reaction rates. Spontaneous reactions are the degradation reactions microbial organisms utilize to degrade benzene as the energy to allow such a reaction is accessible from a thermodynamic standpoint (Atkins and de Paula 2006; Chang 2007; Stryer 1988; Ulrich 2004).

Standard free energy (also known as Gibbs free energy or Gibbs energy), denoted as G, is a measure used to determine the spontaneity of a chemical process. ΔG denotes the change in this standard free energy for the given chemical reaction. When ΔG is a negative value, the reaction is considered exergonic and will proceed spontaneously. Note that this change in standard free energy (ΔG) is merely a reference between the free energy of the products (G_{final}) and the free energy of the reactants ($G_{initial}$) as opposed to the absolute value of the standard free energy of each compound in question. Change in free energy (ΔG) is typically written in the units of kJ/mol (Atkins and de Paula 2006; Chang 2007; Stryer 1988; Ulrich 2004).

Changes in standard free energy can be calculated by a modified version of the second law of thermodynamics and is shown as Equation 2-1 below:

$$\Delta G = \Delta H - T\Delta S \text{ (Eqn. 2-1)}$$

In which ΔH refers to the change in enthalpy, T is the temperature in which the reaction occurs (in Kelvin), and ΔS is the change on entropy. The change in entropy (ΔS) of the universe increases with each spontaneous reaction as defined by the second law of thermodynamics. Since the temperature (T) is written in a Kelvin scale, it can only be a positive value or 0. As a result, the second term of the equation (TΔS) cannot be a negative value. As mentioned previously, a reaction is spontaneous when the value of ΔG is less than 0 or is negative. Therefore, the first terms of the equation which represents the change in enthalpy (ΔH) must be lesser than the second term, TΔS, for the chemical process to occur spontaneously. Table 2-1 below displays the stoichiometric equations as well as the standard free energy changes (ΔG°) for benzene biodegradation under different conditions with varying terminal electron acceptors (Atkins and de Paula 2006; Chang 2007; Stryer 1988; Ulrich 2004).

Table 2-1 – Stoichiometric Equations and Standard Free Energy Changes (ΔG°) of Various Redox Conditions (modified from Vogt et al. 2011).

Oxidized Substance (e- Donor)	Reduced Substance (e- Acceptor)	Stoichiometric Equation	ΔG° (kJ/mol)
ClO_3^-	Cl^-	$C_6H_6 + 5ClO_3^- + 3H_2O \rightarrow 6HCO_3^- + 5Cl^- + 6H^+$	-3,813
O_2	H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O \rightarrow 6\ HCO_3^- + 6H^+$	-3,173
Fe^{3+}	Fe^{2+}	$C_6H_6 + 18\ H_2O + 30\ Fe^{3+} \rightarrow 6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	-3,070
NO_3^-	N_2	$C_6H_6 + 6\ NO_3^- \rightarrow 6\ HCO_3^- + 3\ N_2$	-2,978
NO_3^-	NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O \rightarrow 6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	-2,061
SO_4^{2-}	H_2S	$C_6H_6 + 3\ H_2O + 3.75\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 0.375\ H^+$	-185
CO_2	CH_4	$C_6H_6 + 6.75\ H_2O \rightarrow 2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	-116

Of the three redox conditions explored in this thesis, nitrate-reduction is approximately one order of magnitude more thermodynamically favorable than sulfate-reducing and methanogenic conditions. However, Vogt et al. commented in their review paper that the growth of benzene degrading cultures need not simply correlate to whichever redox condition yields the greatest amount of standard free energy given by an electron acceptor (Vogt et al. 2011). Other factors such as the species of benzene degrading microorganisms present and the availability of substrate(s)

and terminal electron acceptor(s) are commonly the dominating factors governing the possibility and rates of benzene biodegradation.

2.4 – Anaerobic Benzene Biodegradation Pathways

Identification and isolation of individual bacteria species capable of anaerobic benzene biodegradation is a challenge (Phelps et al. 1998). As shown in Section 2.6 of this chapter, most studies demonstrating anaerobic benzene biodegradation rely on a consortium of microbial organisms found in soil or aquifer sediments and groundwaters. To date, only a handful of pure bacteria cultures were demonstrated to perform anaerobic biodegradation. The ability for researchers to identify and pinpoint a certain benzene degradation pathway is often confounded by this challenge in determining which microorganism is responsible for the benzene degradation to begin with (Phelps et al. 1998). Published studies often rely on the detection of ^{13}C- or ^{14}C-labelled intermediates created during the transformation of ^{13}C- or ^{14}C-labelled benzene to validate or further refine proposed pathways. Other technologies and analytical methods such as stable isotope probing (SIP) or stable isotope fractionalization (SIF) in combination with gene sequencing were also instrumental throughout several studies at elucidating the initial benzene activation mechanism. Unfortunately, the inclusion of these technologies was beyond the scope of this thesis.

To date, the exact activation mechanisms during anaerobic benzene biodegradation are still not fully understood (Phelps et al. 1998). Since the late 1980s, three activation reactions and pathways have been proposed and discussed at length: (a) benzene methylation, (b) benzene hydroxylation, and (c) benzene carboxylation (Coates et al. 2001; Foght 2008; Ulrich 2004; Vogt et al. 2011). These three mechanisms of anaerobic attack on the stable structure ring of benzene utilize processes of ring saturation, β-oxidation, and/or ring cleavage reactions (Foght 2008; Harwood and Gibson 1997). Regardless of the difference in specifics between these three pathways, the benzene contaminant is eventually transformed into a common and central metabolite labelled as benzoyl-coenzyme A (or benzoyl-CoA) which is incorporated into cellular biomass or oxidized to acetyl-CoA and eventually mineralized to carbon dioxide (Foght 2008; Harwood et al. 1998; Harwood and Gibson 1997; Luo et al. 2014). In terms of assessing degradation pathways by detecting catabolic genes, no general or universal gene has been attributed to the formation of the central metabolite benzoyl-CoA from benzene as of yet (Meckenstock et al. 2016). Further scientific progress in molecular detection of these genes can be a promising direction to elucidate the benzene degradation pathways in the future. Figure 2-1

below depicts an overview of these three activation mechanisms. The following three subsections address the specifics of each of the three degradation pathways.

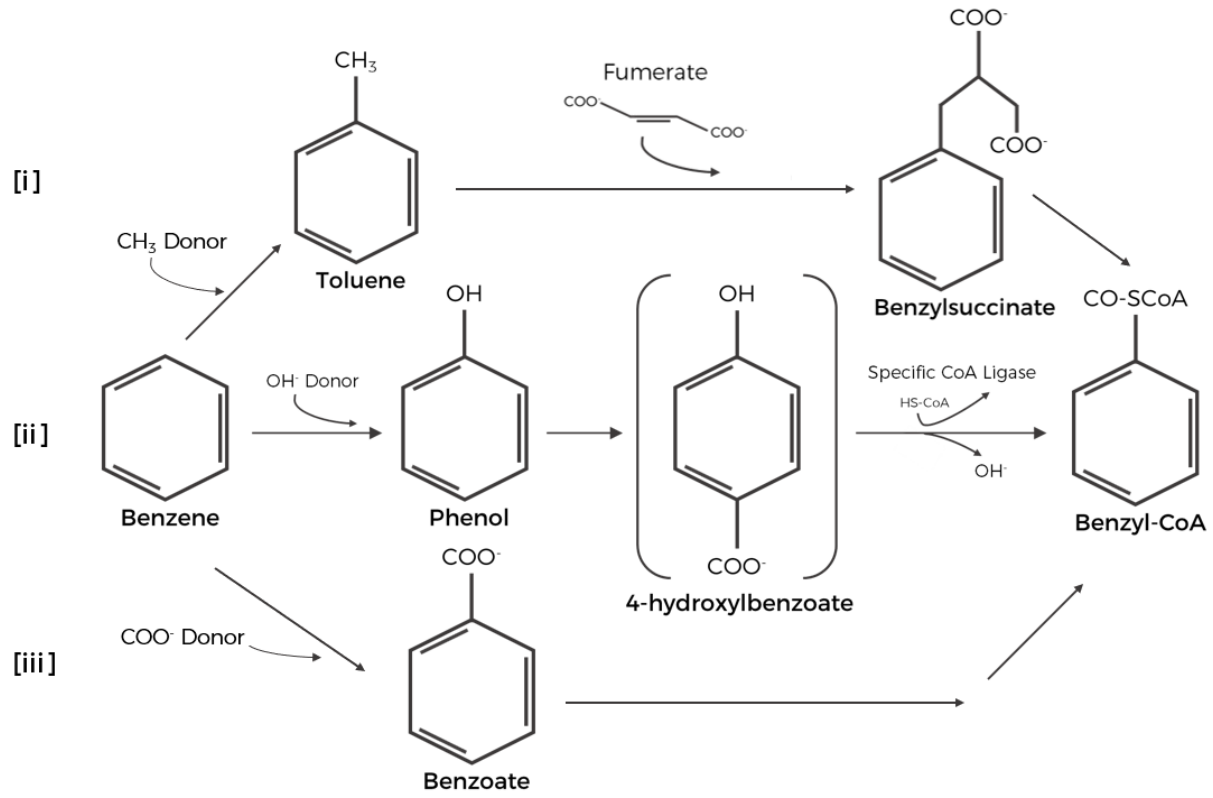

Figure 2-1 - Three Proposed Anaerobic Benzene Initial Activation Mechanisms. [i] Benzene methylation to toluene [ii] Benzene hydroxylation to phenol [iii] Benzene carboxylation to benzoate. (adapted from Vogt et al. (2011), Foght (2008), Ulrich, (2004), and Coates et al. (2002)).

2.4.1 – Benzene Methylation to Toluene

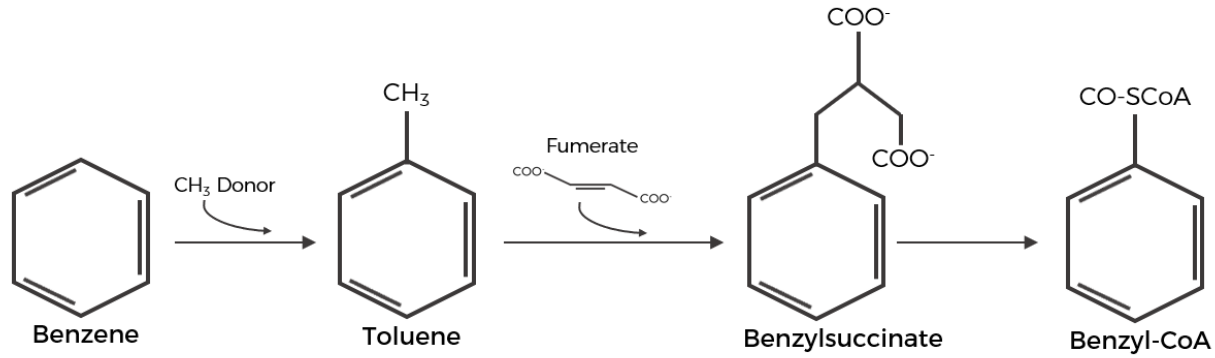

Figure 2-2 - Benzene Methylation to Toluene (adapted from Vogt et al. 2011, Fougt 2008, Ulrich, 2004, and Coates et al. 2002).

The first benzene degradation pathway to be discussed is benzene methylation in which toluene is formed as an intermediate and is depicted as pathway [i] in Figure 2-1 or individually within Figure 2-2. In some literature, benzene methylation to toluene can also be referred to as "alkylation of benzene to toluene". For the purposes of consistency, this thesis will use the term benzene methylation which includes benzene alkylation. The most direct form of evidence in support of benzene methylation exist in the form of utilizing radio-labeled benzene and the subsequent recovery of labeled toluene (Dong et al. 2017; Foght 2008).

Coates et al. 2002 and Vogt et al. 2011 credit benzene methylation to be initiated via a Friedel-Crafts type reaction which is exergonic and rely on a unique biological methyl donor to convert benzene to toluene. These methyl donors can include methyl-tetrahydrofolate, S-adenosylmethionine, or a cobalamin protein (Coates et al. 2002; Foght 2008; Vogt et al. 2011). Following the formation of toluene, fumarate addition catalyzed by the enzyme benzylsuccinate synthase (BSS) to toluene leads to a distinct characteristic compound known as benzylsuccinate as an intermediate (Foght 2008; Vogt et al. 2011). As with the other degradation pathways, benzylsuccinate will be converted to the common intermediate benzyl-CoA. As mentioned before, a sensible strategy in verifying benzene methylation can include detection of the intermediate toluene, but can also include benzylsuccinate or by detecting "presence or expression of bssA-like genes or the induction or activity of BSS" (Vogt et al. 2011).

It should be noted that the benzylsuccinate, a key intermediate under the proposed benzene methylation pathway, has never been detected in benzene-degradation experiments. In this regard, no evidence is provided to support benzene methylation as the initial activation mechanism (Dong et al. 2017). While this pathway has been discussed repeatedly in journal articles and review papers regarding benzene degradation, the only studies identified in this literature review (Section 2.6) to suggest benzene methylation as a possible pathway for observed benzene biodegradation was performed by Ulrich et al. (2005) and Taubert et al. (2012). Protein based stable isotope probing (protein-SIP) was employed in the latter study on a benzene-degrading and sulfate-reducing enrichment culture. Aside from classifying potential benzene degraders, Taubert et al. identified proteins affiliated to enzymes BbsA and BbsB which are associated with degrading benzylsuccinate. As a result, Taubert et al. tentatively proposed benzene methylation as the upper pathway responsible for the degradation demonstrated in that study.

In a recent 2018 journal article, Atashgahi et al. discredits benzene activation by methylation to toluene for four reasons. First, no direct proteins associated with benzene methylation has been discovered in proteogenomic analysis of benzene degrading cultures; second, no pure cultured demonstrating benzene degradation seem to employ benzene methylation; third, the key product benzylsuccinate has not been detected in conjunction of the methylation pathway (as discussed in the previous paragraph); and fourth, some anaerobic benzene grading cultures fail to degrade toluene (Atashgahi et al. 2018).

2.4.2 – Benzene Hydroxylation to Phenol

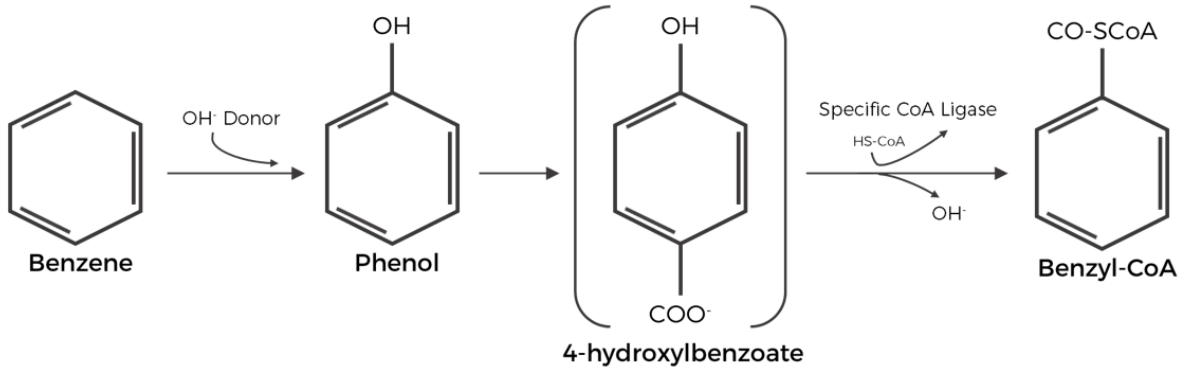

Figure 2-3 - Benzene Hydroxylation to Phenol (adapted from Vogt et al. 2011, Fougt 2008, Ulrich, 2004, and Coates et al. 2002).

The second benzene degradation pathway to be discussed is benzene hydroxylation in which phenol is formed as an intermediate and is depicted as pathway [ii] in Figure 2-1 or individually within Figure 2-3.

In the benzene hydroxylation pathway, benzene is initially activated to phenol as the initial step via the use of a hydroxyl donor. Under methanogenic conditions, Grbić-Galić and Vogel (1986) postulated the hydroxyl donor to be H_2O. Under nitrate-reducing conditions, with *Dechloromonas aromatica*, Chraborty et al. (2005) suggested that a hydroxyl free radical performs as the hydroxyl donor. Regardless, this phenol subsequently undergoes carbon dioxide dependent carboxylation to form 4-hydroxybenzoate (Vogt et al. 2011). Lastly, the 4-hydroxybenzoate becomes activated by a specific CoA ligase, allowing for the removal of the hydroxyl group, and effectively reducing the 4-hydroxybenzoate to benzyl-CoA (Coates et al. 2002).

One challenge with elucidating the benzene hydroxylation pathway involves phenol. Specifically, phenol can also be formed abiotically from benzene within culture media when iron- or sulfate-reducing microorganisms contact air. Vogt et al. (2011) suspects that hydroxyl radicals are generated by oxidation of iron during sample work up or sampling and will react with benzene to produce trace amounts of phenol. Laban et al. (2009) used this mechanism to explain the presence of 2-hydroxybenzoate and 4-hydroxybenzoate observed in their study and to ultimately eliminate benzene hydroxylation as the initial activation mechanism in that study.

In support of benzene hydroxylation, Zhang et al. (2013) published a benzene degradation study which utilized *Geobacter metallireducens* under Fe(III)-reducing conditions. Inclusion of ^{18}O-labelled H_2O resulted in ^{18}O-labelled phenol. Furthermore, deletion of genes PpsA and PpcB which are key subunits in the enzymes responsible for the degradation of phenol resulted in a cessation in benzene degradation. Zhang et al. concludes benzene hydroxylation to be the most likely activation mechanism responsible for the demonstrated degradation.

In 2018, Keller et al. tentatively attributed benzene hydroxylation initiated via monooxygenase as the initial activation mechanism for the observed degradation, but the stable isotope fractionization data did not yield conclusive results and further investigation is required to validate benzene hydroxylation in that case.

The following studies in the literature review (Section 2.6) discusses or attribute benzene hydroxylation as the initial activation mechanism for the reported benzene degradation: Grbić-Galić and Vogel (1987), Harwood and Gibson (1997), Ulrich et al. (2005), Laban et al. (2009), Duo et al. (2010), Zhang et al. (2013), and Keller et al. (2018).

2.4.3 – Benzene Carboxylation to Benzoate

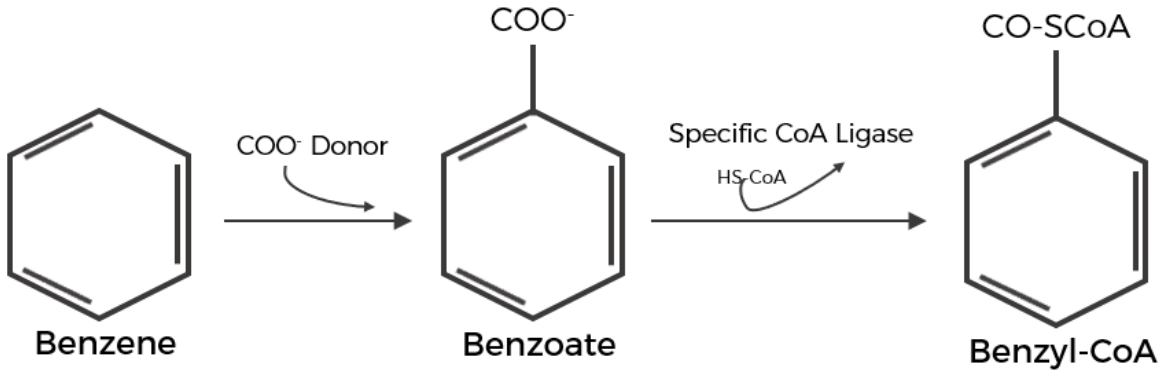

Figure 2-4 - Benzene Carboxylation to Benzoate (adapted from Vogt et al. 2011, Fougt 2008, Ulrich, 2004, and Coates et al. 2002).

The third and final benzene degradation pathway is benzene carboxylation in which benzoate is formed as an intermediate and is depicted as pathway [iii] in Figure 2-1 or individually within Figure 2-4.

Benzene carboxylation is perhaps the most straight-forward of the three proposed degradation pathways and occurs in two steps. First, a carboxyl donor initiates carboxylation of benzene into benzoate. Initially, the carboxy donor was thought to be bicarbonate, which is commonly used as a buffering agent in the anaerobic media for benzene degradation studies. In 2001, Phelps et al. exposed ^{13}C-radiolabeled bicarbonate with un-labeled benzene in a degradation experiment. However, the benzoate intermediate that was formed did not possess a ^{13}C radiolabeled signature (Phelps et al. 2001). This suggests that the carboxyl donor is not bicarbonate or related to carbon dioxide, but may be derived from benzene itself (Foght 2008; Vogt et al. 2011). It is for this very reasoning in which Coates et al. (2002) argues against benzene carboxylation as the initial activation mechanism. Foght (2008) proposed that this conversion of benzene into benzoate may involve more than one enzymatic step but for the purposes of clarity and simplicity, this transformation is only shown as one step in Figure 2-1 and 2-4. Second, a specific CoA ligase activates the newly created benzoate and produces benzoyl-CoA (Coates et al. 2002; Foght 2008; Vogt et al. 2011).

In 2009, Abu Laban et al. identified a benzene-induced protein known as “anaerobic benzene carboxylase” or AbcA in a microcosm study with *Aromatoleum aromaticum*. The authors of that study intentionally induced the production of the AbcA enzyme with benzene in the absence

of phenol or benzoate to suggest a relation between AbcA and benzene carboxylation. Subsequently, differential proteome analysis of this gene cluster also identified a "phenylphosphate carboxylase γ-subunit-like protein" labeled as AbcD linked to benzene carboxylation (Abu Laban et al. 2009; Meckenstock et al. 2016). Afterwards, the study performed by Luo et al. (2014) also identified similar gene clusters of AbcA and AbcD while the microcosm study involving *Ferroglobus placidus* by Holmes et al. (2011) only observed the enzyme AcbA (Holmes et al. 2011; Meckenstock et al. 2016). In 2017, van der Waals et al. performed a biofilm study within a retentostat set up. Following active benzene degradation, benzoic acid was an observed intermediate as well as the carboxylase encoding gene abcA during gene sequencing. Due to the absence of phenol or toluene, van der Waals et al. suggested benzene carboxylation to be the initial activation mechanism for their study.

While benzene carboxylation is simple in nature, it is not overly supported with evidence in literature nor does it come without challenges in proving. Direct carboxylation of benzene is thermodynamically difficult due to the high dissociation energy of the carbon-hydrogen bond (473kJ/mol) (Meckenstock et al. 2016). Another obstacle includes the fact that benzoyl-CoA is the activated form of benzoate and is a common intermediate within the anaerobic degradation pathway of other aromatic compounds including toluene and phenol – creating obstacles in differentiating against benzene hydroxylation or methylation. Furthermore, some studies have also suggested benzoate to be an intermediate formed during phenol degradation (Vogt et al. 2011). As a result, elucidating the benzene carboxylation pathway becomes fairly convoluted as it is difficult to determine if the presence of a benzoate metabolite is formed due to direct carboxylation of benzene, or whether it is formed as a metabolite during the downstream stages of degradation due to toluene in benzene methylation or phenol in benzene hydroxylation (Vogt et al. 2011).

The following studies in the literature review (Section 2.6) attribute benzene hydroxylation as the initial activation mechanism for the observed benzene degradation: Laban et al. (2009), Duo et al. (2010), Luo et al. (2014), and Dong et al. (2017).

2.5 – Complexity with Anaerobic and Saline Benzene Biodegradation

Available literature concerning the biodegradation of benzene is vast. However, most of these studies relate to the aerobic biodegradation and existing studies specific to anaerobic benzene biodegradation is scarce. Winfrey et al. may have been the first study to suggest the possibility for the biodegradation of benzene in the absence of oxygen in 1982. In that study, concepts of utilizing

sulfate-reduction and methanogenesis were proposed (Winfrey et al. 1982). To date, the only organisms capable of anaerobically degrading BTEX compounds are bacteria but work involving anaerobic fungi are also being explored (Johnson et al. 2003). So far, only a handful of pure cultures capable of anaerobic benzene biodegradation has been isolated. Naturally, these pure cultures are instrumental to the elucidation of degradation pathways, intermediates, and gene encoding of key enzymes (Foght 2008). More commonly, studies in this field are associated with microbial populations of mixed cultures or undefined consortia derived from contaminated soils, aquifer sediments, or groundwaters. With the use of a variety of analytical chemical equipment, these samples containing a mixed microbial population are still significant in the work to validate or reject proposed degradation pathways (Foght 2008). The experiment in this thesis is of the latter variety.

One common difficulty regarding anaerobic benzene biodegradation involves lengthy incubation times (upwards of multiple years even after the culture had been enriched for years prior). In comparison to degradation studies of other PHC compounds, such as toluene also within the BTEX family, or in comparison to aerobic degradation studies of benzene, these slow degradation rates over longer resident times are logistical challenges which occasionally mislead researchers to conclude a lack of degradation (Johnson et al. 2003; Weiner and Lovley 1998).

A second layer of difficulty arises when the concept of salinity is considered. Existing research regarding the biodegradation of petroleum hydrocarbons in hypersaline environments is sparse (Al-Mailem et al. 2013). These papers often describe the ability of aerobic halophilic and halotolerant microbial organisms to biodegrade polycyclic aromatic hydrocarbons, simple aromatic compounds, and *n*-alkanes. The applications towards the degradation of light aromatic hydrocarbons (such as benzene) is far more limited (Berlendis et al. 2010). In the literature review performed in preparation of this thesis, only a handful of studies exploring benzene biodegradation on the presence of salinity were found. No research specifically involving a salinity factor when considering anaerobic biodegradation of benzene was discovered at the time of writing this thesis.

Over the past three decades, scholars around the world arrived at varying findings and offered differing proposed mechanisms regarding the effect that salinity has on benzene bioremediation. An increase in salinity results in a decrease of hydrocarbon solubility, therefore translating to a lower substrate availability to microorganisms. This may be the reason in which the earliest studies of this field assumed an inverse relationship between salinity and hydrocarbon

biodegradation (Al-Mailem et al. 2010). In contrast, studies such as the 1988 report published by Kerr and Capon claimed no relationship between the two factors (Kerr and Capone 1988) while other researchers such as Diaz et al. and Yang et al. proposes that the presence of salinity accelerates hydrocarbon bioremediation (Al-Mailem et al. 2010; Kerr and Capone 1988; Yang et al. 2000). Regardless of the varying conclusions and diversity in findings, it is apparent that the phenomenon of biodegrading benzene in an anaerobic and saline condition has respectable layers of complexity involving the microbial consortium, substrate in question, and specific on-site conditions.

2.6 – Literature Review on Anaerobic Benzene Biodegradation

Since the earliest recorded instances of anaerobic benzene biodegradation such as the study performed by Winfrey et al. (1982), researchers from all over the world had suggested the possibility of utilizing a variety of terminal electron acceptors to degrade benzene with microorganisms. In the myriad of studies available, oxygen, nitrate, sulfate, manganese, iron, and carbon dioxide (methanogenic) are the most commonly utilized terminal electron acceptors. Since the experiment in this thesis explores nitrate-, sulfate-reducing, and methanogenic conditions to achieve anaerobic benzene biodegradation, the literature review below will only discuss the history of these three redox conditions. By no way are the following sections meant to be a comprehensive review of all performed studies in this field, but merely as a guide which glosses over the nearly four decade long journey of anaerobic benzene biodegradation and to provide similarities between experimental set-ups and trends of degradation rates both successful and otherwise.

2.6.1 – Anaerobic Benzene Biodegradation under Nitrate-Reducing Conditions

2.6.1.1 – General Overview

In terms of bioremediation, especially in-situ bioremediation, utilizing nitrate as the terminal electron acceptor is an attractive option for several reasons. First, of the bacteria capable of anoxic growth, denitrifiers commonly have a higher growth yield. Secondly, current genetic manipulations and understanding are comparatively more advanced in most denitrifying bacteria. Third and lastly, nitrate is more economic and soluble in comparison to other candidate electron acceptors (Kao and Borden 1997).

Initial studies attempting benzene biodegradation under nitrate-reducing conditions were unsuccess but ultimately paved the way for subsequent studies with relatively more progress in

this field (Kao and Borden 1997; Kazumi et al. 1997; Langenhoff et al. 1996; Reinhard et al. 1997). In 1998, Nales et al. was the first to demonstrate benzene biodegradation under nitrate-reducing conditions with a microcosm study. Coates et al. (2001) and Chakraborty et al. (2005) followed with comparable experiments reporting similar results but both had also identified a strain of *Dechloromonas* (labeled as RCB) as the responsible biodegrader. Ulrich and Edwards published two studies (2003 and 2005) and were the first to consider degradation pathways by reporting both toluene and benzoate as intermediates via isotope trapping. Ulrich et al. (2005) suggested benzene methylation to be the initial activation mechanism for the degradation observed in the latter experiment. Similarly, Duo et al. (2010) demonstrated *Bacillus cerus* as a benzene degrader and linked both benzene hydroxylation and carboxylation as possible degradation pathways due to the presence of phenol and benzoate as intermediates. Van der Zaan et al. (2012) utilized a chemostat configuration and demonstrated degradation under nitrate-reducing conditions by a diverse microbial culture primarily of *Peptococcaeceae*, but DNA-SIP (stable isotope probing) also identified *Rhodocycclaceae* and *Burkholderiaceae* to be associated with downstream degradation as well. Luo et al. (2014) suggested a syntrophic relationship between a member in *Peptococcaeceae* and an *Azoarcus* strain in which the former degraded benzene to benzoate and the latter is a denitifyer and degraded benzoate. Van der Waals et al. (2017) also identified *Peptococcaeceae* as well as a bacterium within the *Anaerolineaceae* to be the active degraders in a retentostat study. By combining the presence of benzoic acid, the absence of phenol, and identifying the carboxylase gene abcA during gene sequencing, van der Waals et al. attributed benzene carboxylation to be the initial activation mechanism responsible for the observed biodegradation. Lastly, Keller et al. 2018 performed a microcosm study converting a former sulfate-reducing culture toward nitrate-reducing conditions and achieved an average degradation rate of 10.1 μM/d along with nitrate utilization and nitrite generation molar ratios comparable to literature. 16s rRNA sequencing identified *Azoarcus* and *Simplicispira* belonging to *Rhodocyclaceae* and *Dokdonella* of *Xanthomonadaceae* to be the most abundant operational taxonomic units. Stable isotope fractionalization (SIF) was performed in attempts to elucidate a specific degradation pathway but generally yielded fruitless results. Keller et al. tentatively suggested benzene hydroxylation initialed via monooxygenase as the activation mechanism as this pathway was suggested for previous experiments with the same culture in the past.

Table 2-2 on the following page provides an overview of the studies discussed in this literature review which utilized nitrate as the terminal electron acceptor in benzene degradation. Section 2.6.1.2 provides a summary of each of these publications on an individual basis. If the reader is not interested in this level of detail, they are encouraged to skip ahead to Section 2.6.2 (page 38) which address benzene biodegradation under sulfate-reducing conditions. Conversely, the reader is also suggested to seek out the published studies themselves should they be interested in the specific articles.

Table 2-2 – Overview of Benzene Degradation Studies Under Nitrate-reducing Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Langenhoff et al.	1996	Continuous-flow packed bed-column	No	None Identified	None Suggested	Soil sediments: River Rhine, Wageningen, Netherlands; sugar beet wastewater
Kao and Borden	1996	Microcosm study	No	None Identified	None Suggested	Contaminated aquifer materials: Rocky Point, North Carolina; wastewater activated sludge: Chapel Hill, North Carolina; contaminated sediments: Traverse City, Michigan and Sleeping Bears Dunes, Michigan
Reinhard et al.	1997	In-situ treatment with injected slugs of treated groundwater	No	None Identified	None Suggested	Seal Beach Naval Weapons Stations, California
Kazumi et al.	1997	Microcosm study	No	None Identified	None Suggested	Contaminated Aquifer Sediments: Sleeping Bears Dunes, Michigan; Fresh Kills landfill: New York / New Jersey harbor
Nales et al.	1998	Microcosm Study	Yes	None Identified	None Suggested	Contaminated sediments: terminal site in South Carolina; oil refinery in Oklahoma
Coates et al.	2001	Microcosm study	Yes	*Dechloromonas* strain - RCB	None Suggested	Contaminated Sediments: Ptotmac River, Maryland
Ulrich and Edwards	2003	Microcosm study, previously enriched for 6 years	Yes	None Identified through Gene Sequencing	None Suggested	Derived from Nales et al.; contaminated sediments from Cartwright Gas Station and land farm, Toronto, Ontario
Chakraborty et al.	2005	Microcosm study	Yes	*Dechloromonas* strain - RCB	None Suggested	Source Unknown

Table 2-2 (Cont.) – Overview of Benzene Degradation Studies Under Nitrate-reducing Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Ulrich et al.	2005	Microcosm study. Previously enriched for 8 years. Employed isotope trapping, identified benzoate and toluene as an intermediate	Yes	None Identified	Benzene Methylation	Contaminated sediments from Cartwright Gas Station, Toronto, Ontario
Duo et al.	2010	Microcosm study, phenol and benzoate identified as intermediates	Yes	*Bacillis cerus*	Benzene Hydroxylation and Benzene Carboxylation	Source Unknown
Van der Zaan et al.	2012	Chemostat study. Capable to interchanging electron acceptor	Yes	*Peptococcaceae, Rhodocyclaceae, and Burkholderiaceae*	None Suggested	Benzene contaminated site in northern Netherlands
Luo et al.	2014	Microcosm study, explored benzene and benzoate co-contamination	Yes	Member in *peptococcaceae* and an *Azoarcus* strain	Benzene Carboxylation	Contaminated sediments from Cartwright Gas Station, Toronto, Ontario
Van der Waals et al.	2017	Rententostat study.	Yes	*Peptococcaceae* and *Anaerolineaceae*	Benzene Carboxylation	From van der Zaan et al. 2012: Benzene contaminated site in northern Netherlands
Keller et al.	2018	Microcosm study. Stable isotope fractionization.	Yes	*Azoarcus* and *Simplicispira* belonging to *Rhodocyclaceae* and *Dokdonella* of *Xanthomonadaceae*	Benzene Hydroxylation initiated by Monooxygenase (tentative)	From Vieth et al. 2005: Former hydrogenation plant in Zietz, Germany

2.6.1.2 – Detailed Summary of Individual Publications

Langenhoff et al. performed a continuous-flow packed bed-column experiment in 1996 to achieve anaerobic benzene biodegradation. Each column had a flow rate of 3.5mL/hr, a retention time of 10 hours, and utilized a different terminal electron acceptor for degradation. These columns contained a mix culture of microorganisms found in anaerobic soil sediments originating from the Rhine River near Wageningen, Netherlands, which was polluted by toluene, benzene, and naphthalene, and from granular sludge that is rich in anaerobic bacteria obtained from an up-flow anaerobic sludge blanket reactor used for the treatment of sugar beet wastewater (Langenhoff et al. 1996). Each column had an initial and influent concentration of 25 μM benzene as well as 10 mM of nitrate in the form of $NaNO_3$. No significant benzene degradation was observed in the nitrate-reducing column and it was subsequently decommissioned between day 200 and 300 of operation in favor of testing the performance of another electron acceptor in the experiment (Langenhoff et al. 1996).

In 1996, Kao and Borden published a study regarding the potential for biodegradation of BTEX compounds under nitrate-reducing conditions. This study included microcosms originating from a range of locations including: aquifer materials from four fuel-oil contaminated sites from Rocky Point, North Carolina; activated sludge from a waste-water treatment plant at Chapel Hill, North Carolina; sediment originating from Traverse City, Michigan and was supplied by Dr. Steve Hutchins in his similar study; and sediments from a contaminated plume in Sleeping Bear Dunes, Michigan (Kao and Borden 1997). 20 g of core material were placed in 70 mL bottles. Nitrate concentrations were initialized at 55 mg/L $NaNO_3$ and benzene concentration was supplied between 1.5 and 3 mg/L. This study reported degradation rates of all BTEX compounds under aerobic conditions and varying degrees of success of toluene, ethylbenzene, and xylene degradation within nitrate-reducing conditions in microcosm studies upwards of 300 days (Kao and Borden 1997). Unfortunately, benzene was concluded to be recalcitrant under nitrate-reducing conditions for all samples and microcosms (Kao and Borden 1997).

Reinhard et al. published a 1997 study in which slugs of treated groundwater were amended with various concentrations of BTEX compounds and electron acceptors, and purposefully injected into a gasoline contaminated aquifer at the Seal Beach Naval Weapons Stations in southern California for potential in-situ remediation (Reinhard et al. 1997). This was the first

instance in which in-situ incubation was identified in this literature review. The test zone consisted of a modified sampling device capable of accepting the experiment's series of injectants and is of an unconfined radius containing an estimated 470 to 1,700 L of groundwater throughout the course of the trials (Reinhard et al. 1997). The first three series of test were performed to determine a baseline of the geochemical conditions on site as well as to define the hydraulic properties on site. During this time, the existing BTEX contamination on site were reported to have been depleted as the carbon source for biodegradation. The subsequent trials involved amending the injectant with a known BTEX concentrations. The recovered BTEX compounds during sampling of the following trials can be compared against the known BTEX content provided in the injectant (Reinhard et al. 1997). Two series of tests explored the degradation of BTEX compounds under nitrate-reducing conditions. In these trials, benzene was also amended at 241 and 255 µg/L. Furthermore, 226.8 and 37.2 mg/L of nitrate was supplied in the injectant. The amount of nitrate provided was intentionally greater than the theoretical demand required to bioremediate the given substituents. Each test was approximately 80 days in length. While toluene, ethylbenzene, m-xylene, and o-xylene underwent varying degrees of degradation, benzene was reported to remain recalcitrant (Reinhard et al. 1997).

Harwood and Gibson published a paper in 1997 which did not work directly on the degradation of benzene, but instead, it discussed the nitrate-reducing biodegradation pathways for two compounds later suggested to be intermediates of benzene biodegradation - benzoate (formed during the carboxylation pathway) and 4-hydroxybenzoate (hydroxylation pathway). This report included a description of a phototrophic bacterium *Rhodopseudomonas palustris* and two denitryfying species: *Thauera aromatica* and *Azoarcus evansii* which were identified to be capable of benzoate and 4-hydroxybenzoate degradation under nitrate-reducing conditions (Harwood and Gibson 1997).

In 1997, Kazumi et al. obtained aquifer sediments from petroleum contaminated aquifers at Sleeping Bear Dunes National Lakeshore, a national lakeshore at Empire, Michigan. Using these samples, 160 mL microcosms in serum bottles were developed containing 50 g of sediment containing a microbial community and 75 mL of mineral media (Kazumi et al. 1997). The samples from Michigan experienced lag phases between 360 to 420 days and were incubated for a total of 520 to 590 day. No benzene removal was reported for samples relying on nitrate-reducing conditions. Lastly, Kazumi et al. also retrieved estuarine sediments near the Fresh Kills landfill in

the New York / New Jersey harbor and developed microcosms containing a 10:90 sediment:media ratio. Again, no degradation was reported under nitrate-reducing conditions while degradation occurred under methanogenic, sulfate-, and ferric-reducing conditions (Kazumi et al. 1997). It is noteworthy that no biodegradation occurred even though nitrate-reducing conditions is considered more thermodynamically favorable in comparison to the other conditions in which degradation was observed.

In this literature review, the first instance of success nitrate-reducing benzene biodegradation was identified in the 1998 study published performed by Nales et al. Samples were collected between May to July 1995, incorporated into 250 mL bottles containing an initial benzene concentration of 150 μM, and incubated for a period of 1.5 years (Nales et al. 1998). In one part of the experiment, sediments were taken from below the groundwater level of a terminal site in South Carolina. With nitrate concentrations between 7 and 8 mM, a lag period of less than 8 days was reported. The initial degradation rate was greater than 20 μM/day and the max degradation rate was between 35-36 μM/day during the incubation period (Nales et al. 1998). Of the microcosms developed from sediments collected from this location, an average of only 20% of ^{14}C radiolabeled benzene was recovered as $^{14}CO_2$. The remaining unaccounted 80% was presumed to have been sequestered into sediments (Nales et al. 1998). Lastly, degradation was not sustained as benzene degradation stopped after approximately three additions of benzene. In the second part of this report, sediments were taken from an oil refinery in Oklahoma. The specific location was described to be close to the surface, highly anaerobic, and chronically exposed to hydrocarbons (Nales et al. 1998). With an initial nitrate concentration of 7 mM, a lag period of less than 130 days was reported. Initial benzene degradation rates were between 3.1 and 3.3 μM/day and the max degradation rate was 27 μM/day (Nales et al. 1998). An average of 71% of ^{14}C-benzene was recovered as $^{14}CO_2$ for microcosms developed from sediments of this site (Nales et al. 1998). Degradation was reported to be sustained, suggesting benzene degradation occurred repeated with each refeed of benzene until the experiment was decommissioned.

In 2001, Coates et al. published a report regarding the isolation and characterization of a *Dechloromonas* strain - labelled as RCB. Strain RCB originated from sediments collected from the Potomac River, Maryland for its active microbial population capable of anaerobic hydrocarbon degradation as well as chlorate- and perchlorate-reducing capabilities (Coates et al. 2001). Strain RCB was demonstrated to oxidize benzene within nitrate-reducing conditions (10 mM). 45% of

^{14}C-labelled benzene was accounted for as $^{14}CO_2$ after incubation periods of 6 days while 47% remain undegraded within the culture medium. This accounts for 92% of the original ^{14}C radiolabeled benzene. The remaining amount was assumed to be incorporated into biomass (Coates et al. 2001). Furthermore, this study reported the reduction of 843 ± 64 μM nitrate for the oxidation of 163 ± 19 μM benzene - resulting in greater than 83% of the theoretical ratio of benzene oxidation coupled with nitrate-reduction (Coates et al. 2001). Lastly, cell number increases were concomitant with decreases of benzene concentration. Conversely, minimal growth occurred during benzene depletion and this observed growth was assumed to be due to the carry over results from previous trials (Coates et al. 2001).

In a 2003 experiment, Ulrich and Edwards utilized microcosms derived from the samples collected and described in the study published by Nales et al. (1998). Nine mixed cultures were enriched for a period of six years in which benzene was the sole carbon source and amended with their respective terminal electron acceptors. Specifically, this included a total of three nitrate-reducing enrichment cultures sourced from three separate sites (Ulrich and Edwards 2003). The most notable sample was sourced from a gas station and was capable of a max degradation rate of 13 μM/day at an initial benzene concentration of 240 μM. The electron acceptor to donor ratio was reported as 8.2 which compared favorably to the theoretical value of 9.75 (Ulrich and Edwards 2003). The remaining microcosms originated from a gas station and land farm. Their max degradation rate was 6 μM/day and 1 μM/day at initial concentrations of 150 and 170 μM respectively. The electron acceptor:donor ratios were 10 and 17 respectively (Ulrich and Edwards 2003). Genetic sequencing unfortunately did not yield any matches with known cultured bacteria.

Chakraborty et al. (2005) experimented with the previously mentioned versatile isolate labelled *Dechloromonas* strain RCB capable of anaerobic biodegradation of all BTEX compounds in conjunction with various terminal electron acceptors (Chakraborty et al. 2005). This study utilized 10 mL of liquid culture in nitrate-reducing conditions. *Dechloromonas* strain RCB was able to anaerobically degrade 56% of benzene to CO_2 within 25 hours. After a period of 4 days, only 10 μM remained from the original 35 μM, resulting in an approximate 71% removal (Chakraborty et al. 2005). Furthermore, in the absence of nitrate, this isolate was demonstrated to utilize chlorate as an alternative electron acceptor. In that instance, Chakraborty et al. reported 64% benzene removal after a period of 25 hours (Chakraborty et al. 2005).

In 2005, Ulrich et al. published a study involving the detection of transient metabolites created during anaerobic benzene biodegradation under nitrate-reducing conditions in attempts to elucidate a specific degradation pathway as suggested by previous literature. Microcosms were developed from contaminated soil and groundwater obtained from a decommissioned retail gas station in Toronto, Ontario. These samples had benefitted from eight years of enrichment in which benzene was the sole carbon source amended to concentrations between 130 and 1,100 μM as well as having endured multiple successful transfers to mineral media solutions. The terminal electron acceptor was nitrate given in the form of sodium nitrate and amended to between 2 to 5 mM (Ulrich et al. 2005). In this study, Ulrich et al. employed isotope trapping - a novel concept unseen in other studies throughout the literature review to enhance the detection of benzene degradation metabolites. In previous experiments, it had been noted that benzoate was a transient metabolite formed during degradation. As a result, unlabeled benzoate (the trap) was intentionally added into the microcosms. The carbon source provided exist in the form of ^{13}C-radiolabeled benzene. In theory, the benzoate trap will cause a buildup of ^{13}C-radiolabeled benzoate and any other upstream metabolites, and the detection of these products would aid in establishing an indisputable link towards the radiolabeled benzene substrate (Ulrich et al. 2005). While the specific degradation rates were not the focus of this experiment, Ulrich et al. reported benzene content to decrease from 210 μM to 11 μM in 11 days for samples that did not receive the benzoate trap. Conversely, the samples which received the benzoate trap (added on Day 2) had degradation reported to decrease from 220 μM to under 11μM in 32 days (Ulrich et al. 2005). By comparing the development of radiolabeled metabolites between microcosms which received and did not receive the isotope trap, Ulrich et al. reported that both toluene and benzoate were intermediates formed during nitrate-reducing conditions of benzene biodegradation as the trap successfully inhibited degradation progress. Furthermore, the formation and subsequent disappearance of these metabolites were then analyzed with a temporal context. Overall, the disappearance of benzene translated to a gradual increase in toluene, which then further decreased in concentration and benzoate was noticed to become dominant. Ulrich et al. concluded that the pathway seen in this nitrate-reducing condition is most likely benzene methylation as it is consistent with the transformation of benzene to toluene, and then toluene to benzoate (Ulrich et al. 2005).

Duo et al. (2010) performed serial dilutions on microcosms created from gasoline contaminated sediments which consisted of mixed bacteria to obtain an isolated strain. 16s rDNA

gene sequencing indicated that this isolate had 99.3% similarity to a member of genus *Bacillis cereus* (Dou et al. 2010). Under nitrate-reducing conditions, *B. cereus* completely degraded 150 mg/L of benzene within a 25 day incubation period. The maximum degradation rate was 5.16 mg/L·day. Duo et al. commented that this rate was greater to those of related studies (Dou et al. 2010). Interestingly, the previous microcosms containing mixed cultures from which this isolate was developed from had suffered toxic effects with benzene content greater than 80 mg/L, however, the same was not observed with the pure bacterium *B. cereus*. Approximately 9.88 moles of nitrate was consumed per 1 mole of benzene degraded, indicating that nitrate was indeed reduced to nitrite, but only partial mineralization to nitrogen gas afterwards (Dou et al. 2010). The use of gas chromatography–mass spectrometry (GC-MS) indicated that both phenol and benzoate were intermediates produced during degradation, linking implications to the benzene hydroxylation pathway (due to phenol) and the carboxylation pathway to benzoate.

In 2012, van der Zaan et al. utilized a chemostat configuration with a continuous culture inoculated from contaminated soil originating from a site in northern Netherlands to achieve benzene degradation in the presence of 2.5 mM of nitrate. Upwards of 80 μM of benzene was repeatedly degraded in an incubation period of over 250 days and a rate constant of 0.7 day^{-1} was reported when nitrate was the terminal electron acceptor (van der Zaan et al. 2012). DNA-SIP (stable isotope probing) utilized ^{13}C-labelled benzene discovered the coexistence of multiple species within the chemostat. *Peptococcaceae* was identified to be the dominant benzene consumers while bacteria related to *Rhodocyclaceae* and *Burkholderiaceae* also demonstrated responsibility to the benzene degradation process (van der Zaan et al. 2012). As a result, van der Zaan suggests benzene degradation to occur within syntrophic relationships between the microbial culture as opposed one sole species governing biodegradation. This hypothesis was further reinforced when nitrate was substituted with other electron acceptors such as chlorate, sulfate, and ferric iron. While degradation occurred to a lesser extent than under nitrate-reducing conditions, the possibility of biodegradation under a variety of terminal electron acceptors suggest the presence of a diverse microbial community. High performance liquid chromatography (HPLC) and GC-MS analysis did not identify the presence of other commonly seen degradation intermediates such as acetate, benzoate, and phenol (van der Zaan et al. 2012). In terms of specific degradation pathways, van der Zaan et al. made no hypothesis regarding the process of initial benzene activation.

Ribosomal and mRNA sequencing of a benzene-degrading, nitrate-reducing mixed culture led Luo et al. (2014) to hypothesize a syntrophic relationship between the benzene degrading capabilities of a member of the *peptococcaceae* family in the order *Clostridiales* which initiates benzene degradation to benzoate and followed by a nitrate-reducing benzoate-degrader identified as a *Azoarcus* strain. Similar to the work performed by Ulrich et al. (2005), the cultures in this study had been maintained since 1997 through clone libraries or growth experiments and originate from the Cartwright gas station in Toronto. These parent cultures had previously been shown to contain operational taxonomic units belonging to families such as *Peptococcaeceae*, *Rhodocyclaceae*, and *Burkholderiaceae*, as well as the phyla *Chlorobi* and *Planctomycetes* (Luo et al. 2014). It is noteworthy that the greatest cell number increased was observed for *Peptococcaeceae* via polymerase chain reaction (PCR) analysis during benzene metabolism. In this study, different amendment configurations of benzene (128 μM) and benzoate (128 μM) were explored in conjunction with 2 mM of nitrate as the terminal electron acceptor. On average, benzene degradation rates were between 5 and 10 μM/day with a consumption between 10 to 14 moles of nitrate consumed per mole of benzene degraded which comparable favorable to the theoretical stoichiometric expectation for the oxidation of benzene to carbon dioxide coupled with the incomplete reduction of nitrate to nitrite (as opposed to the complete reduction of nitrate to nitrogen). This was further supported by the transient nitrite observed to have accumulated throughout the course of the experiment. Following benzene degradation, some of this accumulated nitrite was eventually reduced to nitrogen gas, in which fatty acids or other carbohydrates released from biomass or the sulfide present in the mineral media were suspected to perform as the electron donors (Luo et al. 2014). In terms of benzoate, degradation rate of this substrate were reportedly 20 times greater in comparison to benzene. This proved intriguing as these cultures were never amended with benzoate or have used it as the sole carbon source. Furthermore, the addition of benzoate before benzene did not provide addition stimulation for benzene degradation as the researchers originally expected. Lag phases in this situation were between 20 to 30 days which is similar to or greater than lag phases of cultures transferred onto fresh medium without the benefit of benzoate enrichment period (Luo et al. 2014). Ultimately, this leads Luo et al. to hypothesize that one organism (*peptococcaceae*) work in a syntrophic manner to degrade the benzene to benzoate and another organism (*Azoarcus*) degraded the benzoate under denitrifying conditions.

In 2017, van der Waals et al. employed the continuous culture described in van der Zaan (2012) for a biofilm experiment via a retentostat set up. This continuous culture had been enriched on benzene as the substrate and nitrate as the terminal electron acceptor for over 14 years at this point (van der Waals et al. 2017). In attempts to maximize activity from the benzene degrading culture, the retentostat set up employed a filtration finger which retains biomass by recirculating liquid between the reactor vessel and the filtration finger while minimizing loss to the effluent vessel. Van der Waal et al. explored dilution rates between 0.25 and 2 day^{-1} and repeatedly demonstrated the ability of benzene degradation to below detection limit when the reservoir concentration ranged between 105 and 615 µM although a proportional relationship between inflow concentrate and residual benzene concentration was also reported. A threshold of 615 µM was concluded as the upper limit to which the microbial community can no longer handle the benzene load (van der Waals et al. 2017). 16s rRNA gene sequencing identified *Peptococcaceae* and *Anaerolineaceae* and the carboxylase encoding abcA gene to be an active part of the benzene activation process within the biofilm. Together with the observation of benzoic acid at concentrations upwards of 0.18uM in the reactor vessel and the absence of phenol, van der Waal et al. concludes benzene carboxylation to be the initial activation pathway.

Continuing the work performed by Vieth et al., Keller et al. (2005) published a study which utilized filling materials and benzene degrading and sulfate-reducing contaminated groundwater from the previously described former hydrogenation plant near Zeitz, Germany. A column study was operational since 2006 but conversion to anoxic, specifically nitrate-reducing, conditions began in 2012. These columns contained 2.5 mM and 5 mM of nitrate (Keller et al. 2018). As this was a microcosm study, column sediments were next transferred to bottles resulting in ≤10 mM nitrate and ≤650 µM benzene. Over a 310 day incubation period, an average degradation rate of 10.1 µM/d was achieved. Furthermore, 13 moles of nitrate were consumed, and 8 moles of nitrite generated, per mole of benzene degraded (Keller et al. 2018). Keller et al. commented that these ratios compared well against the theoretical expectations.16s rRNA gene sequencing identified genus *Azoarcus* and *Simplicispira* belonging to *Rhodocyclaceae* and *Dokdonella* of *Xanthomonadaceae* to be the most abundant operational taxonomic unit (OUT). Lastly, Keller et al. applied stable isotope fractionalization in attempts to elucidate the potential benzene activation mechanism occurring with the observed biodegradation. Fractionization data suggests that observed degradation within the nitrate- and sulfate-reducing conditions are performed by

different organisms. The authors tentatively suggest benzene hydroxylation initiated by monooxygenase as this pathway was responsible for previous experiments with the same cultures but require further investigation to confirm (Keller et al. 2018).

2.6.2 – Anaerobic Benzene Biodegradation under Sulfate-Reducing Conditions

2.6.2.1 – General Overview

Instances of applying sulfate as the terminal electron acceptor in anaerobic benzene biodegradation predate experiments using nitrate. Utilizing sulfate in this manner provides several advantages: sulfate does not react with reduced metabolites such as iron (II) or sulfide, and sulfate is not consumed in abiological processes unrelated to benzene degradation (Anderson and Lovley 2000). In terms of in-situ treatment on field sites, sulfate does not form precipitates with iron (III) oxides which can potentially clog aquifer flow pathways. In comparison to oxygen, sulfate has twice the capacity to accept electrons and is more soluble than oxygen, allowing for a greater amount to be injected. Lastly, the forms in which sulfate can be injected into a contaminated aquifer are relatively economic (Anderson and Lovley 2000).

Edwards and Grbić-Galić (1992), Lovley et al. (1995), Phelps et al. (1996), Nales et al. (1998), Ulrich and Edwards (2003) demonstrated the ability of sulfate-reducing microorganisms to degrade benzene. In some of those studies, inclusion of radiolabeled benzene has been observed to mineralize to end products such as carbon dioxide. Lengthy lag phases had been observed in these studies: such as 84 days (Phelps et al. 1998) and upwards of 120 days (Kazumi et al. 1997). Reinhard et al. (1997) was the first to attempt in-situ remediation involving injected slugs of treated groundwater. Unfortunately, over 90% of benzene was retained throughout a 60-day incubation period. The loss of benzene observed was concluded to be due to dispersion and dilution as opposed to any real degradation activity. Anderson and Lovley (2000) performed another in-situ treatment via sulfate injection and confidently reported that sulfate was the electron acceptor via comparison to a bromide tracer. In this study, approximately 83% of benzene was removal after 84 days of treatment. Phelps et al. (1998) was the first to identify candidate sulfate-reducing benzene degrading microorganisms, *Thiomicrospira* sp. and *Desulfobacula toluolica*, via microcosm experiments. In 2005, Vietz et al. utilized SIF to access the bioremediation of a heavily contaminated hydrogenation plant in Zeitz, Germany. Laban et al. (2009) identified *Pelotomaculum isophthalicum* and *Desulfomonile tiedjei* as sulfate-reducing benzene degraders and were the first to test co-substrates in the form of toluene and phenol but reported a lack of

degradation, ultimately leading the study to favorably suggest benzene carboxylation as the initial activation mechanism. Taubert et al. (2012) continued the work of Veith et al. (2005) and employed protein based stable isotope probing (protein-SIP) on the enrichment culture and identified proteins affiliated to the family *Peptococcaceae* with the genera *Desulfotomaculum* and *Pelotomaculum* to be the primary benzene degraders. Furthermore, a link between sulfide accumulation and cessation of benzene degradation was also encountered in this study. Dong et al. published a 2017 study relying on microcosms derived from the Laban et al. (2009) experiment and did not favor the commonly three activation mechanism but suggested a single cell process occurring within a novel member of the *Pelotomaculum* genus as the initial activation mechanism.

Table 2-3 on the following page provides an overview of the studies discussed in this literature review which utilized sulfate as the terminal electron acceptor in benzene degradation. Section 2.6.2.2 provides a summary of each of these publications on an individual basis. If the reader is not interested in this level of detail, they are encouraged to skip ahead to Section 2.6.3 (page 52) which address benzene biodegradation under methanogenic conditions. Conversely, the reader is also suggested to seek out the published studies themselves should they be interested in the specific articles.

Table 2-3 – Overview of Benzene Degradation Studies Under Sulfate-reducing Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Edwards and Grbić-Galić	1992	Microcosm Study	Yes	None Identified	None Suggested	Soil sediments: Seal Beach, California
Loveley et al.	1995	Microcosm Study	Yes	None Identified	Direct oxidation to CO_2	Contaminated soil sediments: Shelter Island San Diego Bay, California
Langenhoff et al.	1996	Continuous-flow packed bed-column	No	None Identified	None Suggested	Soil sediments: River Rhine, Wageningen, Netherlands; sugar beet wastewater
Phelps et at.	1996	Microcosm Study	Yes	None Identified	None Suggested	Contaminated deep water sediments: Gulf of California, Mexico
Kazumi et al.	1997	Microcosm study	No	None Identified	None Suggested	Contaminated Aquifer Sediments: Sleeping Bears Dunes, Michigan; Fresh Kills landfill: New York / New Jersey harbor
Reinhard et al.	1997	In-situ treatment with injected slugs of treated groundwater	No	None Identified	None Suggested	Seal Beach Naval Weapons Stations, California
Nales et al.	1998	Microcosm Study	Yes	None Identified	None Suggested	Contaminated sediments: terminal site in South Carolina; oil refinery in Oklahoma
Phelps et at.	1998	Microcosm Study	Yes	*Thiomicrospira sp.* and *Desulfobacula toluolica*	None Suggested	Contaminated deep water sediments: Gulf of California, Mexico
Andersen and Lovley	2000	In-situ treatment via sulfate injection	Yes	None Identified	None Suggested	Oil Refinery in Ponka City, Oklahoma

Table 2-3 (Cont.) – Overview of Benzene Degradation Studies Under Sulfate-reducing Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Ulrich and Edwards	2003	Microcosm study, previously enriched for 6 years	Yes	None Identified	None Suggested	Derived from Nales et al.; contaminanted sediments from Cartwright Gas Station and landfarm, Toronto, Ontario
Vieth et al.	2005	Use of isotope fractionation to access the progress of in-situ degradation	Yes	None Identified	None Suggested	Former hydrogenation plant: Zeitz, Germany
Laban et al.	2009	Microcosm Study. Tested toluene and phenol co-substrates	Yes	*Pelotomaculum isophthalicum* and *Desulfomonile tiedjei*	Benzene Carboxylation	Former coal gasification plant: Gliwice, Poland
Herrmann et al.	2010	Microcosm Study. Utilized DNA-SIP to identify bacteria.	Yes	*Cryptanaerobacter* / *Pelotomaculum* (within *Peptococcaceae*) and *Epsilonproteobacteria*	None Suggested	From Vieth et al. 2005: Former hydrogenation plant near Zeitz, Germany
Taubert et al.	2012	Microcosm study	Yes	Family *Peptococcaceae* with the genera *Desulfotomaculum* and *Pelotomaculum*	Benzene Methylation	From Vieth et al. 2005: Former hydrogenation plant near Zeitz, Germany
Dong et al.	2017	Microcosm Study	Yes	Suggested novel member within genus *Pelotomaculum*	Suggested an unknown single cell process	Derived from Laban et al. 2009: Former coal gasification plant: Gliwice, Poland
Keller et al.	2018	Microcosm Study	Yes	*Pelotomaculum*	None Suggested	From Vieth et al. 2005: Former hydrogenation plant near Zeitz, Germany

2.6.2.2 – Detailed Summary of Individual Publications

The earliest instance of sulfate-reducing benzene biodegradation identified in this literature review was published by Edwards and Grbić-Galić in 1992. In this study, subsurface sediments were collected from Seal Beach, California. 100 g of sediment along with 160 mL mineral media were placed in 250 mL anaerobic bottles resulting in a headspace with of approximately 50 mL. The initial benzene concentration ranged between 40 to 200 μM in these six microcosms and sulfate content was supplied at 20 mM (Edwards and Grbić-Galić 1992). The initial lag phase for most microcosms were between 30 to 60 days, but at the highest tested benzene concentration (200uM), the lag phase increased to between 70 and 100 days. Initial benzene degradation rate also increased as benzene concentration increased, up to 140 μM (Edwards and Grbić-Galić 1992). The greatest initial degradation rate was observed in 140 μM benzene, with a rate of 3.7 μM/day. The 200 μM benzene microcosms had the slowest rate, at 0.4μM/day (Edwards and Grbić-Galić 1992). 90% of ^{14}C-radiolabeled benzene was reported to be recovered in the form of $^{14}CO_2$. Lastly, Edwards et al. noted that the sulfate content decreased (from 20 to 19.5 mM) and is relatively consistent with the theoretical amount of sulfate expected to be reduced based on the amount of benzene oxidized, but this experiment could not exclude the possibly that CO_2 behaved as a terminal electron acceptor in some capacity and allowed for simultaneous methanogenic benzene degradation (Edwards and Grbić-Galić 1992).

In 1995, Lovely et al. published a study demonstrating impressive benzene biodegradation capabilities with convincing evidence linking degradation to sulfate-reducing conditions. The sediments containing the microbial community in this experiment were collected from a petroleum hydrocarbon impacted harbor known as Shelter Island in San Diego Bay, California. An initial microcosm was first amended with toluene (20 μM) and a less significant amount of benzene (1 μM). After 14 days of incubation, the toluene was reported to have disappeared, but no benzene had been degraded. By Day 55, approximately half of this initial benzene had disappeared and by Day 59, all the benzene was depleted (Lovley et al. 1995). Benzene was resupplied three consecutive times with degradation occurring more rapidly and without a lag phase. Afterwards, these microcosms were used to inoculate larger serum bottles containing more sediments from the San Diego Bay (Lovley et al. 1995). A series of biodegradation trials were performed at this step which further enriched the microcosms to benzene and sulfate-reducing conditions. The steps,

data, and results of these trials will not be discussed here but the reader is referred to the original publication should they wish for further details. 10 mL aliquots from these serum bottles were placed in 25 mL serum bottles for the main microcosm experiment. Each microcosm contained 5 mM of sulfate and 125 μM of benzene. Lovely et al. reported four complete cycles of benzene depletion and subsequent re-addition occurred within 12 days of incubation (Lovley et al. 1995). Furthermore, inclusion of ^{14}C-radiolabeled benzene resulted in 92% conversion to $^{14}CO_2$. At one point, benzene metabolism abruptly stopped. Analysis of sulfate concentration revealed that the amount of electron acceptor has dropped below 100 μM from the original 5 mM. The sediments immediately resumed degrading benzene upon the re-supply of sulfate (Lovley et al. 1995). At another point of the experiment, molybdate, an effective and selective inhibitor of sulfate reduction in sediments, was added which immediately ceased benzene uptake and CO_2 production (Lovley et al. 1995). This was further confirmation that the enriched microcosms relied on sulfate as the terminal electron acceptor. Lastly, this study did not detect the production of common extracellular intermediates such as phenol, benzoate, p-hydroxybenzoate, cyclohexane, and acetate from the radiolabeled benzene. Lovely et al. suggests that the benzene has been oxidized directly into carbon dioxide within the single celled sulfate-reducing microorganisms of this experiment rather than the degradation pathways commonly suggested by literature of this field (Lovley et al. 1995).

Langenhoff et al. performed a continuous-flow packed bed-column experiment in 1996 to achieve anaerobic benzene biodegradation. Each column had a flow rate of 3.5mL/hr, a retention time of 10 hours, and utilized a different redox condition for degradation. These columns contained a mix culture of microorganisms found in anaerobic soil sediments originating from the Rhine River near Wageningen, Netherlands, which was polluted by toluene, benzene, and naphthalene, and in granular sludge that was rich in anaerobic bacteria as it was obtained from an up-flow anaerobic sludge blanket reactor used for the treatment of sugar beet wastewater (Langenhoff et al. 1996). Each column had an initial and influent concentration of 25 μM benzene as well as 10 mM of sulfate in the form of Na_2SO_4. After 425 days of operation, no significant benzene degradation was reported under sulfate-reducing conditions (Langenhoff et al. 1996).

Phelps et al. collected deep-water sediments from hydrocarbon seeps in the Guaymas Basin within the Gulf of California of Mexico. These sediments were reported to have a strong hydrocarbon smell and was sulfidogenic. Microcosms were initially enriched and prepared in

60 mL serum bottles, with 30 mM of sulfate and approximately 100 µM of benzene, toluene, o-, m- , and p-xylene each as the sole carbon source. Subsequently, upon exhaustion of these carbon sources, each microcosm was re-amended with only 100 µM benzene (Phelps et al. 1996). Benzene degradation began after a lag phase of 84 days. By Day 120, degradation has lowered the benzene concentration to approximately 10 µM. The substrate was immediately re-amended to over 125 µM, and subsequently re-degraded to approximately 15 µM by Day 150 (Phelps et al. 1996). In terms of stoichiometry, 45.1 µM of benzene was utilized concurrent with 214 µM of sulfate, accounting for 127% of the theoretical value. This additional difference was suspected to be the result of benzene utilization in cell synthesis (Phelps et al. 1996). Further evidence for sulfate-reducing conditions occurred during the addition of molybdate, a specific inhibitor in sulfate-reducing bacteria. A complete loss of activity was reported in the active cultures which received molybdate. Lastly, 98% of ^{14}C-radiolabeled benzene was recovered in the form of $^{14}CO_2$ (Phelps et al. 1996).

In 1997, Kazumi et al. obtained aquifer sediments from petroleum impacted aquifers at Sleeping Bear Dunes National Lakeshore, a national park at Empire, Michigan and sediments from Seal Beach, California. Using these samples, 160mL serum bottles were developed containing 50 g of sediment containing a microbial community and 75 mL of mineral media (Kazumi et al. 1997). The samples from Michigan were experienced lag phases between 360 to 420 days and were incubated for a total of 520 to 590 day. 20% benzene removal was reported under sulfate-reducing conditions. The samples from California underwent a 120 day lag phase and 320 days of incubation and resulted in 56% benzene degradation when utilizing sulfate as the terminal electron acceptor (Kazumi et al. 1997). Lastly, Kazumi et al. also retrieved estuarine sediments near the Fresh Kills landfill in the New York / New Jersey harbor and developed bottles containing a 10:90 sediment:media ratio. From these samples, 31% benzene removal was reported under sulfate-reduction. The lag phases was between 60 and 100 days while the total incubation period was 180 to 210 days in length (Kazumi et al. 1997).

Reinhard et al. (1997) published a study in which slugs of treated groundwater which were amended with various known concentrations of BTEX compounds and electron acceptors, and injected into a gasoline impacted aquifer at the Seal Beach Naval Weapons Stations in southern California to observe for potential in-situ remediation (Reinhard et al. 1997). This was the first instance in which sulfate-reducing in-situ remediation was attempted in this literature review. The

test zone consisted of a modified sampling device capable of accepting the experiment's series of injectants and is of an unconfined radius containing an estimated 470 to 1,700 L of groundwater throughout the course of the trials (Reinhard et al. 1997). The first three series of test were performed to determine a baseline of the geochemical conditions on site as well as to define the hydraulic properties on site. During this time, the existing BTEX contamination on site were reported to have been depleted as the carbon source for biodegradation. The subsequent trials involved amending the injectant with known BTEX concentrations. The recovered BTEX compounds during sampling in the following trials were compared against the known BTEX content provided in this injectant (Reinhard et al. 1997). Two series of tests explored the degradation of BTEX compounds under sulfate-reducing conditions. In these trials, benzene was also amended at 204 and 250 µg/L. Furthermore, 44.6 and 15.9 mg/L of sulfate was supplied in the injectant. The amount of sulfate provided was intentionally greater than the theoretical demand required to bioremediate the given contaminants. The first test was 60 days and the second test was over 75 days in duration (Reinhard et al. 1997). While toluene, ethylbenzene, m-xylene, and o-xylene underwent varying degrees of degradation, benzene only displayed a downward trend in terms of remaining concentration. Figures provided in the publishing shows an approximate 90% and 80% retention of benzene in the two respective trials (Reinhard et al. 1997). However, Reinhard et al. suggested this decrease in benzene content is most likely due to dispersion and dilution occurring in the unconfined incubation zone as opposed to any active microbial biodegradation activity.

Nales et al. published a study in 1998 demonstrating benzene biodegradation under sulfate-reducing conditions. Samples were collected between May and July 1995, incorporated into 250 mL bottles containing an initial benzene concentration of 150 µM, and incubation period of 1.5 years (Nales et al. 1998). In one part of the experiment, sediments were taken from below the groundwater level from a terminal site in South Carolina. With sulfate concentrations between 23 and 27 mM, a lag period of less than 2 to 8 days was reported. The initial degradation rate was between 24 and 58 µM/day and the maximum residual degradation rate was between 32 and 33 µM/day during the incubation period (Nales et al. 1998). Of the microcosms developed from sediments collected from this location, an average of only 20% of ^{14}C-radiolabeled benzene was recovered as $^{14}CO_2$. The remaining unaccounted 80% was presumed to have been sequestered into

sediments (Nales et al. 1998). Degradation was not sustained as microcosm depletion of benzene stopped after approximately three additions of benzene.

In 1998, Phelps et al. published another study regarding the characterization of a consortium of microbial bacteria capable of benzene degradation under sulfate-reducing conditions. As before, this consortium originated from sediment obtained in Guaymas Basin, in the Gulf of California. The research group had demonstrated repeated benzene degradation via multiple dilutions and benzene re-additions over the course of three years which continually enriched the microbial culture. By the time of this experiment, these cultures only contain approximately 10^{-7} of the original sediments (Phelps et al. 1998). The degradation of approximately 150μM of benzene was demonstrated over a two-week incubation period. The report confidently suggests sulfate to be the terminal electron acceptor for two reasons. First, sulfate reduction with benzene disappearance occurred at the theoretical stoichiometric proportions. Second, the addition of molybdate (a sulfate-reducing inhibitor), had interrupted benzene degradation in samples which were amended with molybdate at the midpoint of the incubation period. Mineralization of radiolabeled benzene into $^{14}CO_2$ had also been achieved (Phelps et al. 1998). However, specifics such as the percentage yield into $^{14}CO_2$, degradation rates, and initial sulfate concentrations were not reported was it is the isolation and characterization of the sulfate-reducing consortium that was the focus of this publication. The cloning and sequencing analysis of this experiment had identified 12 unique small subunits of rRNA genes consisting of a broad diversity within the domain Bacteria (Phelps et al. 1998). The most notable results are as follows: three proteobacteria with 98.4% similarity to *Thiomicrospira* sp., approximately 85% similarity to *Campylobacter* and *Wolinella* (Phelps et al. 1998). In terms of non-proteobacterial clones, two falls within the order *Cytophagales* of the *Bacteroides* and *Cytophaga* group with 90.5% and 89.8% similarity to *Anaeroflexus maritimus*. Another had 99% similarity to *Exiguobacterium aurantiacum* (Phelps et al. 1998). Lastly four clones were identified to be within the family of *Desulfobacteriaceae*. One of which shown a 95% similarity to a known aromatic hydrocarbon degrader - *Desulfobacula toluolica* (Phelps et al. 1998).

Anderson and Lovley published the findings of a field study which documented the stimulation of sulfate-reducing microorganisms for in-situ bioremediate of benzene in a petroleum impacted aquifer. Sulfate was favored as the electron acceptor due to its ease of addition into groundwater and relatively inexpensive cost. This pilot project was located downgradient from a

major petroleum refinery in Ponca City, Oklahoma which suffered long term contamination (over 50 years) and resulted in an aquifer that is highly reduced and methanogenic as well as containing a considerable degree of contaminated hydrocarbons (benzene concentrations upwards of 100μM) (Anderson and Lovley 2000). Two rows of 20 injection wells (40 in total) were placed in a rectangular pattern, spanning the width of 30.5 m. The slotted screens of the well were placed at depths ranging between 2.7 to 3.7 m below ground surface. The pumping solution contained 8. mM sodium sulfate and 1.6 mM potassium bromide (serves as an inert tracer). Each injection well was reported to receive 19 L of sulfate solution per day (Anderson and Lovley 2000). After 84 days of sulfate injection, the treatment zone was estimated to be 176 m in length. Baseline benzene concentrations ranged from 10 to 105 μM but averaged 55 μM of all observation wells before the sulfate injection. Abrupt increases for both downstream sulfate and bromide concentrations were observed, but the relative ratio between increases in both sulfate and the inert tracer bromide suggest a significant removal of sulfate along the flow path and therefore indicated sulfate to perform as the terminal electron acceptor in the stimulated benzene biodegradation (Anderson and Lovley 2000). In terms of remediation, all observations wells experienced a decrease in benzene concentration, with one well reaching a non-detectable limit from the initial 63 μM. Overall, the report summarizes a removal average of 83% of the initial benzene concentrations in comparison to pre-treatment (Anderson and Lovley 2000).

In a 2003 experiment, Ulrich and Edwards utilized microcosms derived from the samples collected and described in the study published by Nales et al. (1998). Nine mixed cultures were enriched for a period of six years in which benzene was the sole carbon source and enriched on various redox conditions. Specifically, this included a total of four sulfate-reducing enrichment cultures sourced from four separate sites (Ulrich and Edwards 2003). The two most notable sample was sourced from an oil refinery and gas station. These samples were capable of a maximum degradation rate of 11 and 9 μM/day at an initial concentration of 300 and 180 μM respectively. The electron acceptor to donor ratio were also reported as 3.3 and 4.0 which compared favorably to the theoretical value of 3.49 (Ulrich and Edwards 2003).

Vieth et al. published a 2005 experiment exploring the use of SIF to monitor and access potential in-situ bioremediation at a former hydrogenation plant in Zeitz, Germany. Historic benzene production had led to widespread BTEX contamination (upwards of 850 mg/L benzene) situated over two partially connected aquifers consisting of heterogeneous materials

(Vieth et al. 2005). During biochemical reactions (such as bioremediation), lighter isotopes (^{12}C) react more readily than heavier isotopes (^{13}C). This results in an eventual accumulation of the heavier isotopes in the residual fraction of the substrate (benzene in this case). The SIF process involves analyzing shifts in the stable isotope ratios ($^{13}C/^{12}C$) to quantify biodegradation (Meckenstock et al. 2016). These stable isotope ratios are then expressed in δ notations as $\delta^{13}C$ values which are percentage values. By applying a ^{13}C isotope signature onto the contaminants, Vieth et al. were able to assess both the temporal and spatial aspects of in-situ biodegradation occurring in this hydrogeologically complicated site. Sulfate concentrations in uncontaminated portions of the aquifers averaged 1 g/L while sampling at the center of the BTEX plume indicated sulfate content to be only a few milligrams per liter. As a result, Vieth et al. suggested sulfate-reduction to be the dominant redox condition for benzene biodegradation. The most significant benzene decreases occurred within the eastern portion of the plume, and superficially only at the upper and lower fringes of the plume. At depths between 10.5 and 13.0 m below ground level, the benzene concentration was reportedly 3.5 and 2.8 mg/L with isotope signatures of -26.9 and -27.0% respectively. However, at a depth of 14.5 m, benzene content increased significantly to 90.1 mg/L and a carbon isotope ratio of -28.1%. The benzene concentration at 15.5 m below ground level dropped once again to 1.9 mg/L and with an isotope signature of -26.3% (Vieth et al. 2005). While this study did not focus on the specific degradation rates achieved on this site, it had demonstrated that in-situ sulfate-reducing biodegradation is possible and that SIF can be a viable means to assess the bioremediation progress of a benzene plume in geologically or hydrogeologically complicated field sites.

After 15 successful transfers over an unspecified time period, Abu Laban et al. (2009) developed a successful benzene degrading and sulfate-reducing culture from soil collected at a former coal gasification site in Gliwice, Poland. The samples reported in this study were capable of completely degrading 350 μM of benzene during an incubation period of under 60 days and reduced 1.2 mM of sulfate to HS^- (corresponding to 88.8% of the theoretical stoichiometric value) (Abu Laban et al. 2009). Furthermore, the degradation of ^{13}C-radiolabeled benzene and formation of 1.8 mM of $^{13}CO_2$ translated to only 83.6% of the expected theoretical carbon dioxide yield. 5 mM of molybdate was introduced in some samples after 38 day of incubation. This sulfate-reduction inhibitor halted benzene degradation and carbon dioxide production. Laban et al. continued by testing toluene and phenol as co-substrates and reported neither substances were

degraded co-metabolically (Abu Laban et al. 2009). The most notable results from 16s rRNA gene sequencing yielded a 95% similarity to *Pelotomaculum isophthalicum* within the Gram-positive family *Peptococcaceae* as well as a 93.3% similarity to the dehalogenating bacterium *Desulfomonile tiedjei* within the deltaproteobacterial family *Synthrophaeceae* (Abu Laban et al. 2009). Phenol, 2-hydroxybenzoate, 4-hydroxybenzoate, and benzoate were reported to be metabolites formed during degradation. The lack of phenol degradation during the co-metabolism portion of the study led Laben et al. to rule out benzene hydroxylation as the initial degradation pathway. Similarly, coupling the lack of effect in which toluene had on benzene degradation with the absence of benzylsuccinate as a metabolite suggested that benzene methylation was also not the initial degradation pathway. As a result, due to benzoate being observed as a metabolite, Laban et al. concluded the publication by tentatively favoring benzene carboxylation as the initial activation mechanism in these sulfate-reducing cultures (Abu Laban et al. 2009).

In 2010, Herrmann et al. developed microcosms derived from coarse sand particles (from a contaminated field site in Zeitz, Germany) colonized by microbial communities which previously demonstrated anaerobic benzene biodegradation. These microcosms were amended with benzene concentrations ($^{13}C_6$-radiolaballed and unlabeled) between 588 and 744 µM. Degradation rates were reported to be successful between 3.6 and 5.7 µM/day for both the labelled and unlabeled benzene over an incubation period of under 200 days (Herrmann et al. 2010). Sulfide was observed to form throughout the course of degradation. Conversely, sulfide was not formed in the abiotic controls in which benzene loss was minimal, indicating sulfide to be a product of active biodegradation under sulfate-reducing conditions. However, a greater amount of sulfate was consumed in comparison to expected values suggested by the theoretical chemical reaction discussed in the article. Herrmann et al. assumed this extraneous sulfide content to be formed during precipitation which created a coating of iron sulfide on the sand particles within the microcosms. It is noteworthy that if this assumption is correct, this would lead to an underestimation of sulfate-reduction occurring within the microcosm (Herrmann et al. 2010). Analysis of radiolabeled isotope signatures resulted in a high correlation between radiolabeled benzene and the carbon dioxide formed. Furthermore, no lag phase was observed as the ^{13}C signature carbon dioxide was detected after 14 days of incubation. Herrmann et al. reported an average of 95% of ^{13}C-labelled benzene to be recovered in the form of carbon dioxide, suggesting

significant mineralization capabilities in these microcosms (Herrmann et al. 2010). Lastly, methane with the same radio-signature was also detected and an approximate 5 μM of methane was formed per 100 μM of benzene. DNA-SIP (stable isotope probing) involved comparing DNA of ^{13}C-benzene-incubated microcosms against ^{12}C-benzene-incubated microcosms. A phylotype belonging to *Cryptanaerobacter* / *Pelotomaculum* group of the *Desulfotomaculum* cluster within the family *Peptococcaceae* had been identified to consume the supplied benzene. A less dominant phylotype belonging to *Epsilonproteobacteria* had also been identified (Herrmann et al. 2010). Herrmann et al. suggest that not one singular organism was responsible for the oxidation of benzene and sulfate-reduction. Instead, the *Cryptanaerobacter* / *Pelotomaculum* group initiated the first step of benzene degradation but does not completely oxidize the substrate but releases reduced metabolites which can include hydrogen, acetate, or other fermentation products of lower molecular masses. Subsequently, secondary bacteria may use these reduced metabolites for their respective metabolism. The identified *Epsilonproteobacteria* was suspected of behaving as such a hydrogen scavenger (Herrmann et al. 2010).

Taubert et al. (2012) employed a metagenome-based functional metaproteomic approach, protein-based stable isotope probing (protein-SIP), to analyze a benzene degrading sulfate-reducing enrichment culture. SIP had been a method utilized to analyze elemental fluxes between individual strains of a microbial culture. In the SIP process, a substrate in question is labeled with a heavy isotope (commonly ^{13}C or ^{15}N). These isotopes are then used as a metabolic tracer when it is incorporated into different classes of biomolecules and allow certain metabolic activities to be marked. Recently, SIP has been applied to amino acids, phospholipid-derived fatty acids, DNA, RNA, and proteins (Taubert et al. 2012). When protein-SIP is paired with high-resolution mass spectrometry, mass shifts regarding the previously mentioned heavy isotope labels within peptides can be detected and quantified. If a community sequence catalog or reference is available, this technology can achieve functional and phylogenetic classification in a microbial community (Taubert et al. 2012). In this study, Taubert et al. continued with the work of Herrmann et al. (2010) and utilized the same benzene-degrading, sulfate-reducing microbial culture (originally from Vieth et al. (2005)) originated from a contaminated former hydrogenation planet in Zeitz, Germany. Unfortunately, employing protein-SIP was the focal point of this study, and data related to degradation rates and degradation kinetics were not included. Through the incorporation of ^{13}C, protein SIP, and mass spectrometry, Taubert et al. identified three functional

groups associated with benzene degradation. The first group was dominated by phylotype *Clostridiales*, with over 95% of the proteins affiliated to the family *Peptococcaceae* with the genera *Desulfotomaculum* and *Pelotomaculum*. This group was believed to be responsible for direct benzene activation (Taubert et al. 2012). The second group consisted of Deltaproteobacteria, specifically assigned to the genus *Desulfobacca* within the family of *Syntrophaceae*. Taubert et al. suggested this group to be responsible in degrading fermentation products. The third and last group was largely heterogeneous and contained a large proportion of proteins related to *Bacteroidetes* and *Chlorobi*. This group was suggested to simply behave as scavengers and feed off the dead cells of the previous two groups (Taubert et al. 2012). In terms of degradation pathways, Taubert et al. identified proteins affiliated with BbsA and BbsB, a key enzyme in the downstream metabolite of toluene. Taubert et al. tentatively suggested benzene methylation to be the initial activation mechanism.

In 2017, Dong et al. published a journal article outlining 16s rRNA gene sequencing on active sulfate-reducing benzene degraders and hypothesized to be responsible by a single cell process via a novel member within the genus *Pelotomaculum*. This contrasts against *Pelotomaculum thermopropionicum* (the only other member in the genus *Pelotomaculum* which has been sequenced) which relies on a syntrophic relationship with partner microbial organism(s) as it is incapable of sulfate-reduction (Dong et al. 2017). The microbial culture studied within this experiment was the same as the one described in Labal et al. (2009). The soil sediments originated from a former coal gasification plant in Gliwice, Poland and enriched with 0.5 mM of benzene as the sole carbon source and 10 mM of sulfate as the electron acceptor. According to the publication dates of these papers, it is assumed that this culture has benefitted from such enrichment for approximately 8 years. The degradation pathway and related genomics were the focus of this study as opposed to the benzene degradation kinetics, as a result, data related to degradation rates were not provided. Conversely, Dong et al. was interested in the initial activation reaction for the degradation observed. Methylation to toluene was ruled out when genes related to enzymes such as benzylsuccinate synthase (Bss) or genes for beta-oxidation of benzylsuccinate to benzoyl-CoA were not identified (Dong et al. 2017). Similarly, hydroxylation to phenol was also concluded as improbably as genes related to enzymes for phenol degradation were lacking in a genetic context (Dong et al. 2017). Direction carboxylation to benzoate was the last of the three common proposed degradation pathway and therefore a likely candidate as it was associated with Laban et al. 2009.

As a result, Dong et al. searched for "genes encoding for the proposed anaerobic benzene carboxylase (AbcA and AbcD)" which were previously shown as responsible for the carboxylation of benzene to benzoate. However, only one gene sharing a 33% similarity to the gene AbcA was identified and no genes related to AbcD were found. Dong et al. concluded that this novel member in *Pelotomaculum* does not employ the above three initial activation pathways, but instead, relies on a so far unknown, anaerobic strategy for benzene activation (Dong et al. 2017).

Continuing the work performed by Vieth et al. (2005), Keller et al. published a study which utilized filling materials and benzene contaminated groundwater from the previously described former hydrogenation plant near Zeitz, Germany. A column study was operational since 2006 but conversion to anaerobic, specifically nitrate-reducing conditions began in 2012 (Keller et al. 2018). As this was a sulfate-reducing microcosm study, column sediments were next transferred to bottles containing ≤650 μM benzene and a conversion back to 0.2 g/L sodium sulfate also occurred. No specific data were provided regarding the degradation capabilities of these sulfate-reducing microcosms as the nitrate-reducing microcosms were the focus on this paper. 16s rRNA gene sequencing identified genus *Pelotomaculum* to be the most abundant operational taxonomic unit (Keller et al. 2018). Lastly, Keller et al. mentioned applying SIF in attempts to elucidate the potential benzene activation mechanism occurring with the observed biodegradation. Fractionization data suggests that observed degradation within the nitrate- and sulfate-reducing conditions are performed by different organisms. The authors did not arrive at a conclusion regarding a specific degradation pathway.

2.6.3 – Anaerobic Benzene Biodegradation under Methanogenic Conditions

2.6.3.1 – General Overview

Bioremediation of benzene contaminated sites often aim to utilize oxygen, nitrate, and sulfate as the terminal electron acceptors as these compounds comparatively thermodynamically more favorable to oxidize benzene (Vogt et al. 2011) and are consistently shown to provide a greater degradation rate in most available literature. However, a petroleum hydrocarbon release in reality often means that the ample amount of substrate released exceeds the supply of the previously listed electron acceptors available within the soil. Unless the environmental engineers or scientists purposefully resupply the contaminated soils or aquifer with oxygen, nitrate, sulfate, etc., the site will be rendered methanogenic (Van Beelen and Van Keulen 1990). Under methanogenic conditions or methanogenesis, biodegradation is not limited by the requirement of

an exogeneous electron acceptor (Luo et al. 2016). This section describes studies which intentionally created conditions in benzene biodegradation experiments that are methanogenic.

An additional challenge in methanogenic studies relate to the difficulty in isolating the specific benzene degraders within a methanogenic consortium. While pure strains of nitrate- and sulfate-reducers can be enriched with benzene as the sole carbon source and eventually observed to degrade benzene within its cellular activity, this is not the case for methanogenic degraders. Specifically, methanogenic consortia often do not derive sufficient energy from benzene alone to make the initial activation thermodynamically favorable. As a result, a syntrophic partner in the methanogenic consortia is often required (Foght 2008; Ulrich and Edwards 2003). Therefore, a difficulty arises in terms of gene sequencing to accurately identify both the specific microorganism responsible for the benzene degradation, as well as its syntrophic partner(s) within the whole of the microbial community.

Available literature suggests that the concept of benzene biodegradation under methanogenic conditions predate that of both nitrate- and sulfate- reducing conditions via the two related studies published by Vogel and Grbić-Galić in 1986 and 1987 in which benzene was observed to degrade while carbon dioxide was formed. Radiolabeled carbon dioxide yielded a production of phenol with the same radio-signature. These studies may be the first attempts to elucidate the benzene degradation pathway under methanogenic conditions, in which the authors suggested benzene hydroxylation in the latter paper. In the follow years, several studies also depicted experiments in which benzene was observed to degrade under methanogenesis (Van Beelen and Van Keulen 1990; Kazumi et al. 1997). Weiner and Lovley published a methanogenic experiment in 1998 which shown rapid degradation rates with no lag phase but also the observation that phenol, acetate, and propionate were metabolites formed during degradation. The authors did not suggest benzene hydroxylation even with the formation of phenol. Ulrich and Edwards (2003) were the first to identify candidate microcosms - *Dehalospirillum multivorans* and *Methanobacterium formicicum*. In 2005, Ulrich et al. utilized isotope trapping, a novel idea thus far in this field, to identify phenol, benzoate, and toluene as transient intermediates formed during methanogenic degradation. Ulrich et al. suggested benzene methylation in parallel or in series with benzene hydroxylation to be responsible as the initial activation mechanism. Sakai et al. 2009 identified *Synthrophus gentianae* and *Sedimentibacter* sp. within methanogenic degradation from soil sediments originating from pristine lotus fields. Lastly, Luo et al. (2016) had

found *Syntrophobacterales*, *Desulfobacterales*, and *Desulfuromonadales* to show promising methanogenic degradation capabilities as well.

Table 2-4 on the following page provides an overview of the studies discussed in this literature review which benzene degradation occurred under methanogenic conditions. Section 2.6.3.2 provides a summary of each of these publications on an individual basis. If the reader is not interested in this level of detail, they are encouraged to skip ahead to Section 2.6.4 (page 63) which address benzene biodegradation under other redox conditions. Conversely, the reader is also suggested to seek out the published studies themselves should they be interested in the specific articles.

Table 2-4 – Overview of Benzene Degradation Studies Methanogenic Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Vogel and Grbić-Galić	1986	Microcosm study. Observed the formation of phenol.	Yes	None Identified	None Suggested	Sewage sludge: origin unknown
Grbić-Galić and Vogel	1987	Microcosm study. Observed the formation of phenol.	Yes	None Identified	Benzene Hydroxylation	Derived from Vogel and Grbić-Galić 1986: Sewage sludge: origin unknown
Van Beelan and Van Keulen	1988	Microcosm study	Yes	None Identified	None Suggested	Contaminated river sediments: River Merwede, Gorinchem, Netherlands
Langenhoff et al.	1996	Continuous-flow packed bed-column	No	None Identified	None Suggested	Soil sediments: River Rhine, Wageningen, Netherlands; sugar beet wastewater
Kazumi et al.	1997	Microcosm study	Yes	None Identified	None Suggested	Contaminated Aquifer Sediments: Sleeping Bears Dunes, Michigan; Fresh Kills landfill: New York / New Jersey harbor
Weiner and Lovley	1998	Microcosm study. Rapid degradation with no lag phase. Phenol, acetate, and propionate observed as metabolites.	Yes	None Identified	None Suggested	Contaminated aquifer sediments: Ponca City, Oklahoma
Ulrich and Edwards	2003	Microcosm study, previously enriched for 6 years	Yes	*Dehalospirillum multivorans* and *Methanobacterium formicicum*	None Suggested	Derived from Nales et al.; contaminated sediments from Cartwright Gas Station and land farm, Toronto, Ontario

Table 2-4 (Cont.) – Overview of Benzene Degradation Studies Methanogenic Conditions

Author(s)	Year	Noteworthy Experiment Setup and Observations	Success of Biodegradation	Dominant Microorganism(s) Identified	Suggested Degradation Pathway	Sample Source
Ulrich et al.	2005	Microcosm study. Employed Isotope trapping to confirm that phenol, toluene, and benzoate as an intermediate	Yes	None Identified	Benzene Methylation in parallel or series with Benzene Hydroxylation	Contaminated soil sediments: former oil refinery, Oklahoma
Sakai et al.	2009	Microcosm study	Yes	*Synthrophus gentianae* and *Sedimentibacter* sp.	None Suggested	Pristine soil sediments: lotus field, unknown location
Luo, et al.	2016	Microcosm study	Yes	*Syntrophobacterales, Desulfobacterales*, and *Desulfuromonadales*	None Suggested	Contaminated soil sediments: former oil refinery, Oklahoma and former gas station, Toronto, Ontario

2.6.3.2 – Detailed Summary of Individual Publications

In 1986, Vogel and Grbić-Galić published a methanogenic study with cultures originating from sewage sludge which had been utilizing ferulic acid as the carbon source and could mineralize the substrate to carbon dioxide and methane. Afterwards, these samples were further amended with benzene (six refeeds) as the carbon source for a period of one year. The microcosms consisted of culture tubes containing 10 mL of mineral media and 3.0 mM benzene (Vogel and Grbić-Galić 1986). Vogel and Grbić-Galić observed the formation of ^{18}O-radiolabeled metabolites in the form of phenol via the ^{18}O-radiolabelled water of the media. However, the produced phenol content was only 2.5% as opposed to the theoretical 8% expected. Furthermore, no specific degradation rates, lag phases, and final concentrations of benzene were provided in this journal article (Vogel and Grbić-Galić 1986). This experiment may be the first attempt at methanogenic benzene biodegradation.

Grbić-Galić and Vogel returned with a consecutive publication in 1987 with microcosms originating from the same source as their 1986 study. In this experiment, the microcosms were enriched on 15 mM of benzene (and a trace amount of methanol) for a period of 9 months before the onset of the main experiment (Grbić-Galić and Vogel 1987). These 9 months were considered a pre-experimental period. As the microbial cultures were reliant on ferulate as the sole carbon source before that point, a lag phase of 11 days was observed when the carbon source was switched to 15 mM benzene. After three months, the cultures had adapted to benzene as the new carbon source and substrate transformation began immediately after each refeed (Grbić-Galić and Vogel 1987). During this pre-experimental period, Grbić-Galić and Vogel reported that the cultures had depleted over half of the benzene and the percentage of methane in produced gas was approximately 60.3%, comparable to the theoretical 62.5% expected. No degradation rates were provided other than the completely disappearance of benzene after 34 days (Grbić-Galić and Vogel 1987). The main experiment reported in this journal article possessed an incubation period of 60 days with the benzene concentration supplied at 1.5 and 3 mM as well as a combination of benzene and methanol also at 1.5 and 3 mM. The benzene used was ^{14}C-radiolabeled benzene to trace the production to $^{14}CO_2$. The focus on this experiment once again was not the specific degradation rates but instead the CO_2 content within the headspace of the samples at both the onset and conclusion of the 60 day incubation period. Grbić-Galić and Vogel reported the $^{14}CO_2$ content increased from 0.002% to 5.8% and from 0.85% to 5.4% in the 1.5 and

3 mM benzene cultures respectively between the start and end of the incubation period. In terms of the cultures with both benzene and methanol, $^{14}CO_2$ content increased from 0.25% to 2.6% and 0.05% to 2.0% for the 1.5 and 3 mM sample sets respectively (Grbić-Galić and Vogel 1987). These low percentages of carbon dioxide production indicated an incomplete conversion of benzene to mineralization. The presence of methanol appeared to hindered the methanogenic biodegradation of benzene but was not believed to have performed as an electron acceptor. Again, no specific degradation rates or data related to the degradation potential was supplied by this journal article. Lastly, phenol was observed to be an intermediate formed along with trace amounts of cyclohexane and propanoic acid. The presence of phenol as the intermediate byproduct led Grbić-Galić and Vogel to suggest the methanogenic pathway to be related to benzene hydroxylation in which benzene is oxidized to phenol, then subsequently to cyclohexane, to propanoic acid, and finally mineralized to carbon dioxide and methane (Grbić-Galić and Vogel 1987).

In 1988, Van Beelan and Van Keulen collected methanogenic river sediments in a harbor at the River Merwede near the Dutch town of Gorinchem. The initial experiment tested microcosms at 1 μg/L of benzene and an incubation period of 63 days during which only 37 to 43% of the given benzene was degraded (Van Beelen and Van Keulen 1990). Furthermore, only a small fraction of this benzene was converted to carbon dioxide via analysis of ^{14}C-radiolabeled carbon: 5% at day 6 and dropped to 3.5% at the end of the 63 day incubation period. Sorption of benzene to the inner wall of the rubber stopper of the microcosms and incomplete degradation of benzene to intermediates such a phenol as opposed to complete mineralization was given as an explanation as to why benzene disappearance was far greater than observed benzene mineralization (Van Beelen and Van Keulen 1990). The initial rate of mineralization was reported at 5% of the total benzene content per day. In a second portion of the experiment, Van Beelan and Van Keulen amended the microcosms with 100 μg/L of benzene. They had noted the same rate of initial degradation, concluding that no significant adaptation occurred for the microbial community towards benzene biodegradation under methanogenic conditions (Van Beelen and Van Keulen 1990).

Langenhoff et al. performed a continuous-flow packed bed-column experiment in 1996 to achieve anaerobic benzene biodegradation. Each column had a flow rate of 3.5 mL/hr, a retention time of 10 hours, and utilized a different redox condition for degradation. These columns contained a mix culture of microorganisms found in anaerobic soil sediments originating from the Rhine

River near Wageningen, Netherlands, which was polluted by toluene, benzene, and naphthalene, and in granular sludge that was rich in anaerobic bacteria as it was obtained from an up-flow anaerobic sludge blanket reactor used for the treatment of sugar beet wastewater (Langenhoff et al. 1996). Each column had an initial and influent concentration of 25 μM benzene. After 525 days of operation, no benzene disappearance was reported under methanogenic conditions (Langenhoff et al. 1996).

In 1997, Kazumi et al. obtained aquifer sediments from petroleum contaminated aquifers at Sleeping Bear Dunes National Lakeshore, a national park at Empire, Michigan and sediments from Seal Beach, California. Using these samples, 160 mL serum bottles were developed containing 50 g of sediment containing a microbial community and 75 mL of mineral media (Kazumi et al. 1997). The samples from Michigan experienced lag phases between 360 to 420 days and were incubated for a total of 520 to 590 day. 32% benzene removal was reported for samples relying methanogenic conditions. Lastly, Kazumi et al. also retrieved estuarine sediments near the Fresh Kills landfill in the New York / New Jersey harbor and developed bottles containing a 10:90 sediment:media ratio. From these samples, 58% benzene removal was reported under methanogenic conditions. The lag phases were between 60 and 100 days while the total incubation period was 180 to 210 days (Kazumi et al. 1997).

Weiner and Lovley published a methanogenic degradation study in 1998. Microcosm samples were developed using sediments originating from an aquifer in Ponca City, Oklahoma which had been plagued by hydrocarbon contamination for over 50 years, of which benzene was reported to be the principle hydrocarbon at concentrations between 130 and 640 μM (Weiner and Lovley 1998). ^{14}C-radiolabeled benzene was added to the microcosms to determine the degradation byproducts. After only 13 days, 53% of the radiolabeled benzene had been mineralized to carbon dioxide and methane (Weiner and Lovley 1998). To study the metabolism of the microbial community further, radiolabeled benzene was added once again. After this re-feed, the ^{14}C-benzene was observed to immediately mineralize into $^{14}CO_2$ and $^{14}CH_4$ with no lag phase. After a period of slightly over 15 days, more than 50% of the benzene had been recovered in the form of CO_2 and CH_4 (Weiner and Lovley 1998). Furthermore, 80% of the ^{14}C-radiolabeled benzene that had been recovered, was in the form of $^{14}CH_4$. This compares favorably against the theoretical 62.5% according to methanogenic biodegradation of benzene suggesting that the degradation occurring in this experiment was methanogenic (Weiner and Lovley 1998). Next,

isotope trapping studies were conducted and the potential intermediates during this degradation included phenol, acetate, and propionate (Weiner and Lovley 1998). This study is perhaps the first documented case in which methanogenic degradation of benzene occurred not only rapidly, but with no apparent lag phase. Weiner and Lovley suggested two possible explanations. First, the site in which the sediments were collected had suffered a long history of benzene contamination (over 50 years) at high concentrations. The site had most likely depleted the other terminal electron acceptors which are more thermodynamically favorable to reduce, leaving the site extensively methanogenic and causing the microbial consortium to have adapted likewise. Second, other studies often perform a series of lab manipulations to the sediment samples during the creation of microcosm samples. These actions can include filtration or dilution with enrichment media. Conversely, this experiment focused on minimal disturbance of collected sediments and extra precautions were taken to ensure that the samples remained anaerobic during transport and subsequent lab manipulations. Weiner and Lovley suggested this careful treatment of sediments may have preserved the delicate consortia required for methanogenic benzene biodegradation (Weiner and Lovley 1998).

In a 2003 experiment, Ulrich and Edwards utilized microcosms derived from the samples collected and described in the study published by Nales et al. (1998). Nine mixed cultures were enriched for a period of six years in which benzene was the sole carbon source and amended with their respective terminal electron acceptors. Specifically, this included two methanogenic enrichment cultures sourced from two separate sites (Ulrich and Edwards 2003). These two samples were sourced from a gas station and oil refinery, and reported a max degradation rate of 75 μM/day and 33 μM/day at an initial concentration of 820 and 990 μM respectively (Ulrich and Edwards 2003). It should be noted that based on this metric, the methanogenic microcosms unexpectedly outperformed both the nitrate and sulfate microcosms by over an order of magnitude. The methane generation ratio were respectively reported as 3.2 and 4.0 which compared favorably to the theoretical value of 3.60 (Ulrich and Edwards 2003). Genetic sequencing yielded a 99% match to the bacteria *Dehalospirillum multivorans* and a 97% match to the archaea *Methanobacterium formicicum* in two samples (Ulrich and Edwards 2003).

In 2005, Ulrich et al. returned with another publication. This study involved the detection of transient metabolites created during anaerobic benzene biodegradation under methanogenic conditions in hopes of elucidating a specific degradation pathway as suggested by previous

literature. The microcosms developed in this experiment contained soil and groundwater obtained from an oil refinery in Oklahoma. These samples had benefitted from eight years of enrichment in which benzene was the sole carbon source amended to concentrations between 130 and 1,100 μM as well as having endured multiple successful transfers to defined mineral media solutions (Ulrich et al. 2005). In this study, Ulrich et al. employed isotope trapping to enhance the detection of benzene degradation metabolites. In pervious experiments, it had been noted that benzoate was a transient metabolite formed during degradation. As a result, unlabeled benzoate (the trap) was intentionally added into the microcosms. The carbon source provided exist in the form of ^{13}C-radiolabeled benzene. In theory, the benzoate trap will cause a buildup of ^{13}C-radiolabeled benzoate and any other upstream metabolites, and detection of these products would aid in establishing an indisputable link towards the radiolabeled benzene substrate (Ulrich et al. 2005). While the specific degradation rates were not the focus of this experiment, Ulrich et al. did observe that the benzene content had decreased from 330 μM to under 11 μM in 18 days for samples that did not receive the benzoate trap. Conversely, the samples which received the benzoate trap (added on Day 1) had degradation reported to decrease from 410 μM to under 11 μM in 22 days (Ulrich et al. 2005). By comparing the development of radiolabeled metabolites between microcosms which received and did not receive the isotope trap, Ulrich et al. reported that phenol, toluene, and benzoate were intermediates formed during methanogenic of benzene biodegradation. Furthermore, the formation and subsequent disappearance of these metabolites were then analyzed with a temporal context. Initially, phenol concentration increased and subsequently decreased, suggesting it to be a mere degradation intermediate. An increase in toluene content was also noticed but not to the degree of the nitrate-reducing microcosms (see Section 2.6.1.2). Lastly, benzoate concentration did not peak during the depletion of benzene, therefore indicating benzoate to be a downstream metabolite of phenol and/or toluene (Ulrich et al. 2005). Ulrich et al. concluded that the pathway seen in this methanogenic condition is most likely benzene methylation (due to the presence of toluene) in parallel or series with benzene hydroxylation (due to the presence of phenol). Since microcosms utilizing methanogens to degrade hydrocarbons usually comprise of a consortia consisting of many different species, it was very possible that different microorganisms can utilize different degradation pathways simultaneously (Ulrich et al. 2005).

In 2009, Sakai et al. obtained methanogenic sediment samples from a pristine lotus field. After a lag phase of 100 days, the microcosms began degrading benzene (initially at 0.10 mM) and

complete degradation was achieved by Day 194 (Sakai et al. 2009). Six successful benzene refeeds occurred afterwards to concentrations between 0.10 and 0.20 mM and degradation commenced with no lag phases during each re-amendment. These six cycles of degradation occurred between Days 194 and 474 (Sakai et al. 2009). Methane production was observed throughout this period. Specifically, the study reported that the degradation of 1 mole of benzene produced an average of 4.2 moles of methane, which compares favorably against the theoretical value of 3.75 moles of methane for methanogenic degradation (Sakai et al. 2009). On Day 474, the microbial community was subjected to DNA-SIP analysis. The results included a 85.1% match to the closest identified bacterium, *Syntrophus gentianae* (Sakai et al. 2009). Sakai et al. constructed a bacterial clone library and an archael clone library. 62 clones were created in the bacterial clone library, with 60% of these clones belonging to either a novel cluster in *Firmicutes*, Hasda-A (which was an almost full length 16S rRNA gene sequence identified by denaturing gel gradient electrophoresis (DGGE)), or a clone closely related to *Sedimentibacter* sp. in *Firmicutes* (Sakai et al. 2009). In terms of the archael clone library, 83% of the clones were reported to belong to either *Methanomicrobiales* or *Methanosarcinales* (Sakai et al. 2009). Sakai et al. concluded that a consortium of fermenters, aceticlastic methanogens, and hydrogentrophic methanogens were most likely working on conjunction to sequentially degrade benzene in their samples. The researchers further suggest that a similar syntrophic relations exist in other methanogenic cultures as methanogens can only degrade compounds with low molecular weight and no known methanogens are found to degrade aromatic compounds solely and directly (Sakai et al. 2009; Vogt et al. 2011).

Luo, Devine, and Edwards published a 2016 study utilizing microcosms previously demonstrated by the Edwards lab at the University of Toronto. This journal article involved 16 methanogenic microcosms which also originated from an oil refinery in Oklahoma and soil samples from a former local gas station and had benefitted from over 15 years of enrichment on benzene. During this time, the cultures had endured multiple transfers and developed many subcultures for diverse experiments. These cultures were reportedly capable of consistently degrading benzene at rates ranging from 1.4 to 25 μM/d and produced quantities of methane and carbon dioxide which were stoichiometrically comparable (Luo et al. 2016). Previous 16S rRNA gene sequencing from these cultures yielded a clone library dominated by methanogens corresponding to *Deltaproteobacterium*, and distantly related to *Syntrophobacterales*, *Desulfobacterales*, and *Desulfuromonadales* (Luo et al. 2016). The specific benzene degradation

kinetics were not the focus of this journal article. Instead, Luo et al. aimed to find common traits between these 16 methanogenic cultures. Via the previously mentioned 16S rRNA clone library in conjunction with pyrotag 16S rRNA amplicon sequencing, metagenome sequencing, and quantitative polymerase chain reaction (QPCR), an operational taxonomic unit under *Deltaproteobacteria* designated as ORM2 has been consistently identified in all 16 of the methanogenic cultures and analyzed to be 84 to 86% similar to *Syntrophus* or *Desulfobacterium* sp.. This similarity can be explained by the fact that these cultures either share a common origin or had experienced intermixing transfers in the past (Luo et al. 2016).

2.6.4 – Anaerobic Benzene Biodegradation under Other Conditions

Jahn et al. explored the effects of AQDS (9,10-anthraquinone-2,6-disulfonic acid) on benzene degradation under Fe(III)-reducing conditions. This study explored benzene concentrations between 120 and 150 μM (Jahn et al. 2005). One culture was enriched in the presence of AQDS, contained a lag phase of 16 days, and complete degradation was achieved within 77 days of incubation. A second culture was not enriched with AQDS. The lag phase demonstrated by this culture was lengthier at 61 days. After 115 days of incubation, degradation slowed and ceased before complete benzene depletion by Day 162. These cultures utilized only 82% and 74% of the theoretically expected amount of electron acceptor (Jahn et al. 2005).

In 2011, Holmes et al. utilized a hyperthermophilic pure culture of *Ferroglobus placidus* to anaerobically degrade benzene under iron (III) (Fe(III)) reducing conditions with evidence towards the benzene carboxylation degradation pathway. While Fe(III) reducing conditions was not the focus of this thesis, the work outlined in this article was notable as the only benzene degrading pure cultured that had been isolated at that point were nitrate- and chlorate- reducing cultures (Holmes et al. 2011). After one year of enrichment, Holmes et al. demonstrated 0.66 mM benzene loss with the accumulation of 23.33 mM Fe(II) during an incubation period of less than 80 days. The author reported that these values compared favorably to the theoretical stoichiometry for benzene oxidation coupled with Fe(III) reduction. Furthermore, analysis of radiolabeled compounds indicated the maximum conversion of 81.2% ^{14}C-benzene to ^{14}C-CO_2. Benzoate concentrations upwards of 10.5 mM were also detected whereas toluene and phenol were not observed. Further evidence towards benzene carboxylation to benzoate as the primary degradation pathway exists in the form of gene transcription analysis in which putative carboxylase (used in

benzoate degradation) genes were expressed to a higher degree in benzene grown samples in comparison to benzoate grown samples (Holmes et al. 2011).

Zhang et al. published a 2013 study detailing the degradation of 0.25 mM benzene with *Geobacter metallireducens* under Fe(III) reducing conditions (50 mM). Phenol was detected as a metabolite but benzoate was not, leading to a hypothesis that benzene degradation occurs via hydroxylation in these samples. To further investigate the formation of phenol as an intermediate, Zhang et al. introduced ^{18}O-radiolabeled H_2O into the media and discovered the formation of ^{18}O-radiolabeled phenol via anaerobic enzymatic reactions of *G. metallireducens*. Next, the genes PpsA and PpcB, were removed. As these are two subunits of the key enzymes required for phenol metabolism, the hypothesis in that hydroxylation to phenol as the initial action mechanism was validated when benzene degradation ceased (Zhang et al. 2013).

2.7 – Literature Review on Benzene Biodegradation in the Presence of Salinity

As previously mentioned, no research has been collected at the time of writing this thesis which involves the biodegradation of benzene in both saline and anaerobic conditions. The following instances of benzene biodegradation occur in varying salinity levels but occurred in an aerobic environment and therefore assumed to rely on oxygen as the terminal electron acceptor. Salinity concentrations can be expressed in several ways. In this section, the summary of each study will use the native manner in which salinity concentrations were expressed in that publication. On the next page, Table 2-5 serves to provide a convenient basis of comparison between these units of saline measurements.

Table 2-5 – Comparison Between Units of Salinity Concentrations

M or $\frac{\text{mole}}{\text{L}}$ NaCl	**% NaCl** (Approximate)	**$\frac{\text{g}}{\text{L}}$ NaCl** (Approximate)
0.25	1.5	14.6
0.50	2.9	29.2
0.75	4.4	44.8
1.0	5.8	58.4
2.0	11.7	116.9
3.0	17.5	175.3
4.0	23.4	233.8
5.0	29.2	292.2

In 2003, Nicholson and Fathepure collected soil samples from Seminole County in Oklahoma. The microbial culture in these samples underwent 7 to 8 months of continuous enrichment in which benzene was the sole carbon source, during which degradation averaged 12 μM/d (Nicholson and Fathepure 2004). At the conclusion of this enrichment period, the microbial community was adapted into 50 mL serum bottles consisting of 45 mL mineral media and 5 mL of the enriched culture. With an initial salinity level of 2.5 M NaCl, these samples repeatedly degraded all benzene (between 200 to 300 μM) within 2.5 weeks (Nicholson and Fathepure 2004). Following this success, Nicholson and Fathepure tested different salinity levels ranging from 0 to 4 M NaCl. At 0.5, 1.0, 2.0, and 2.5 M NaCl, 25 to 30 μmol benzene was degraded in the 50 mL bottles within 7 to 14 days, with 1.0 M NaCl being the most rapid (Nicholson and Fathepure 2004). No degradation was reported for the 0, 3.0 and 4.0 M NaCl salinity levels (Nicholson and Fathepure 2004). This inability to degrade at 0 M NaCl coupled with the degradation observed between 0.5 to 2.5 M NaCl suggests that the enrichment culture obtained are true halophiles. No reason was given as to why degradation did not occurred at 3.0 and 4.0 M NaCl.

Nicholson and Fathepure published a more extensive paper in 2006. In this study, soil samples from Great Salt Plains National Wildlife Refuge, Oklahoma were enriched on benzene and salinity. In 160 mL serum bottles containing 45 mL mineral media and 5 mL enriched culture and at 2.5 M NaCl, 220-300 μM of benzene were repeatedly and completely degraded within 7 to

10 days at room temperature within an incubation period of over 150 days (Nicholson and Fathepure 2005). In this experiment, ^{14}C-radiolabeled benzene was provided as the carbon source and 33% of which was reported to be converted to $^{14}CO_2$ (Nicholson and Fathepure 2005). Further testing involved investigating the effect temperature had on benzene biodegradation. The greatest degradation rate was 6.44 µM/d occurring at 30°C, followed by 5.96 µM/d occurring at 45°C. 50°C appeared to be the slowest at 0.77 µM/d (Nicholson and Fathepure 2005). Lastly, this experiment tested salinity concentrations of 0, 0.5, 1.0, 2.0, 2.5, 3.0, and 4.0 M NaCl. Each bottle was supplied with 21 to 23 µmole of benzene as the sole carbon source. The most efficient degradation was reported at the 1.0, 2.0, and 2.5 M NaCl concentrations and complete benzene biodegradation was achieved within 7 days (Nicholson and Fathepure 2005). Salinity levels 0, 0.5, and 3.0 M NaCl required up to 2 weeks for complete benzene removal while the greatest salinity concentration, 4.0 M NaCl, required approximately 4 weeks (Nicholson and Fathepure 2005). Nicholson and Fathepure proposed that at the highest salinity level, the increase salt content decreases benzene solubility, resulting in less accessibility for the microorganisms to reach the benzene in the mineral media. This explanation was supported as testing showed 68% of the benzene was partitioned into the headspace at 4.0 M NaCl in comparison to the 41% in the 1.0 M NaCl sample (Nicholson and Fathepure 2005).

In 2006, Li et al. published an aerobic benzene biodegradation study centered on a psychrotolerant and moderately haloalkaliphilic bacteria belonging to genus *Planococcus*. This microorganism was labelled as strain ZD22 and originated from the Daqing oil field, in the northern Heilongjiang Province of China (Li et al. 2006). In comparison to North America, the Heilongjiang Province is considered the Asian sister province of Alberta, Canada due to similar climates patterns. 100 mL of enriched isolate culture samples were placed in 500 mL serum bottles each amended with 2 mM of benzene as the sole carbon source. Salinity concentrations ranged between 0% and 26% NaCl (Li et al. 2006). ZD22 fully degraded 2mM of benzene within an incubation period of 3 days for salinity concentrations ranging between 5 to 20% NaCl. The optimal NaCl concentration was reported to be 10%. No degradation was reported for NaCl content at 0% and above 25% (Li et al. 2006). Furthermore, effects of temperatures were also studied: 0, 8, 15, 20, 25, 30, and 37°C. Serum bottles incubated at temperatures between 8°C and 30°C were capable of biodegrading 2 mM of benzene within 5 days. Li et al. reported the optimal temperature to be 20°C (requiring 45 hours for degradation) but 15°C and 25°C were comparable as well,

requiring approximately 52 and 64 hours respectively. This paper classified ZD22 as a psychrotolerant microorganism, but complete benzene biodegradation required approximately 97 hours at 8°C. Li et al. commented that this was a lower but acceptable degradation rate.

Sei and Fathepure developed and enriched a mix culture of microorganisms collected from the hypersaline sediments of Rozel Point at Great Salt Lake, Utah in 2009. After a period of enrichment, these cultures were placed within 50 mL serum bottles containing 2.5 M NaCl and benzene concentrations between 20 and 25 μM benzene. The samples were repeatedly capable of complete benzene biodegradation within 3 to 5 days (Sei and Fathepure 2009). Further testing involved the same bottle volumes and re-amendment to the same benzene concentrations but varying salinity concentrations between 0 and 5 M NaCl were explored. The samples at 1 and 2 M NaCl were reported to biodegrade all available benzene within 2 weeks. 3 weeks was required for bottles at 0, 3, and 4 M NaCl. The 5 M NaCl concentration proved most detrimental to degradation as it required upwards of 6 weeks (Sei and Fathepure 2009). In regards to the greater saline presence, Sei and Fathepure reported the degradation rates to be 1.69 μM/d at 4 M NaCl and 0.84 μM/d at 5 M NaCl (Sei and Fathepure 2009).

Berlendis et al. isolated two aerobic microbial bacteria in the *Marinobacter* genus in 2009. *Marinobacter vinifirmus* originates from the hypersaline industrial wastewater of a tartaric acid production plant and *Marinobacter hydrocarbonoclasticus* was isolated from Mediterranean seawaters in proximity to a petroleum refinery (Berlendis et al. 2010). While the focus of this study was the biodegradation of toluene by these two isolates, applications to degrade other BTEX compounds were also included at the latter portions of the paper. After a 3 day incubation period at 60 g/L NaCl, strain *M. vinifirmus* was reported to have completely degraded 100% of available benzene. In contrast, after 7 days of incubation at the same salinity level, *M. hydrocarbonoclasticus* only degraded approximately 10% of the available benzene (Berlendis et al. 2010). No specific values involving degradation rate, initial and final benzene concentrations or amounts were reported.

In 2010, Al-Mailem et al. published an aerobic benzene biodegradation study related to salinity. This paper claimed that isolates from the *Haloferax*, *Halobacterum*, and *Halococcus* strains were obtained from the hypersaline coast areas of the Arabian Gulf and were be capable of aerobic benzene degradation at salinity levels of 1, 2, 3, and 4 M NaCl (Al-Mailem et al. 2010).

However, no specific degradation rates, initial and final benzene concentration, or microcosm sizes were included in this report.

Soil samples from the Wadi El-Natrum soda lakes in proximity to Cairo, Egypt were obtained by Hassan et al. in 2012. 100 mL flasks containing 60 mL of mineral media along with 3 mL of liquid culture consisting of an isolate labelled as *Alcanivorax* sp. HA03 and 1.5 mM NaCl were capable of completely degrading 2 mM benzene within a 60 hour incubation period. The reported degradation rate in this initial enrichment period was 2.85µM/d (Hassan et al. 2012). Subsequently, the aerobic benzene degrading abilities of *Alcanivorax* sp. HA03 was tested under a range of salinity concentrations: 3, 7, 10, and 15% NaCl. Each flash was amended with 20 µmole of benzene. The degradation rates are as follows: 2.85, 1.5, 1.00, and 0.74 µM/d for the 3, 7, 10, and 15% NaCl concentrations respectively (Hassan et al. 2012). The degradation of benzene, an aromatic compound, expanded the understanding of *Alcanivorax sp*. as it was only previously demonstrated to degrade aliphatic hydrocarbons.

In 2013, Al-Mailem published another study. *Marinobacter sedimentarum* and M*arinobacter flavismaris* were isolates obtained from the hypersaline soils and waters in the southern and northern coasts of Kuwait. This paper commented on the growth of *M. sedimentarum* and *M. flavimaris* to be "good" when benzene was the sole carbon source during aerobic degradation (Al-Mailem et al. 2013). Optimal degradation was observed in salinities ranging between 1 to 1.5 M NaCl but a salinity level up to 5 M was also examined (Al-Mailem et al. 2013). Once again, no specific degradation rates, initial and final benzene concentration, or microcosm sizes were included in this report.

2.8 – Conclusion

The physical, chemical, and partitioning properties of benzene results in sorption to subsurface soils but also a high solubility which can allow significant off-site migration. In conjunction with its carcinogenic and toxic nature, this mobility causes benzene to be a concerning environmental contaminant. Biodegradation with a microbial community is an attractive option in tackling benzene contamination due to its lower cost, limited on-site management, non-intrusive nature, and ability to handle residual amounts of contaminant which challenges traditional means of remediation. However, one major problem of biodegradation is the stalling point between aerobic and anaerobic biodegradation, in which oxygen depletion forces the microbial community to rely upon other redox conditions which decreases in terms of thermodynamic favourability. To

date, three major benzene degradation pathways have been proposed, discussed, supported, and critiqued in literature: benzene methylation to toluene, hydroxylation to phenol, and carboxylation to benzoate.

Anaerobic benzene biodegradation has been a topic studied and demonstrated through experiments since the early 1980's. This literature review explored those of nitrate-, sulfate-reducing, and methanogenic conditions. The earlier publications simply focused on the possibility of benzene biodegradation under these redox conditions and reported varying rates of degradation. The subsequent studies increased in scope and complexity, and began considering concepts of co contaminants, intermediate by-products, metabolic pathways, and bacterial species responsible for degradation within a microbial community. Over the past four decades, technologies such as radiolabeling benzene and analysis with GC-MS to follow its transformation to downstream metabolites, isotope trapping to validate the presence of certain intermediates, and SIF to quantify in situ degradation have become staple analytical techniques in this topic. Application of 16s rRNA gene sequencing and DNA-SIP (or protein-SIP) has allowed researchers to identify and discover individual bacteria species involved with the biodegradation process.

In reality, benzene contamination within the oil and gas industry is often further convoluted as PHC impacted field sites commonly possess salt as a co-contaminant. The available studies which concern both salt and benzene only demonstrate aerobic degradation. In these experiments, technologies similar to the ones previously mentioned were also employed to quantify rates of degradation as well as to identify microbial organisms capable of degradation. This plethora of literature provides both reasons in which salt may stimulate biodegradation but may also inhibited biodegradation. In general, an optimal salt concentration was reported in each publication for which the greatest benzene biodegradation rate was observed.

Altogether, this chapter serves to provide the reader with the context of anaerobic benzene biodegradation in the presence of salinity. In the preparation of this thesis, no research had been identified which address both anaerobic conditions as well as a saline co-contaminant within the topic of benzene biodegradation. In the next chapter, the experimental set-up and analytical methods used will be discussed.

2.9 – Chapter 2 References

Abu Laban, N., Selesi, D., Jobelius, C., and Meckenstock, R. U. (2009). "Anaerobic Benzene Degradation by Gram-Positive Sulfate-Reducing bacteria." *FEMS Microbiology Ecology*, 68(3), 300–311.

Al-Mailem, D. M., Eliyas, M., and Radwan, S. S. (2013). "Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts." *Extremophiles*, 17(3), 463–470.

Al-Mailem, D. M., Sorkhoh, N. A., Al-Awadhi, H., Eliyas, M., and Radwan, S. S. (2010). "Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf." *Extremophiles*, 14(3), 321–328.

Anderson, R. T., and Lovley, D. R. (2000). "Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer." *Environmental Science and Technology*, 34(11), 2261–2266.

Atashgahi, S., Hornung, B., Van Der Waals, M. J., Da Rocha, U. N., Hugenholtz, F., Nijsse, B., Molenaar, D., Van Spanning, R., Stams, A. J. M., Gerritse, J., and Smidt, H. (2018). "A benzene-degrading nitrate-reducing microbial consortium displays aerobic and anaerobic benzene degradation pathways." *Scientific Reports*, Springer US, 8(1), 1–12.

Atkins, P., and de Paula, J. (2006). *Physical Chemistry for the Life Sciences*. W. H. Freeman and Company, New York, NY.

Atlas, R. M. (1981). "Microbial Degradation of Petroleum Hydrocarbons : an Environmental Perspective." *Microbiological reviews*, 45(1), 180–209.

Becalski, A. (2014). "Chapter 20 - Processing Contaminants: Benzene." *Encyclopedia of Food Safety*, 376–380.

Van Beelen, P., and Van Keulen, F. (1990). "The Kinetics of the Degradation of Chloroform and Benzene in Anaerobic Sediment From the River Rhine." *Hydrobiological Bulletin*, 24(1), 13–21.

Berlendis, S., Cayol, J. L., Verhé, F., Laveau, S., Tholozan, J. L., Ollivier, B., and Auria, R. (2010). "First Evidence of Aerobic Biodegradation of BTEX Compounds by Pure Cultures of Marinobacter." *Applied Biochemistry and Biotechnology*, 160(7), 1992–1999.

CCME. (2004). *Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health: Benzene*.

Chakraborty, R., O'Connor, S. M., Chan, E., and Coates, J. D. (2005). "Anaerobic Degradation of Benzene , Toluene , Ethylbenzene , and Xylene Compounds by Dechloromonas Strain RCB." *Applied and Environmental Microbiology*, 71(12), 8649–8655.

Chang, R. (2007). *Physical Chemistry for the Biosciences*. Univ Science Books, Sausalito, CA.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Bender, K. S., and Achenbach, L. A. (2001). "Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas." *Letters to Nature*, 411(6841), 1039–1043.

Coates, J. D., Chakraborty, R., and McInerney, M. J. (2002). "Anaerobic Benzene Biodegradation - A New Era." *Research in Microbiology*, 153(10), 621–628.

Dong, X., Dröge, J., von Toerne, C., Marozava, S., McHardy, A. C., and Meckenstock, R. U. (2017). "Reconstructing Metabolic Pathways of a Member of the Genus Pelotomaculum Suggesting its Potential to Oxidize Benzene to Carbon Dioxide with Direct Reduction of Sulfate." *FEMS Microbiology Ecology*, 93(3), 1–9.

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., and Wang, J. (2010). "Anaerobic Benzene Biodegradation by a Pure Bacterial Culture of Bacillus cereus Under Nitrate Reducing Conditions." *Journal of Environmental Sciences*, The Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, 22(5), 709–715.

Edwards, E. A., and Grbić-Galić, D. (1992). "Complete Mineralization of Benzene by Anaerobic Microorganisms under Strictly Anaerobic Conditions." *American Society for Microbiology*, 58(8), 2663–2666.

Foght, J. (2008). "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects." *Journal of Molecular Microbiology and Biotechnology*, 15(2–3), 93–120.

Grbić-Galić, D., and Vogel, T. M. (1987). "Transformation of Toluene and Benzene by Mixed Methanogenic Cultures." *Applied and Environmental Microbiology*, 53(2), 254–260.

Harwood, C. S., Burchhardt, G., Herrmann, H., and Fuchs, G. (1998). "Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway." *FEMS Microbiology Reviews*, 22(5), 439–458.

Harwood, C. S., and Gibson, J. (1997). "Shedding light on anaerobic benzene ring degradation: A process unique to prokaryotes?" *Journal of Bacteriology*, 179(2), 301–309.

Hassan, H. A., Rizk, N. M., Hefnaway, M. A., and Awad, A. A. (2012). "Isolation and Characterization of Halophilic Aromatic and Chloroaromatic Degrader from Wadi El-

Natrun Soda." *Life Science Journal*, 9(3), 1565–1570.

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H. H., and Vogt, C. (2010). "Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing." *Environmental Microbiology*, 12(2), 401–411.

Holmes, D. E., Risso, C., Smith, J. A., and Lovley, D. R. (2011). "Anaerobic oxidation of benzene by the hyperthermophilic archaeon Ferroglobus placidus." *Applied and Environmental Microbiology*, 77(17), 5926–5933.

Jahn, M. K., Haderlein, S. B., and Meckenstock, R. U. (2005). "Anaerobic Degradation of Benzene, Toluene, Ethylbenzene, and o-Xylene in Sediment-Free Iron-Reducing Enrichment Cultures." *Applied and Environmental Microbiology*, 71(6), 3355–3358.

Johnson, S. J., Woolhouse, K. J., Prommer, H., Barry, D. A., and Christofi, N. (2003). "Contribution of anaerobic microbial activity to natural attenuation of benzene in groundwater." *Engineering Geology*, 70(3–4), 343–349.

Kao, C.-M., and Borden, R. C. (1997). "Site-Specific Variability in BTEX Biodegradation Under Denitrifying Conditions." *Ground Water*, 35(2), 305–311.

Kazumi, J., Caldwell, M. E., Suflita, J. M., Lovley, D. R., and Young, L. Y. (1997). "Anaerobic degradation of benzene in diverse anoxic environments." *Environmental Science and Technology*, 31(3), 813–818.

Keller, A. H., Kleinsteuber, S., and Vogt, C. (2018). "Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics." *Microbial Ecology*, Microbial Ecology, 75(4), 941–953.

Kerr, R. P., and Capone, D. G. (1988). "The effect of salinity on the microbial mineralization of two polycyclic aromatic hydrocarbons in estuarine sediments." *Marine Environmental Research*, 26(3), 181–198.

Knovel. (2008). "Basic Physical Properties of Chemical Compounds." *Knovel Critical Tables (2nd Edition)*.

Langenhoff, A. A. M., Zehnder, A. J. B., and Schraa, G. (1996). "Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns." *Biodegradation*, 7(3), 267–274.

Li, H., Liu, Y. H., Luo, N., Zhang, X. Y., Luan, T. G., Hu, J. M., Wang, Z. Y., Wu, P. C., Chen, M. J., and Lu, J. Q. (2006). "Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic Planococcus sp. strain ZD22." *Research in Microbiology*, 157(7), 629–636.

Lovley, D. R., Coates, J. D., Woodward, J. C., and Phillips, E. J. P. (1995). "Benzene Oxidation Coupled to Sulfate Reduction." *American Society for Microbiology*, 61(3), 953–958.

Luo, F., Devine, C. E., and Edwards, E. A. (2016). "Cultivating Microbial Dark Matter in Benzene-Degrading Methanogenic Consortia." *Environmental Microbiology*, 18(9), 2923–2936.

Luo, F., Gitiafroz, R., Devine, C. E., Gong, Y., Hug, L. A., Raskin, L., and Edwards, E. A. (2014). "Metatranscriptome of an Anaerobic Benzene-Degrading, Nitrate-Reducing Enrichment Culture Reveals Involvement of Carboxylation in Benzene Ring Activation." *Applied and Environmental Microbiology*, 80(14), 4095–4107.

Lyons, W. C., Plisga, G. J., and Lorenz, M. D. (2016). "Aqueous Solubility Data for Selected Petroleum Products." *Standard Handbook of Petroleum and Natural Gas Engineering (3rd Edition)*.

Meckenstock, R. U., Boll, M., Mouttaki, H., Koelschbach, J. S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A. M. (2016). "Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons." *Journal of Molecular Microbiology and Biotechnology*, 26(1–3), 92–118.

Nales, M., Butler, B. J., and Edwards, E. A. (1998). "Anaerobic benzene biodegradation: A microcosm survey." *Bioremediation Journal*, 2(2), 125–144.

Nicholson, C. A., and Fathepure, B. Z. (2004). "Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions." *Applied and Environmental Microbiology*, 70(2), 1222–1225.

Nicholson, C. A., and Fathepure, B. Z. (2005). "Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma." *FEMS Microbiology Letters*, 245(2), 257–262.

Phelps, C. D., Kazumi, J., and Young, L. Y. (1996). "Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction." *FEMS Microbiology Letters*, 145(3), 433–437.

Phelps, C. D., Kerkhof, L. J., and Young, L. Y. (1998). "Molecular characterization of a sulfate-

reducing consortium which mineralizes benzene." *FEMS Microbiology Ecology*, 27(3), 269–279.

Phelps, C. D., Zhang, X., and Young, L. Y. (2001). "Use of Stable Isotopes to Identify Benzoate as a Metabolite of Benzene Degradation in a Sulphidogenic Consortium." *Environmental Microbiology*, 3(9), 600–603.

Reinhard, M., Shang, S., Kitanidis, P. K., Orwin, E., Hopkins, G. D., and Lebron, C. A. (1997). "In Situ BTEX Biotransformation under Enhanced Nitrate- and Sulfate-Reducing Conditions." *Environmental Science & Technology*, 31(1), 28–36.

Sakai, N., Kurisu, F., Yagi, O., Nakajima, F., and Yamamoto, K. (2009). "Identification of putative benzene-degrading bacteria in methanogenic enrichment cultures." *Journal of Bioscience and Bioengineering*, The Society for Biotechnology, 108(6), 501–507.

Sei, A., and Fathepure, B. Z. (2009). "Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake." *Journal of Applied Microbiology*, 107(6), 2001–2008.

Shiu, W. Y., Ma, K. C., Mackay, D., Seiber, J. N., and Wauchope, R. D. (1990). "Solubilities of pesticide chemicals in water. Part I: Environmental physical chemistry." *Reviews of environmental contamination and toxicology*, 116, 1–13.

Stryer, L. (1988). *Biochemistry*. W. H. Freeman and Company, New York, NY.

Taubert, M., Vogt, C., Wubet, T., Kleinsteuber, S., Tarkka, M. T., Harms, H., Buscot, F., Richnow, H. H., Von Bergen, M., and Seifert, J. (2012). "Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing, benzene-degrading microbial consortium." *ISME Journal*, 6(12), 2291–2301.

Ulrich, A. C. (2004). "Characterization of Anaerobic Benzene-Degrading Cultures." University of Toronto.

Ulrich, A. C., Beller, H. R., and Edwards, E. A. (2005). "Metabolites detected during biodegradation of 13C 6-benzene in nitrate-reducing and methanogenic enrichment cultures." *Environmental Science and Technology*, 39(17), 6681–6691.

Ulrich, A. C., and Edwards, E. A. (2003). "Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures." *Environmental Microbiology*, 5(2), 92–102.

Vallero, D. (2008). "Chapter 6 - Air Pollution Systems and Processes." *Fundamentals of Air Pollution (4th Edition)*, 154–198.

Vieth, A., Kästner, M., Schirmer, M., Weiß, H., Gödeke, S., Meckenstock, R. U., and Richnow, H. H. (2005). "Monitoring In Situ Biodegradation of Benzene and Toluene by Stable Carbon Isotope Fractionation." *Environmental Toxicology and Chemistry*, 24(1), 51–60.

Vogel, T. M., and Grbić-Galić, D. (1986). "Incorporation of Oxygen from Water into Toluene and Benzene during Anaerobic Fermentative Transformation." *Applied and Environmental Microbiology*, 52(1), 200–202.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). "Anaerobic benzene degradation by bacteria." *Microbial Biotechnology*, 4(6), 710–724.

van der Waals, M. J., Atashgahi, S., da Rocha, U. N., van der Zaan, B. M., Smidt, H., and Gerritse, J. (2017). "Benzene degradation in a denitrifying biofilm reactor: activity and microbial community composition." *Applied Microbiology and Biotechnology*, Applied Microbiology and Biotechnology, 101(12), 5175–5188.

Weiner, J. M., and Lovley, D. R. (1998). "Rapid Benzene Degradation in Methanogenic Sediments from a Petroleum-Contaminated Aquifer." *American Society for Microbiology*, 64(5), 1937–1939.

Wilson, S., Card, G., and Haines, S. (2009). "Table 3.3 - Properties of Some Commonly Occurring Vapours." *Ground Gas Handbook*, Whittles Publishing.

Winfrey, M. R., Beck, E., Boehm, P., and Ward, D. M. (1982). "Impact of Crude Oil on Sulphate Reduction and Methane Production in Sediments Impacted by The Amoco Cadiz Oil Spill." *Marine Environmental Research*, 7(3), 175–194.

Yang, L., Lai, C.-T., and Shieh, W. K. (2000). "Biodegradation of Dispersed Diesel Fuel Under High Salinity Conditions." *Water Research*, 34(13), 3033–3314.

Yaws, C. L. (2014). "Table 118 - Henry's Law Constant for Compound in Water - Organis Compounds." *Yaws' Critical Property Data for Chemical Engineers and Chemists*.

van der Zaan, B. M., Saia, F. T., Stams, A. J. M., Plugge, C. M., de Vos, W. M., Smidt, H., Langenhoff, A. A. M., and Gerritse, J. (2012). "Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntrophic process." *Environmental Microbiology*, 14(5), 1171–1181.

Zhang, T., Tremblay, P. L., Chaurasia, A. K., Smith, J. A., Bain, T. S., and Lovley, D. R. (2013). "Anaerobic benzene oxidation via phenol in Geobacter metallireducens." *Applied and Environmental Microbiology*, 79(24), 7800–7806.

CHAPTER 3

Experimental Set-Up, Analytical Procedures, and Data Interpretation

3.1 – Introduction

This chapter consists of three parts. In the first component, Section 3.2, the origins and experimental set up of the microcosms (or treatments) are provided in detail. In addition, the procedures of the manipulations or maintenance work involving these treatments throughout the Culture Enrichment period and Salinity Experiment are also described. These include the anaerobic media mix (AMM) recipe, benzene, nitrate, sulfate, salt (re)amendment, and the designation system used to label the treatments.

In the second portion, Section 3.3, the various procedures and instrumental set-ups of the analytical methods used in this experiment are discussed. These analytical methods include utilizing a gas chromatography system equipped with a flame ionization detector (GC-FID) along with a purge and trap variant (P+T GC-FID) to monitor the benzene content in the headspace of the treatment bottles. Additional analytical methods include utilizing an ion chromatography (IC) system to determine nitrate, nitrite, and sulfate levels within the AMM and a gas chromatography system with a thermal conductivity detector (GC-TCD) to monitor headspace methane content the methanogenic treatment bottles.

Section 3.4 is the last component of this chapter and details the calculation methods used to interpret the results for the discussion component of Chapters 4 and 5. Terms such as degradation cycle, initial degradation, residual degradation, re-equilibration period, overall degradation rate, maximum degradation rate, lag phase, and terminal electron acceptor utilization ratio are key metrics used in the discussion of the following chapters. A formal definition and the calculation process of each of these terms is provided in Section 3.4.

3.2 – Experimental Set-up

3.2.1 – Microbial Culture Origin and Microcosm Set up

The clay and sand sediments from which the microcosms of this thesis were derived, originated from uncontaminated sediment cores collected during the construction of a groundwater monitoring well research transect in 2006. This project was located in what is currently known as the South Tailings Pond on the Suncor Energy Inc. mine lease, approximately 35 km north of Fort McMurray, Alberta, Canada (Holden 2012). The wells were installed across a sand channel flow system immediately downgradient of the South Tailings Pond. These subsurface sediments were collected during borehole drilling (0.11 m casing on a 1503 Nodwell Sonic Drill Rig). During

drilling, polycarbonate resin sleeves lined the drill stem and encapsulated produced core during the drilling process in attempt to preserve the subsurface redox conditions. These cores were then transported to the University of Alberta, and placed in a -20°C freezing unit belonging to the Cold Regions Geoenvironmental Research Facility (Holden 2012). Following geological classification of the collected sediment cores, a radial arm saw was used to cut the core into slices containing the same sediment material (clay or sand). Special care was given to measuring the moisture content of these samples during the thawing process and subsequently maintained at those native moisture levels to prevent potential complications associated with sediment drying such as precipitation of mineral phases (Holden 2012). The newly obtained sand and clay sediments were then incorporated into microcosm studies involving various PHC substrates and redox conditions. These treatments have endured multiple rounds of dilution and benefitted from years of enrichment on carbon sources including benzene since its creation. Should the reader be interested in more details on this process, they are referred to the 2012 Ph. D thesis of Alexander Holden, which discussed in detail the origins of these sediments and microbial cultures as well as the first laboratory experiments involved with these samples.

In regard to the experiment of this thesis, the necessary hardware such as serum bottles, butyl rubber stoppers, and crimp and caps were washed with ultrapure water and autoclaved for microcosm set-up. Microcosm set up was performed within an anaerobic glove box. The airlock chamber and anaerobic glove box (model AALC) were purchased from Coy Laboratory Products (Grass Lake, Michigan). All necessary materials were placed inside the airlock. Prepackaged sterile consumables that are sealed received a needle sized hole punctured into the packaging to allow for proper gas sparging. Within the airlock, two sparge cycles with N_2 gas followed by one cycle of a gas mixture containing 5% CO_2, 5% H_2, and a N_2 balance occurred before entering the anaerobic glove box. These gas tanks were purchased from Praxair (Edmonton, AB).

In the Salinity Experiment, microcosms consist of 30 mL of anaerobic media mix (see Section 3.2.2) containing the microbial culture and trace particles of the original sediments, and housed within a 60 mL glass serum bottle purchased from Fisher Scientific (Toronto, ON). As microcosm set-up occurred within the anaerobic glove box, the remaining 30 mL headspace of the microcosm consisted of the gas mixture previously described (5% CO_2, 5% H_2, and a N_2 balance). When necessary, calculated volumes of NaCl and/or electron acceptor stock solution is added to the microcosm via a 1 mL syringe (Fischer Scientific, Toronto, ON) (see Sections 3.2.3 through

3.2.6). The microcosms were stoppered with a butyl rubber septum and sealed with a crimp and cap system also purchased from Fischer Scientific. Below, Figure 3-1 displays the appearance of a typical microcosm bottle in the Culture Enrichment period.

Figure 3-1 – Typical Appearance of a Treatment Bottle. In this picture, microcosm CNB[1] of the Culture Enrichment period is shown. Notable features of this picture include the anaerobic media mix containing the microbial culture as well as trace amounts of the original clay sediments, headspace (approximately greater than the liquid volume due to repeated IC analysis), and rubber butyl stopper and crimp and cap stopper system to seal the bottle. Picture taken on May 31, 2017.

3.2.2 – Anaerobic Media Mix Recipe

The anaerobic media mix (AMM) recipe used in this experiment was developed and shared by the Edwards lab at the University of Toronto. This recipe is summarized below.

The AMM consists of a combination of eight separate master mixes (MM1 through MM8) which must be created first. It should be noted that MM7 cannot be used after several months post-preparation. Each of the eight master mixes contain the listed compounds and are prepared as follows:

MM1 - Phosphate Buffer (100x):

- 20.96 g KH_2PO_4, 42.85 g K_2HPO_4
- Dissolve in 1 L of ultrapure water

MM2 - Salt Solution (100x):

- 53.5 g NH_4Cl, 7 g $CaCl_2 \cdot 6H_2O$ (or 4.79 g $CaCl_2 \cdot 2H_2O$), 2 g $FeCl_2 \cdot 4H_2O$
- Dissolve in 1 L of ultrapure water

MM3 - Trace Minerals (500x):

- 0.3 g H_3BO_3, 0.1 g ZnCl, 0.073 g $(NH_4)_6Mo_7O_{24}\cdot 7H_2O$, 0.75 g $NiCl_2\cdot 6H_2O$, 1 g $MnCl_2\cdot 4H_2O$, 0.1 g $CuCl_2\cdot 2H2O$, 1.5 g $CoCl_2\cdot 6H_2O$, 0.02 g Na_2SeO_3, 0.1 g $Al_2(SO_4)_3\cdot 18H_2O$
- Add 1 mL 1 M H_2SO_4
- Dissolve in 1 L of ultrapure water

MM4 - Magnesium Sulfate Solution (500x)

- 62.5 g/L $MgSO^4\cdot 7H_2O$ or minimizing sulfate reductions 50.8 g/L $MgCl_2\cdot 6H_2O$

MM5 - Redox Indicator

- 1 g/L resazurin

MM6:

- 20 g $NaHCO_3$, 100 mL ultrapure water in 160 mL serum bottle
- Cover with foil and autoclave
- Sparge with N_2 for 15 minutes and seal with crimp

MM7 – Frozen Aliquots of Vitamins (10000x) and Working Stock (100x):

- 0.02 g biotin, 0.02 g folic acid, 0.1 g pyridoxine HCl, 0.05 g riboflavin, 0.05 g thiamine, 0.05 g nicotinic acid, 0.05 g pantothenic acid, 0.05 g PABA, 0.05 g cyanocobalamin i.e. vitamin B12, 0.05 g thioctic (lipoic) acid, 1 g coenzyme M
- Dissolve in 1 L of ultrapure water
- Adjust pH to 7 with 2 N NaOH
- Freeze in 1 mL aliquots
- To make working stocks, autoclave a foil-wrapped 160 mL serum bottle and crimp, then prepare with a 250 mL beaker, hyper-filtered water, 10 mL syringe, 2x 0.22 um filters, and the frozen stock into the glovebox. Dilute the stock 1:100 into ultrapure water and filter sterilize into the serum bottle. Seal and crimp.

MM8 - Amorphous Ferrous Sulfide 2 g/L

- Deoxygenate 2.5 L of ultrapure water by cycling in the airlock and leaving in glovebox for over 1 hour
- Weigh out 19.6 g $(NH_4)_2Fe(SO_4)_2\cdot 6H_2O$ i.e. (ferrous ammonium sulfate)
- Weight out 12 g $Na_2S\cdot 9H_2O$ (i.e. sodium sulfide)

- Place the two compounds and a 1 L Erlenmeyer flask with a stopper into the glovebox
- Pour 500 mL ultrapure water into the 1 L Erlenmeyer flask and add $Na_2S \cdot 9H_2O$. Mix till dissolved
- Add the $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. Place the Erlenmeyer flask immediately into the airlock and cycle to evacuate the H_2S being formed
- Return the Erlenmeyer flask to the glovebox and allow the precipitate to settle for 24 hours
- Decant the supernatant and wash the precipitate with 500 mL ultrapure water. Allow precipitate to settle for another 24 hours. Repeat thrice to remove free sulfide in the water
- Resuspend to 500 mL with ultrapure water and dispense into five 160 mL serum bottles. Crimp and autoclave the serum bottles.

After these eight master mixes were prepared, the following procedure were performed to create the AMM.

- Mix 955 mL ultrapure water, 10 mL MM1, 10 mL MM2, 2 mL MM3, 2 mL MM4, and 1 mL MM5
- Wrap the end of the bottle with tinfoil and autoclave
- Ethanol rinse the work surface, surrounding areas, and any other object which may come into physical contact with the following steps
- Prepare a N_2 sparging station with the necessary tubing and regulator. Ensure that all the lining equipment which may contact the media bottle must be sterile and autoclaved.
- Lift the tinfoil from the bottle and gently insert the sparging tube into the media. Close off the opening at the top of the bottle by wrapping the tinfoil around the tubing (hand tighten)
- Sparge the bottle with N_2 gas for a minimum of half an hour or until the media has transformed from a deep shade of blue or purple to a clear or slightly pink hue. Figure 3-2 below depicts the naturally pink color of AMM.

Figure 3-2 – Treatment Bottle Demonstrating the Natural Pink Color Hue of AMM

- The reason for this color changed is caused by resazurin of MM5. When exposed to oxygen, resazurin remains a deep shade of blue or purple. Upon deoxygenation, this color will lighten and become clear. When the deoxygenation process reaches anaerobic conditions associated with sulfate-reducing conditions of the redox ladder (or lower), the AMM may take on a pink hue.
- Remove the sparging equipment carefully and cap the bottle
- Cycle the bottle in the glovebox to remove any trace amounts of oxygen during the capping process
- Withdraw and deposit 10 mL of MM6, 7, and 8 into the media within the glove box
- Wait until the media remains consistently clear or slightly pink in color. This may take upwards of two weeks. Afterwards the AMM is ready for use

3.2.3 – Benzene Stock Solution and Benzene Amendment

Neat benzene was kept in a 100 mL amber vial (Fischer Scientific, Toronto, ON) as a stock solution. This benzene was HPLC grade and of ≥99.9% purity (Sigma Aldrich, Oakville, ON).

Every benzene (re)amendment accounted for the existing benzene concentration within the AMM of a treatment bottle. The required volumes of the benzene stock for reamendment were calculated based on the mass difference between the exisiting and target benzene concentrations, the density of benzene (0.877 $g \cdot cm^{-3}$), and the dimensionless Henry's Law constant (0.2289) (Becalski 2014; Knovel 2008; Wilson et al. 2009). This desired volume was injected into treatment bottles with the use of a 10 µL glass syringe from Hamilton Co. (Reno, NV) after flushing with methanol.

3.2.4 – NaCl Stock Solution and NaCl Amendment

NaCl stock solutions were housed within 60 mL serum bottles (Fischer Scientific, Toronto, ON) and contain 30 mL of ultrapure water. All glassware and accessories were cleaned and autoclaved as described in Section 3.2.1 prior to use. To create a 1 M stock solution, 1.75 g of ≥ 99.0% sodium chloride (NaCl) (Fischer Scientific, Toronto, ON) was placed within the serum bottle. The bottles were stirred until the added compound had completely dissolved, closed with a rubber butyl stopper, and sealed with a crimp and cap system (Fischer Scientific, Toronto, ON). The stock solution was then autoclaved and sparged for 1 hour by a 5% CO_2, 5% H_2, and N_2 balance gas mixture (Praxair, Edmonton, AB). NaCl stock solutions were stored within the anaerobic glove box.

The Salinity Experiment explored benzene biodegradation under a range of salt concentrations: 0, 0.5, 1.0, and 2.0 g/L NaCl. Below, Table 3-1 tabulates the volume of 1 M NaCl stock amended to treatment bottles at each salt level.

Table 3-1 – Volumes of 1 M NaCl Stock at Each Salt Concentration

Salt Concentration	Volume of 1 M NaCl Stock
0.0 g/L	None
0.5 g/L	0.26 mL
1.0 g/L	0.51 mL
2.0 g/L	1.03 mL

The microcosms and necessary syringe and needles were cycled into the anaerobic glove box as outlined in Section 3.2.1. A sterile work surface within the glove box was created by wiping with an ethanol solution. The rubber butyl stoppers of the microcosm and salt stock was also wiped with the ethanol solution. The corresponding volumes of 1 M NaCl solution were withdrawn with a syringe and needle and injected into the microcosm in accordance with Table 3-1. A new syringe and needle were used for each microcosm regardless of salt concentrations or redox condition.

3.2.5 – Nitrate Stock Solution and Nitrate Amendment

Nitrate stock solutions were housed within 60 mL serum bottles (Fischer Scientific, Toronto, ON) and contain 30 mL of ultrapure water. All glassware and accessories were cleaned and autoclaved as described in Section 3.2.1 prior to use. To create a 1 M stock solution, 2.55 g of 98+% sodium nitrate ($NaNO_3$) (Fischer Scientific, Toronto, ON) was placed within the serum

bottle. The bottles were stirred until the added compound had completely dissolved, stoppered with a butyl rubber stopper, and sealed with a crimp and cap system (Fischer Scientific, Toronto, ON). The stock solution was then autoclaved and sparged for 1 hour by a 5% CO_2, 5% H_2, and a N_2 balance gas mixture (Praxair, Edmonton, AB). Nitrate stock solutions were stored within the anaerobic glove box.

The target nitrate concentration for nitrate-reducing treatments was 5 mM. The reasoning for this target concentration is discussed in Chapter 4. For fresh microcosms with no nitrate, 0.15 mL of the 1 M nitrate stock was injected into the microcosm with same process as salt amendment (Section 3.2.4). For existing microcosms which required nitrate reamendment, volumes of the stock solution were calculated based on IC data for existing nitrate content, the mass difference between existing nitrate and the target concentration, and the volume within the treatment bottle.

3.2.6 – Sulfate Stock Solution and Sulfate Amendment

Sulfate stock solutions were housed within 60 mL serum bottles (Fischer Scientific, Toronto, ON) and contain 30 mL of ultrapure water. All glassware and accessories were cleaned and autoclaved as described in Section 3.2.1 prior to use. To create a 1 M stock solution, 4.26 g 99.99% of sodium sulfate (Na_2SO_4) (Fischer Scientific, Toronto, ON) was placed within the serum bottle. The bottles were stirred until the added compound dissolved, stoppered with a rubber butyl stopper, and sealed with a crimp and cap system (Fischer Scientific, Toronto, ON). The stock solution was then autoclaved and sparged for 1 hour by a 5% CO_2, 5% H_2, and N_2 balance gas mixture (Praxair, Edmonton, AB). Sulfate stock solutions were stored within the anaerobic glove box.

The target sulfate concentration for sulfate-reducing treatments was 15 mM. The reasoning for this target concentration is discussed in Chapter 4. For fresh microcosms with no sulfate, 0.45 mL of the 1 M sulfate stock was injected into the microcosm with same process as salt amendment (Section 3.2.4). For existing microcosms which required sulfate reamendment, volumes of the stock solution were calculated based on IC data for existing sulfate content, the mass difference between existing sulfate and the target concentration, and the volume within the treatment bottle.

3.2.7 – Microcosm Designations

During the Culture Enrichment period, 12 microcosm or treatment bottles were dedicated to anaerobic benzene biodegradation (four bottles for each of the three redox conditions). The bottles are listed in Table 3-2 below.

Table 3-2 – Treatments of the Culture Enrichment Period

Nitrate-Reducing	Sulfate-Reducing	Methanogenic
CNB^1	CSB^1	CMB^1
CNB^2	CSB^2	CMB^2
SNB^1	SSB^1	SMB^1
SNB^2	SSB^2	SMB^2

The first letter in each designation refers to the whether the treatment bottle was originally derived from clay or sand sediments: "C" for clay and "S" for sand. Section 3.2.1 summarizes the sampling process in which these source materials were collected.

The second letter refers to the three redox conditions for which this thesis explores: "N" for nitrate-reducing, "S" for sulfate-reducing, and "M" for methanogenic conditions.

The third letter is "B", indicating benzene degradation. All treatments bottles in this experiment utilize benzene as the sole carbon source. This third letter simply served to differentiate against treatment bottles which degrade other PHC compounds (i.e. toluene) which was not apart of this thesis.

In the second phase, the Salinity Experiment, 66 treatment bottles were dedicated for anaerobic benzene biodegradation when influenced by salinity (22 treatments per redox condition) (Table 3-3). Within each 22 treatment bottle set, 13 contain a live microbial culture while the remaining nine served as controls. Each set contained the following:

- Quadruplets at 0.0 g/L NaCl;
- Triplicates at 0.5 g/L NaCl;
- Triplicates at 1.0 g/L NaCl;
- Triplicates at 2.0 g/L NaCl;
- Triplicates of 0.0 g/L NaCl sodium azide kill controls;
- Triplicates of 1.0 g/L NaCl sodium azide kill controls; and,
- Triplicates of media controls amended with terminal electron acceptor.

Table 3-3 – Treatments of the Salinity Experiment

	Nitrate-Reducing	Sulfate-Reducing	Methanogenic
0.0 g/L NaCl	N0.0A / N0.0B / N0.0C / N0.0D	S0.0A / S0.0B / S0.0C / S0.0D	M0.0A / M0.0B / M0.0C / M0.0D
0.5 g/L NaCl	N0.5A / N0.5B / N0.5C	S0.5A / S0.5B / S0.5C	M0.5A / M0.5B / M0.5C
1.0 g/L NaCl	N1.0A / N1.0B / N1.0C	S1.0A / S1.0B / S1.0C	M1.0A / M1.0B / M1.0C
2.0 g/L NaCl	N2.0A / N2.0B / N2.0C	S2.0A / S2.0B / S2.0C	M2.0A / M2.0B / M2.0C
0.0 g/L NaCl Sodium Azide	N0.0A SA / N0.0B SA/ N0.0C SA	S0.0A SA / S0.0B SA/ S0.0C SA	M0.0A SA / M0.0B SA/ M0.0C SA
1.0 g/L NaCl Sodium Azide	N1.0A SA / N1.0B SA/ N1.0C SA	S1.0A SA / S1.0B SA/ S1.0C SA	M1.0A SA / M1.0B SA/ M1.0C SA
0.0 g/L NaCl Media Only	N0.0A MED / N0.0B MED / N0.0C MED	S0.0A MED / S0.0B MED/ S0.0C MED	M0.0A MED / M0.0B MED/ M0.0C MED

3.3 – Analytical Procedures

In this section, the four analytical methods used throughout the Culture Enrichment period and the Salinity Experiment will be discussed. Two instruments were employed for headspace benzene monitoring. A GC-FID was used from May 10, 2017 to September 11, 2018 but instrument error resulted in a switchover to a P+T GC-FID until the conclusion of the experiment on May 22, 2019. An IC system was operated to monitor the nitrate, nitrite, and sulfate levels within the treatments bottles. Lastly, a GC-TCD was utilized for monitoring headspace methane content.

3.3.1 – Gas Chromatography - Flame Ionization Detector

Benzene measurements were performed with an Agilent 7890A Gas Chromatography (GC) system equipped with a flame ionization detection (FID) utilizing an Agilent HP-5 (19091J-413) column of the following dimensions: 30 m × 320 µm × 0.2 µm. The column flow was set to 4.5 mL/min under a pressure of 17.735 psi. The oven temperature gradient was as follows: 50°C for 2.5 minutes and an equilibration time of 3 minutes. The purge flow to split vent rate was set at 40 mL/min starting at 0.5 minutes. Total run time was approximately 3 minutes.

The detector was maintained at 250°C, and the inlet injection port at 250°C. H_2 gas was used as the source of the FID and set at a flow rate of 45 mL/min and air flow at 450 mL/min. The makeup / carrier flow gas was helium and set to 25 mL/min. The injector split-less had a septum purge flow of 3 mL/min under a pressure of 17.735 psi. The lower detection limit of the GC-FID for benzene analysis was approximately 0.25 mg/L.

Injection volumes were 200 µL and collected with a Hamilton Co. (Reno, NV) 250 µL gas syringe with a Chaney Adapter (model # 702/750) designed for repeated headspace withdraws.

A standard curve was developed during each monitoring to interpret the data of each injection of the treatment bottles. This standard curve consisted of benzene standards prepared in the previous day containing benzene concentrations at 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, and 30.0 mg/L. For the purposes of demonstrating this concept, Table 3-4 displays the injection data related to the benzene standards on November 18, 2017's monitoring session.

Table 3-4 – GC-FID Standard Curve Data – Example Set

Standard Benzene Concentration (mg/L)	Retention Time (min)	Area
0.5	1.313	348.6
1.0	1.310	631.2
2.0	1.311	1,461.6
3.0	1.311	2,088.27
5.0	1.312	3,562.41
10.0	1.311	6,289.27
20.0	1.313	12,747.9
30.0	1.311	16,716

Next, the area counts of the benzene standards were plotted against the benzene concentrations. A line of best fit containing a vertical intercept of 0 was developed and the resulting equation was used as the standard curve for the remaining data. This is shown in Figure 3-3 below.

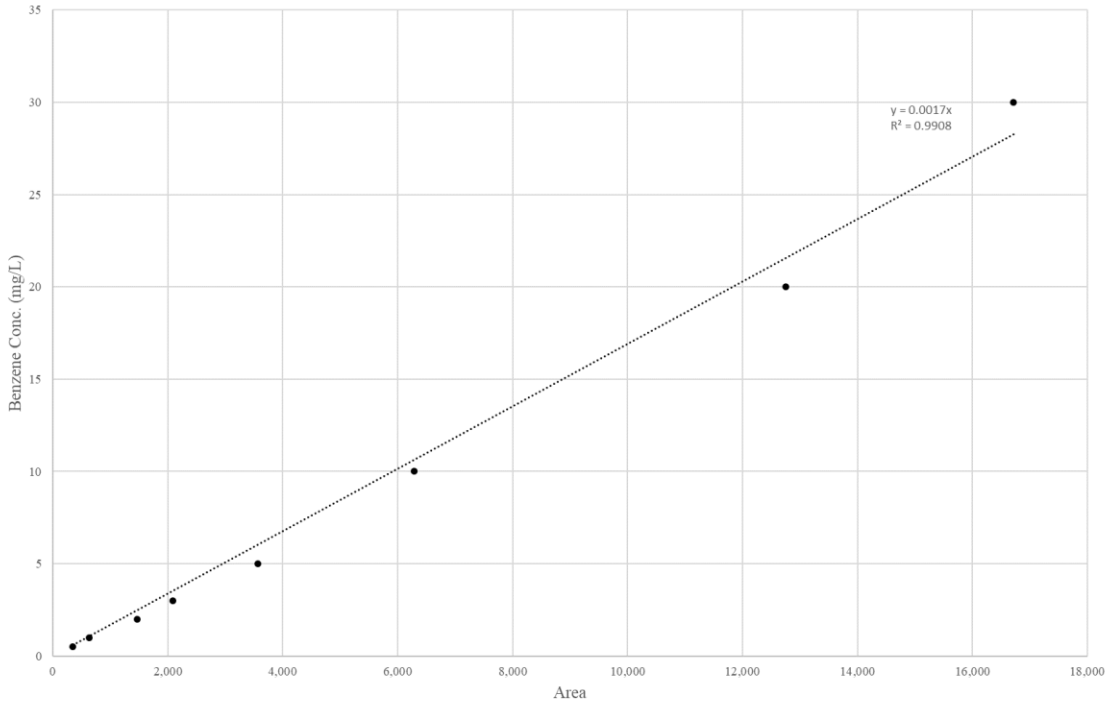

Figure 3-3 – GC-FID Standard Curve – Example Set. Benzene concentration (●) of each standard injection.

In this example, a standard curve equation of "Y = 0.0017X" was developed. The Y-variable corresponds to the benzene concentration in the injected sample, while the X-variable is the area count obtained from the GC-FID. The area counts of each injection was then used in this equation to calculate a headspace benzene concentration. The dimensionless Henry's law coefficient was used in to obtain the benzene concentration within the liquid fraction of the treatment bottle. For example, if the GC-FID indicated a sample had an area count of "5,162.32", then the following calculation were made:

$$\text{Headspace Conc.} = 0.0017 \times 5{,}162.32 = 8.15 \ \frac{\text{mg Benzene}}{\text{L headspace}}$$

$$\text{Liquid Conc.} = \frac{\text{Headspace Conc.}}{\text{Dimensionless Henry's Law Constant}} = \frac{8.15 \text{ mg/L}}{0.192125} = 42.42 \ \frac{\text{mg Benzene}}{\text{L media}}$$

A series of tests involving standards with decreasing benzene content were tested at the beginning of the experiment. These tests indicated the GC-FID to have a lower detection limit of approximately 0.25 mg/L.

3.3.2 – Purge and Trap Gas Chromatography - Flame Ionization Detector

Benzene measurements were also performed with a Hewlett Packard 6890m Series Gas Chromatography (GC) system equipped with a Hewlett Packard 7695 Purge and Trap (P+T) Concentrator and flame ionization detection (FID) utilizing an Agilent Durabond DB-5MS UI (122-5512UI) column of the following dimensions: 15 m × 0.250 µm × 0.25 µm. The column flow was set to 7.4 mL/min under a pressure of 5 psi. The oven temperature gradient was as follows: 36°C for 4 minutes, with a first ramp of 5°C/min to 150°C with no hold time. The second ramp was 15°C/min until 240°C and held for 6 minutes, resulting in a total run time of 38.8 minutes per injection. The detector was maintained at 250°C. The inlet injection port was configured to inlet injection port at 200°C, 5 psi, and a total flow of 380 mL/min. The split ratio was 50:10 with a split flow of 369 mL/min with the gas saver option enabled at 20.0 mL/min at the 2.00 minute mark. H_2 gas was used as the source of the FID and set at a flow rate of 35 mL/min and air flow at 350 mL/min. The makeup / carrier flow gas was helium and set to a flow rate of 35 mL/min. The lower detection limit of the P+T GC-FID for benzene analysis was approximately 0.5 mg/L.

Unlike the GC-FID, the P+T GC-FID required liquid injections from 48 mL glass vials (Fischer Scientific, Toronto, ON). For this type of analysis, 0.5 mL of AMM were withdrawn from treatment bottles with a 1 mL glass syringe (Fischer Scientific, Toronto, ON) inside the anaerobic glove box, filtered through glass wool, and injected into the 48 mL glass vial filled with ultrapure water. This resulted in a 1/96x dilution factor for the analyzed sample. The standard curves for the P+T GC-FID involved benzene standards of 0.1, 0.5, 1.0, 2.0, and 4.0 mg/L. Aside from considering the dilution factor, the process to calculate the benzene content within each injection is similar to the process described in Section 3.3.1.

A series of tests involving benzene standards with decreasing benzene content were tested when analysis with the P+T GC-FID began. These tests indicated the P+T GC-FID to have a lower detection limit of approximately 0.5 mg/L.

3.3.3 – Ion Chromatography

Nitrate, nitrite, and sulfate analysis was measured by ion chromatography (IC) via a Dionex ICS 2100 system equipped by a Dionex™ IonPac™ AS18 IC column. Effluent flow was set at 0.25 mL/min with an effluent generator enabled at a concentration of 32.00 mM. The oven temperature was maintained at 30°C while the conductivity detector temperature was set at 35°C. Detection was achieved with suppressed conductivity using an anion self-regeneration suppresser

(ASRS 2 mm, Auto-Suppression mode, 20 mA current). Background or baseline conductivity was allowed stabilize for approximately 2 hours and lower than 1 µS, and system backpressure was approximately 2000 psi.

Dionex seven anion mix (057590) (Thermo Fisher Scientific, Edmonton, AB) was used as a stock solution and diluted to create a standard calibration curve during each monitoring session. This stock solution contained 20 mg/L fluoride, 100 mg/L chloride, 100 mg/L nitrite, 100 mg/L bromide, 100 mg/L nitrate, 200 mg/L phosphate, and 100 mg/L sulfate. The standard curves were developed from the stock solution with dilution factors of 20, 10, 5, and 2.

3.3.4 – Gas Chromatography – Thermal Conductivity Detector

CO_2 measurement were performed with an Agilent 7890A Gas Chromatography (GC) system equipped with a thermal conductivity detector (TCD) utilizing an Agilent HP-PLOT/Q column with the following dimensions: 30 m × 320 µm × 0.2 µm. The oven temperature gradient was as follows: two minutes at 50°C, followed by an increase at the rate of 30°C/min to 150°C which was further maintained for 2 min. Helium was used as carrier gas with the following flow program: 8.83 mL/min for two minutes, and decreasing to 5.67 mL/min until the end of the separation. The total run time was approximately 7.33 minutes. The detector was maintained at 200°C, and the inlet injection port at 300°C. The makeup gas (helium) was set to 5 mL/min. The injector split ratio was set to 5:1 (no gas saver), with a column flow of 8.89 mL/min, split vent flow of 44.4 mL/min, and a septum purge flow of 58.3 mL/min under a pressure of 30 psi.

Injection volumes were 200 µL and collected with a Hamilton Co. (Reno, NV) 250 µL gas syringe with a Chaney Adapter (model # 702/750) designed for repeated withdraws.

The method to analyze methane content within treatment bottles is similar to benzene analysis described in Section 3.3.1. The methane concentrations used in these standards to develop the standard curve were 0%, 10%, 20%, 30%, 40%, and 50% methane with a remaining N_2 balance.

3.4 – Data Interpretation

This section outlines the calculation methods for determining the values of the salient degradation features within the various discussion sections of Chapters 4 and 5. Various temporal aspects such degradation cycles, initial degradation, residual degradation, re-equilibrate periods, and lag phases will be given a formal definition in this section. In addition, methods related to rates

such as the overall degradation rate, maximum degradation rate, and ratios of terminal electron acceptor usage are also discussed.

3.4.1 – Degradation Cycle

A "degradation cycle" is defined as one degradation curve which comprises of the aggregate data collected between one instance of benzene refeed and the next instance of benzene refeed (or the conclusion of the experiment). Benzene refeeds occur when benzene content is consistent which indicates degradation of the microcosm stalled or the observed benzene content is below detection limit of the analytical methods discussed in Section 3.3. An example of degradation cycles is shown in Figure 3-4 below.

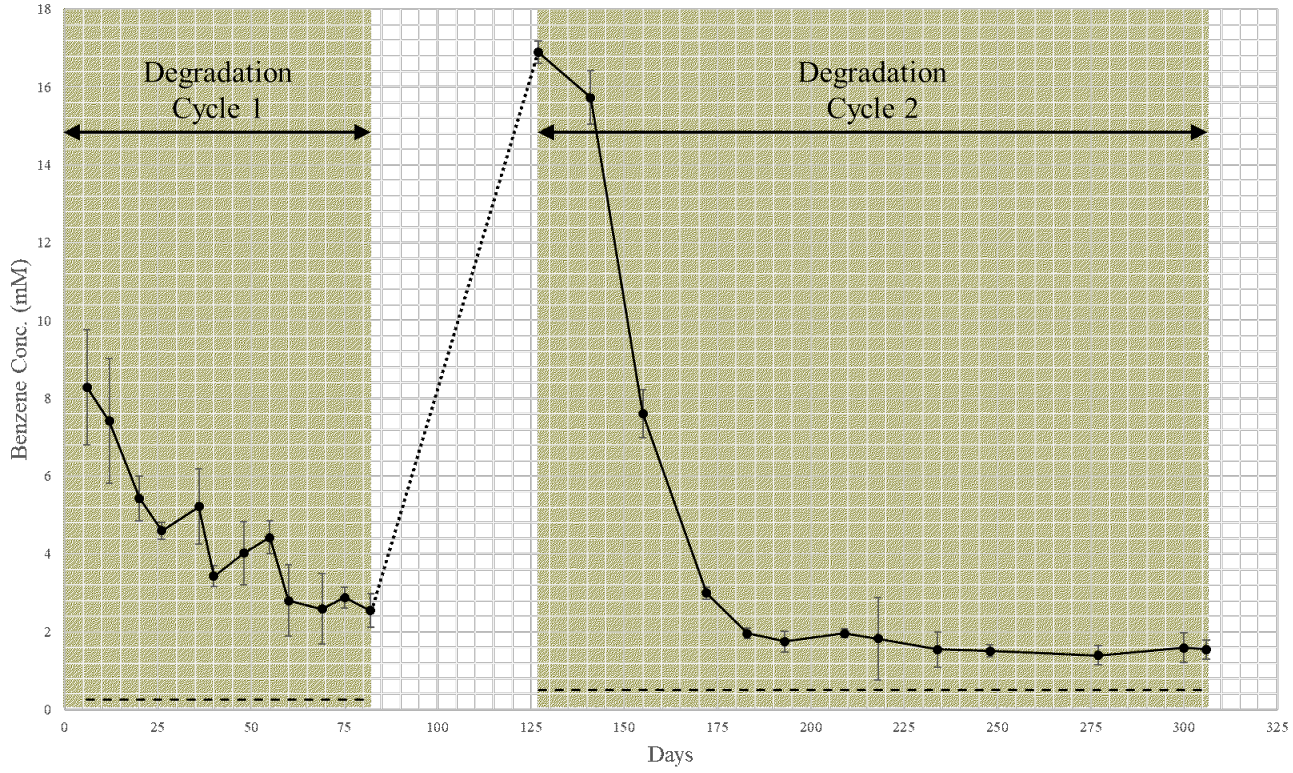

Figure 3-4 – Example of Degradation Cycles. This specific degradation profile is of nitrate-reducing 0.0 g/L NaCl: benzene biodegradation (●). Vertical upwards black dotted lines represent benzene reamendment. Horizontal black dashed line represents a lower detection limit of 0.25 and 0.5 mg/L. Day 0 to Day 306. Two different degradation cycles are highlighted in yellow. Error bars represent one standard of deviation between the quadruplicate samples. Instances in which error bars do no appear indicate a low standard of deviation.

Figure 3-4 depicts the overall degradation cycles observed in a nitrate-reducing microcosm containing 0.0 g/L NaCl in the Salinity Experiment portion of this thesis. As highlighted in yellow, two degradation cycles occurred between Days 0 and 306. In the first degradation cycle, benzene

was biodegraded from 8.3 to 2.5 mg/L between Days 6 and 82. Subsequently, benzene was refed to 16.9 mg/L on Day 127. This marks the beginning of the second degradation cycle, which concluded at a concentration of 1.5 mg/L on Day 306.

3.4.2 – Initial Degradation and Residual Degradation Phases

In most of the overall degradation profiles of the microcosms in both the Culture Enrichment Period (Chapter 4) and Salinity Experiment (Chapter 5), degradation was observed to begin at a linear rate. This period was considered the "initial degradation". The initial degradation often abruptly concluded. A second period, henceforth referred to as the "residual degradation", began. During residual degradation, benzene disappearance occurs at a much lower rate in comparison to the initial degradation and often lasted for an extended period (upwards of hundreds of days) before the benzene content is lowered to below the detection limit. In most cases, this residual degradation was not left to proceed to such a degree as it was more beneficial conclude the degradation cycle, perform a benzene refeed, and obtain another degradation cycle.

Determining the timepoint which marks the end of a initial degradation phase and the onset of the residual degradation phase is an important step in calculating the salient degradation features discussed in the following sections. For the purposes of this thesis, this timepoint is calculated from four consecutive monitoring sessions in which the three benzene concentration changes are less than 5% of the peak benzene concentration of the corresponding degradation cycle.

Once again, the nitrate-reducing 0.0 g/L NaCl microcosm is used as an example to illustrate this concept. Figure 3-5 below is similar to that of Figure 3-4, but the horizontal scale is enlarged to only display the second degradation cycle.

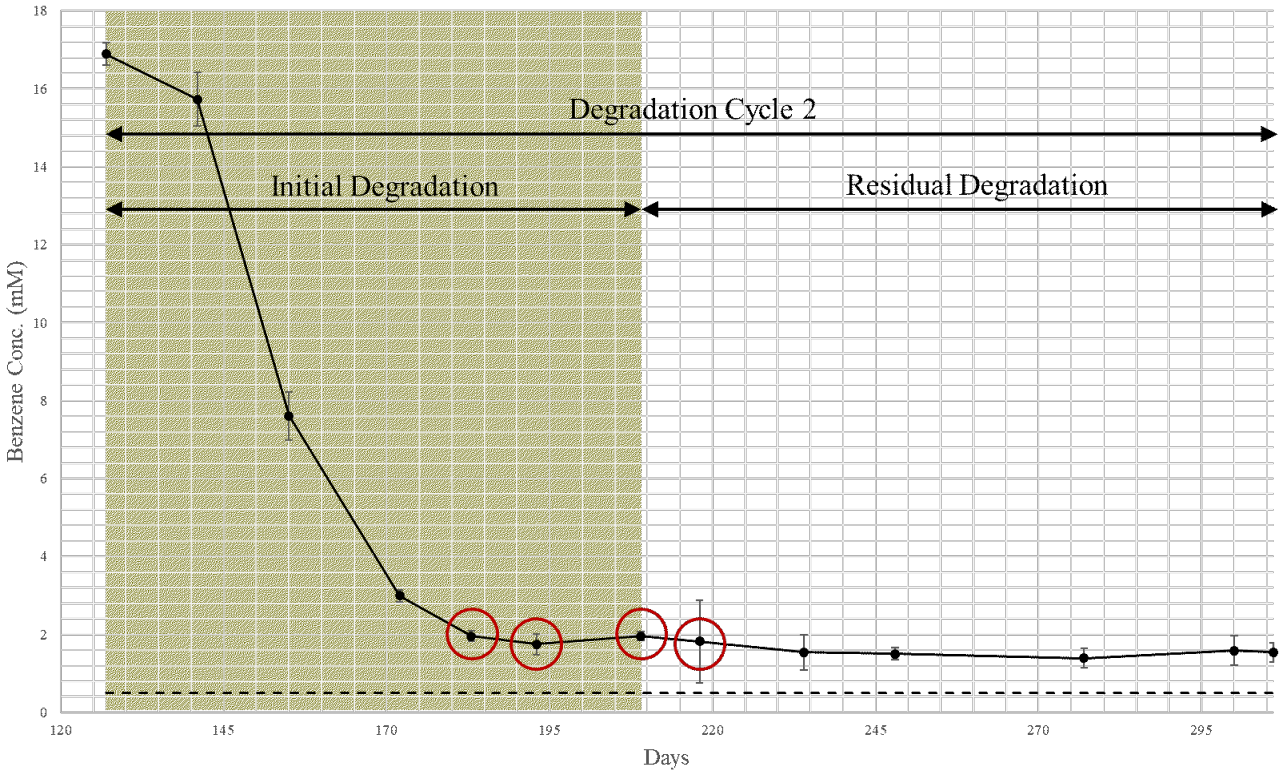

Figure 3-5 – Example of Initial and Residual Degradation Phases. This specific degradation cycle is the second of nitrate-reducing 0.0 g/L NaCl: benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 and 0.5 mg/L. Day 120 to Day 306. Initial degradation highlighted in yellow. Error bars represent one standard of deviation between the quadruplicate samples. Instances in which error bars do no appear indicate a low standard of deviation.

In this second degradation cycle, benzene had been re-fed to a concentration of 16.9 mg/L on Day 127. As seen on Figure 3-5, biodegradation proceeded immediately following this refeed and a linear decrease in benzene content was achieved until the profile unexpectedly tapered off and becomes relatively horizontal (representing residual degradation). In this case, four consecutive benzene monitoring sessions yielded 2.0, 1.7, 2.0, and 1.8 mg/L on Days 183, 193, 209, and 218 respectively. These four monitoring sessions are circled in red.

The benzene difference between the first two monitoring sessions (Days 183 and 193) was 0.3 mg/L (i.e. 2.0 – 1.7 mg/L). This 0.3 mg/L change represented only 1.25% of the initial 16.9 mg/L peak concentration of this cycle. Similarly, the following monitoring sessions yielded -1.24 and 0.79% changes. Together, these four monitoring sessions identified three consecutive benzene content decreases which are less than 5% of the peak concentration. Therefore, a transition between initial degradation and residual degradation has been identified within this time period. In such instances, the third (of four) monitoring session will be considered the official start of the

residual degradation. In this example, the second cycle's initial degradation will be considered to occur between Days 127 and 209 while residual degradation occurred between Days 210 and 306. The two phases are labelled as such in Figure 3-5.

In some cases, this concept of a benzene content change which is less than 5% in four consecutive monitoring sessions was not identified within an entire degradation cycle. This was the case with the first degradation cycle of the microcosm previously shown. In such instances, initial degradation was considered to be the entire degradation cycle and no residual degradation was occurred.

3.4.3 – Re-equilibrate Period

A "re-equilibrate period" had been observed in some degradation profiles. This term is used to describe the phenomenon in which the benzene content observed in a monitoring session immediately following benzene refeed does not reflect the equilibrium or peak concentration in its respective degradation cycle. Instead, benzene content increased until it peaked at a certain amount of days after the refeed. Figure 3-6 depicts an example of a re-equilibrate period in the nitrate-reducing 0.5 g/L NaCl microcosms of the Salinity Experiment.

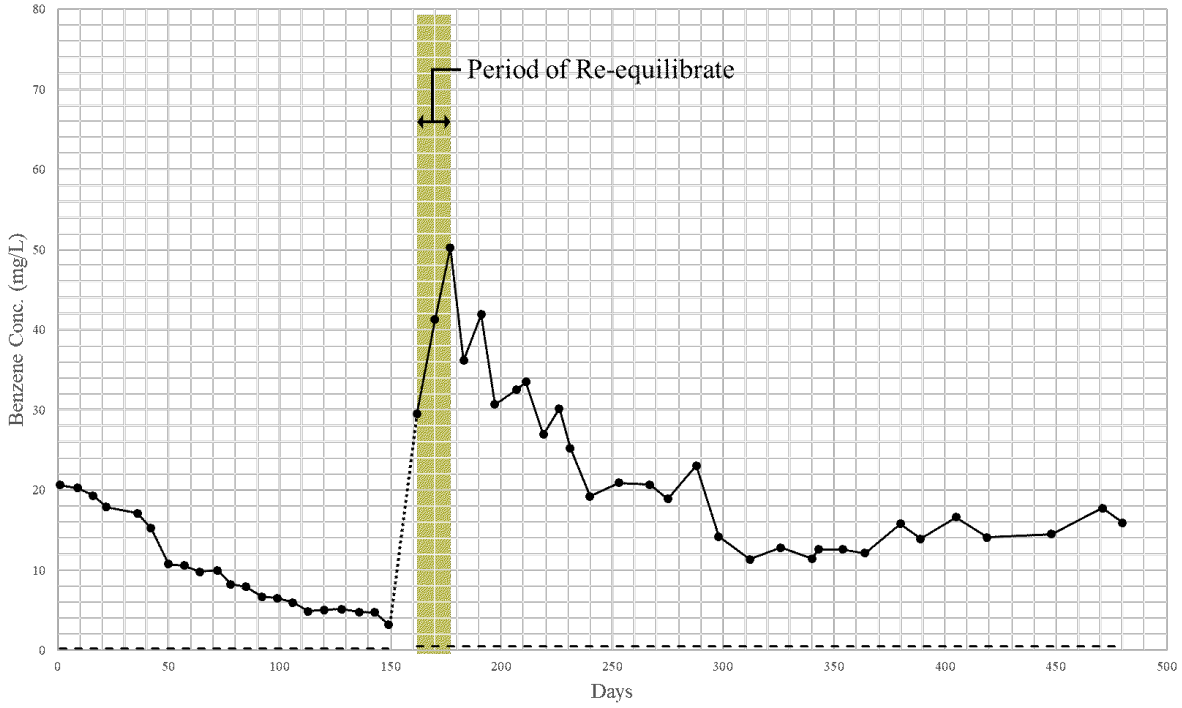

Figure 3-6 – Example of a Re-equilibrate Period. This specific degradation profile is of nitrate-reducing 0.5 g/L NaCl: benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 and 0.5 mg/L. Day 0 to Day 480. The period of re-equilibrate highlighted in yellow.

As shown in Figure 3-6, benzene refeed occurred on Day 149 following the first degradation cycle. On Day 162, benzene concentration was observed to 29.5 mg/L but continually increased until peaking at 50.2 mg/L on Day 177. In this case, the period of re-equilibrate is considered to last 15 days (Days 162 to 177).

Periods of re-equilibrate may exists due to the newly resupplied benzene creating an imbalance between the dissolved benzene in the media, sorption on the sediments, sorption to the inner glass bottle or rubber septa, and volatilization into the headspace. In such instances, the sum benzene content must reach a new equilibrium between these phases within the microcosm. Eventually, a new equilibrium has been achieved within the microcosm and degradation related to microorganisms dominate and lower benzene content. Some microcosms did not exhibit a period of re-equilibrate following benzene refeed. However, in the cases in which such a period did exist, the duration was observed to last an average of two weeks for most microcosms.

3.4.4 – Overall Degradation Rate

With the initial and residual degradation phases defined in Section 3.4.2, aggregate data from monitoring sessions were used to generate a linear trendline with slope value representing the overall degradation occurring during the initial degradation phase. An example of this concept is shown in Figure 3-7 on the next page.

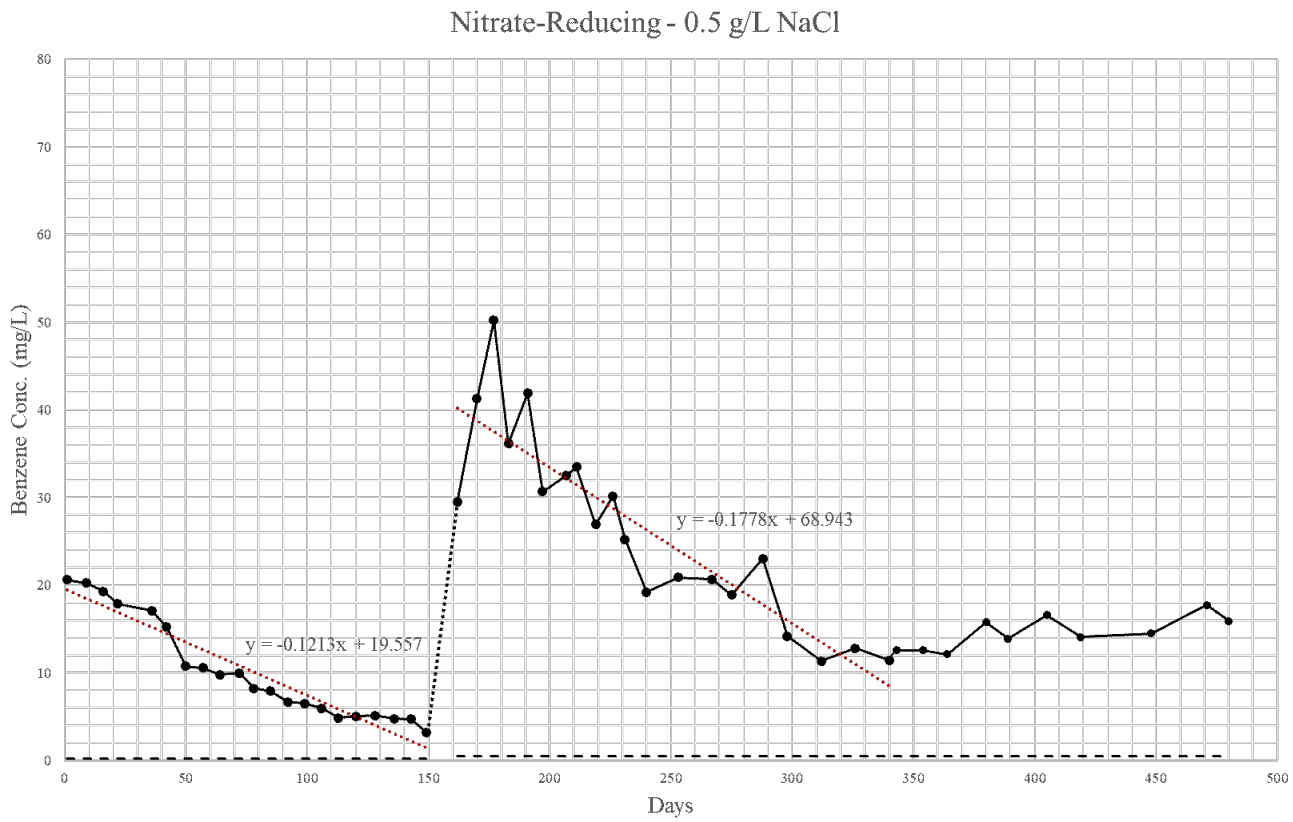

Figure 3-7 – Example of Overall Degradation Rate. This specific degradation profile is of nitrate-reducing 0.5 g/L NaCl Overall Degradation: benzene biodegradation (●). Vertical upwards black dotted line represents benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 and 0.5 mg/L. Red dotted line represents overall trendline. Day 0 to Day 480.

As per the method discussed in Section 3.4.2, the initial degradation phase was identified between Days 0 and 149 for the first degradation cycle, and Days 162 and 340 for the second degradation cycle. Data points corresponding to the re-equilibrate phase were not considered when calculating the overall degradation rate. A linear trendline was established with the slope representing the overall degradation rates. In this case, 0.12 and 0.18 mg/L·d of benzene for the first and second degradation cycles respectively.

3.4.5 – Maximum Degradation Rate

The maximum degradation rates were taken as the largest change in benzene concentration between any four consecutive monitoring sessions and calculated in the same manner as overall degradation rate (Section 3.4.4). The maximum degradation rate can be a useful metric in providing a sense at which initial degradation can proceed upon optimal conditions, caution should be taken during analysis regarding overreliance on this parameter as maximum degradation rate

can be highly influenced by individual outliers or anomalies and analytical or instrumental errors on a day-to-day basis. It represents the potential for degradation under the most ideal of conditions and the maximum degradation rate cannot be expected to be sustained throughout an entire degradation cycle.

3.4.6 – Lag Phase

Reardon et al. (2000) and Mahour (2016) employed lag phase as an additional metric to describe the acclimation period required by the microbial culture when presented with the refeed of substrate or after dilution of transfer of a treatment bottle. In those publications, a "lag phase" was defined as the duration required for the degradation of 2% of the supplied substrate following reamendment (Mahour 2016; Reardon et al. 2000). Following that definition, lag phases were back calculated from the benzene feed concentration (observed during monitoring) and the overall degradation rate (Section 3.4.4) throughout the discussion sections of Chapters 4 and 5.

3.4.7 – Mol Nitrate or Sulfate : Mol Benzene Ratio

Anion analysis with ion chromatography (IC) was conducted intermittently throughout the Culture Enrichment period and the Salinity Experiment due to the destructive nature of this type of analysis and the small volumes of the treatment bottles. When IC data was available, the total moles of terminal electron acceptor (nitrate or sulfate) consumed was compared to the total moles of benzene consumed. Specifically, only the data relating to the benzene concentrations from headspace monitoring sessions occurring closest to the IC monitoring sessions were used to provide the most time accurate differences in both benzene and electron acceptor. These ratio values were compared to theoretically expected values shown in Chapter 2 and discussed in Chapters 4 and 5. For degradation cycles in which multiple electron acceptor reamendments have occurred, the total amount of supplied electron acceptor is considered against the residual amount remaining before each individual reamendment to obtain a total molar amount of electron acceptor utilized in the corresponding degradation cycle.

3.5 – Chapter 3 References

Becalski, A. (2014). “Chapter 20 - Processing Contaminants: Benzene.” *Encyclopedia of Food Safety*, 376–380.

Holden, A. A. (2012). “The Geochemical Evolution of Oil Sands Tailings Pond Seepage, Resulting from Diffusive Ingress Through Underlying Glacial Till Sediments.”

Knovel. (2008). “Basic Physical Properties of Chemical Compounds.” *Knovel Critical Tables (2nd Edition)*.

Mahour, R. (2016). “Chemical and Biological Oxidation of Naphthenic Acids - where Stoichiometry, Kinetics and Thermodynamics Meet.” University of Alberta.

Reardon, K. F., Mosteller, D. C., and Bull Rogers, J. D. (2000). “Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for Pseudomonas putida F1.” *Biotechnology and Bioengineering*, 69(4), 385–400.

Wilson, S., Card, G., and Haines, S. (2009). “Table 3.3 - Properties of Some Commonly Occurring Vapours.” *Ground Gas Handbook*, Whittles Publishing.

CHAPTER 4

The Culture Enrichment Period

4.1 – Introduction and Overview

Recall from Chapter 1 the objective of this thesis, "*to investigate the effect of salinity on anaerobic benzene biodegradation*." To accomplish this objective, existing anaerobic microcosms which previously demonstrated benzene degradation were inherited from other members of the Ulrich laboratory. However, a proper understanding of these microbial cultures' degradation capabilities must be established as a baseline before the salinity factor can be introduced to the experiment. This naturally led to two research questions – one for each phase of the experiment and aimed to achieve the previously mentioned objective. These research questions are as follows:

I. "*What differences in anaerobic benzene biodegradation capabilities exist between treatments based on nitrate-reducing, sulfate-reducing, and methanogenic conditions? Furthermore, are these biodegradation capabilities affected by whether the treatments are derived from clay or sand sediments originating from the same source?"* and,

II. "*For the same treatments in Research Question I, is anaerobic benzene biodegradation also possible under varying salinity conditions? And if it is possible, what is the optimal salinity concentration which yields the greatest degradation rate?"*

In the publications which explored benzene biodegradation under saline conditions, rates or degrees of degradation were not demonstrated equally across the salinity ranges within each experiment. Specifically, a salt concentration between 1.0 and 2.0 M NaCl often outperformed samples at 0 M or even greater concentrations such as 5.0 M NaCl (Al-Mailem et al. 2013; Hassan et al. 2012; Li et al. 2006; Nicholson and Fathepure 2004, 2005; Sei and Fathepure 2009). These conclusions in conjunction with the previously mentioned research questions ultimately led to the hypothesis that *trace amounts of salinity will not impede (but may even stimulate) benzene biodegradation. However, further addition in salinity past an optimum range will result in inhibitory effects towards benzene biodegradability*.

In order to explore the validity of this hypothesis, the Culture Enrichment period solely focused on providing a baseline for the benzene biodegrading capabilities of the microbial cultures under nitrate-, sulfate-reducing, and methanogenic conditions. There was also the additional benefit of further microbial enrichment on benzene as the sole carbon source in conjunction with the respective redox conditions throughout this time. Minor refinements on analytical techniques and processes were also optimized during this period. The results collected during the Culture Enrichment period are reported and discussed here in Chapter 4 which aims to answer Research

Question I. The salinity factor which forms the backbone of this thesis was not introduced during Culture Enrichment, but instead, will be addressed in Chapter 5 which targets Research Question II.

The Culture Enrichment period commenced on May 10, 2017. Treatment bottles were kept stationary in a dark box at 20°C. After 202 days, on November 28, 2017, half of the microcosms were chosen based on their capability to degrade benzene at a greater rate and decommissioned. These microcosms served as a launch point into the second phase of this thesis when their biomasses were incorporated into microcosms of the Salinity Experiment. The latter half of the microcosms which were not consumed at that point received regular monitoring to generate additional data. On November 9, 2018, 548 days after the start of the Culture Enrichment Period, those unchosen microcosms were also decommissioned and utilized in the Salinity Experiment. This marked the official conclusion of the Culture Enrichment Period.

4.2 – Degradation Cycles of the Culture Enrichment Period

Four microcosms (or treatments) were set up within each of the three redox conditions for a total of 12 treatments being dedicated to the Culture Enrichment period (Table 3-2). Each of these treatments underwent between three to five degradation cycles. To keep this chapter succinct, only one overall degradation profile will be shown for each redox condition in Section 4.2 as these treatments exhibited greater rates of degradation and the most salient features of a degradation profile. The reader is referred to Appendix A, which not only showcases the degradation profiles of the remaining eight treatments, but also discusses their notable features and potential stalling points.

4.2.1 – Culture Enrichment: Treatment CNB^2 Results (Nitrate-Reducing)

Microcosm CNB^2 received an incubation period of 205 days throughout the Culture Enrichment period in which two benzene re-feeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure 4-1.

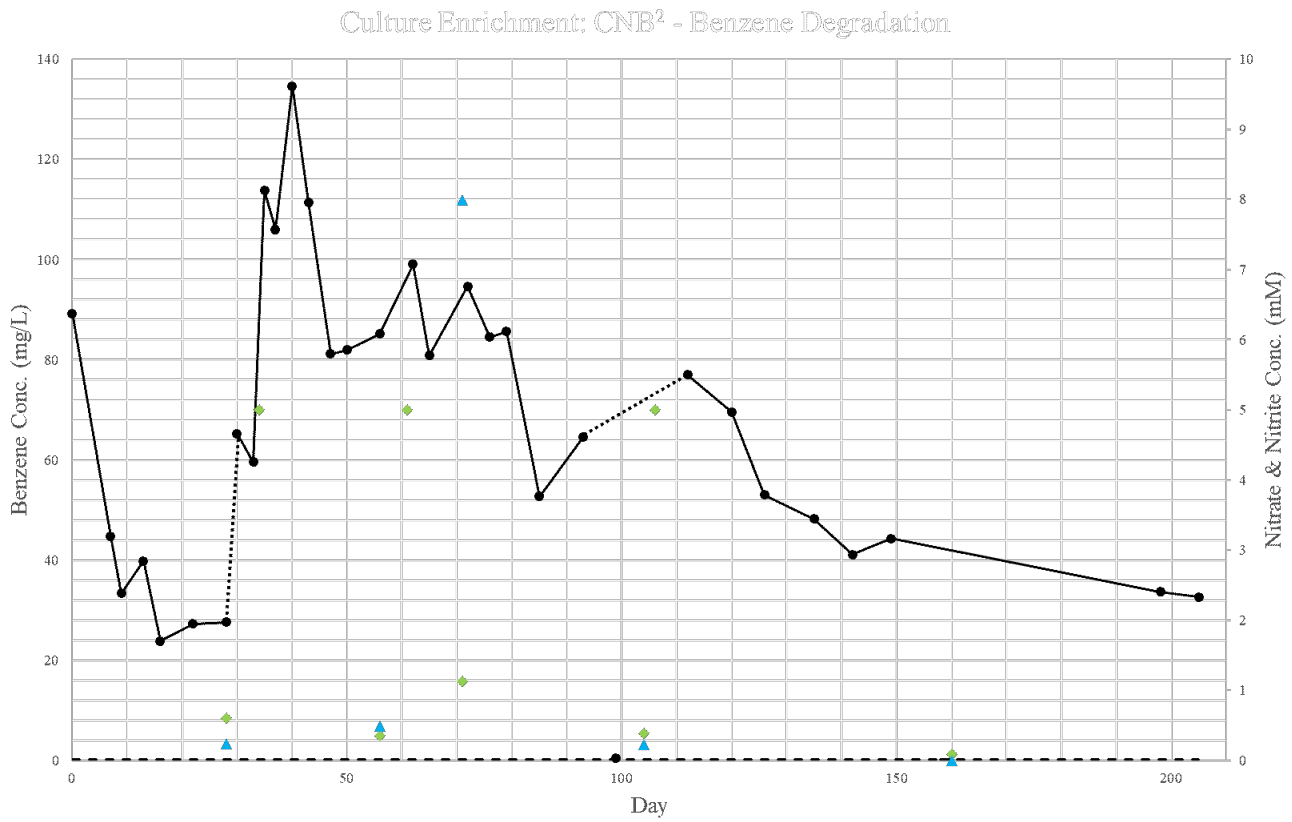

Figure 4-1 – CNB^2 Overall Degradation Profile: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 205.

In the first degradation cycle, CNB^2 demonstrated significant benzene degradation (89.2 to 27.6 mg/L) in 28 days resulting in the utilization of nitrate to 0.2 mM at the end of the cycle. Following nitrate reamendment and benzene refeed which required approximately 11 days to re-equilibrate, the second cycle was capable of decreasing benzene content from approximately 135 mg/L to 52 mg/L over 42 days of incubation. While this second degradation cycle demonstrated promising degradation capabilities, specifically its ability to tolerate a higher benzene concentration not seen in this experiment hitherto, it was prematurely abandoned due to the elevated nitrite content (8.0 mM) observed on Day 71. On Day 97, CNB^2 was diluted with fresh anaerobic media mix (AMM) to alleviate this high nitrite concentration. The third and final degradation cycle began on Day 101 following a benzene refeed and nitrate re-amendment on Day 106. It is noteworthy that no re-equilibrate period was observed on this degradation cycle and benzene content was decreased from 77.0 mg/L to 32.7 mg/L over a 93 day incubation period. Throughout each degradation cycle, nitrate was consistently utilized and required reamendment to

5 mM. Following these three degradation cycles, microcosm CNB^2 was later incorporated into the 0.5, 1.0, and 2.0 g/L NaCl microcosms of the Salinity Experiment.

4.2.2 – Culture Enrichment: Treatment SSB^2 Results (Sulfate-Reducing)

Microcosm SSB^2 received an incubation period of 341 days throughout the Culture Enrichment period in which two benzene re-amendments occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure 4-2.

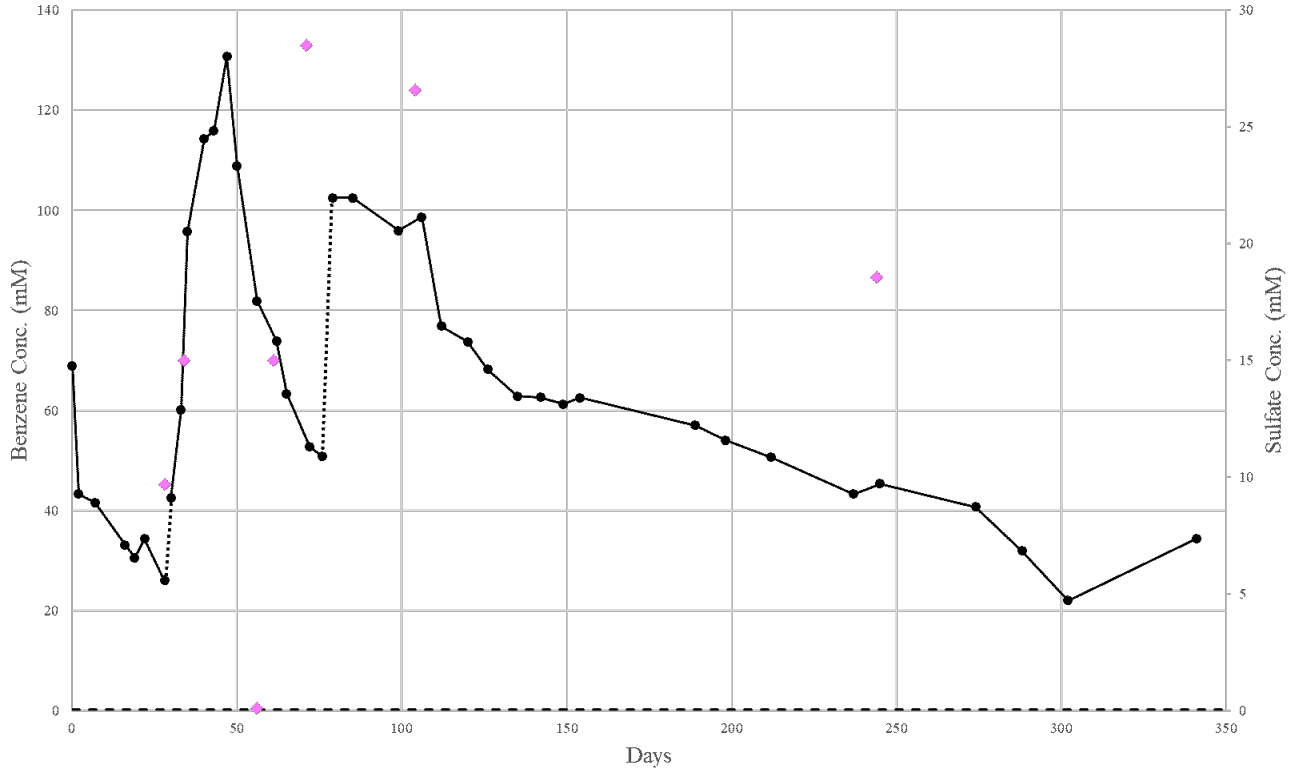

Figure 4-2 – SSB^2 Overall Degradation Profile: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 341.

In the first degradation cycle, benzene content decreased from 68.9 to 26.0 mg/L within 28 days. The second degradation cycle started with a substrate concentration of 42.5 mg/L on Day 30 but increased steadily to 130.7 mg/L by Day 47 and degraded to 50.8 mg/L by Day 76. This is the only instance of the sulfate-reducing microcosms in which the period of re-equilibrate (17 days) was comparable to the time required to degrade benzene back to approximately the initial concentration (29 days). Sulfate content was considerable at 28.5 mM and allowed to be consumed throughout the third degradation cycle without re-amendment. This was most likely due to instrumentation error of the ion chromatography (IC) system on Day 56, which suggested a sulfate

concentration of only 0.1 mM. Therefore, the subsequent 15 mM sulfate re-amendment on Day 61 would not have been necessary and is the most likely explanation for the 28.5 mM concentration on Day 71. As seen in Figure 4-2, consumption of this terminal electron acceptor resumed nevertheless from the elevated amount based on Days 104 and 244's IC analysis within the following cycle. Degradation was not as abrupt in the third cycle in comparison to the previous two cycles but was sustained at a relatively constant rate of degradation for over 250 days – decreasing from 102.5 to 22.0 mg/L. Following these three degradation cycles, microcosm SSB^2 was later incorporated into the 0.0 g/L NaCl microcosm and the sodium azide kill controls of the Salinity Experiment.

4.2.3 – Culture Enrichment: Treatment CMB^2 Results (Methanogenic)

Microcosm CMB^2 received an incubation period of 341 days throughout the Culture Enrichment period in which two benzene re-amendments occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure 4-3.

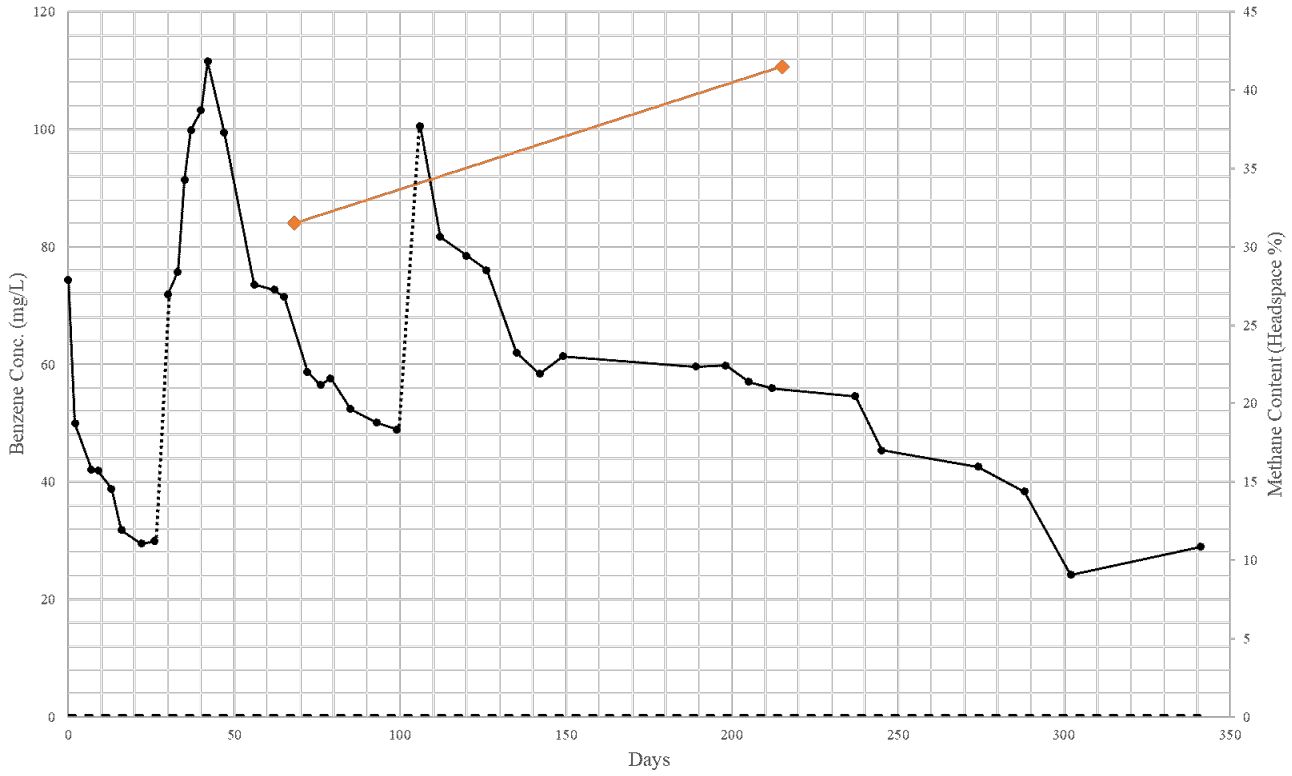

Figure 4-3 – CMB^2 Overall Degradation Profile: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 341.

Considerable degradation appears to have occurred during the first degradation cycle in which benzene content was lowered from 74.4 to 29.9 mg/L over a 26 day period. However, it should be noted that the reading on Day 0 was elevated in comparison to the remaining data within the same degradation cycle. Aside from this treatment, this trend was also observed for the 11 other treatments of the Culture Enrichment period. The analysis performed on this day was the first time in which the gas chromatography flame ionization detection (GC-FID) system was used for headspace analysis. This suggest that human error and suboptimal analytical techniques was the most probable cause for the readings that suggested an exaggerated substrate content.

Following benzene refeed and a re-equilibrate period of 12 days, a peak concentration of 111.5 mg/L was recorded on Day 42 and degradation lowered the substrate content to 48.9 mg/L by Day 99. In the third degradation cycle, the most significant degradation occurred between Days 106 and 142 (100.5 to 58.4 mg/L). Residual degradation dominated for the remainder of the cycle and ended with a final concentration of 28.9 mg/L by the end of the nearly yearlong incubation. Between the latter two degradation cycles, gas chromatography thermal conductivity detector (GC-TCD) analysis observed an increase in methane from 31.5% to 41.5% within the treatment's headspace. Following these three degradation cycles, microcosm CMB^2 was later incorporated into the 0.0 g/L NaCl microcosm and the sodium azide kill controls of the Salinity Experiment.

4.3 – Salient Degradation Features of Culture Enrichment

In this section, the most salient degradation features observed during the Culture Enrichment period are compiled in Table 4-1 on the following page. These features include the concentration to which benzene had been amended during refeed, the overall degradation rates observed in each degradation cycle, the maximum degradation rate observed at any time within a cycle, the period to re-equilibrate following benzene refeed, and the lag phase associated with each degradation cycle. Further along, Tables 4-6 and 4-7 displays data related to the usage of nitrate or sulfate as the terminal electron acceptor throughout each degradation cycle and provides a comparison to the theoretical amount(s) as suggested by the literature review in Chapter 2.

Table 4-1 – Culture Enrichment: Salient Degradation Features

Microcosm	Degradation Cycle: Days[A]	Benzene Conc. Feed (mM)[B]	Overall Degradation (µM/day)[C]	Max Degradation (µM/day) (Between Days)[D]	Re-equilibrate Period (Days)[E]	Lag Phase (Days)[F]
CNB[1]	1: 0-28	0.66	20.4	23.7 (13-22)	9	0.6
	2: 29-61	1.24	42.2	N/A	5	0.6
	3: 61-250	0.10	0.6	1.5 (79-99)	None	3.6
	4: 250-341	0.11	1.5	1.5 (274-341)	None	1.5
CNB[2]	1: 0-28	1.14	23.9	53.4 (0-13)	None	1.0
	2: 29-100	1.72	16.5	71.5 (40-50)	10	2.1
	3: 101-205	0.99	5.0	17.2 (112-135)	None	4.0
SNB[1]	1: 0-28	0.58	19.1	21.0 (2-13)	None	0.6
	2: 29-60	0.60	19.5	19.5 (33-43)	4	0.5
	3: 61-102	0.10	2.6	4.3 (65-79)	None	0.8
	4: 103-249	0.69	1.7	10.3 (120-142)	14	8.0
	5: 250-341	0.42	2.6	2.6 (274-341)	None	3.2
SNB[2]	1: 0-28	0.83	18.2	32.8 (0-9)	None	0.9
	2: 29-102	1.07	11.0	32.9 (56-65)	7	1.9
	3: 103-198	0.64	4.2	13.1 (120-149)	None	3.0
CSB[1]	1: 0-28	1.65	21.8	86.2 (0-9)	None	1.5
	2: 29-99	1.31	14.0	30.7 (40-50)	7	1.9
	3: 100-198	1.44	22.4	39.1 (112-135)	6	1.3
CSB[2]	1: 0-28	0.21	14.3	8.1 (0-9)	None	0.3
	2: 29-93	0.37	25.6	16.0 (40-50)	10	0.1
	3: 94-341	0.28	32.5	9.0 (112-135)	13	0.2
SSB[1]	1: 0-28	0.67	9.9	16.2 (7-16)	None	1.4
	2: 29-76	1.07	12.1	6.0 (35-56)	5	1.8
	3: 77-198	1.47	22.6	24.1 (106-142)	27	1.3

Table 4-1 (Cont.) – Culture Enrichment: Salient Degradation Features

Microcosm	Degradation Cycle: Days[A]	Benzene Conc. Feed (mM)[B]	Overall Degradation (µM/day)[C]	Max Degradation (µM/day) (Between Days)[D]	Re-equilibrate Period (Days)[E]	Lag Phase (Days)[F]
SSB[2]	1: 0-28	0.88	3.6	21.8 (0-16)	None	4.9
	2: 29-76	1.67	48.3	47.9 (47-62)	19	0.7
	3: 77-341	1.31	14.4	17.5 (106-126)	None	1.8
CMB[1]	1: 0-28	1.00	19.2	33.5 (0-9)	None	1.0
	2: 29-65	1.47	27.0	27.0 (40-65)	7	1.1
	3: 66-198	1.26	7.5	15.6 (120-142)	21	3.3
CMB[2]	1: 0-26	0.95	17.5	40.1 (0-9)	None	1.1
	2: 27-99	1.43	13.4	26.7 (42-62)	12	2.1
	3: 100-341	1.29	11.3	14.2 (105-126)	None	2.3
SMB[1]	1: 0-28	0.97	18.3	25.2 (0-13)	None	1.1
	2: 29-65	1.64	30.6	47.2 (43-56)	13	1.1
	3: 66-198	1.73	14.0	23.5 (76-93)	None	2.5
SMB[2]	1: 0-22	0.96	21.7	37.7 (0-9)	None	0.9
	2: 23-76	1.36	16.6	35.7 (40-50)	11	1.6
	3: 77-341	1.24	7.2	25.5 (79-106)	None	3.5

[A] Degradation cycles and duration as defined in Chapter 3, Section 3.4.1.

[B] Maximum benzene concentration observed within each degradation cycle

[C] Overall degradation rate calculated from the initial degradation phase of each degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.4.

[D] The maximum degradation rate observed between any four consecutive monitoring sessions within one degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.5.

[E] Number of days between benzene refeed and the day a maximum benzene concentration was observed within each degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.3.

[F] Number of days required for a 2% decrease from the maximum benzene concentration in each respective degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.6.

4.3.1 – Overall Degradation Rates

The average overall degradation rate is 16.3 ± 10.6 µM/d with a range of 0.6 to 48.3 µM/d throughout the 39 degradation cycles of the Culture Enrichment period. The overall degradation rates are gathered from Table 4-1 and condensed to Table 4-2 or graphically portrayed in Figure 4-4 below.

Table 4-2 – Culture Enrichment: Overall Degradation Rates

Average: 16.3 ± 1.7 µM/d	Clay: 17.7 ± 2.4 µM/d
	Sand: 14.9 ± 2.5 µM/d
Nitrate-Reducing: 12.6 ± 3.0 µM/d	Clay: 15.7 ± 5.6 µM/d
	Sand: 9.9 ± 2.8 µM/d
Sulfate-Reducing: 20.1 ± 3.4 µM/d	Clay: 21.8 ± 2.9 µM/d
	Sand: 18.5 ± 6.5 µM/d
Methanogenic: 17.0 ± 2.0 µM/d	Clay: 16.0 ± 2.8 µM/d
	Sand: 18.1 ± 3.2 µM/d

Values represent the average overall degradation rate plus/minus one standard error.

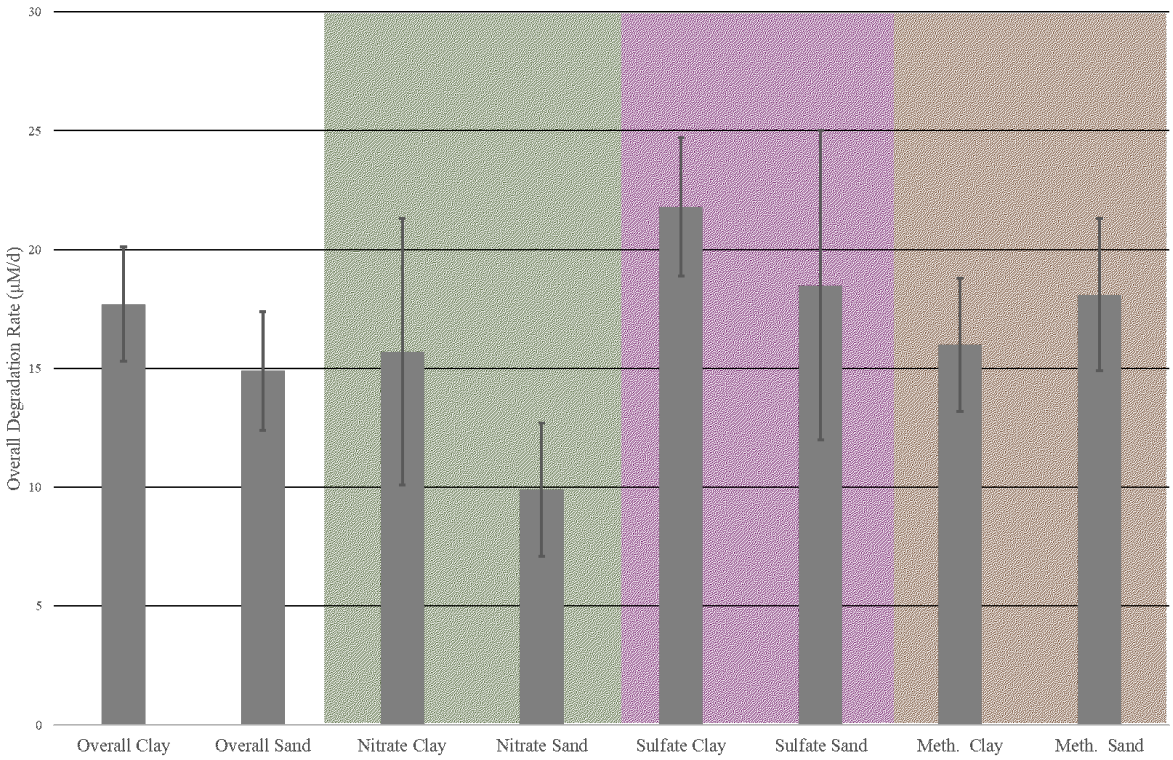

Figure 4-4 – Culture Enrichment: Overall Degradation Rates. Data is shown as an average with regards to specific anaerobic conditions and/or sediment origin. The error bars represent one standard error within its respective criteria.

The methanogenic treatments demonstrated an average rate of 17.0 ± 2.0 µM/d which is the most comparable to the average of all treatments (16.3 ± 1.7 µM/d) (Figure 4-4). The expected intermediates of benzene degradation under methanogenic conditions are bicarbonate (HCO_3^-), methane (CH^4), and the cationic form of hydrogen (H^+) which are not toxic when accumulated in comparison to the by-products such as nitrite (NO_2^-) or bisulfide (HS^-) of nitrate- and sulfate-reducing conditions respectively (Foght 2008; Meckenstock et al. 2016; Ulrich 2004; Ulrich and Edwards 2003; Vogt et al. 2011). This lack for a potential accumulation of compounds which causes adverse effects to microbial communities may be one reason for the greater degradation rate demonstrated by these methanogenic treatments. This overall degradation rate compares favorably to the methanogenic studies performed by Ulrich and Edwards (2003), Ulrich et al. (2005), and Luo et al. (2016) but far greater rates those of other methanogenic experiments (Van Beelen and Van Keulen 1990; Grbić-Galić and Vogel 1987; Sakai et al. 2009).

Alternatively, the sulfate-reducing treatments possessed the greatest overall degradation rates averaging at 20.1 ± 3.4 µM/d (Figure 4-4), which is comparable with the degradation rate reported by Nales et al. (1998) (24 - 58 µM/d). The rates reported by the remaining sulfate-reducing publications were to a lesser degree (Abu Laban et al. 2009; Edwards and Grbić-Galić 1992; Herrmann et al. 2010; Lovley et al. 1995; Phelps et al. 1996, 1998; Ulrich and Edwards 2003).

Of the three anaerobic conditions, the lowest degradation rate of 12.6 ± 3.0 µM/d is associated with the nitrate-reducing treatments. This subpar degradation rate is directly caused by periods in which degradation progress appeared stalled due to suboptimal conditions (Figure 4-1 and Appendix A). There are three potential causes for these occurrences. The first and most direct is benzene depletion. Substantial degradation of the substrate cannot occur if there are insufficient amounts of said substrate to begin with. For treatment CNB^1, the benzene concentration consistently remained between 1 and 2 mg/L from Day 153 to 250 and is one example in which benzene depletion may have inhibited the potential for further biodegradation. Nitrate depletion is the second cause of stalled degradation. A nitrate target concentration of 5 mM was chosen (as opposed to 15 mM for sulfate) in attempts to prevent significant nitrite accumulation. One challenge with a lower target concentration is the potential for unexpected nitrate depletion in the event of accelerated biodegradation when anion analysis is infrequent, which will in turn cause a cessation of further degradation. The stalled period in between Days 154 and 250 of treatment

SNB[1] is mostly likely due to nitrate depletion as a reamendment occurred on Day 106 but only 0.2 mM remained on Day 244. Third and lastly, instances in which degradation abruptly ceases after cycles of successful degradation even in the presence of ample substrate and electron acceptor has been observed (Nales et al. 1998). This overall degradation rate of 12.6 ± 3.0 μM/d by the nitrate-reducing treatments is comparable with the experiments performed by Ulrich and Edwards (2003), Luo et al. (2014), and Keller et al. (2018).

In terms of comparing treatments of differing soil sediments, those of clay origin demonstrated a higher overall degradation rate of 17.6 ± 2.4 μM/d against the sand counterparts (14.9 ± 2.5 μM/d) (Figure 4-4). Specifically, within the three redox conditions, similarities in which clay-derived treatments outperformed those of sand were also evident apart from the methanogenic samples. A two-tailed T-test was performed to determine if a statistically significant difference exists between average of the overall degradation rates of the clay samples against the sand samples. This test yielded a *t*-value of 1.01 and a *p*-value of 0.35 (against a significance value or α-value of 0.05). As a result, the apparent difference in degradation performance between the clay and sand samples cannot currently be conclusively demonstrated. However, three reasons are provided here which may serve as an explanation for the observed differences. The tetrahedral and octahedral basic units in which clays are stacked is separated by an interlayer space which commonly contains organic matter. This organic matter can include nutrients beneficial for microbial growth (Holden 2012). Furthermore, iron (Fe) can also substitute the aluminium (Al) core of octahedral structured clays (Holden 2012) and benzene degradation under iron-reducing conditions has also been demonstrated in literature (Holmes et al. 2011; Jahn et al. 2005; Zhang et al. 2013). Another possible explanation for the greater degradation rates observed from the clay derived treatments is that this isomorphic substitution of iron may have inadvertently allowed degradation linked to iron-reducing conditions in addition to the simultaneous degradation already occurring. Together, the unexpected organic matter and iron content within the clay sediments may have contributed to the greater degradation capabilities exhibited by the clay-derived treatments. Quantification of iron content within the treatment bottles was beyond the scope of this experiment and conclusions regarding this reason for benzene disappearance cannot be made at this time. In terms of physical differences, finer sediments (clay) possess a greater surface area with smaller pore spaces in comparison to coarser sediments (sand) which can enhance substrate bioavailability

due to the greater amount of sorption sites (abiotic losses) as well as a greater area to host microbial organisms (biotic losses) (Haghollahi et al. 2016; Kogbara et al. 2015).

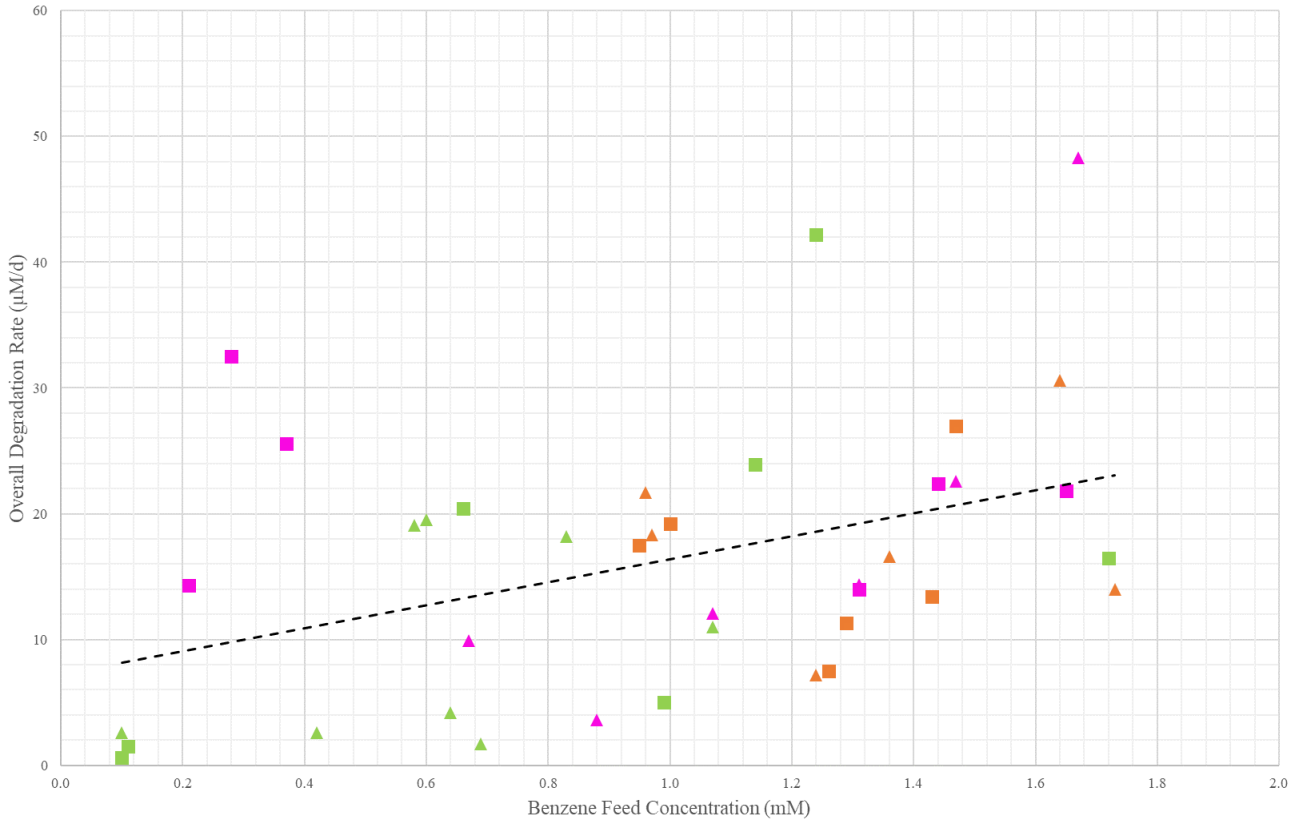

Figure 4-5 – Culture Enrichment Period: Overall Degradation Rate as a Function of Benzene Feed Concentration. Nitrate-reducing clay (■) and sand (▲), sulfate-reducing clay (■) and sand (▲), and methanogenic clay (■) and sand (▲). Dashed lines represent the linear correlation between overall degradation rate and benzene feed concentration.

A linear correlation is shown between benzene feed concentration and overall degradation rate. Specifically, an increase in benzene feed concentration resulting in a proportional increase in overall degradation rate was observed (Figure 4-5). This may be explained by the concept in which the degradation capabilities or substrate tolerance of a microbial community may be underutilized when the feed concentration is low. Furthermore, the ability of the culture within the AMM to access the substrate may also be a governing factor in degradation as benzene trapped within the headspace or sorbed onto the sediments may limit metabolic access (Kogbara et al. 2015). Conversely, excessive benzene content during refeed will result in benzene oversaturation within the AMM and may allow a greater overall degradation rate. This linear correlation is visualized by the dashed lined in Figure 4-5 with the third degradation cycle of CSB^2 (0.28 mM feed and 32.5 µM/d degradation rate) being the only outlier. To summarize, a lower degradation rate may occur within a high feed concentration, such as the third cycle of SMB^1 (14 µM/d rate at 1.73 mM), but

the inverse in which a high degradation rate occurring at a lower feed concentration was not observed. Nevertheless, an upper tolerance until toxic effects exists for all microbial communities for benzene content. One example of such an occurrence was reported by Duo et al. (2010) in which toxic effects above 80 mg/L immediately and irreversibly ceased degradation in the mixed cultures of that experiment. The upper tolerance for benzene tolerance was never reached in both the Culture Enrichment period and the Salinity Experiment of this thesis.

4.3.2 – Maximum Degradation Rates

The maximum degradation rate represents the greatest observed amount of degradation between any four consecutive monitoring sessions within a degradation cycle. The average maximum degradation rate is 25.5 ± 10.6 µM/d with a range of 1.5 to 86.2 µM/d throughout the 39 degradation cycles of the Culture Enrichment period.

Table 4-3 – Culture Enrichment: Maximum Degradation Rates

Average: 25.5 ± 10.6 µM/d	Clay: 28.6 ± 22.4 µM/d
	Sand: 22.5 ± 12.5 µM/d
Nitrate-Reducing: 21.8 ± 19.7 µM/d	Clay: 28.1 ± 26.1 µM/d
	Sand: 17.1 ± 10.9 µM/d
Sulfate-Reducing: 26.9 ± 21.6 µM/d	Clay: 31.5 ± 26.9 µM/d
	Sand: 22.3 ± 12.8 µM/d
Methanogenic: 28.6 ± 9.4 µM/d	Clay: 26.2 ± 9.2 µM/d
	Sand: 31.4 ± 9.0 µM/d

The maximum degradation rates provide insight to the degradation capabilities of each treatment at peak performance with optimal conditions. As a result, these rates are always greater than the overall degradation rates in Table 4-1 and 4-2 as the ideal conditions for degradation cannot be sustained for the entire duration of a degradation cycle. For this reason, the maximum rate is not as robust and instead, overall degradation rate is preferred as the metric for analysis when considering the degradation capabilities of this experiment.

In general, the same trends observed for the overall degradation rates are also seen for the maximum degradation rates. Between the three redox conditions, both sulfate-reducing (26.9 ± 21.6 µM/d) and methanogenic (28.6 ± 9.4 µM/d) conditions demonstrated a maximum degradation rate comparable to the global average. Maximum degradation rate also follows the

previous trend in which treatments originating from clay sediments are comparable to the sand counterparts. Lastly, maximum degradation rate is plotted as a function of benzene feed concentrate in Figure 4-6 below. The linear correlation between these two metrics once again complement that of the overall degradation rate but no outliers are identified in this instance.

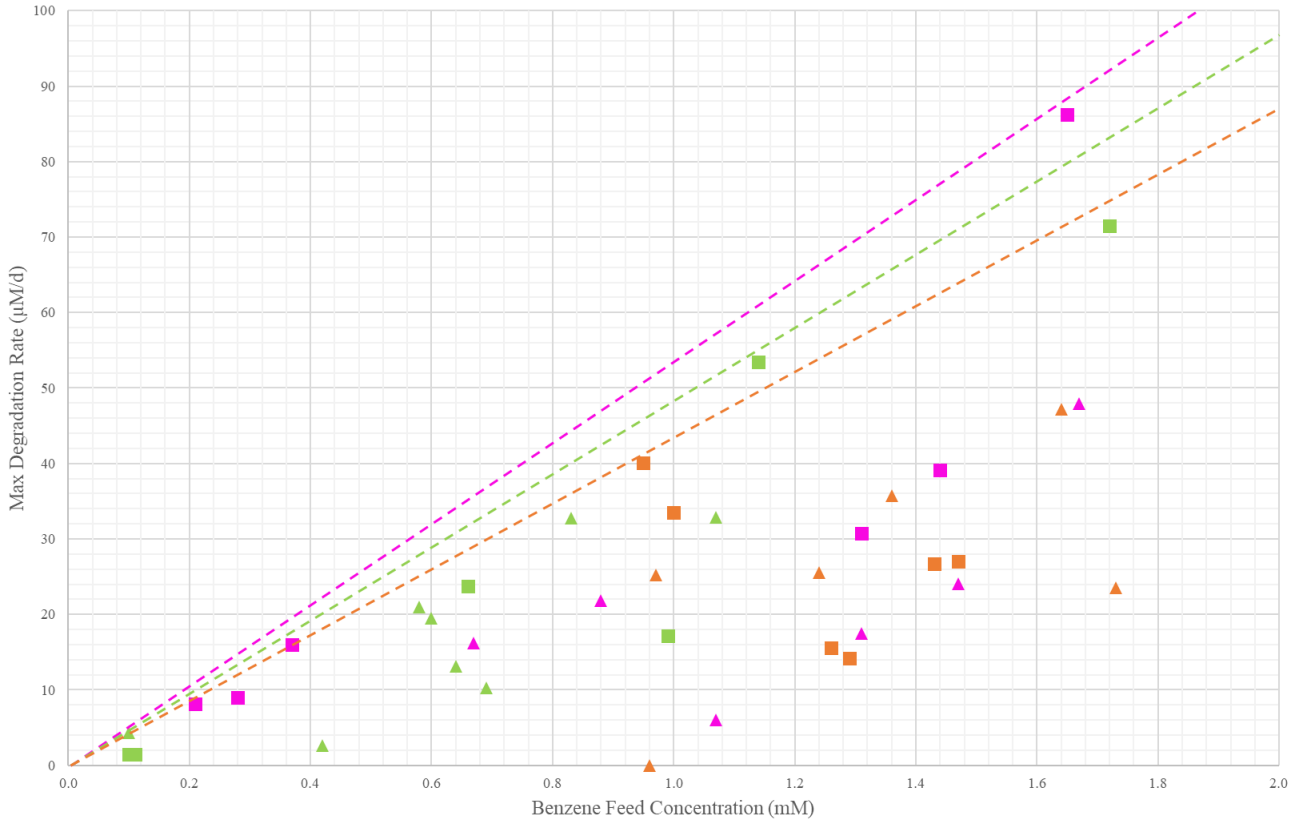

Figure 4-6 – Culture Enrichment Period: Maximum Degradation Rate as a Function of Benzene Feed Concentration. Nitrate-reducing clay (■) and sand (▲), sulfate-reducing clay (■) and sand (▲), and methanogenic clay (■) and sand (▲). Dashed lines represent the linear correlation between overall degradation rate and benzene feed concentration.

4.3.3 – Re-equilibrate Phase

The re-equilibrate phase refers to the period between benzene refeed and the monitoring session in which the highest benzene concentration was observed during the degradation cycle. A re-equilibrate phase was only observed in 13 of the 39 degradation cycles. Instances in which a re-equilibrate phase was non-existent (0 days) refers to occasions where the highest benzene concentration was observed on the monitoring session immediately following the benzene refeed. In most cases, this period required no more than two weeks for the newly supplied benzene to reach a new equilibrium between the dissolved benzene in the AMM, sorption on the sediments, sorption to the inner glass bottle and rubber septa, and volatilization into the headspace. During

periods of re-equilibrate, benzene content will appear to continually increase during the subsequent monitoring sessions immediately following substrate refeed until a new peak or equilibrium concentration is achieved and degradation processes can dominate to begin lowering the benzene concentration in the AMM and developing a new degradation curve. While biodegradation is expected to occur during this period, the experimental set-up is not designed to discriminate between the increasing benzene content and the biotic decreases. For the purposes of simplicity, benzene biodegradation is considered to officially start at the following initial degradation phase.

During the Culture Enrichment period, re-equilibrate phases ranged between 0 and 27 days with an average of 5.1 ± 6.9 days. Below, Table 4-4 displays all the re-equilibrate phases based on redox conditions and sediment origins.

Table 4-4 – Culture Enrichment: Re-equilibrate Phases

Average: 5.1 ± 6.9 d	Clay: 5.3 ± 5.9 d
	Sand: 5.0 ± 7.6 d
	Benzene < 0.5 M: 3.3 ± 5.3 d
	Benzene > 0.5 M: 5.5 ± 7.1 d
Nitrate-Reducing: 3.3 ± 4.5 d	Clay: 3.4 ± 4.2 d
	Sand: 3.1 ± 4.8 d
Sulfate-Reducing: 7.3 ± 8.4 d	Clay: 6.0 ± 4.8 d
	Sand: 6.7 ± 7.7 d
Methanogenic: 5.3 ± 7.0 d	Clay: 6.7 ± 7.8 d
	Sand: 4.0 ± 5.7 d

On average, re-equilibrate phases for methanogenic treatments most closely resemble that of the overall average and sulfate-reducing treatments required the longest amount of time (7.3 ± 8.4 days). Interestingly, as discussed in the previous sections, nitrate-reducing treatments provided the lowest overall and maximum degradation rates, but the shortest re-equilibrate phases were still observed in these samples (3.3 ± 4.5 days). Furthermore, benzene feed concentration appeared to influence re-equilibrate phase duration as samples fed with a concentration less than 0.5 mM has a period of 3.3 ± 5.3 days while treatments greater than this concentration had a lengthier period of 5.5 ± 7.1 days. Lastly, no significant difference was observed between clay (5.3 ± 5.9 days) and sand-derived (5.0 ± 7.6 days) treatments.

4.3.4 – Lag Phase

Lag phases related to a 2% decrease in benzene content has been back-calculated from the overall degradation rate (Mahour 2016; Reardon et al. 2000). During the Culture Enrichment period, these lag phases averaged 1.8 ± 1.5 days and ranged between 0.1 and 8.0 days. A prolonged lag phases occur when a treatment received a greater dosage of benzene during refeed but was only capable of a lower overall degradation rate. One example of such an occurrence is the 8.0 day lag phase during the second degradation cycle of SNB[1], in which a refeed concentration of 0.69 mM corresponded to an overall degradation rate of merely 1.72 μM/d. Below, Table 4-5 displays the lag phases of the Culture Enrichment period.

Table 4-5 – Culture Enrichment: Lag Phases

Average: 1.8 ± 1.5 d	Clay: 1.5 ± 1.0 d
	Sand: 2.1 ± 1.8 d
	Benzene < 0.5 M: 1.3 ± 1.3 d
	Benzene > 0.5 M: 2.0 ± 1.5 d
Nitrate-Reducing: 2.2 ± 1.9 d	Clay: 1.9 ± 1.3 d
	Sand: 2.4 ± 2.4 d
Sulfate-Reducing: 1.4 ± 1.2 d	Clay: 0.9 ± 0.7 d
	Sand: 2.0 ± 1.4 d
Methanogenic: 1.8 ± 0.9 d	Clay: 1.8 ± 0.8 d
	Sand: 1.8 ± 0.9 d

In terms of the three redox conditions, nitrate-reducing, methanogenic, then sulfate-reducing treatments demonstrated the lengthiest lag phases (2.2 ± 1.9, 1.8 ± 0.9, and 1.4 ± 1.2 days respectively). The nitrate-reducing treatments contained the shortest re-equilibrate phases but conversely the most prolonged lag phases. This is most likely due to the lower overall degradation rates of these samples as discussed in Section 4.3.1. Sand-derived microcosms exhibited lengthier lag phases for the average of all 39 degradation cycles, nitrate-, and sulfate-reducing conditions but was comparable at 1.8 days for the methanogenic samples. When microcosms were refed with a benzene content greater than 0.5 mM, lag phases of 2.0 ± 1.5 days were encountered while short durations of 1.3 ± 1.3 days were associated with feed concentrates lesser than 0.5 mM. Below, Figure 4-7 was developed to further evaluate the influence in which benzene refeed concentration

has on the length of lag phases. Unlike Figures 4-5 (overall degradation rate) and 4-6 (maximum degradation rate), no linear correlation is apparent between lag phase and benzene feed concentration. The data points shown here appear consistently random with no discernable pattern between the three anaerobic conditions nor sediment origins. This may suggest that lag phases are more limited by the specific degradation capabilities of the microbial community as opposed to the physical and chemical set-up of the treatments.

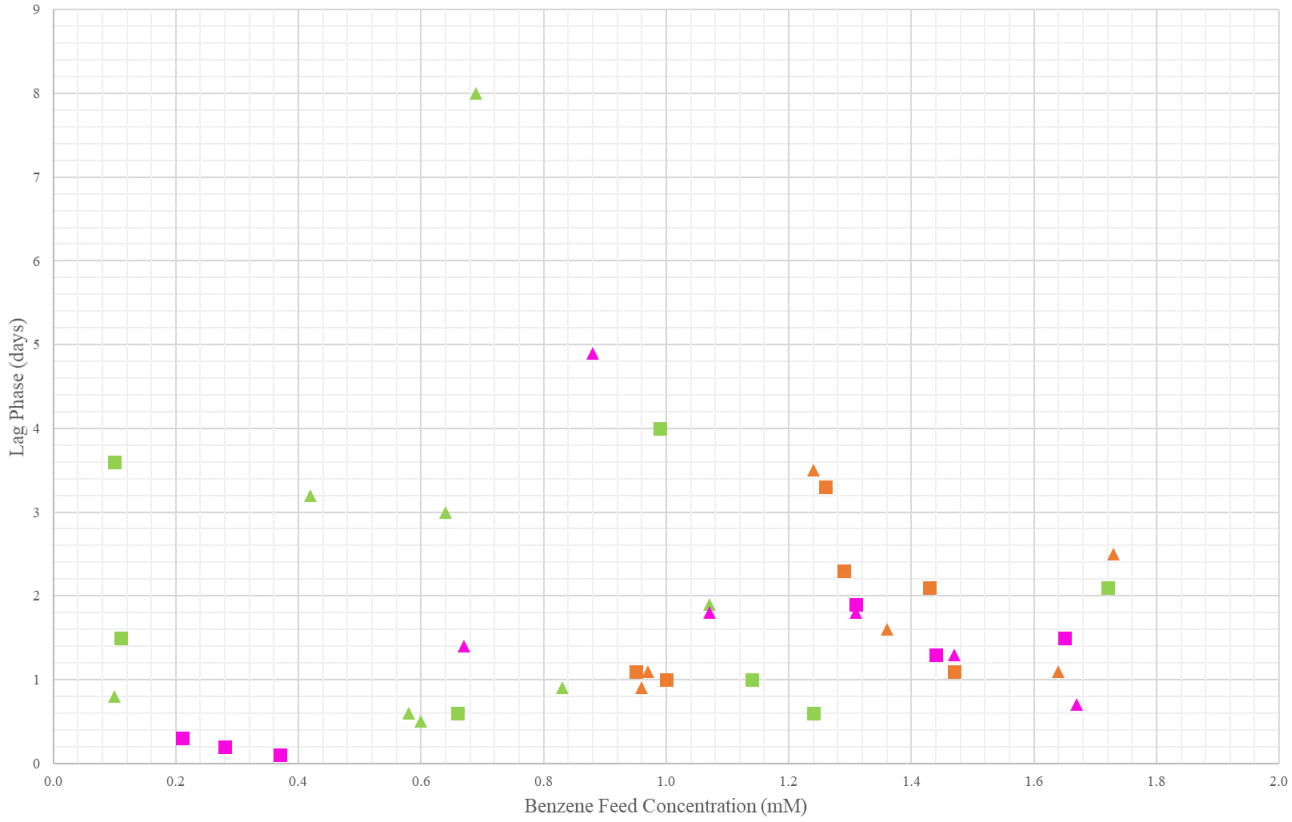

Figure 4-7 – Culture Enrichment Period: Lag Phase as a Function of Benzene Feed Concentration. Nitrate-reducing clay (■) and sand (▲), sulfate-reducing clay (■) and sand (▲), and methanogenic clay (■) and sand (▲).

4.3.5 – Electron Acceptor Utilization Ratios

In this section, nitrate and sulfate utilization rates will be discussed for the nitrate- and sulfate-reducing treatments. Terminal electron acceptor concentrations were monitored via anion analysis throughout the degradation cycles. The optimal nitrate concentration for this experiment was chosen as 5 mM to allow for ample benzene biodegradation without constant nitrate reamendment while avoiding the possibility for nitrite accumulation leading to toxic effects (Ulrich 2004). The sulfate target concentration was greater at 15 mM as bisulfide accumulation, although toxic at higher concentrations, is of a lesser concern. Anion analysis via ion

chromatography (IC) was intermittently performed at the midpoints and conclusion of every degradation cycle to evaluate changes in nitrate, nitrite, and sulfate content and determine the need for electron acceptor re-amendment. Due to the destructive nature of IC analysis and the small volumes of each microcosm, employing the IC system for this purpose occurred infrequently in comparison to the headspace analysis to determine benzene content. These changes in nitrate and sulfate concentration is mapped against the decrease in benzene to yield Table 4-6 (nitrate) and 4-7 (sulfate) below which displays the observed ratio of terminal electron acceptor utilized for the benzene consumed during biodegradation.

In terms of the incomplete reduction of nitrate to nitrite, several theoretical ratios of molar nitrate reduction per molar benzene oxidization had been suggested by literature. These ratio values are displayed in chemical reactions 1 through 3 below:

(Foght 2008; Vogt et al. 2011):

$$C_6H_6 + \mathbf{15}NO_3^- + 3H_2O \rightarrow 6HCO_3^- + 15NO_2^- + 6H^+ \text{ (1)}$$

(Ulrich and Edwards 2003):

$$C_6H_6 + 2.56H_2O + \mathbf{9.75}NO_3^- + 0.44NH_4^+ \rightarrow 3.81HCO_3^- + 9.75NO_2^- + 0.44C_5H_7O_2N + 6H^+ \text{(2)}$$

(Ulrich 2004):

$$C_6H_6 + 3.72H_2O + \mathbf{7.80}NO_3^- + 0.72NH_4^+ + 2.88CO_2 \rightarrow 5.28HCO_3^- + 7.8NO_2^- + 0.72C_5H_7O_2N + 6H^+ \text{ (3)}$$

Conversely, complete reduction of nitrate to nitrogen gas has also been suggested by literature and are shown in the following chemical reactions. These ratio values of 5.23 and 6 are obtained from the chemical reactions (4 and 5) below:

(Foght 2008; Meckenstock et al. 2016; Vogt et al. 2011):

$$C_6H_6 + \mathbf{6}NO_3^- \rightarrow 6HCO_3^- + 3N_2 \text{ (4)}$$

(Ulrich 2004):

$$C_6H_6 + 2.43H_2O + \mathbf{2.53}NO_3^- + 0.87NH_4^+ + 3.48CO_2 \rightarrow 5.23HCO_3^- + 1.92N_2 + 0.87C_5H_7O_2N + 3.12H^+ \text{ (5)}$$

Table 4-6 – Culture Enrichment: Nitrate Usage Ratios of Nitrate-Reducing Microcosms

				Ratio mol Nitrate : mol Benzene	
Microcosm	Degradation Cycle	Days	Benzene Conc. Fed (mM)	Observed	Theoretical[A]
CNB[1]	2	29 - 61	1.24	31.5	to NO_3^-: 7.80[A] 9.75[B] 15[C] to N_2: 2.53[A] 6[D]
	3	61 - 250	0.12	4.9	
CNB[2]	2	29 - 100	1.72	7.7	
	3	101 - 205	0.99	11.7	
SNB[1]	2	29 – 60	0.60	38.6	
	3	61 – 102	0.10	95.6	
	4	103 – 249	0.69	13.8	
	5	250 – 341	0.42	28.0	
SNB[2]	2	29 – 102	1.07	23.6	
	3	103 – 198	0.64	4.0	

[A] (Ulrich 2004)
B (Ulrich and Edwards 2003)
C (Foght 2008; Vogt et al. 2011)
D (Foght 2008; Meckenstock et al. 2016; Vogt et al. 2011)

The treatment and individual degradation cycles of nitrate-reducing treatments during the Enrichment Period yielded nitrate : benzene ratios between 4.0 and 95.6. Specifically, the ratios of 4.0, 4.9, and 7.7 of SNB[2] (third cycle), CNB[1] (second cycle), and CNB[2] (second cycle) respectively compares most favorably against the expected theoretical value of 2.53 or 6 when nitrate undergoes a complete reduction to nitrogen gas (N_2) (Table 4-6).

In contrary, the ratios of 7.7, 11.7, and 13.8 of CNB[2] (second cycle), CNB[2] (third cycle), and SNB[1] (fourth cycle) respectively compares most favorably against the expected theoretical value of 7.80, 9.75, or 15 when nitrate undergoes an incomplete reduction to nitrite (NO_2^-) (Table 4-6).

Radiolabeling nitrate and employing stable isotope probing (SIP) to observe its conversion to either nitrite or nitrogen gas may provide insight regarding which specific nitrate related redox condition dominated in these treatments but was beyond the scope of this thesis. Based on the

available anion analysis data (Figure 4-1 and Appendix A), nitrite accumulation was not encountered beyond a reading of 0.6 mM on Day 56 of CNB[1] and SNB[2]. On one hand, these observations can suggest the reduction of nitrate to nitrogen gas, and therefore, the observed ratios between 11.7 to 95.6 will be in gross excess of the expected nitrate utilization. This exceedance would translate of a 95 to 1,493% increase of the theoretical value, resulting over an entire order magnitude in excess.

On the other hand, the lack of nitrite accumulation need not indicate the conversion of nitrate to nitrogen gas but can still support the concept of nitrate-reduction to nitrite. A diverse microbial culture within the microcosm may exist which can readily metabolize the produced nitrite at a greater rate than nitrate to nitrite generation, resulting in the lack of an observable nitrite accumulation. This concept of syntrophic relations and utilization of downstream metabolites to target transient nitrite is suggested in several studies (Dou et al. 2010; Luo et al. 2014; van der Zaan et al. 2012).

Although the electron acceptor utilization ratios throughout the Culture Enrichment period are elevated in comparison to the theoretical values regardless of the reduction of nitrate towards nitrite or nitrogen gas, these values are comparable to some reported in literature (Dou et al. 2010; Keller et al. 2018; Luo et al. 2014; Ulrich and Edwards 2003). The ratios developed in this section will be used as a baseline comparison to those ratios demonstrated by the nitrate-reducing treatments in the Salinity Experiment.

Table 4-7 – Culture Enrichment: Sulfate Usage Ratios of Sulfate-Reducing Microcosms

				Ratio mol Sulfate : mol Benzene	
Microcosm	Degradation Cycle	Days	Benzene Conc. Fed (mM)	Observed	Theoretical
CSB[1]	2	29-99	1.31	108.3	3.49[A] 3.75[B]
CSB[2]	2	29-93	0.37	11.4	
SSB[1]	2	29-76	1.07	100.7	
	3	77-198	1.47	1.6	
SSB[2]	2	29-76	1.67	23.8	
	3	77-341	1.31	13.6	

[A] (Ulrich and Edwards 2003)
[B] (Foght 2008; Meckenstock et al. 2016; Ulrich 2004; Ulrich and Edwards 2003; Vogt et al. 2011)

Several theoretical ratios of sulfate reduction per benzene oxidization has been reported by literature (written in bold). A theoretical ratio of 3.49 sulfate reduction per benzene oxidization is shown in chemical reaction 6 below:

(Ulrich and Edwards 2003):

$$C_6H_6 + 2.91H_2O + \mathbf{3.49}SO_4^{2-} + 0.088NH_4^+ \rightarrow 5.56HCO_3^- + 1.74H_2S + 1.74HS^- + 0.088C_5H_7O_2N + 0.77H^+ \quad (6)$$

An additional sulfate-reduction reaction with the exclusion of ammonium and cyanoacetate containing a ratio value of 3.75 have also been proposed in literature:

(Foght 2008; Ulrich 2004; Ulrich and Edwards 2003; Vogt et al. 2011):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3^- + 1.88H_2S + 1.88HS^- + 0.38H^+ \quad (7)$$

(Foght 2008):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3 + 1.88H_2S + 1.88HS^- + 0.38H^+ \quad (8)$$

(Meckenstock et al. 2016):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3^- + 3.75HS^- + 2.25H^+ \quad (9)$$

The treatment and individual degradation cycles of sulfate-reducing treatments during the Enrichment Period yielded sulfate : benzene ratios between 1.6 and 108.3 (Table 4-7). These ranges represent 45% to 3,103% of the theoretical value of 3.49. Furthermore, these ranges also

represent 43% to 2,888% of the theoretical value of 3.75. Only four of the six obtained ratios during the Culture Enrichment period remained within one order of magnitude in comparison to the theoretical values. The greatest outliers were a ratio of 108.3 and 100.7 from the second cycles of CSB^1 and SSB^1 respectively.

The ratio values observed during the Culture Enrichment period do not compare favorably against the ratios in literature. Ulrich and Edwards (2003) achieved a sulfate-reduction ratio of 3.3 and 4.0 while Phelps et al. (1996) managed to obtain a value of 4.74. These ratios are only in slightly in excess to the theoretical value of 3.49 or 3.75. Contrarily, Abu Laban et al. (2009) managed to utilize less than expected sulfate and only reduced 88% of the theoretical amount of sulfate.

One possible explanation for the underestimated utilization of sulfate is the concept of a syntrophic consortium discussed by Herrmann et al. (2010), van der Zaan (2012), and Luo et al. (2014). In this concept, not one singular microbial organism is responsible for benzene degradation and sulfate-reduction. Instead, one species of microbial organism may be responsible for benzene activation to produce a downstream metabolite. Other organisms may respond in a syntrophic manner to degrade this metabolite and reduce sulfate. This process may occur multiple instances until mineralization and extraneous sulfate utilization will occur in comparison to the expected theoretical values since reduction occurred not only for benzene, but for the degradation of potential downstream metabolites as well. Although the electron acceptor utilization ratios throughout the Culture Enrichment period are extremely elevated in comparison to the theoretical values, the ratios developed in this section will be used as a baseline for comparison to those ratios demonstrated in the Salinity Experiment (Chapter 5).

4.4 – Conclusions

The following conclusions were gleaned from the 548 day Culture Enrichment period in which 39 degradation cycles occurred across nitrate-, sulfate-reducing, and methanogenic benzene degrading treatments:

- Nitrate-reducing treatments degraded benzene at an average overall rate of 12.6 ± 3.0 µM/d across 15 degradation cycles.
    - Slowest of the three investigated redox conditions.
    - Depletion of nitrate or benzene most likely responsible for stalled degradation.

- Sulfate-reducing treatments degraded benzene at an average overall rate of 20.1 ± 3.4 μM/d across degradation 12 cycles.
- Methanogenic-reducing treatments degraded benzene at an average overall rate of 17.0 ± 2.0 μM/d across 12 degradation cycles.
- Clay- and sand-derived treatments degraded benzene at comparable rates (17.7 ± 2.4 and 14.9 ± 2.5 μM/d respectively). A T-test with *t*-value of 1.01, *p*-value of 0.35, and α significant-value of 0.05 does not allow for a conclusive statement in which clay samples outperform the sand samples.
- A strong linear correlation exists between benzene feed concentrate and both overall degradation rate as well as maximum degradation rate. Specifically, an increase in the concentrated of benzene often led to a proportional increase in these two metrics.
- Lag phases averaged 1.8 ± 1.5 days, with sand-derived treatments having a slightly longer lag phase than clay-derived treatments.
- Observed electron acceptor utilization ratios per mole of benzene degraded were elevated compared to theoretical values in most degradation cycles. These unexpected nitrate and sulfate losses can be due to degradation of downstream metabolites by a diverse microbial culture.

4.5 – Chapter 4 References

Abu Laban, N., Selesi, D., Jobelius, C., and Meckenstock, R. U. (2009). "Anaerobic Benzene Degradation by Gram-Positive Sulfate-Reducing bacteria." *FEMS Microbiology Ecology*, 68(3), 300–311.

Al-Mailem, D. M., Eliyas, M., and Radwan, S. S. (2013). "Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts." *Extremophiles*, 17(3), 463–470.

Van Beelen, P., and Van Keulen, F. (1990). "The Kinetics of the Degradation of Chloroform and Benzene in Anaerobic Sediment From the River Rhine." *Hydrobiological Bulletin*, 24(1), 13–21.

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., and Wang, J. (2010). "Anaerobic Benzene Biodegradation by a Pure Bacterial Culture of Bacillus cereus Under Nitrate Reducing Conditions." *Journal of Environmental Sciences*, The Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, 22(5), 709–715.

Edwards, E. A., and Grbić-Galić, D. (1992). "Complete Mineralization of Benzene by Anaerobic Microorganisms under Strictly Anaerobic Conditions." *American Society for Microbiology*, 58(8), 2663–2666.

Foght, J. (2008). "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects." *Journal of Molecular Microbiology and Biotechnology*, 15(2–3), 93–120.

Grbić-Galić, D., and Vogel, T. M. (1987). "Transformation of Toluene and Benzene by Mixed Methanogenic Cultures." *Applied and Environmental Microbiology*, 53(2), 254–260.

Haghollahi, A., Fazaelipoor, M. H., and Schaffie, M. (2016). "The effect of soil type on the bioremediation of petroleum contaminated soils." *Journal of Environmental Management*, 180, 197–201.

Hassan, H. A., Rizk, N. M., Hefnaway, M. A., and Awad, A. A. (2012). "Isolation and Characterization of Halophilic Aromatic and Chloroaromatic Degrader from Wadi El-Natrun Soda." *Life Science Journal*, 9(3), 1565–1570.

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H. H., and Vogt, C. (2010). "Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing." *Environmental Microbiology*, 12(2), 401–411.

Holden, A. A. (2012). “The Geochemical Evolution of Oil Sands Tailings Pond Seepage, Resulting from Diffusive Ingress Through Underlying Glacial Till Sediments.”

Holmes, D. E., Risso, C., Smith, J. A., and Lovley, D. R. (2011). “Anaerobic oxidation of benzene by the hyperthermophilic archaeon Ferroglobus placidus.” *Applied and Environmental Microbiology*, 77(17), 5926–5933.

Jahn, M. K., Haderlein, S. B., and Meckenstock, R. U. (2005). “Anaerobic Degradation of Benzene, Toluene, Ethylbenzene, and o-Xylene in Sediment-Free Iron-Reducing Enrichment Cultures.” *Applied and Environmental Microbiology*, 71(6), 3355–3358.

Keller, A. H., Kleinsteuber, S., and Vogt, C. (2018). “Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics.” *Microbial Ecology*, Microbial Ecology, 75(4), 941–953.

Kogbara, R. B., Ayotamuno, J. M., Worlu, D. C., and Fubara-Manuel, I. (2015). “A Case Study of Petroleum Degradation in Different Soil Textural Classes.” *Recent Patents on Biotechnology*, 9(2), 108–115.

Li, H., Liu, Y. H., Luo, N., Zhang, X. Y., Luan, T. G., Hu, J. M., Wang, Z. Y., Wu, P. C., Chen, M. J., and Lu, J. Q. (2006). “Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic Planococcus sp. strain ZD22.” *Research in Microbiology*, 157(7), 629–636.

Lovley, D. R., Coates, J. D., Woodward, J. C., and Phillips, E. J. P. (1995). “Benzene Oxidation Coupled to Sulfate Reduction.” *American Society for Microbiology*, 61(3), 953–958.

Luo, F., Gitiafroz, R., Devine, C. E., Gong, Y., Hug, L. A., Raskin, L., and Edwards, E. A. (2014). “Metatranscriptome of an Anaerobic Benzene-Degrading, Nitrate-Reducing Enrichment Culture Reveals Involvement of Carboxylation in Benzene Ring Activation.” *Applied and Environmental Microbiology*, 80(14), 4095–4107.

Mahour, R. (2016). “Chemical and Biological Oxidation of Naphthenic Acids - where Stoichiometry, Kinetics and Thermodynamics Meet.” University of Alberta.

Meckenstock, R. U., Boll, M., Mouttaki, H., Koelschbach, J. S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A. M. (2016). “Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons.” *Journal of Molecular Microbiology and Biotechnology*, 26(1–3), 92–118.

Nales, M., Butler, B. J., and Edwards, E. A. (1998). “Anaerobic benzene biodegradation: A microcosm survey.” *Bioremediation Journal*, 2(2), 125–144.

Nicholson, C. A., and Fathepure, B. Z. (2004). “Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions.” *Applied and Environmental Microbiology*, 70(2), 1222–1225.

Nicholson, C. A., and Fathepure, B. Z. (2005). “Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma.” *FEMS Microbiology Letters*, 245(2), 257–262.

Phelps, C. D., Kazumi, J., and Young, L. Y. (1996). “Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction.” *FEMS Microbiology Letters*, 145(3), 433–437.

Phelps, C. D., Kerkhof, L. J., and Young, L. Y. (1998). “Molecular characterization of a sulfate-reducing consortium which mineralizes benzene.” *FEMS Microbiology Ecology*, 27(3), 269–279.

Reardon, K. F., Mosteller, D. C., and Bull Rogers, J. D. (2000). “Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for Pseudomonas putida F1.” *Biotechnology and Bioengineering*, 69(4), 385–400.

Sakai, N., Kurisu, F., Yagi, O., Nakajima, F., and Yamamoto, K. (2009). “Identification of putative benzene-degrading bacteria in methanogenic enrichment cultures.” *Journal of Bioscience and Bioengineering*, The Society for Biotechnology, 108(6), 501–507.

Sei, A., and Fathepure, B. Z. (2009). “Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake.” *Journal of Applied Microbiology*, 107(6), 2001–2008.

Ulrich, A. C. (2004). “Characterization of Anaerobic Benzene-Degrading Cultures.” University of Toronto.

Ulrich, A. C., and Edwards, E. A. (2003). “Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures.” *Environmental Microbiology*, 5(2), 92–102.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). “Anaerobic benzene degradation by bacteria.” *Microbial Biotechnology*, 4(6), 710–724.

van der Zaan, B. M., Saia, F. T., Stams, A. J. M., Plugge, C. M., de Vos, W. M., Smidt, H., Langenhoff, A. A. M., and Gerritse, J. (2012). “Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a

syntrophic process." *Environmental Microbiology*, 14(5), 1171–1181.

Zhang, T., Tremblay, P. L., Chaurasia, A. K., Smith, J. A., Bain, T. S., and Lovley, D. R. (2013). "Anaerobic benzene oxidation via phenol in Geobacter metallireducens." *Applied and Environmental Microbiology*, 79(24), 7800–7806.

CHAPTER 5

The Salinity Experiment

5.1 – Introduction and Overview

Recall from Chapter 1 the objective of this thesis, "*to investigate the effect of salinity on anaerobic benzene biodegradation.*" Subsequently, two research questions were developed to guide the research and accomplish this objective:

I. "*What differences in anaerobic benzene biodegradation capabilities exist between treatments based on nitrate-reducing, sulfate-reducing, and methanogenic conditions? Furthermore, are these biodegradation capabilities affected by whether the treatments were derived from clay or sand sediments originating from the same source?"*

The first phase of the experiment, the Culture Enrichment period, served to answer this question. It provided a baseline for degradation performance and trends to be expected from the microbial cultures on hand. The work undertaken and results obtained from this period were discussed at length in Chapter 4. Therefore, only Research Question II remains:

II. "*For the same treatments in Research Question I, is anaerobic benzene biodegradation also possible under varying salinity conditions? And if it is possible, what is the optimal salinity concentration which yields the greatest degradation rate?"*

In the available publications which explored benzene biodegradation under saline conditions, degrees of degradation were not demonstrated equally across the salinity ranges within each experiment. Specifically, a salinity concentration between 1.0 and 2.0 M NaCl often outperformed identical samples at 0 M or even greater concentrations such as 5.0 M NaCl (Al-Mailem et al. 2013; Hassan et al. 2012; Li et al. 2006; Nicholson and Fathepure 2004, 2005; Sei and Fathepure 2009). These conclusions in conjunction with the previously mentioned research questions ultimately led to the hypothesis that *trace amounts of salinity will not impede (but may even stimulate) benzene biodegradation. However, further addition in salinity past an optimum range will result in inhibitory effect towards benzene biodegradability*.

The Salinity Experiment is the second phase and backbone of this thesis. It aims to examine the same features of degradation as seen during Culture Enrichment within an additional context of salinity. Specifically, the salt concentrations explored were 0.0, 0.5, 1.0, and 2.0 g/L NaCl within the anaerobic media mix (AMM). These salt concentrations are relatively miniscule in comparison to those of the saline aerobic benzene biodegradation studies discussed in Chapter 2 (upwards of 100 g/L NaCl). However, as this is the first known experiment to study the effects of salt during anaerobic benzene biodegradation, a smaller and conservative salinity ceiling was taken in attempt

for achievable degradation results. The Salinity Experiment commenced on November 28, 2017, proceeded for a duration of 480 days, and concluded on March 22, 2019.

5.1.1 – Transition from the Culture Enrichment Period to the Salinity Experiment

On November 28, 2017, 202 days after the start of the Culture Enrichment period, half the treatment cultures were chosen based on their ability to degrade benzene at a greater rate and decommissioned. These microcosms served as an initial launch point into the Salinity Experiment. The biomasses from treatment bottles of the same redox condition (nitrate-, sulfate-reducing, and methanogenic) were pooled (biomass resuspension following centrifuging in the collection process) regardless of which sediment it was originally derived (clay or sand) and used to create treatments of 0.5, 1.0, and 2.0 g/L NaCl.

On May 17, 2018, 372 days after the start of the Culture Enrichment period, the remaining half of the treatment cultures, previously unchosen, were also decommissioned. In a similar fashion, the biomasses of those treatment bottles were used to create the 0.0 g/L NaCl, 0.0 g/l NaCl sodium azide kill control, and 1.0 g/L NaCl sodium azide kill control treatments of the Salinity Experiment.

During the Culture Enrichment period, varying analysis (in types and quantity) of treatment bottles between redox conditions resulted in different amounts of available liquid culture and biomasses. All available microbial culture content were used during this transition but ultimately contained different dilution ratios to develop the same overall volume which was split equally between all the bottles. These dilution ratios were approximately 50% existing culture with 50% fresh AMM. The dilution ratios and treatment lineages are graphically displayed as Figure 5-1 through 5-3 on the following page.

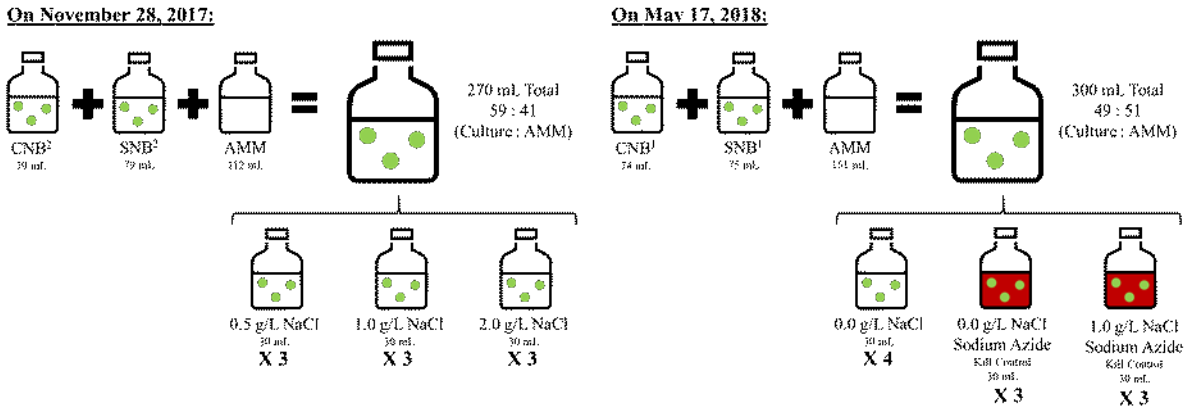

Figure 5-1 – Nitrate-Reducing Treatments: Microcosm Lineages and Dilution Factors

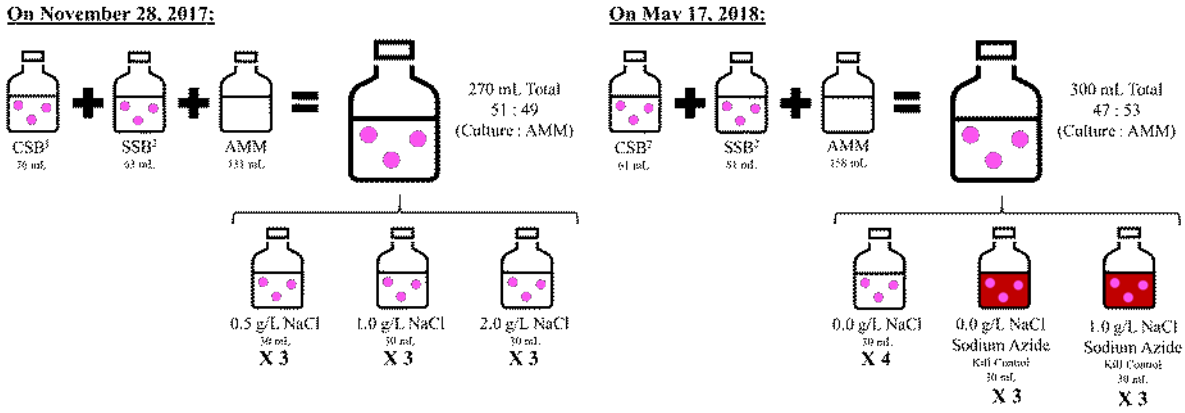

Figure 5-2 – Sulfate-Reducing Treatments: Microcosm Lineages and Dilution Factors

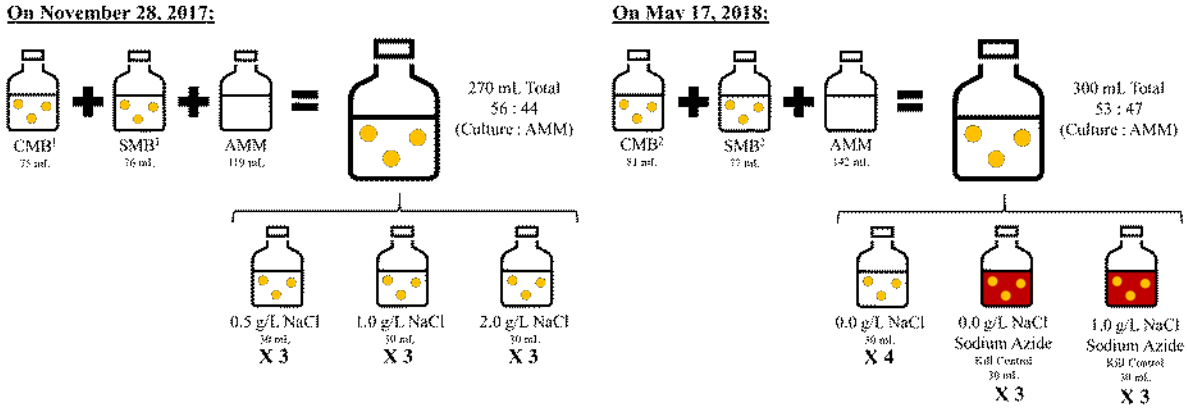

Figure 5-3 – Methanogenic Treatments: Microcosm Lineages and Dilution Factors

5.1.2 – Treatment Setup and Controls of the Salinity Experiment

A total of 66 treatment bottles were developed and dedicated to the Salinity Experiment (22 for each of the three redox conditions). Of the 22 treatment bottles, 13 contain a live microbial culture for benzene degradation, and the remaining nine served as experimental controls. Treatment bottles were kept stationary in a dark box at 20°C. For each redox condition, the treatment bottles are as follows:

- Quadruplets at 0.0 g/L NaCl;
- Triplicates at 0.5 g/L NaCl;
- Triplicates at 1.0 g/L NaCl;
- Triplicates at 2.0 g/L NaCl;
- Triplicates sodium azide kill controls at 0.0 g/L NaCl;
- Triplicates sodium azide kill controls at 1.0 g/L NaCl; and,
- Triplicates of media controls amended with terminal electron acceptor.

The nine control treatment bottles consist of six sodium azide kill controls and three media only controls which serve to quantify any benzene loss extraneous to biodegradation. These abiotic losses may include sorption to sediments within the AMM to produced precipitates such as iron (II) sulfide, sorption to the inner glass walls of the bottle, sorption to the underside of the rubber butyl stopper, and chemical oxidation. For the purpose of this experiment, the crimp and cap apparatus used to install the rubber butyl stopper is assumed to hold an airtight seal within the treatment bottle and prevent any loss of benzene outside the treatment bottle. No visual observations or analytical data from any monitoring sessions throughout both the Culture Enrichment period and the Salinity Experiment contradicts this assumption.

The sodium azide kill controls were developed from active benzene biodegrading treatments of the Culture Enrichment period (see Figures 5-1 through 5-3), amended with a sodium azide concentration of 1 g/L, and autoclaved once per day for three consecutive days to prevent any active metabolism of the microbial culture within. On the following page, Figure 5-4 depicts the benzene content profile of the nitrate-amended, 0.0 g/L NaCl, sodium azide kill control.

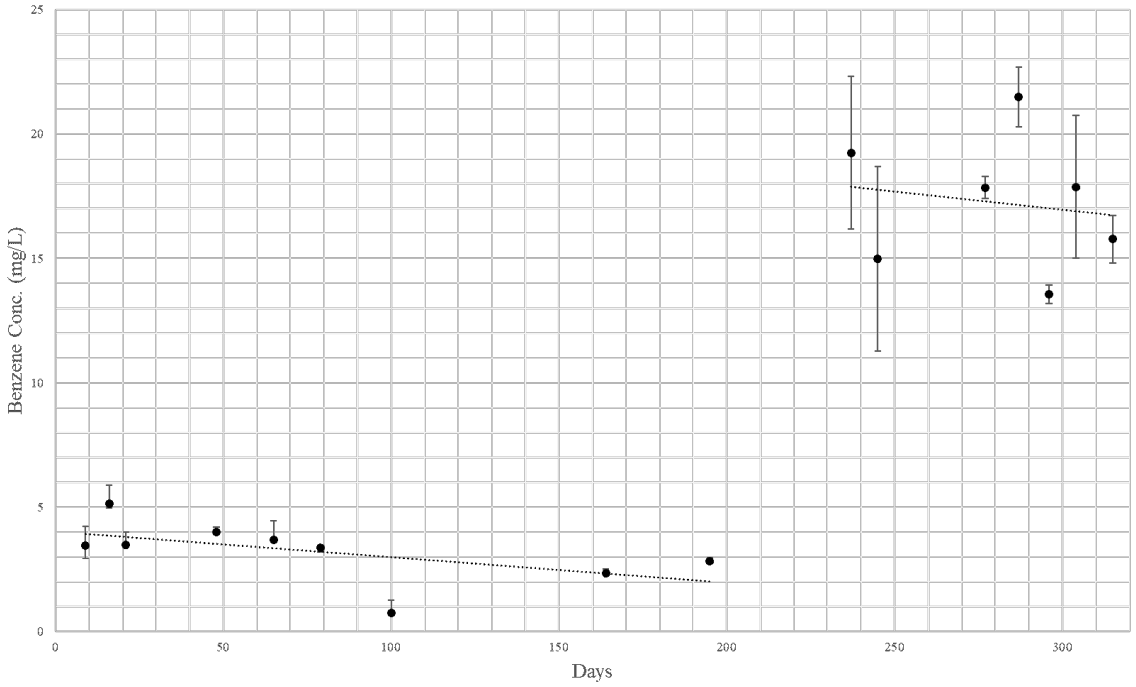

Figure 5-4 – Salinity Experiment: Nitrate-Reducing, 0.0 g/L NaCl, Sodium Azide Kill Control: Abiotic Benzene Losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation between the triplicate treatment bottles. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

In parallel, media controls were also developed. Unlike sodium azide kill controls, these media controls do not contain a benzene degrading culture. Instead, media controls consist solely of fresh AMM amended with the same concentration of terminal electron acceptor as the live degrading treatments (5 mM sodium nitrate for nitrate-reducing treatments and 15 mM sodium sulfate for sulfate-reducing treatments). The methanogenic media controls contained only AMM. These media controls were also autoclaved once per day for a duration of three days. On the next page, Figure 5-5 depicts the benzene content profile of the nitrate-amended, 0.0 g/L NaCl, media control.

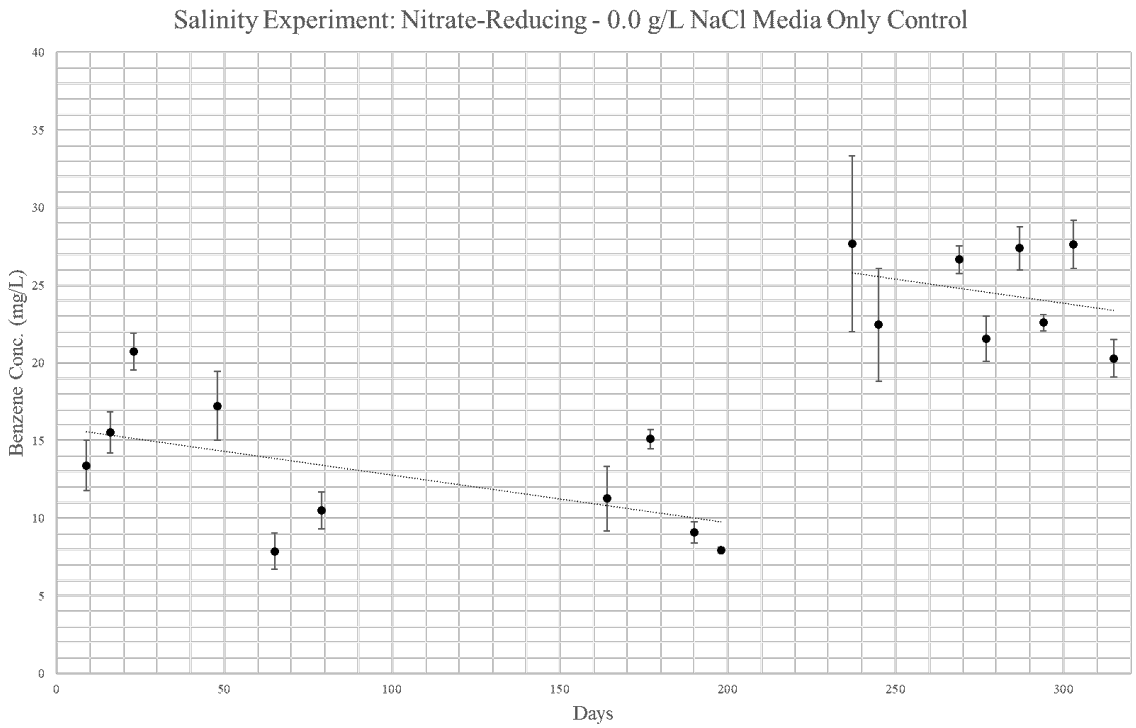

Figure 5-5 – Salinity Experiment: Nitrate-Reducing, 0.0 g/L NaCl, Media Control: Abiotic Benzene Losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation between the triplicate treatment bottles. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

As seen in Figures 5-4 and 5-5, the controls underwent two "degradation cycles" within the 325 day "incubation" period. Benzene refeed occurred on Day 232 to a higher substrate concentration to evaluate the effect of a greater benzene concentration on abiotic losses. In comparison to treatment bottles with a live microbial culture and active biodegradation, no significant benzene biodegradation occurred for both the sodium azide kill and media controls but a downward trend in benzene content is demonstrated for these control treatments. This gradual decrease in benzene is most likely due to the sum of abiotic losses previously described. In efforts to keep Chapter 5 succinct, the nitrate-amended, 1.0 g/L NaCl, sodium azide kill control, as well as the benzene profiles of both the sulfate-reducing and methanogenic sodium azide and media controls will not be shown here. The benzene profiles of these remaining seven control treatments are displayed in Appendix B. Conversely, the remaining data and figures shown in this chapter are not adjusted by these controls as the demonstrated abiotic losses will be minor in comparison to the benzene disappearance due to active biodegradation during the initial degradation period.

5.2 – Degradation Cycles of the Salinity Experiment

Within the three redox conditions (nitrate-, sulfate-reducing, and methanogenic), four salinity levels were explored within the Salinity Experiment: 0.0, 0.5, 1.0, and 2.0 g/L NaCl. This translates to 12 biodegradation profiles. To develop these biodegradation profiles, treatment conditions were set-up in triplicates (quadruplets in the case of 0.0 g/L NaCl), and the average value of these triplicates during each analysis or monitoring session was used to create one data point within the overall biodegradation profile. As a result, 39 treatment bottles were dedicated to the Salinity Experiment (13 for each redox condition).

Each of these treatments underwent two or three degradation cycles for a total of 27 degradation cycles. For the purposes of keeping this chapter succinct, a singular figure compiling the 0.0, 0.5, 1.0, and 2.0 g/L NaCl treatments will be shown for each redox condition in the following pages as Figures 5-6 through 5-8. These degradation profiles are placed in a side-by-side manner for ease in comparison between different salinity levels. With the exception of the 0.0 g/L NaCl treatments, the vertical axis' (benzene, nitrate, nitrite, sulfate, or methane concentration) are set to the same scale for cross comparison. Appendix C showcases all 12 degradation profiles on an individual and enlarged scale for a detailed view of each treatment.

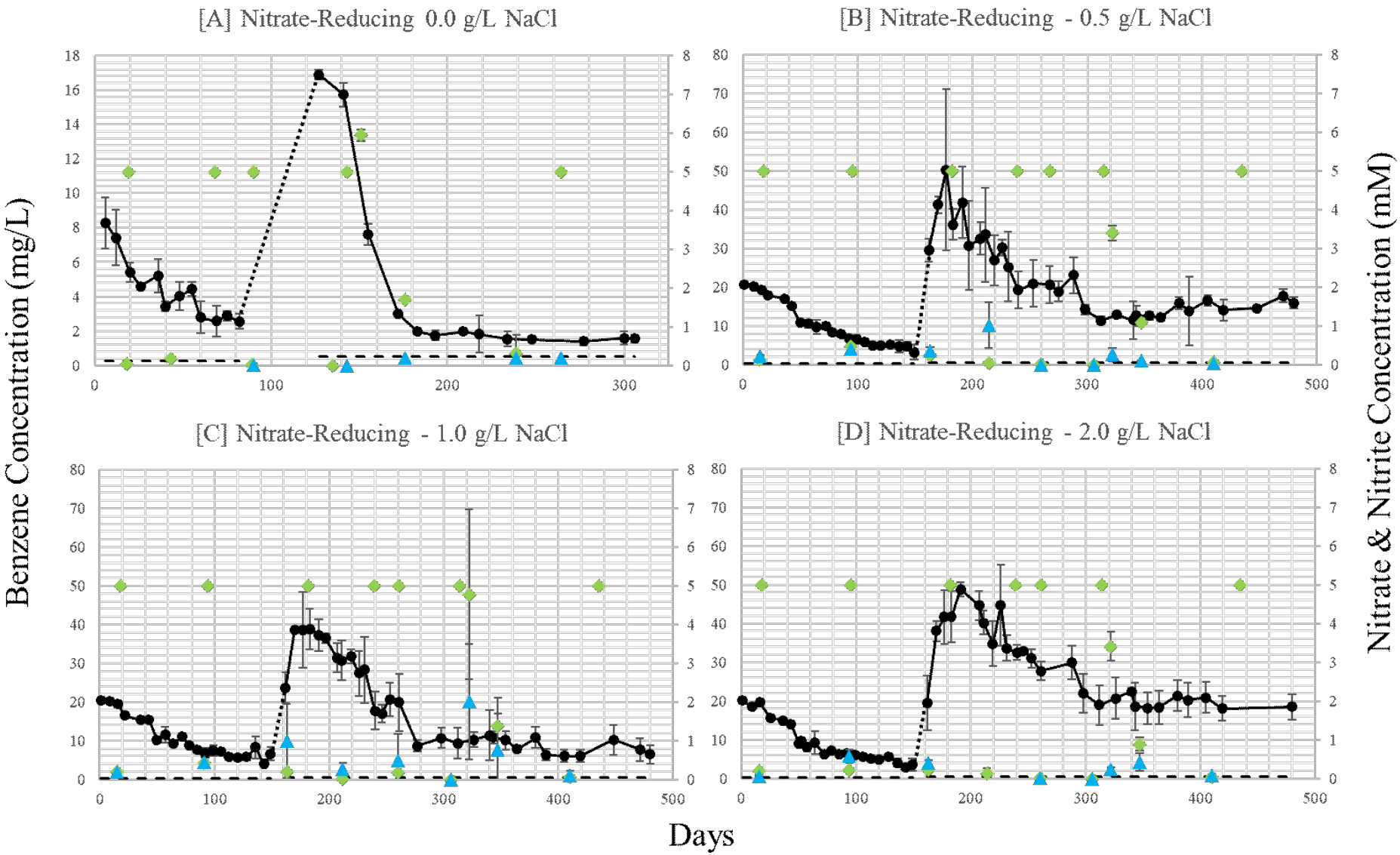

Figure 5-6 – Salinity Experiment: Nitrate-Reducing Treatments: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates.

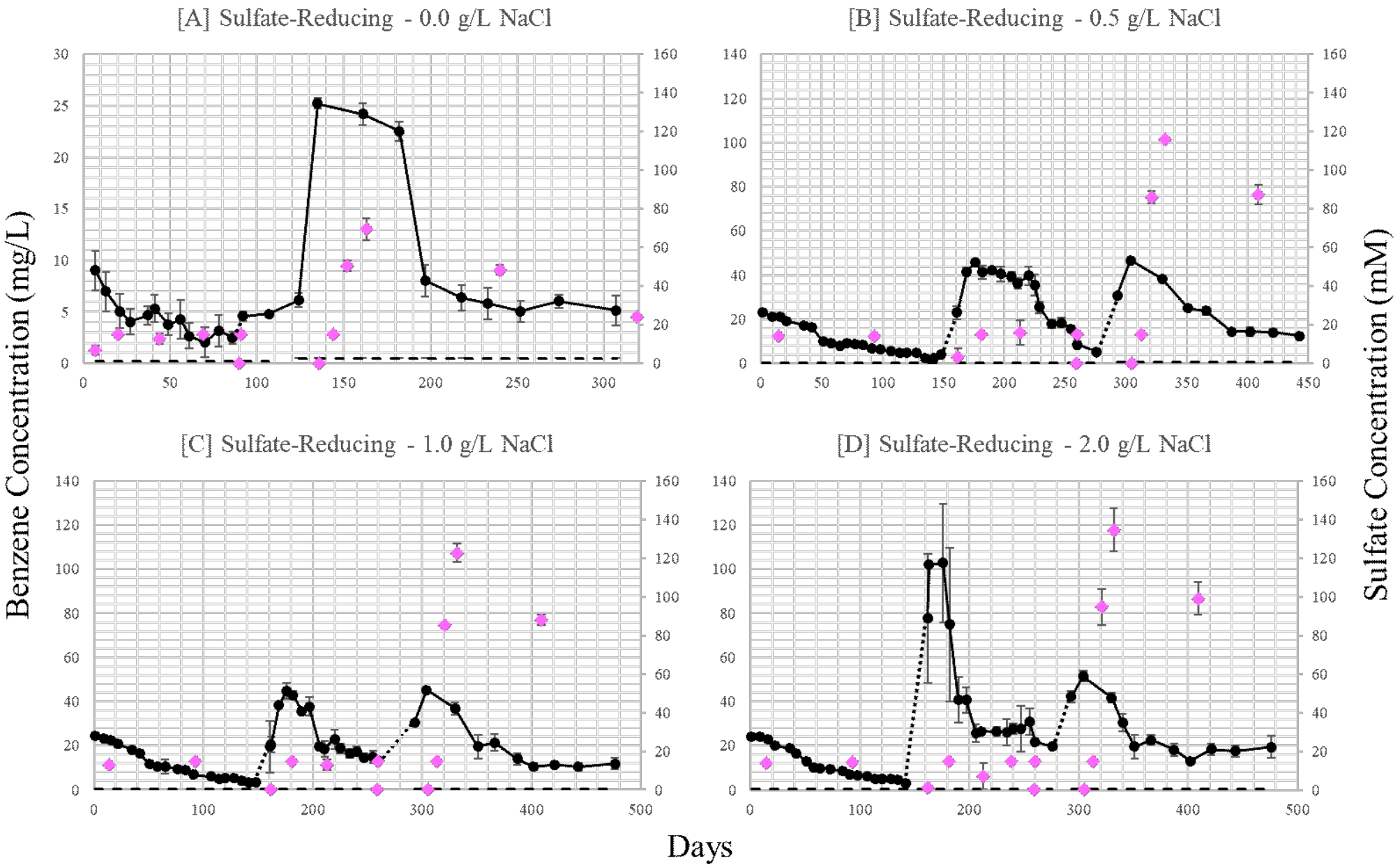

Figure 5-7 – Salinity Experiment: Sulfate-Reducing Treatments: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates.

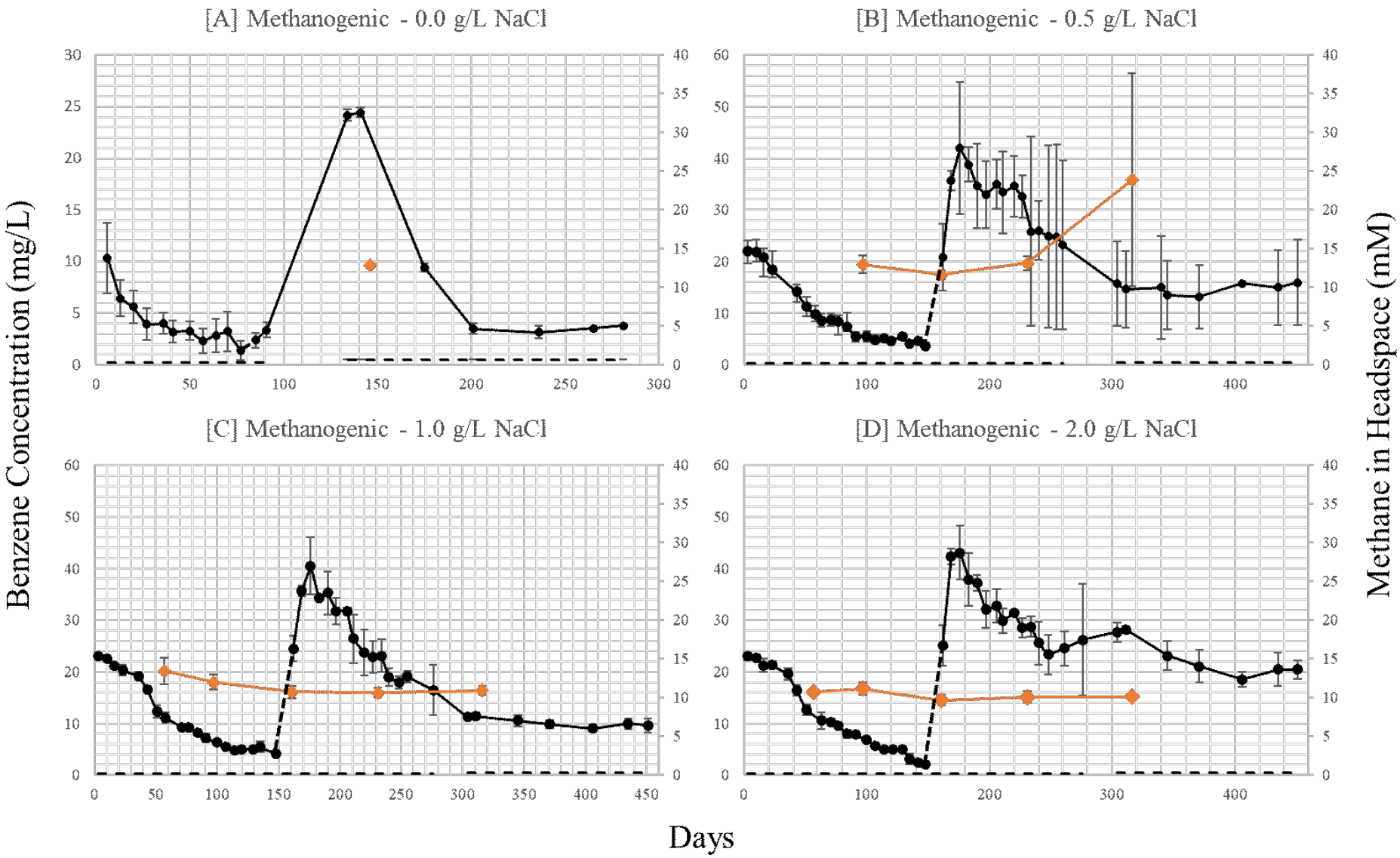

Figure 5-8 – Salinity Experiment: Methanogenic Treatments: Methane content (◆) and benzene biodegradation (●).Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates.

5.3 – Salient Degradation Features of the Salinity Experiment

In this section, the most salient degradation features observed during the Salinity Experiment are compiled in Table 5-1 below. These features include the concentration to which benzene had been amended during refeed, the overall degradation rates observed in each degradation cycle, the maximum degradation rate observed at any time within a cycle, the period to re-equilibrate following benzene refeed, and the lag phase associated with each degradation cycle. Further along, Tables 5-8 and 5-9 displays data related to the usage of nitrate or sulfate as the terminal electron acceptor throughout each degradation cycle and provides a comparison to the theoretical amount(s) as suggested by literature.

Values in Table 5-1 are based on the individual degradation profiles of Figures 5-6 through 5-8 or those in Appendix C. Those figures were plotted from the average benzene, nitrate, nitrite, sulfate, or methane values obtained from the triplicates of each analytical session.

Table 5-1 – Salinity Experiment: Salient Degradation Features

Microcosm	Degradation Cycle: Days[A]	Benzene Conc. Feed (mM)[B]	Overall Degradation (μM/day)[C] [R^2]	Max Degradation (μM/day) (Between Days)[D]	Re-equilibrate Period (Days)[E]	Lag Phase (Days)[F]
Nitrate 0.0 g/L NaCl	1: 0-89	0.11	0.93 [0.81]	2.5 (6-26)	None	2.4
	2: 90-306	0.22	2.70 [0.84]	4.3 (127-172)	None	1.6
Nitrate 0.5 g/L NaCl	1: 0-160	0.26	1.55 [0.92]	4.3 (36-57)	None	3.4
	2: 161-480	0.64	2.28 [0.79]	9.5 (177-197)	15	5.6
Nitrate 1.0 g/L NaCl	1: 0-160	0.26	1.92 [0.93]	3.2 (43-64)	None	2.7
	2: 161-480	0.50	2.34 [0.78]	8.1 (219-240)	8	4.3
Nitrate 2.0 g/L NaCl	1: 0-160	0.26	1.42 [0.85]	5.3 (43-57)	None	3.7
	2: 161-480	0.62	1.53 [0.58]	6.4 (226-246)	29	8.1
Sulfate 0.0 g/L NaCl	1: 0-90	0.12	0.85 [0.74]	3.1 (7-27)	None	2.8
	2: 91-307	0.32	1.98 [0.73]	4.5 (161-218)	43	3.2
Sulfate 0.5 g/L NaCl	1: 0-148	0.30	1.77 [0.90]	5.1 (35-58)	None	3.4
	2: 149-276	0.59	3.87 [0.62]	15.2 (220-239)	15	3.0
	3: 277-443	0.60	2.63 [0.77]	5.1 (304-387)	11	4.6

Table 5-1 (Cont.) – Salinity Experiment: Salient Degradation Features

Microcosm	Degradation Cycle: Days[A]	Benzene Conc. Feed (mM)[B]	Overall Degradation (μM/day)[C] [R^2]	Max Degradation (μM/day) (Between Days)[D]	Re-equilibrate Period (Days)[E]	Lag Phase (Days)[F]
Sulfate 1.0 g/L NaCl	1: 0-148	0.31	2.09 [0.93]	4.6 (35-58)	None	3.0
	2: 149-260	0.57	4.77 [0.78]	12.6 (190-211)	15	2.4
	3: 261-476	0.58	3.34 [0.83]	5.6 (304-366)	11	3.5
Sulfate 2.0 g/L NaCl	1: 0-142	0.31	2.14 [0.93]	5.0 (36-58)	None	2.9
	2: 143-276	1.32	19.89 [0.74]	39.6 (176-197)	14	1.3
	3: 277-476	0.66	3.24 [0.72]	8.5 (304-351)	11	4.1
Meth. 0.0 g/L NaCl	1: 0-77	0.13	1.13 [0.73]	3.7 (6-27)	None	2.3
	2: 78-281	0.31	1.99 [0.70]	4.3 (134-201)	56	3.1
Meth. 0.5 g/L NaCl	1: 0-148	0.28	2.74 [0.97]	3.5 (43-63)	None	2.0
	2: 149-451	0.54	2.19 [0.93]	6.2 (220-240)	14	4.9
Meth. 1.0 g/L NaCl	1: 0-148	0.30	2.37 [0.97]	5.0 (36-58)	None	2.5
	2: 149-451	0.52	2.62 [0.92]	5.1 (206-227)	14	4.0
Meth. 2.0 g/L NaCl	1: 0-148	0.29	1.95 [0.95]	4.3 (36-63)	None	3.0
	2: 149-451	0.55	2.18 [0.81]	6.2 (176-197)	14	5.0

[A] Degradation cycles and duration as defined in Chapter 3, Section 3.4.1.

[B] Maximum benzene concentration observed within each degradation cycle

[C] Overall degradation rate calculated from the initial degradation phase of each degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.4.

[D] The maximum degradation rate observed between any four consecutive monitoring sessions within one degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.5.

[E] Number of days between benzene refeed and the day a maximum benzene concentration was observed within each degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.3.

[F] Number of days required for a 2% decrease from the maximum benzene concentration in each respective degradation cycle. Detailed explanation and calculation process discussed in Chapter 3, Section 3.4.6.

5.3.1 – Overall Degradation Rate

From the Salinity Experiment, 27 degradation cycles exploring benzene biodegradation within nitrate-, sulfate-reducing, and methanogenic conditions at salinity concentrations of 0.0, 0.5, 1.0, and 2.0 g/L NaCl were obtained. The average overall degradation rate was 2.9 ± 3.4 µM/d within a range of 0.85 to 19.89 µM/d. Parallel to Section 4.3.1 and Figure 4-5 of the previous chapter, a strong linear correlation was also observed between benzene feed concentration and the resulting overall degradation during the Salinity Experiment. This is shown in Figure 5-9 below.

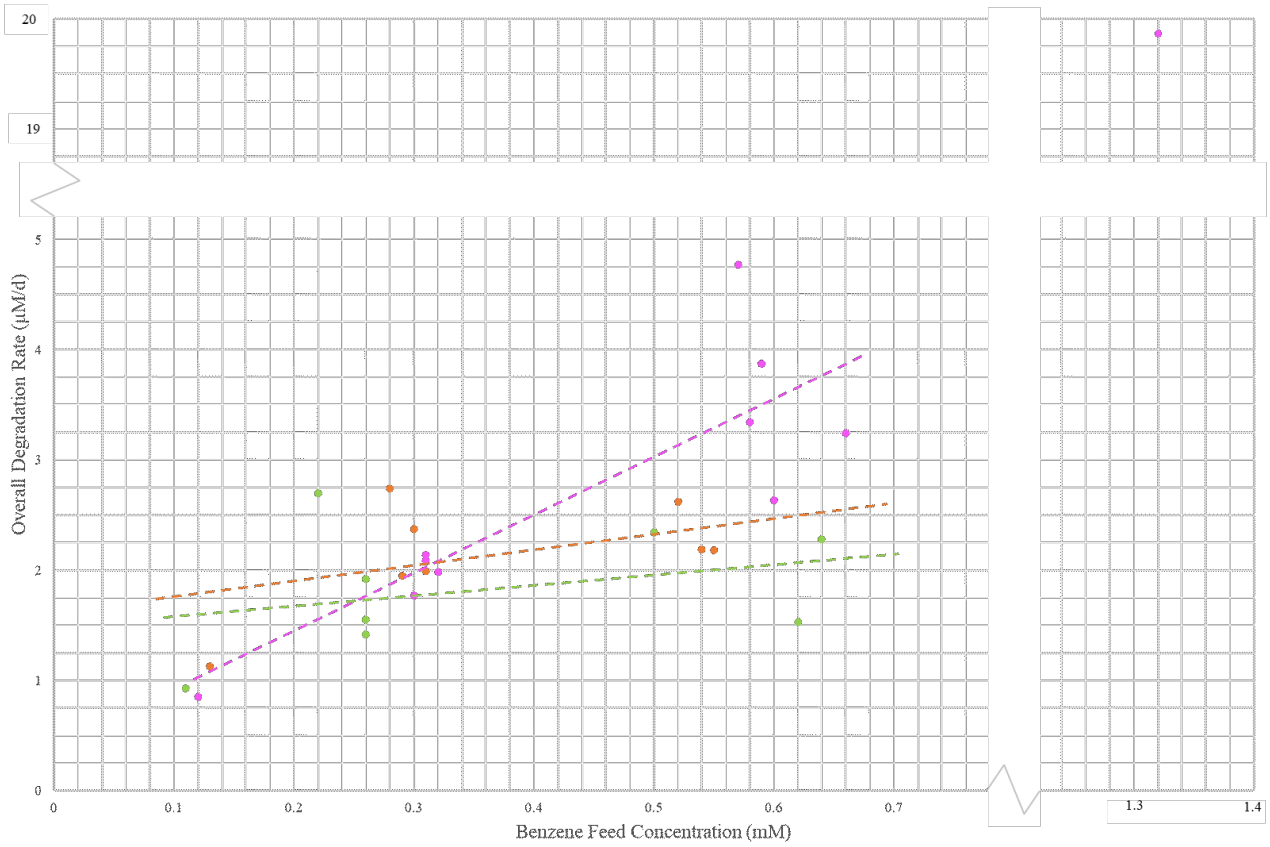

Figure 5-9 – Salinity Experiment: Overall Degradation Rate as a Function of Benzene Feed Concentration. Nitrate-reducing (●), sulfate-reducing (●), and methanogenic (●) treatments. Dotted lines represent the linear correlation between overall degradation rate and benzene feed concentration.

At lower benzene concentrations, available and accessible substrate is most likely degraded without limitations on terminal electron acceptor or suboptimal microbial culture count within the AMM. Specifically, the lower degradation rate is simply due to the lower benzene content within the treatment bottle. Conversely, excessive benzene concentrations (before the onset of toxic effects) can oversaturate the AMM and result in a greater degradation rate due to the accessibility

of the benzene for biodegradation. In that case, the rate limiting factor would be the limited cell count of the microbial culture itself. The only outlier for this trend is the second degradation cycle of the sulfate-reducing 2.0 g/L NaCl treatment in which a benzene feed concentration of 1.32 mg/L resulted in an overall degradation rate of 19.89 μM/d. The axis' of Figure 5-9 are scaled in a manner show this linear correlation but excludes this outlier.

It is also evident in Figure 5-9 that benzene refeeds resulted in substrate concentrations within three clusters of approximately 0.12, 0.3, and 0.6 mM throughout the Salinity Experiment. As the relationship between substrate feed concentration and degradation rate is previously established, only treatments with similar benzene content is considered when comparing the biodegradation abilities between salinity concentrations. In Table 5-2 and Figure 5-10 below, the overall rates for degradation cycles in which a benzene feed concentration ranging between 0.2 and 0.4 mM are tabulated and plotted. Similarly, Table 5-3 and Figure 5-11 shows the overall degradation rates for a higher benzene feed concentration: 0.5 to 0.7 mM.

Table 5-2 – Salinity Experiment: Overall Degradation Rates (0.2 – 0.4 mM Benzene Feed Conc.)

Benzene Feed Concentration: 0.2 – 0.4 mM	
Average: **2.1 ± 0.4** μM/d	0.0 g/L NaCl: **2.2 ± 0.3** μM/d
	0.5 g/L NaCl: **2.0 ± 0.5** μM/d
	1.0 g/L NaCl: **2.1 ± 0.2** μM/d
	2.0 g/L NaCl: **1.8 ± 0.3** μM/d
Nitrate-Reducing: **1.9 ± 0.5** μM/d	0.0 g/L NaCl: **2.7** μM/d
	0.5 g/L NaCl: **1.6** μM/d
	1.0 g/L NaCl: **1.9** μM/d
	2.0 g/L NaCl: **1.4** μM/d
Sulfate-Reducing: **2.0 ± 0.1** μM/d	0.0 g/L NaCl: **2.0** μM/d
	0.5 g/L NaCl:**1.8** μM/d
	1.0 g/L NaCl: **2.1** μM/d
	2.0 g/L NaCl: **2.1** μM/d
Methanogenic: **2.3 ± 0.3** μM/d	0.0 g/L NaCl: **2.0** μM/d
	0.5 g/L NaCl: **2.7** μM/d
	1.0 g/L NaCl: **2.4** μM/d
	2.0 g/L NaCl: **2.0** μM/d

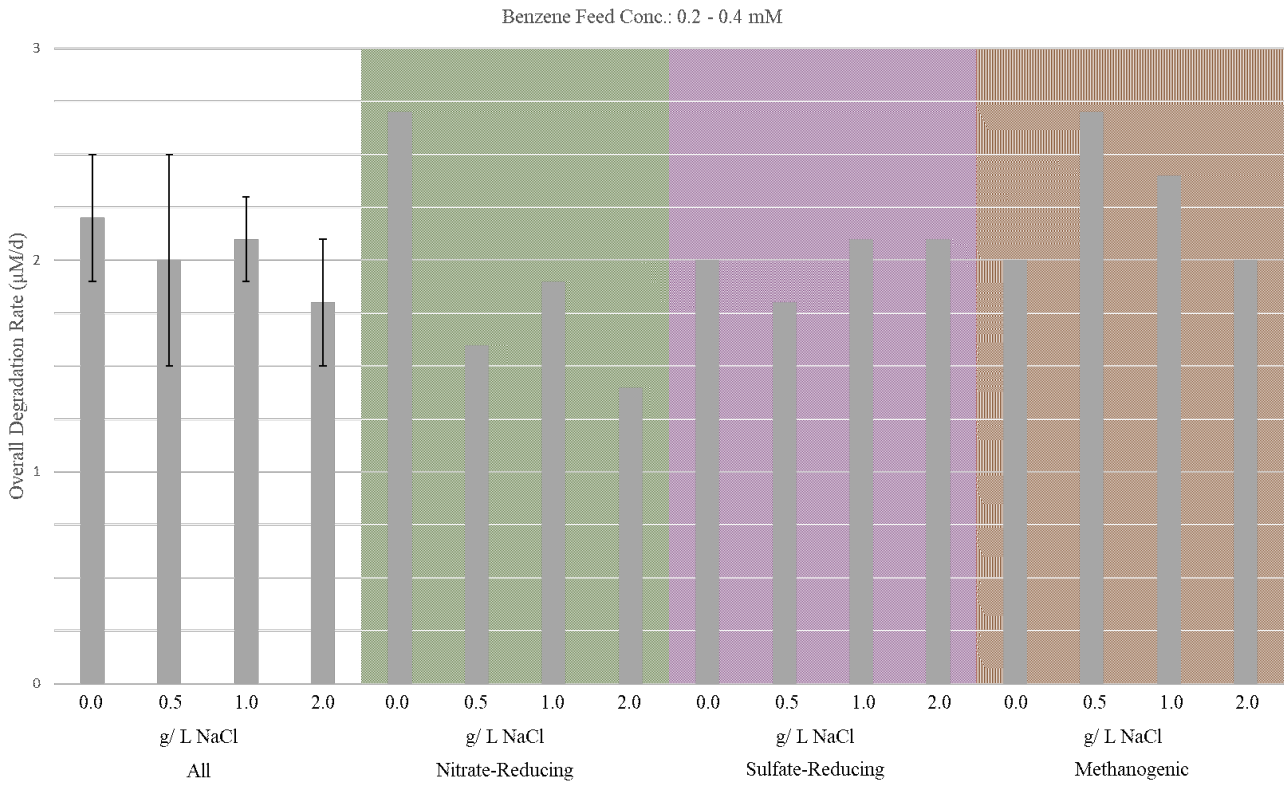

Figure 5-10 – Salinity Experiment: Overall Degradation Rates (0.2-0.4 mM Benzene). Data is organized based on anaerobic condition and salinity concentrations. Error bars represent one standard of deviation within its respective criteria. Values within individual redox conditions do not contain error bars because these degradation rates were calculated from one individual degradation profile.

Table 5-3 – Salinity Experiment: Overall Degradation Rates (0.5 – 0.7 mM Benzene Feed Conc.)

Benzene Feed Concentration: 0.5 – 0.7 mM	
Average: **2.8 ± 0.9** µM/d	0.5 g/L NaCl: **2.7 ± 0.7** µM/d
	1.0 g/L NaCl: **3.3 ± 0.9** µM/d
	2.0 g/L NaCl: **2.3 ± 0.7** µM/d
Nitrate-Reducing: **2.1 ± 0.4** µM/d	0.5 g/L NaCl: **2.3** µM/d
	1.0 g/L NaCl: **2.3** µM/d
	2.0 g/L NaCl: **1.5** µM/d
Sulfate-Reducing: **3.6 ± 0.7** µM/d	0.5 g/L NaCl: **3.3** µM/d
	1.0 g/L NaCl: **4.1** µM/d
	2.0 g/L NaCl: **3.2** µM/d
Methanogenic: **2.3 ± 0.2** µM/d	0.5 g/L NaCl: **2.2** µM/d
	1.0 g/L NaCl: **2.6** µM/d
	2.0 g/L NaCl: **2.2** µM/d

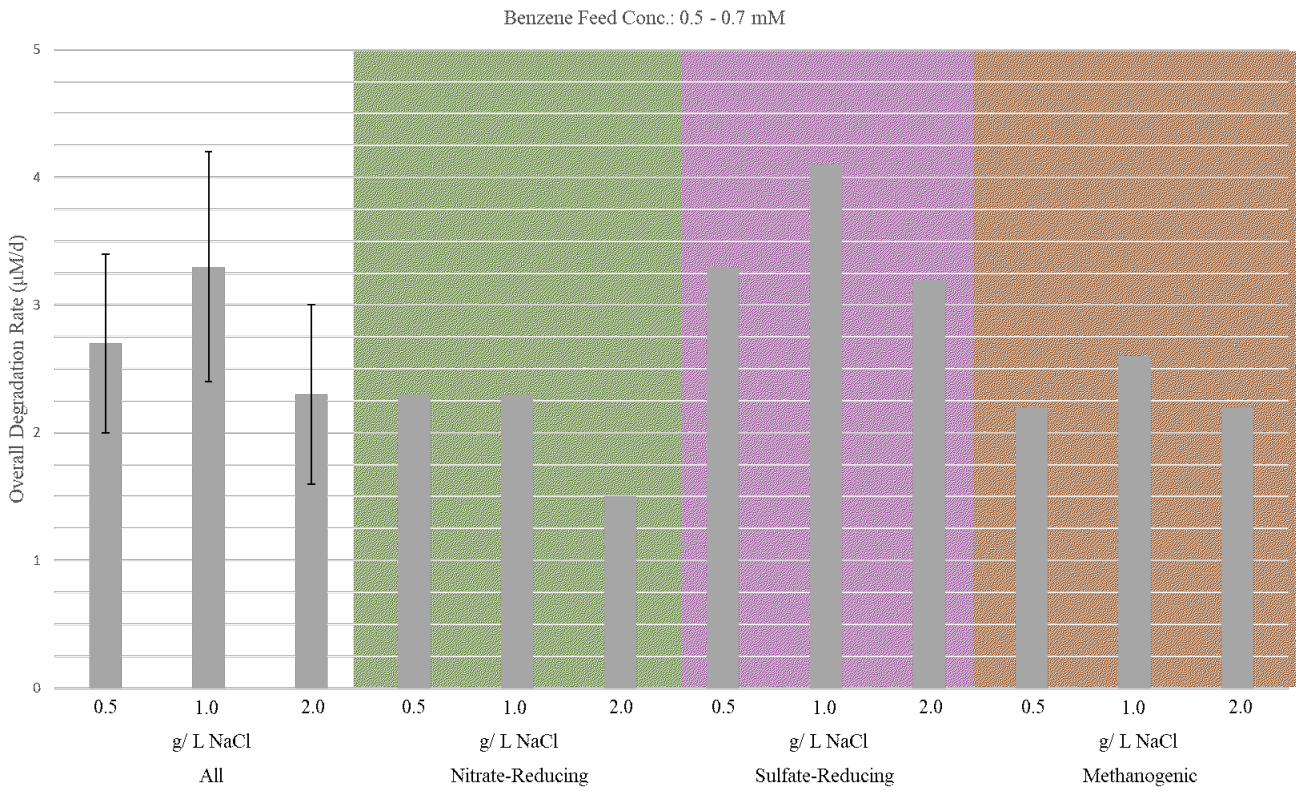

Figure 5-11 – Salinity Experiment: Overall Degradation Rates (0.5-0.7 mM Benzene). Data is organized based on anaerobic condition and salinity concentrations. Error bars represent one standard of deviation within its respective criteria. Values within individual redox conditions do not contain error bars because these degradation rates were calculated from one individual degradation profile.

As seen in Figure 5-10 corresponding to treatments with 0.2 to 0.4 mM of initial benzene, nitrate-reducing treatments degraded benzene at the greatest rate within 0.0 g/L NaCl (2.7 µM/d) but degradation performance decreased with additional salinity. When substrate concentration was increased to an elevated range of 0.5 to 0.7 mM, both 0.5 and 1.0 g/L NaCl demonstrated a rate of 2.3 µM/d. The 2.0 g/L NaCl treatment exhibited the slowest overall degradation rates in both circumstances.

For sulfate-reducing treatments, no significant differences in degradation was observed within the lower benzene range and an average rate of 2.0 ± 0.1 µM/d was achieved. However, in the 0.5 to 0.7 mM range, the 1.0 g/L NaCl treatment outperformed those of 0.5 and 2.0 g/L NaCl. Furthermore this 1.0 g/L NaCl sulfate-reducing treatment demonstrated the greatest overall degradation rate (4.1 µM/d) of the three redox conditions within this higher feed concentration.

In the lower benzene range of the methanogenic treatments, degradation was greatest at 0.5 and 1.0 g/L NaCl (2.7 and 2.4 µM/d respectively). Within the higher benzene concentrations, overall degradation rate also peaked at 1.0 g/L salinity level (2.6 µM/d).

The overall degradation rates seen in the Salinity Experiment are less than those of the Culture Enrichment period (recall 12.6 ± 11.3, 20.1 ± 11.3, and 17.0 ± 6.8 µM/d for the nitrate-, sulfate-reducing, and methanogenic treatments of Chapter 4). At a cursory glance, the inclusion of salt may be a likely explanation for the difference in degradation capabilities for the reasons discussed below. However, if the inclusion of a salt factor is the only reason for such a difference, then benzene biodegradation performance should be comparable for the treatments with 0.0 g/L NaCl within the Salinity Experiment and respective counterparts within the Culture Enrichment period. As this was not the case, the dilution rates (approximately 50% based on Figures 5-1 through 5-3) applied to microbial cultures to develop the treatment bottles for this phase of the experiment is most likely the only reason for diminished biodegradation capabilities. Although the Salinity Experiment was incubated for a total period of 548 days, it appears more time is required for the microbial culture to reacclimate and cultivate to its full potential after such an extensive dilution.

Numerous studies explored benzene or other petroleum hydrocarbon biodegradation in the presence of salinity. Ulrich et al. (2009) and Børresen and Rike (2007) reported that trace concentrations of NaCl (<0.3% w/w) causes a stimulatory effect on degradation. A possible explanation for this effect may be that the salt content provides an ionically balanced medium for microbial growth, or cause the medium to disperse clays and therefore create a greater surface area allowing for increased bioavailability and access to nutrients (Ulrich et al. 2009). Throughout the literature review, studies which explored a salinity range for aerobic benzene degradation often reported an optimal concentration which yielded a greater degradation rate. In those cases, a salt content between 1.0 and 2.0 M NaCl often outperformed identical samples at 0 M or even greater concentrations such as 5.0 M NaCl (Al-Mailem et al. 2013; Hassan et al. 2012; Li et al. 2006; Nicholson and Fathepure 2004, 2005; Sei and Fathepure 2009). However, further increases in salt content past this optimal range often leads to inhibitory effects. In general, salt creates a high osmotic potential within the microbe's environment and changes the "solubility or sorption of toxic or essential ions" which directly inhibits metabolic activity (Ulrich et al. 2009). Excessive salt is believed to disrupt tertiary protein structures and denature enzymes of cell dehydration

within microbial communities (Ulrich et al. 2009). Furthermore, it is suspected that salinity may also deceases the accessibility of organic matter within the sediments to the microbial community (Qin et al. 2012).

Across the three redox conditions, 1.0 g/L NaCl was identified as the optimal salinity concentration which consistently yielded a higher overall degradation rate. There were specific instances, as seen in Figures 5-10 and 5-11, in which treatments at 0.5 or 2.0 g/L NaCl provided a comparable degradation rate, but generally does not outperform the 1.0 g/L NaCl treatments. In the previous paragraph, possible explanations in which salt can stimulate or inhibit biodegradation were provided. Unfortunately, the simple set-up of the Salinity Experiment cannot discriminate between these possible effects. The results presented in this chapter only show the cumulative effects, both stimulatory and inhibitory, of salt on the microbial community's ability to biodegrade benzene under anaerobic conditions. Further experiments involving smaller salinity intervals and exploring a greater salinity range can elucidate the optimal salinity concentration for anaerobic benzene biodegradation; but the available data tentatively suggest this range to be approximately 1.0 g/L NaCl.

5.3.2 – Maximum Degradation Rate

The maximum degradation rate represents the greatest observed amount of degradation between any four consecutive monitoring sessions within a degradation cycle. The average maximum degradation rate is 7.1 ± 7.0 µM/d with a range of 2.5 to 39.6 µM/d throughout the 27 degradation cycles of the Salinity Experiment. This information is tabulated in Tables 5-4 and 5-5 based on benzene feed concentration, and graphically displayed as Figure 5-12.

Table 5-4 – Salinity Experiment: Max. Degradation Rates (0.2 – 0.4 mM Benzene Feed Conc.)

Benzene Feed Concentration: 0.2 – 0.4 mM	
Average: **4.5 ± 0.6** μM/d	0.0 g/L NaCl: **4.4 ± 0.1** μM/d
	0.5 g/L NaCl: **4.3 ± 0.7** μM/d
	1.0 g/L NaCl: **4.3 ± 0.8** μM/d
	2.0 g/L NaCl: **4.9 ± 0.4** μM/d
Nitrate-Reducing: **4.3 ± 0.7** μM/d	0.0 g/L NaCl: **4.3** μM/d
	0.5 g/L NaCl: **4.3** μM/d
	1.0 g/L NaCl: **3.2** μM/d
	2.0 g/L NaCl: **5.3** μM/d
Sulfate-Reducing: **4.8 ± 0.3** μM/d	0.0 g/L NaCl: **4.5** μM/d
	0.5 g/L NaCl: **5.1** μM/d
	1.0 g/L NaCl: **4.6** μM/d
	2.0 g/L NaCl: **5.0** μM/d
Methanogenic: **4.3 ± 0.5** μM/d	0.0 g/L NaCl: **4.3** μM/d
	0.5 g/L NaCl: **3.5** μM/d
	1.0 g/L NaCl: **5.0** μM/d
	2.0 g/L NaCl: **4.3** μM/d

Table 5-5 – Salinity Experiment: Max. Degradation Rates (0.5+ mM Benzene Feed Conc.)

Benzene Feed Concentration: 0.5+ mM	
Average: **8.0 ± 3.1** μM/d	0.5 g/L NaCl: **9.0 ± 3.9** μM/d
	1.0 g/L NaCl: **7.9 ± 3.0** μM/d
	2.0 g/L NaCl: **7.0 ± 1.0** μM/d
Nitrate-Reducing: **8.0 ± 1.3** μM/d	0.5 g/L NaCl: **9.5** μM/d
	1.0 g/L NaCl: **8.1** μM/d
	2.0 g/L NaCl: **6.4** μM/d
Sulfate-Reducing: **9.4 ± 3.9** μM/d	0.5 g/L NaCl: **10.2** μM/d
	1.0 g/L NaCl: **9.1** μM/d
	2.0 g/L NaCl: **8.5** μM/d
Methanogenic: **5.8 ± 0.5** μM/d	0.5 g/L NaCl: **6.2** μM/d
	1.0 g/L NaCl: **5.1** μM/d
	2.0 g/L NaCl: **6.2** μM/d

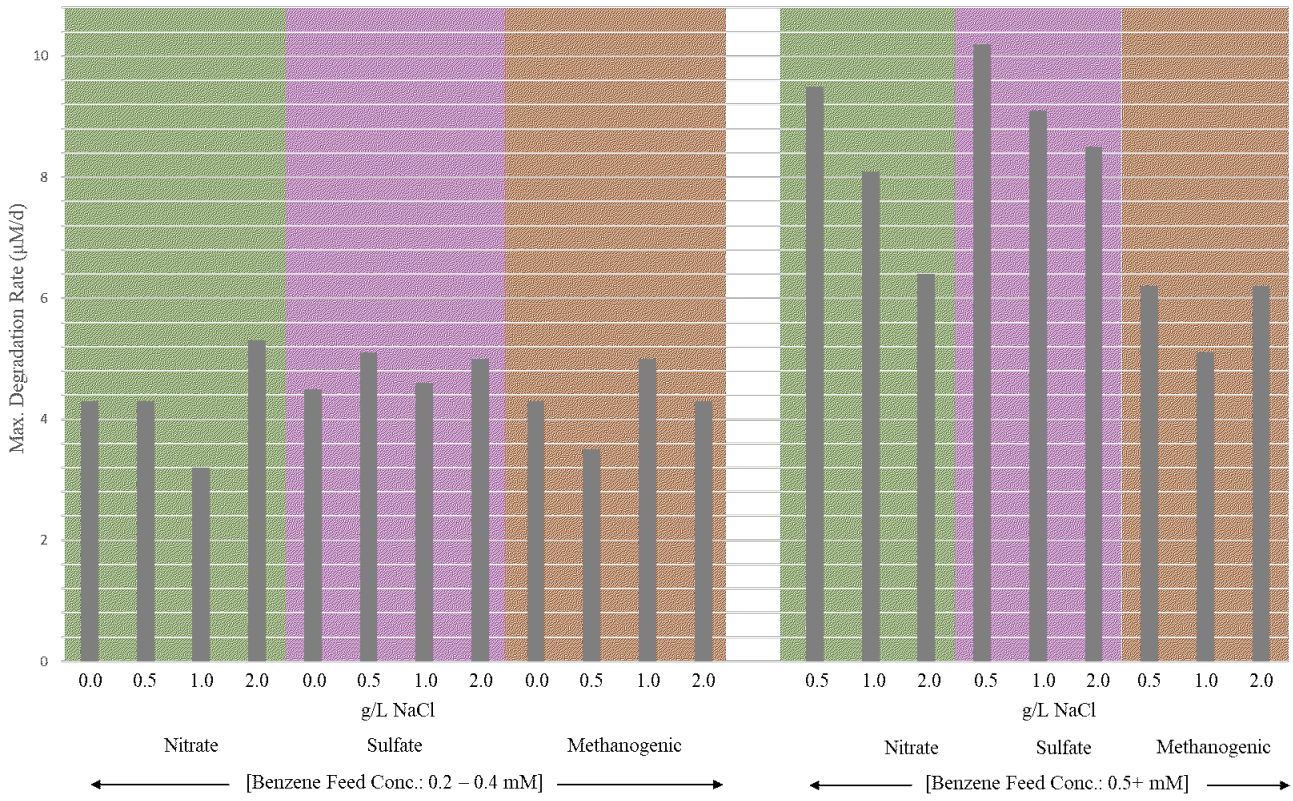

Figure 5-12 – Salinity Experiment: Maximum Degradation Rates. Data is organized based on redox condition, salinity concentrations and benzene feed concentration.

These maximum degradation rates represent the degradation capabilities of each treatment within the most optimal conditions provided during the Salinity Experiment. Therefore, the rates provided in this section will always be greater in comparison to those in Section 5.3.1, but is not as robust as a metric for comparison as it only considers four consecutive data points as opposed to the degradation cycle in its entirety.

Within the lower benzene concentration range (0.2 to 0.4 mM), a distinctive salinity concentration resulting in the greatest maximum degradation rate does not appear obvious. In contrary, the rates remained consistent (4.5 ± 0.6 µM/d) across the three redox conditions. The only outliers are the nitrate-reducing 1.0 g/L NaCl and methanogenic 0.5 g/L NaCl treatments with a maximum degradation rate of 3.2 and 3.5 µM/d respectively. Regarding the higher benzene feed concentration (more than 0.5 mM), increasing salt content inhibits maximum degradation rates for the nitrate- and sulfate-reducing treatments. Lastly for the methanogenic treatments, a salt content

of 1.0 g/L NaCl resulted in the lowest maximum rate whilst the 0.5 and 2.0 g/L NaCl both demonstrated a rate of 6.2 μM/d.

5.3.3 – Re-equilibrate Phase

The re-equilibrate period is the required time in between benzene refeed and the day in which a maximum benzene concentration was observed within any degradation cycle. This parameter represents the necessary time for a new equilibrium to be establish after benzene refeed. During the Salinity Experiment, the re-equilibrate period averaged 10.0 ± 13.6 days with a range of 0 to 56 days. In Table 5-6 below, the re-equilibrate phases are tabulated based on redox conditions and salinity.

Table 5-6 – Salinity Experiment: Re-equilibrate Phases

Average: **10.0 ± 13.6** days	0.0 g/L NaCl: **16.5** ± **23.6** days*
	0.5 g/L NaCl: **7.9 ± 6.9** days*
	1.0 g/L NaCl: **6.9 ± 6.3** days*
	2.0 g/L NaCl: **9.7 ± 10.0** days*
Nitrate-Reducing: **6.5 ± 9.9** days	0.0 g/L NaCl: **0** days
	0.5 g/L NaCl: **7.5** days
	1.0 g/L NaCl: **4.0** days
	2.0 g/L NaCl: **14.5** days
Sulfate-Reducing: **10.9 ± 11.9** days	0.0 g/L NaCl: **21.5** days
	0.5 g/L NaCl: **8.7 ± 6.3** days*
	1.0 g/L NaCl: **8.7 ± 6.3** days*
	2.0 g/L NaCl: **8.3 ± 6.0** days*
Methanogenic: **12.3 ± 17.8** days	0.0 g/L NaCl: **28.0** days
	0.5 g/L NaCl: **7.0** days
	1.0 g/L NaCl: **7.0** days
	2.0 g/L NaCl: **7.0** days

*One standard of deviation given for datasets calculated from three or more datapoints

On average, the methanogenic treatments had the lengthiest period of re-equilibrate (12.3 ± 17.8 days) while the nitrate-reducing treatments demonstrated the shortest (6.5 ± 9.9 days). Between salinity concentrations, the 0.0 g/L NaCl salt level contained the greatest period of 16.5

± 23.5 days. The remaining three salt concentrations have comparable re-equilibrate phases (6.9 to 7.9 days). Only 14 of the 27 degradation cycles of the Salinity Experiment demonstrated a re-equilibrate period. In general, these periods lasted no longer than two weeks with the exception of nitrate-reducing 2.0 g/L NaCl (second cycle), sulfate-reducing, 0.0 g/L NaCl (first cycle), and methanogenic 0.0 g/L NaCl (second cycle).

5.3.4 – Lag Phase

Lag phases correlating to a 2% decrease in benzene concentration were back-calculated from the overall degradation and benzene feed concentration in Table 5-1 (Mahour 2016; Reardon et al. 2000). These lag phases are tabulated in Table 5-7 below, averaged 3.4 ± 1.4 days, and ranged between 1.3 and 8.1 days across the 27 degradation cycles of the Salinity Experiment.

Table 5-7 – Salinity Experiment: Lag Phase

Average: **3.4 ± 1.4** days	0.0 g/L NaCl: **2.6 ± 0.5** days*
	0.5 g/L NaCl: **3.8 ± 1.2** days*
	1.0 g/L NaCl: **3.2 ± 0.7** days*
	2.0 g/L NaCl: **4.0 ± 2.0** days*
Nitrate-Reducing: **4.0 ± 1.9** days	0.0 g/L NaCl: **2** days
	0.5 g/L NaCl: **4.5** days
	1.0 g/L NaCl: **3.5** days
	2.0 g/L NaCl: **5.9** days
Sulfate-Reducing: **3.1 ± 0.8** days	0.0 g/L NaCl: **2.8** days
	0.5 g/L NaCl: **3.7 ± 0.7** days*
	1.0 g/L NaCl: **3.0 ± 0.4** days*
	2.0 g/L NaCl: **2.8 ± 1.1** days*
Methanogenic: **3.4 ± 1.1** days	0.0 g/L NaCl: **2.7** days
	0.5 g/L NaCl: **3.5** days
	1.0 g/L NaCl: **3.3** days
	2.0 g/L NaCl: **4.0** days

*One standard of deviation given for datasets calculated from three or more datapoints

Nitrate-reducing treatments demonstrated the lengthiest lag phases (4.0 ± 1.9 days) among the three redox conditions while sulfate-reducing and methanogenic treatments demonstrated

comparable lag phases (3.1 ± 0.8 and 3.4 ± 1.1 days respectively). With the influence of salinity, the shortest lag phases were observed for treatments containing 1.0 g/L NaCl except for sulfate-reducing samples. As explained in Section 5.3.1, this salinity concentration was identified to biodegrade benzene at the greatest rate. Since lag phase is calculated inversely proportional to overall degradation rate, treatments at 1.0 g/L will expectedly exhibit shorter lag periods.

5.3.5. – Electron Acceptor Utilization Ratios

Parallel to Section 4.3.5 of the previous chapter, the nitrate- and sulfate- utilization ratios of the Salinity Experiment will be discussed here. These ratios are calculated based on concentrations of nitrate, nitrite, and sulfate analyzed via an ion chromatography (IC) system. Changes in these anions are then mapped against decreases in benzene observed via headspace analysis to develop the ratios. Table 5-8 displays the terminal electron utilization ratios for the nitrate-reducing treatments while Table 5-9 tabulates those of the sulfate-reducing treatments.

Recall the theoretical nitrate : benzene ratios of 7.80, 9.75, and 15 associated with the incomplete reduction of nitrate to nitrite in the chemical reactions 1 through 3 below:

(Foght 2008; Vogt et al. 2011):

$$C_6H_6 + \mathbf{15}NO_3^- + 3H_2O \rightarrow 6HCO_3^- + 15NO_2^- + 6H^+ \text{ (1)}$$

(Ulrich and Edwards 2003):

$$C_6H_6 + 2.56H_2O + \mathbf{9.75}NO_3^- + 0.44NH_4^+ \rightarrow 3.81HCO_3^- + 9.75NO_2^- + 0.44C_5H_7O_2N + 6H^+ \text{(2)}$$

(Ulrich 2004):

$$C_6H_6 + 3.72H_2O + \mathbf{7.80}NO_3^- + 0.72NH_4^+ + 2.88CO_2 \rightarrow 5.28HCO_3^- + 7.8NO_2^- + 0.72C_5H_7O_2N + 6H^+ \text{ (3)}$$

Conversely, complete reduction of nitrate to nitrogen gas has also been suggested by literature and are shown in the following chemical reactions. These ratio values of 5.23 and 6 are obtained from the chemical reactions (4 and 5) below:

(Foght 2008; Meckenstock et al. 2016; Vogt et al. 2011):

$$C_6H_6 + \mathbf{6}NO_3^- \rightarrow 6HCO_3^- + 3N_2 \text{ (4)}$$

(Ulrich 2004):

$$C_6H_6 + 2.43H_2O + \mathbf{2.53}NO_3^- + 0.87NH_4^+ + 3.48CO_2 \rightarrow 5.23HCO_3^- + 1.92N_2 + 0.87C_5H_7O_2N + 3.12H^+ \text{ (5)}$$

Table 5-8 – Salinity Experiment: Usage Ratios of Nitrate-Reducing Microcosms

				Ratio mol Nitrate : mol Benzene	
Microcosm	Degradation Cycle	Days	Benzene Conc. Fed (mM)	Observed	Theoretical
0.0 g/L NaCl	1	0 – 89	0.11	133.2	to NO_3^-: 7.80[A] 9.75[B] 15[C] to N_2: 2.53[A] 6[D]
	2	90 – 306	0.22	28.6	
0.5 g/L NaCl	1	0 – 160	0.26	42.2	
	2	161 – 480	0.64	29.6	
1.0 g/L NaCl	1	0 – 160	0.26	27.3	
	2	161 – 480	0.50	38.5	
2.0 g/L NaCl	1	0 – 160	0.26	43.4	
	2	161 – 480	0.62	48.1	

[A] (Ulrich 2004)
B (Ulrich and Edwards 2003)
C (Foght 2008; Vogt et al. 2011)
D (Foght 2008; Meckenstock et al. 2016; Vogt et al. 2011)

As seen in Table 5-8, the individual degradation cycles of nitrate-reducing treatments during the Salinity Experiment yielded nitrate : benzene ratios between 27.3 and 133.2. Apart from 0.0 g/L NaCl's first cycle, all the observed ratios are within one magnitude greater than the theoretical values regardless of incomplete or complete reduction of nitrate.

In terms of the benzene biodegradation under sulfate-reducing conditions, a theoretical ratio of 3.49 sulfate reduction per benzene oxidization of had been suggest by literature:

(Ulrich and Edwards 2003):

$$C_6H_6 + 2.91H_2O + \mathbf{3.49}SO_4^{2-} + 0.088NH_4^+ \rightarrow 5.56HCO_3^- + 1.74H_2S + 1.74HS^- + 0.088C_5H_7O_2N + 0.77H^+ \quad (6)$$

Conversely, additional sulfate-reduction reactions with the exclusion of ammonium and cyanoacetate containing a ratio value of 3.75 have also been proposed in literature:

(Foght 2008; Ulrich 2004; Ulrich and Edwards 2003; Vogt et al. 2011):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3^- + 1.88H_2S + 1.88HS^- + 0.38H^+ \quad (7)$$

(Foght 2008):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3 + 1.88H_2S + 1.88HS^- + 0.38H^+ \quad (8)$$

(Meckenstock et al. 2016):

$$C_6H_6 + 3H_2O + \mathbf{3.75}SO_4^{2-} \rightarrow 6HCO_3^- + 3.75HS^- + 2.25H^+ \quad (9)$$

As seen in Figure 5-7 and Appendix C, IC analysis on Days 259 and 305 indicated sulfate depletion. Due to these readings, sulfate reamendments to 15 mM were performed on the following days. Further analysis on Days 321 and 332 yielded data suggesting sulfate content were elevated upwards of 134 mM (approximately 9 times the target concentration of 15 mM). In hindsight, the analysis performed on Days 259 and 305 yielded abnormally and unexpectedly low results. These two monitoring sessions occurred during a period in which the faculty shared IC system demonstrated instrumentation errors and was decommissioned for maintenance within the following weeks. The results obtained on Days 259 and 305 most likely indicated a falsely low sulfate content due to instrumentation error. Therefore, the two subsequent sulfate reamendments would have unnecessarily elevated the sulfate content to the excessive levels seen in following monitoring IC sessions on Days 321 and 332. These elevated sulfate concentrations did not appear to have created a toxic environment for the microbial community and cease degradation. However, the possibility in which it does affect the resulting electron acceptor usage ratios cannot be ignored.

Table 5-9 – Salinity Experiment: Sulfate Usage Ratios of Nitrate-Reducing Microcosms

				Ratio mol Sulfate : mol Benzene	
Microcosm	Degradation Cycle	Days	Benzene Conc. Fed (mM)	Observed	Theoretical[A]
0.0 g/L NaCl	1	0-90	0.12	519.0	3.49[A] 3.75[B]
	2	91-307	0.32	186.1	
0.5 g/L NaCl	1	0-148	0.30	193.1	
	2	149-276	0.59	31.4	
	3	277-443	0.60	93.6	
1.0 g/L NaCl	1	0-148	0.31	53.2	
	2	149-260	0.57	39.9	
	3	261-476	0.58	106.8	
2.0 g/L NaCl	1	0-142	0.31	47.4	
	2	143-276	1.32	22.1	
	3	277-476	0.66	119.3	

[A] (Ulrich and Edwards 2003)
[B] (Foght 2008; Meckenstock et al. 2016; Ulrich 2004; Ulrich and Edwards 2003; Vogt et al. 2011)

As seen in Table 5-9, the treatment and individual degradation cycles of sulfate-reducing treatments during the Salinity Experiment yielded sulfate : benzene ratios between 22.1 and 519.0. In parallel to the ratios obtained during the Culture Enrichment period, these values excessively exceed the theoretical values of 3.49 and 3.75.

The first possible explanation of for these elevated ratios for both nitrate- and sulfate-reducing treatments is simply instrument error – specifically the anion analysis with ion chromatography (IC) on Days 260 and 306. The data collected from those two instances were near 0 mM for nitrate, nitrite, and sulfate. If accurate, these values suggest an almost complete depletion of nitrate even with a nitrate reamendment occurring three weeks prior. Such a sudden extent of terminal electron acceptor usage is an outlier throughout the two-year incubation of these treatments. In conjunction with the fact that the IC was decommissioned for several weeks of maintenance during this time, and subsequent anion analysis measurements indicating values

which were relatively normal, the data collected from Days 260 and 306 might be unreliably lower due to instrumental malfunction. These lower electron acceptor values would therefore suggest an exaggerated consumption and ultimately lead to elevated ratios as seen in Table 5-8 and 5-9.

A second explanation for the elevated ratios can be explained by the concept of microbial community with syntrophic relations regarding the degradation of benzene and degradation of subsequent downstream metabolites as introduced by Dou et al. (2010), van der Zaan (2012), and Luo et al. (2014). While nitrate or sulfate is reduced within the initial activation of benzene by one microbial organism, additional amounts of these compounds may be necessary for the further oxidization of the subsequent downstream metabolites by other microbial organisms for the eventual mineralization of the substrate. In their 2014 article, Luo et al. hypothesize that one organism (*peptococcaceae*) work in a syntrophic manner to degrade the benzene to benzoate and another organism (*Azoarcus*) was responsible for degrading the benzoate under denitrifying conditions. In the 2010 study, Herrmann et al. suggest that not one singular organism was responsible for the oxidation of benzene and sulfate-reduction. Instead, the *Cryptanaerobacter / Pelotomaculum* group initiated the first step of benzene degradation but does not completely oxidize the substrate but releases reduced metabolites which can include hydrogen, acetate, or other fermentation products of lower molecular masses. Subsequently, secondary bacteria used these reduced metabolites for their respective metabolism and may also reduce sulfate. This process may occur multiple instances until mineralization which leads extraneous sulfate utilization to occur in comparison to the expected theoretical values since reduction occurred not only for benzene, but for the degradation of potential downstream metabolites as well. A concept in which a plethora of organisms in a microbial community utilizing nitrate or sulfate as a terminal electron acceptor is one possible explanation for the observed elevated ratios in Table 5-8 and 5-9.

5.4 – Conclusions

The Salinity Experiment explored nitrate-, sulfate-reducing, and methanogenic conditions for benzene biodegradation within salinity concentrations of 0.0, 0.5, 1.0, and 2.0 g/L NaCl. 27 degradation cycles were demonstrated and yielded the following conclusions:

- Nitrate-reducing treatments degraded benzene at an average overall rate of 1.8 ± 0.5 µM/d across 8 degradation cycles.
    - Lowest rate of the three anoxic conditions

    - Most likely due to depletion of nitrate and/or benzene, causing stalled degradation
- Sulfate-reducing treatments degraded benzene at an average overall rate of 4.2 ± 5.1 µM/d across 11 degradation cycles.
- Methanogenic treatments degraded benzene at an average overall rate of 2.1 ± 3.4 µM/d across 8 degradation cycles.
    - Methane accumulation was not observed.
- Within the lower initial benzene concentrations of 0.2 to 0.4 mM, the optimal salinity concentration for the greatest overall degradation differed between redox conditions:
    - Nitrate-reducing – 0.0 g/L NaCl at 2.7 µM/d
    - Sulfate-reducing – 1.0 and 2.0 g/L NaCl at 2.1 µM/d
    - Methanogenic – 0.5 g/L NaCl at 2.5 µM/d
- Within the higher initial benzene concentrations of 0.5 to 0.7 mM, the greatest overall degradation rate was 1.0 g/L NaCl for all three redox conditions.
- Lag phases averaged 3.4 ± 1.4 days with the lengthiest observed for the highest salt content (2.0 g/L NaCl) at 4.0 ± 2.0 days.
- The observed electron acceptor utilization ratios per mole of benzene degraded were comparable to those observed during the Culture Enrichment period, but also elevated to the theoretical values reported in literature.
    - These unexpected nitrate and sulfate losses is most likely due to degradation of downstream metabolites by a diverse microbial culture.
    - Instrumentation error resulting in excessive electron acceptor re-amendment.

5.5 – Chapter 5 References

Al-Mailem, D. M., Eliyas, M., and Radwan, S. S. (2013). "Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts." *Extremophiles*, 17(3), 463–470.

Foght, J. (2008). "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects." *Journal of Molecular Microbiology and Biotechnology*, 15(2–3), 93–120.

Hassan, H. A., Rizk, N. M., Hefnaway, M. A., and Awad, A. A. (2012). "Isolation and Characterization of Halophilic Aromatic and Chloroaromatic Degrader from Wadi El-Natrun Soda." *Life Science Journal*, 9(3), 1565–1570.

Li, H., Liu, Y. H., Luo, N., Zhang, X. Y., Luan, T. G., Hu, J. M., Wang, Z. Y., Wu, P. C., Chen, M. J., and Lu, J. Q. (2006). "Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic Planococcus sp. strain ZD22." *Research in Microbiology*, 157(7), 629–636.

Mahour, R. (2016). "Chemical and Biological Oxidation of Naphthenic Acids - where Stoichiometry, Kinetics and Thermodynamics Meet." University of Alberta.

Meckenstock, R. U., Boll, M., Mouttaki, H., Koelschbach, J. S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A. M. (2016). "Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons." *Journal of Molecular Microbiology and Biotechnology*, 26(1–3), 92–118.

Nicholson, C. A., and Fathepure, B. Z. (2004). "Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions." *Applied and Environmental Microbiology*, 70(2), 1222–1225.

Nicholson, C. A., and Fathepure, B. Z. (2005). "Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma." *FEMS Microbiology Letters*, 245(2), 257–262.

Qin, X., Tang, J. C., Li, D. S., and Zhang, Q. M. (2012). "Effect of salinity on the bioremediation of petroleum hydrocarbons in a saline-alkaline soil." *Letters in Applied Microbiology*, 55(3), 210–217.

Reardon, K. F., Mosteller, D. C., and Bull Rogers, J. D. (2000). "Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for Pseudomonas putida F1." *Biotechnology and Bioengineering*, 69(4), 385–400.

Sei, A., and Fathepure, B. Z. (2009). “Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake.” *Journal of Applied Microbiology*, 107(6), 2001–2008.

Ulrich, A. C. (2004). “Characterization of Anaerobic Benzene-Degrading Cultures.” University of Toronto.

Ulrich, A. C., and Edwards, E. A. (2003). “Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures.” *Environmental Microbiology*, 5(2), 92–102.

Ulrich, A. C., Guigard, S. E., Foght, J. M., Semple, K. M., Pooley, K., Armstrong, J. E., and Biggar, K. W. (2009). “Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater.” *Biodegradation*, 20(1), 27–38.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). “Anaerobic benzene degradation by bacteria.” *Microbial Biotechnology*, 4(6), 710–724.

CHAPTER 6

Conclusions and Future Directions

6.1 – Engineering Significance and Conclusions

Environmental releases of benzene are mostly attributed to atmospheric deposition, above- and under-ground storage tank leakages, seepage from improperly designed waste disposal sites, and spillage of oil and gasoline during storage, transportation, and handling within the energy sector and industrial production (CCME 2004). Each year in Canada, an estimated 34 kilotonnes of benzene is released into the environment (Government of Canada, Environment Canada 1993). Traditional remediation methods such as dig-and-dump, pump-and-treat, and soil vapor extractions provide effective means in eliminating the bulk benzene content within a contaminated plume. However, these methods are costly, demand greater on-site management, and are more intrusive to the site. A common remediation strategy is to pair these conventional methods with intrinsic bioremediation. While intrinsic biodegradation is more time consuming and removes benzene at a lower rate, it is an attractive remediation option due to lower costs, lack of on-site operation, and non-intrusive nature in treating residual contamination which often challenges traditional means of remediation. Furthermore, it is effective in targeting the residual benzene content which the previously mentioned methods are often challenged by. However, this form of biodegradation is often stalled when the high oxygen demand imposed by the benzene load renders the subsurface oxygen deficient. It is this shift towards anaerobic biodegradation or reliance on other redox conditions with the next readily available terminal electron acceptor which often stalls continual degradation (Song et al. 1990; U. S. Environmental Protection Agency (EPA) 2000). To further complicate this problem, many contaminated sites in the petroleum industry are also plagued by elevated salt content due to incidental releases of hypersaline produced waters which simultaneously contain both benzene and salt (Ulrich et al. 2009). Available literature on this topic suggest salt to inhibit the metabolism of microbial cultures as it may disrupts tertiary protein structures and denatured enzymes of cells (Ulrich et al. 2009) and decrease the accessibility of soil organic matter to the microbes (Qin et al. 2012). To date, no research has been identified to examine the biodegradation of benzene under both **saline** and **anaerobic** conditions. Developing an understanding of this topic is the first step in relying on biodegradation in this type of contamination. In the future, applications of this technology can be as simple as cataloguing the microbial species capable of saline anaerobic benzene biodegradation and comparing that to the in-situ and indigenous microbial culture of a field site to draw conclusions for intrinsic biodegradation expectations. Furthermore, an even more ambitious application can include

enriching an active degradation culture as a remediation tool which environmental engineers can release onto a field site to kickstart biodegradation. To this end, the objective of this thesis was *to investigate the effect of salinity on anaerobic benzene biodegradation*. From this goal, two research questions were developed to guide the research:

I. *"What differences in anaerobic benzene biodegradation capabilities exist between treatments based on nitrate-reducing, sulfate-reducing, and methanogenic conditions? Furthermore, are these biodegradation capabilities affected by whether the treatments were derived from clay or sand sediments originating from the same source?"* and,

II. *"For the same treatments in Research Question I, is anaerobic benzene biodegradation also possible under varying salinity conditions? And if it is possible, what is the optimal salinity concentration which yields the greatest degradation rate?"*

The only available literature on benzene biodegradation in the presence of salinity are experiments performed under aerobic conditions. In those studies, degrees of degradation were not demonstrated equally across all salinity ranges. Conversely, certain intervals of salt concentrations resulted in greater degradation rates. These conclusions in conjunction with the previously mentioned research questions ultimately led to the hypothesis that *trace amounts of salinity will not impede (but may even stimulate) benzene biodegradation. However, further addition in salinity past an optimum range will result in inhibitory effect towards benzene biodegradability*.

An experiment with two phases was set up to explore the validity of this hypothesis and anaerobic benzene biodegradation was demonstrated in three redox conditions (nitrate-, sulfate-reducing, and methanogenic). The 548 day Culture Enrichment period contained 39 degradation cycles and explored differences between treatments originating from clay and sand sediments. The Salinity Experiment lasted for 479 days and tested for differences in benzene biodegradation capabilities of the same treatments in the presence of salinity, specifically 0.0, 0.5, 1.0, and 2.0 g/L NaCl. The most salient findings of the Culture Enrichment period and Salinity Experiment are listed below.

Nitrate-Reducing Treatments:

- Overall degradation rate of 12.6 ± 3.0 μM/d across 15 degradation cycles during the Culture Enrichment Period.
- Overall degradation rate of 1.8 ± 0.5 μM/d across 8 degradation cycles during the Salinity Experiment.

- Demonstrated the slowest rate of the three redox conditions.
- Depletion of nitrate, depletion of benzene, or accumulation of nitrite caused inhibitory effects on benzene degradation and most likely limited the reported degradation rates.

Sulfate-Reducing Treatments:

- Overall degradation rate of 20.1 ± 3.4 μM/d across degradation 12 cycles during the Culture Enrichment period.
- Overall degradation rate of 4.2 ± 5.1 μM/d across 11 degradation cycles during the Salinity Experiment.
- Instrument error led to excessive sulfate reamendment but degradation was not observed to be inhibited by elevated sulfate content.

Methanogenic Treatments:

- Overall degradation rate of 17.0 ± 2.0 μM/d across 12 degradation cycles during the Culture Enrichment period.
- Overall degradation rate of 2.1 ± 3.4 μM/d across 8 degradation cycles during the Salinity Experiment.
- Methane accumulation was not observed. This may be due to the constant gas withdrawal due to headspace analysis for benzene monitoring.

Degradation Rates and Benzene Concentration

- A linear correlation was also observed between the concentration to which treatments were fed and the resulting degradation rates (overall and maximum).
- Specifically, higher degradation rates can result in treatments fed with greater benzene content. But the converse of low benzene concentrations resulting in high degradation rates was not observed.
- This trend was most likely due to the microbial culture's accessibility to benzene when the substrate was oversaturated in the anaerobic media mix.

Differences Between Clay- and Sand-Derived Treatments:

- Clay-derived treatments outperformed the sand counterparts during the Culture Enrichment period (17.7 ± 2.4 and 14.9 ± 2.5 μM/d respectively).
- A T-test was performed to determine whether a statistical significance exists between the average overall degradation rates of the clay samples against the sand samples. A *t*-value of

1.01, *p*-value of 0.35, and α significance value of 0.05 does not conclusively suggest that the clay samples outperform the sand counterparts.

- But the apparent differences observed in this experiment can be explained by three reasons:
- The interlayer spaces between the tetrahedral or octahedral base units of clay commonly contain organic matter which can include nutrients beneficial for microbial growth.
- Iron can substitute the aluminium core of octahedral structured clays. This isomorphic substitution of iron may result in benzene biodegradation linked with iron-reducing conditions simultaneous to the degradation already occurring.
- Finer sediments (clays) possess a greater surface area with smaller pore spaces in comparison to coarser sediments (sands). The increased quantity of substrate sorption sites and area to host microbial organisms may enhance biodegradability.

Differences Due to Salinity:

- Within the lower initial benzene concentrations of 0.2 to 0.4 mM, the optimal salinity concentration for the greatest overall degradation differed between redox conditions:
  - Nitrate-reducing – 0.0 g/L NaCl at 2.7 μM/d
  - Sulfate-reducing – 1.0 and 2.0 g/L NaCl at 2.1 μM/d
  - Methanogenic – 0.5 g/L NaCl at 2.5 μM/d
- Within the higher initial benzene concentrations of 0.5 to 0.7 mM, the optimal salinity concentration for the greatest overall degradation was 1.0 g/L NaCl for all three redox conditions.
- Trace salt content may enhance biodegradation by providing an ionically balanced medium for microbial growth or cause the medium to disperse clays and therefore create a greater surface area allowing for increased bioavailability and access to nutrients.
- However, elevated salt concentrations past the optimal point can create a high osmotic potential within the microbe's environment and inhibit the solubility and sorption of toxic, essential ions, and organic matter.
- Excessive salt is also believed to disrupt tertiary protein structures and denature enzymes of cell dehydration within microbial communities.

Electron Acceptor Ratios:

- The observed electron acceptor utilization ratios per mole of benzene degraded were comparable to those between the Culture Enrichment period and the Salinity Experiment but

were elevated by approximately one order of magnitude to the theoretical values reported in literature.

- These unexpected nitrate and sulfate losses is most likely due to degradation of downstream metabolites by a diverse microbial culture.
- Instrumentation error resulting in excessive electron acceptor re-amendment for the sulfate-reducing treatments.

6.2 – Future Directions

The work undertaken in this thesis is only the first step in developing an understanding in anaerobic benzene biodegradation in saline environments. As discussed in Chapter 1, sites contaminated with both benzene and salt content is a common occurrence in the oil and gas industry (Ulrich et al. 2009). There is much work that remains before the scientific community can reliably equip anaerobic microbial biodegradation into the arsenal of tools which target saline and petroleum hydrocarbon contaminated sites. In this section, several suggestions are offered for future researchers who may work with the microbial cultures of this thesis. These suggestions are mostly inspired from technologies or procedures reported in other studies and may have suitable applications with the treatments on hand but were not implemented due to the limited scope of this thesis or could not have been applied due to logistical challenges.

Molybdate is an effective and selective inhibitor of sulfate-reduction in sulfate-reducing microbes. In several anaerobic benzene degradation studies, an immediate cease in benzene uptake and CO_2 production was reported when molybdate was added (Abu Laban et al. 2009; Lovley et al. 1995; Phelps et al. 1996, 1998). The addition of this compound onto the sulfate-reducing treatments of this thesis may provide intriguing results, but at the cost of the treatment bottle due to molybdate's irreversible nature. No observations and results suggest the sulfate-reducing treatments to undergo degradation linked with other redox conditions. As a result, stoppage in degradation is expected if molybdate is added in the proper concentration. However, if degradation does not cease, this suggest extraneous processes which result in benzene loss (most likely abiotic) are occurring and further analysis would be required to elucidate the reasoning for this benzene disappearance.

Only four salinity concentrations (0.0, 0.5, 1.0, and 2.0 g/L NaCl) were tested in the Salinity Experiment and the highest overall degradation rate was observed within the 1.0 g/L NaCl salinity

level for benzene biodegradation at higher feed concentrations under the three redox conditions. In comparison to literature, albeit those studies concern aerobic degradation, the salinity range tested here is comparatively low. Several studies with concentrations upwards of 4 M NaCl (234 g/L NaCl) were discussed in Chapter 2 (Al-Mailem et al. 2013; Nicholson and Fathepure 2004, 2005; Sei and Fathepure 2009). Further testing the salinity tolerance of the microbial culture by increasing salt content until biodegradation is completely inhibited will be one task for further researchers. Although there is a large difference in the maximum salt concentration tested in this experiment against those of literature (approximately 117 times less), future experiments with a wider salinity range and smaller salt concentration intervals will be the first step in bridging this gap to draw conclusions between aerobic and anaerobic benzene biodegradation in the presence of salinity.

Methane monitoring via GC-TCD was performed infrequently throughout the Culture Enrichment period and Salinity Experiment. Mapping methane generation against benzene decreases was beyond the initial scope of this project. Instead, methane content in the headspace of the methanogenic treatments were only observed to ensure excessive methane accumulation was not occurring and causing benzene degradation to become thermodynamically unfavorable. As seen in Figure 5-8 and Appendix C, methane content remained relatively consistent (except for the 0.5 g/L NaCl treatment). The limited quantity of methane monitoring sessions does not provide enough data for conclusive results regarding methane generation in this experiment. One possible explanation for the lack of notable methane accumulation is due to the constant benzene headspace monitoring sessions in which 200 µL of gas is withdrawn for each injection. This analysis typically occurred twice per week with upwards of three injections during each session. This lack of methane accumulation can theoretically be occur if the methane generation rate is low and the rate at which headspace gas is withdrawn on a weekly basis is higher. An experiment with a set schedule which limits excessive headspace analysis but also permits the regular monitoring for methane can be the solution to acquiring methane generation rates for these methanogenic treatments for comparison with those of literature.

The fourth and last recommendation involves application of analytical techniques capable of detecting the oxidation of benzene into its metabolites in attempt to elucidate the degradation pathway linked to the demonstrated biodegradation. Utilization of ^{13}C- or ^{14}C-radiolabelled benzene and searching for similarly labeled intermediates is a commonly seen tactic in literature

and may be an appropriate first step in this endeavour with the microbial culture on hand (Chakraborty et al. 2005; Coates et al. 2001; Edwards and Grbić-Galić 1992; Lovley et al. 1995; Nales et al. 1998; Phelps et al. 1996; Ulrich et al. 2005; Ulrich and Edwards 2003; van der Zaan et al. 2012). Special attention should be allocated in the search for toluene, phenol, and benzoate as these intermediates are linked with benzene methylation, hydroxylation, and carboxylation respectively (Coates et al. 2002; Foght 2008; Ulrich 2004; Vogt et al. 2011). Moreover, if trace amounts of these intermediates are discovered, the concept of an isotope trap as described by Ulrich et al. (2005) can be implemented in validating a certain degradation pathway. Other technologies such as stable isotope probing (SIP) and stable isotope fractionalization (SIF) in combination with gene sequencing were also instrumental in other studies for elucidating the initial benzene activation mechanism and identifying microbial species within a culture involved in performing key roles for the degradation process (Chakraborty et al. 2005; Coates et al. 2001; Dou et al. 2010; Keller et al. 2018; Luo et al. 2014; van der Waals et al. 2017; van der Zaan et al. 2012).

6.3 – Chapter 6 References

Abu Laban, N., Selesi, D., Jobelius, C., and Meckenstock, R. U. (2009). "Anaerobic Benzene Degradation by Gram-Positive Sulfate-Reducing bacteria." *FEMS Microbiology Ecology*, 68(3), 300–311.

Al-Mailem, D. M., Eliyas, M., and Radwan, S. S. (2013). "Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts." *Extremophiles*, 17(3), 463–470.

CCME. (2004). *Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health: Benzene*.

Chakraborty, R., O'Connor, S. M., Chan, E., and Coates, J. D. (2005). "Anaerobic Degradation of Benzene , Toluene , Ethylbenzene , and Xylene Compounds by Dechloromonas Strain RCB." *Applied and Environmental Microbiology*, 71(12), 8649–8655.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Bender, K. S., and Achenbach, L. A. (2001). "Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas." *Letters to Nature*, 411(6841), 1039–1043.

Coates, J. D., Chakraborty, R., and McInerney, M. J. (2002). "Anaerobic Benzene Biodegradation - A New Era." *Research in Microbiology*, 153(10), 621–628.

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., and Wang, J. (2010). "Anaerobic Benzene Biodegradation by a Pure Bacterial Culture of Bacillus cereus Under Nitrate Reducing Conditions." *Journal of Environmental Sciences*, The Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, 22(5), 709–715.

Edwards, E. A., and Grbić-Galić, D. (1992). "Complete Mineralization of Benzene by Anaerobic Microorganisms under Strictly Anaerobic Conditions." *American Society for Microbiology*, 58(8), 2663–2666.

Foght, J. (2008). "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects." *Journal of Molecular Microbiology and Biotechnology*, 15(2–3), 93–120.

Government of Canada, Environment Canada, H. and W. C. (1993). *Benzene - Priority Substances List Assessment Report*.

Keller, A. H., Kleinsteuber, S., and Vogt, C. (2018). “Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics.” *Microbial Ecology*, Microbial Ecology, 75(4), 941–953.

Lovley, D. R., Coates, J. D., Woodward, J. C., and Phillips, E. J. P. (1995). “Benzene Oxidation Coupled to Sulfate Reduction.” *American Society for Microbiology*, 61(3), 953–958.

Luo, F., Gitiafroz, R., Devine, C. E., Gong, Y., Hug, L. A., Raskin, L., and Edwards, E. A. (2014). “Metatranscriptome of an Anaerobic Benzene-Degrading, Nitrate-Reducing Enrichment Culture Reveals Involvement of Carboxylation in Benzene Ring Activation.” *Applied and Environmental Microbiology*, 80(14), 4095–4107.

Nales, M., Butler, B. J., and Edwards, E. A. (1998). “Anaerobic benzene biodegradation: A microcosm survey.” *Bioremediation Journal*, 2(2), 125–144.

Nicholson, C. A., and Fathepure, B. Z. (2004). “Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions.” *Applied and Environmental Microbiology*, 70(2), 1222–1225.

Nicholson, C. A., and Fathepure, B. Z. (2005). “Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma.” *FEMS Microbiology Letters*, 245(2), 257–262.

Phelps, C. D., Kazumi, J., and Young, L. Y. (1996). “Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction.” *FEMS Microbiology Letters*, 145(3), 433–437.

Phelps, C. D., Kerkhof, L. J., and Young, L. Y. (1998). “Molecular characterization of a sulfate-reducing consortium which mineralizes benzene.” *FEMS Microbiology Ecology*, 27(3), 269–279.

Qin, X., Tang, J. C., Li, D. S., and Zhang, Q. M. (2012). “Effect of salinity on the bioremediation of petroleum hydrocarbons in a saline-alkaline soil.” *Letters in Applied Microbiology*, 55(3), 210–217.

Sei, A., and Fathepure, B. Z. (2009). “Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake.” *Journal of Applied*

Microbiology, 107(6), 2001–2008.

Song, H. G., Wang, X., and Bartha, R. (1990). "Bioremediation potential of terrestrial fuel spills." *Applied and Environmental Microbiology*, 56(3), 652–656.

U. S. Environmental Protection Agency (EPA). (2000). "Engineered approaches to in situ bioremediation of chlorinated solvents: Fundamentals and field applications." (EPA 542-R-00-008), 1–144.

Ulrich, A. C. (2004). "Characterization of Anaerobic Benzene-Degrading Cultures." University of Toronto.

Ulrich, A. C., Beller, H. R., and Edwards, E. A. (2005). "Metabolites detected during biodegradation of 13C 6-benzene in nitrate-reducing and methanogenic enrichment cultures." *Environmental Science and Technology*, 39(17), 6681–6691.

Ulrich, A. C., and Edwards, E. A. (2003). "Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures." *Environmental Microbiology*, 5(2), 92–102.

Ulrich, A. C., Guigard, S. E., Foght, J. M., Semple, K. M., Pooley, K., Armstrong, J. E., and Biggar, K. W. (2009). "Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater." *Biodegradation*, 20(1), 27–38.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). "Anaerobic benzene degradation by bacteria." *Microbial Biotechnology*, 4(6), 710–724.

van der Waals, M. J., Atashgahi, S., da Rocha, U. N., van der Zaan, B. M., Smidt, H., and Gerritse, J. (2017). "Benzene degradation in a denitrifying biofilm reactor: activity and microbial community composition." *Applied Microbiology and Biotechnology*, Applied Microbiology and Biotechnology, 101(12), 5175–5188.

van der Zaan, B. M., Saia, F. T., Stams, A. J. M., Plugge, C. M., de Vos, W. M., Smidt, H., Langenhoff, A. A. M., and Gerritse, J. (2012). "Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntrophic process." *Environmental Microbiology*, 14(5), 1171–1181.

References

References

Abu Laban, N., Selesi, D., Jobelius, C., and Meckenstock, R. U. (2009). "Anaerobic Benzene Degradation by Gram-Positive Sulfate-Reducing bacteria." *FEMS Microbiology Ecology*, 68(3), 300–311.

Al-Mailem, D. M., Eliyas, M., and Radwan, S. S. (2013). "Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts." *Extremophiles*, 17(3), 463–470.

Al-Mailem, D. M., Sorkhoh, N. A., Al-Awadhi, H., Eliyas, M., and Radwan, S. S. (2010). "Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf." *Extremophiles*, 14(3), 321–328.

Anderson, R. T., and Lovley, D. R. (2000). "Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer." *Environmental Science and Technology*, 34(11), 2261–2266.

Atashgahi, S., Hornung, B., Van Der Waals, M. J., Da Rocha, U. N., Hugenholtz, F., Nijsse, B., Molenaar, D., Van Spanning, R., Stams, A. J. M., Gerritse, J., and Smidt, H. (2018). "A benzene-degrading nitrate-reducing microbial consortium displays aerobic and anaerobic benzene degradation pathways." *Scientific Reports*, Springer US, 8(1), 1–12.

Atkins, P., and de Paula, J. (2006). *Physical Chemistry for the Life Sciences*. W. H. Freeman and Company, New York, NY.

Atlas, R. M. (1981). "Microbial Degradation of Petroleum Hydrocarbons : an Environmental Perspective." *Microbiological reviews*, 45(1), 180–209.

ATSDR. (2007). "Toxicological Profile for Benzene." *Agency for Toxic Substances and Disease Registry (ATSDR)*, (August), 438.

Barker, J. F., Sudicky, E. A., Mayfield, C. I., and Gillham, R. W. (1989). *Petroleum Hydricarbon Contaminantion of Groundwater. Natural Fate and In Situ Remediation, a Summary Report. PACE Report No. 89-5.* Ottawa, Canada.

Becalski, A. (2014). "Chapter 20 - Processing Contaminants: Benzene." *Encyclopedia of Food Safety*, 376–380.

Van Beelen, P., and Van Keulen, F. (1990). "The Kinetics of the Degradation of Chloroform and Benzene in Anaerobic Sediment From the River Rhine." *Hydrobiological Bulletin*, 24(1), 13–21.

Berlendis, S., Cayol, J. L., Verhé, F., Laveau, S., Tholozan, J. L., Ollivier, B., and Auria, R. (2010). "First Evidence of Aerobic Biodegradation of BTEX Compounds by Pure Cultures of Marinobacter." *Applied Biochemistry and Biotechnology*, 160(7), 1992–1999.

CCME. (2004). *Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health: Benzene*.

CCME. (2008). *Canada-Wide Standard for Petroleum Hydrocarbons (PHC) in Soil: Scientific Rationale Supporting Technical Document*.

CCME. (2014). "Canada-wide standards for petroleum hydrocarbons (PHC) in soil." *Ccme*, (January), 1–8.

Chakraborty, R., O'Connor, S. M., Chan, E., and Coates, J. D. (2005). "Anaerobic Degradation of Benzene , Toluene , Ethylbenzene , and Xylene Compounds by Dechloromonas Strain RCB." *Applied and Environmental Microbiology*, 71(12), 8649–8655.

Chang, R. (2007). *Physical Chemistry for the Biosciences*. Univ Science Books, Sausalito, CA.

Chiou, C. T. (1989). "Theoretical COnsiderations of the Partition Uptake of Nonionic Organic COmpounds by Soil Organic Matter." *Reactions and Movement of Organic Chemicals in Soils, SSSA Special Publication 22*, Soil Science Society of America and American Society of Agronomy.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Bender, K. S., and Achenbach, L. A. (2001). "Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas." *Letters to Nature*, 411(6841), 1039–1043.

Coates, J. D., Chakraborty, R., and McInerney, M. J. (2002). "Anaerobic Benzene Biodegradation - A New Era." *Research in Microbiology*, 153(10), 621–628.

Domenico, P. A., and Schwartz, F. W. (1998). "Chapter 19 - Multiphase Fluid Systems." *Physical and Chemical Hydrogeology (2nd Edition)*, 405–416.

Dong, X., Dröge, J., von Toerne, C., Marozava, S., McHardy, A. C., and Meckenstock, R. U. (2017). "Reconstructing Metabolic Pathways of a Member of the Genus Pelotomaculum Suggesting its Potential to Oxidize Benzene to Carbon Dioxide with Direct Reduction of Sulfate." *FEMS Microbiology Ecology*, 93(3), 1–9.

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., and Wang, J. (2010). "Anaerobic Benzene Biodegradation by a Pure Bacterial Culture of Bacillus cereus Under Nitrate Reducing Conditions." *Journal of Environmental Sciences*, The Research Centre for Eco-

Environmental Sciences, Chinese Academy of Sciences, 22(5), 709–715.

Edwards, E. A., and Grbić-Galić, D. (1992). "Complete Mineralization of Benzene by Anaerobic Microorganisms under Strictly Anaerobic Conditions." *American Society for Microbiology*, 58(8), 2663–2666.

English, C. W., and Loehr, R. C. (1991). "Degradation of organic vapors in unsaturated soils." *Journal of Hazardous Materials*, 28(1–2), 55–64.

Fishbein, L. (1985). "An overview of environmental and toxicological aspects of aromatic hydrocarbons IV. Ethylbenzene." *Science of the Total Environment, The*, 44(3), 269–287.

Foght, J. (2008). "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects." *Journal of Molecular Microbiology and Biotechnology*, 15(2–3), 93–120.

Government of Canada, Environment Canada, H. and W. C. (1993). *Benzene - Priority Substances List Assessment Report*.

Government of Canada (GOC). (2015). *Canadian Environmental Protection Act, 1999*.

Grbić-Galić, D., and Vogel, T. M. (1987). "Transformation of Toluene and Benzene by Mixed Methanogenic Cultures." *Applied and Environmental Microbiology*, 53(2), 254–260.

Haghollahi, A., Fazaelipoor, M. H., and Schaffie, M. (2016). "The effect of soil type on the bioremediation of petroleum contaminated soils." *Journal of Environmental Management*, 180, 197–201.

Harwood, C. S., Burchhardt, G., Herrmann, H., and Fuchs, G. (1998). "Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway." *FEMS Microbiology Reviews*, 22(5), 439–458.

Harwood, C. S., and Gibson, J. (1997). "Shedding light on anaerobic benzene ring degradation: A process unique to prokaryotes?" *Journal of Bacteriology*, 179(2), 301–309.

Hassan, H. A., Rizk, N. M., Hefnaway, M. A., and Awad, A. A. (2012). "Isolation and Characterization of Halophilic Aromatic and Chloroaromatic Degrader from Wadi El-Natrun Soda." *Life Science Journal*, 9(3), 1565–1570.

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H. H., and Vogt, C. (2010). "Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing." *Environmental Microbiology*, 12(2), 401–411.

Holden, A. A. (2012). "The Geochemical Evolution of Oil Sands Tailings Pond Seepage, Resulting from Diffusive Ingress Through Underlying Glacial Till Sediments."

Holmes, D. E., Risso, C., Smith, J. A., and Lovley, D. R. (2011). "Anaerobic oxidation of benzene by the hyperthermophilic archaeon Ferroglobus placidus." *Applied and Environmental Microbiology*, 77(17), 5926–5933.

Jacques, A. P. (1990). *National Inventory of Sources and Emissions of Benzene (1985). Report EPS 5/AP/1.* Ottawa.

Jahn, M. K., Haderlein, S. B., and Meckenstock, R. U. (2005). "Anaerobic Degradation of Benzene, Toluene, Ethylbenzene, and o-Xylene in Sediment-Free Iron-Reducing Enrichment Cultures." *Applied and Environmental Microbiology*, 71(6), 3355–3358.

Johnson, S. J., Woolhouse, K. J., Prommer, H., Barry, D. A., and Christofi, N. (2003). "Contribution of anaerobic microbial activity to natural attenuation of benzene in groundwater." *Engineering Geology*, 70(3–4), 343–349.

Kao, C.-M., and Borden, R. C. (1997). "Site-Specific Variability in BTEX Biodegradation Under Denitrifying Conditions." *Ground Water*, 35(2), 305–311.

Kazumi, J., Caldwell, M. E., Suflita, J. M., Lovley, D. R., and Young, L. Y. (1997). "Anaerobic degradation of benzene in diverse anoxic environments." *Environmental Science and Technology*, 31(3), 813–818.

Keller, A. H., Kleinsteuber, S., and Vogt, C. (2018). "Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics." *Microbial Ecology*, Microbial Ecology, 75(4), 941–953.

Kerr, R. P., and Capone, D. G. (1988). "The effect of salinity on the microbial mineralization of two polycyclic aromatic hydrocarbons in estuarine sediments." *Marine Environmental Research*, 26(3), 181–198.

Kirk, R. E., Othmer, D. F., Grayson, M., and Eckroth, D. (1991). *Kirk-Othmer Encylopedia of Chemical Technology (4th Edition)*. John Wiley, New York.

Knovel. (2008). "Basic Physical Properties of Chemical Compounds." *Knovel Critical Tables (2nd Edition)*.

Kogbara, R. B., Ayotamuno, J. M., Worlu, D. C., and Fubara-Manuel, I. (2015). "A Case Study of Petroleum Degradation in Different Soil Textural Classes." *Recent Patents on Biotechnology*, 9(2), 108–115.

Langenhoff, A. A. M., Zehnder, A. J. B., and Schraa, G. (1996). "Behaviour of toluene, benzene

and naphthalene under anaerobic conditions in sediment columns." *Biodegradation*, 7(3), 267–274.

Li, H., Liu, Y. H., Luo, N., Zhang, X. Y., Luan, T. G., Hu, J. M., Wang, Z. Y., Wu, P. C., Chen, M. J., and Lu, J. Q. (2006). "Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic Planococcus sp. strain ZD22." *Research in Microbiology*, 157(7), 629–636.

Lovley, D. R., Coates, J. D., Woodward, J. C., and Phillips, E. J. P. (1995). "Benzene Oxidation Coupled to Sulfate Reduction." *American Society for Microbiology*, 61(3), 953–958.

Luo, F., Devine, C. E., and Edwards, E. A. (2016). "Cultivating Microbial Dark Matter in Benzene-Degrading Methanogenic Consortia." *Environmental Microbiology*, 18(9), 2923–2936.

Luo, F., Gitiafroz, R., Devine, C. E., Gong, Y., Hug, L. A., Raskin, L., and Edwards, E. A. (2014). "Metatranscriptome of an Anaerobic Benzene-Degrading, Nitrate-Reducing Enrichment Culture Reveals Involvement of Carboxylation in Benzene Ring Activation." *Applied and Environmental Microbiology*, 80(14), 4095–4107.

Lyons, W. C., Plisga, G. J., and Lorenz, M. D. (2016). "Aqueous Solubility Data for Selected Petroleum Products." *Standard Handbook of Petroleum and Natural Gas Engineering (3rd Edition)*.

Mackay, D. M., Roberts, P. V, and Cherry, J. a. (1985). "Transport of organic contaminants in groundwater - Distribution and fate of chemicals in sand and gravel aquifers." *Environmental science & technology*, 19(5), 384–92.

Mahour, R. (2016). "Chemical and Biological Oxidation of Naphthenic Acids - where Stoichiometry, Kinetics and Thermodynamics Meet." University of Alberta.

Mancini, S. A., Lacrampe-Couloume, G., and Lollar, B. S. (2008). "Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses." *Environmental Forensics*, 9(2–3), 177–186.

Martins, L. F., and Peixoto, R. S. (2012). "Biodegradation of petroleum hydrocarbons in hypersaline environments." *Brazilian Journal of Microbiology*, 43(3), 865–872.

Meckenstock, R. U., Boll, M., Mouttaki, H., Koelschbach, J. S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A. M. (2016). "Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons." *Journal of Molecular Microbiology and Biotechnology*, 26(1–3), 92–118.

Nales, M., Butler, B. J., and Edwards, E. A. (1998). “Anaerobic benzene biodegradation: A microcosm survey.” *Bioremediation Journal*, 2(2), 125–144.

Nicholson, C. A., and Fathepure, B. Z. (2004). “Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions.” *Applied and Environmental Microbiology*, 70(2), 1222–1225.

Nicholson, C. A., and Fathepure, B. Z. (2005). “Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma.” *FEMS Microbiology Letters*, 245(2), 257–262.

Phelps, C. D., Kazumi, J., and Young, L. Y. (1996). “Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction.” *FEMS Microbiology Letters*, 145(3), 433–437.

Phelps, C. D., Kerkhof, L. J., and Young, L. Y. (1998). “Molecular characterization of a sulfate-reducing consortium which mineralizes benzene.” *FEMS Microbiology Ecology*, 27(3), 269–279.

Phelps, C. D., Zhang, X., and Young, L. Y. (2001). “Use of Stable Isotopes to Identify Benzoate as a Metabolite of Benzene Degradation in a Sulphidogenic Consortium.” *Environmental Microbiology*, 3(9), 600–603.

Qin, X., Tang, J. C., Li, D. S., and Zhang, Q. M. (2012). “Effect of salinity on the bioremediation of petroleum hydrocarbons in a saline-alkaline soil.” *Letters in Applied Microbiology*, 55(3), 210–217.

Reardon, K. F., Mosteller, D. C., and Bull Rogers, J. D. (2000). “Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for Pseudomonas putida F1.” *Biotechnology and Bioengineering*, 69(4), 385–400.

Rebhun, M., Kalabo, R., Grossman, L., Manka, J., and Rav-Acha, C. (1992). “Sorption of organics on clay and synthetic humic-clay complexes simulating aquifer processes.” *Water Research*, 26(1), 79–84.

Reinhard, M., Shang, S., Kitanidis, P. K., Orwin, E., Hopkins, G. D., and Lebron, C. A. (1997). “In Situ BTEX Biotransformation under Enhanced Nitrate- and Sulfate-Reducing Conditions.” *Environmental Science & Technology*, 31(1), 28–36.

Sakai, N., Kurisu, F., Yagi, O., Nakajima, F., and Yamamoto, K. (2009). “Identification of putative benzene-degrading bacteria in methanogenic enrichment cultures.” *Journal of Bioscience and Bioengineering*, The Society for Biotechnology, 108(6), 501–507.

Sei, A., and Fathepure, B. Z. (2009). “Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake.” *Journal of Applied Microbiology*, 107(6), 2001–2008.

Shiu, W. Y., Ma, K. C., Mackay, D., Seiber, J. N., and Wauchope, R. D. (1990). “Solubilities of pesticide chemicals in water. Part I: Environmental physical chemistry.” *Reviews of environmental contamination and toxicology*, 116, 1–13.

Soares, A. A., Albergaria, J. T., Domingues, V. F., Alvim-Ferraz, M. da C. M., and Delerue-Matos, C. (2010). “Remediation of soils combining soil vapor extraction and bioremediation: Benzene.” *Chemosphere*, 80(8), 823–828.

Song, H. G., Wang, X., and Bartha, R. (1990). “Bioremediation potential of terrestrial fuel spills.” *Applied and Environmental Microbiology*, 56(3), 652–656.

Speight, J. G. (2014). *The Chemistry and Technology of Petroleum (5th Edition)*. CRC Press.

Stryer, L. (1988). *Biochemistry*. W. H. Freeman and Company, New York, NY.

Taubert, M., Vogt, C., Wubet, T., Kleinsteuber, S., Tarkka, M. T., Harms, H., Buscot, F., Richnow, H. H., Von Bergen, M., and Seifert, J. (2012). “Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing, benzene-degrading microbial consortium.” *ISME Journal*, 6(12), 2291–2301.

U. S. Environmental Protection Agency (EPA). (2000). “Engineered approaches to in situ bioremediation of chlorinated solvents: Fundamentals and field applications.” (EPA 542-R-00-008), 1–144.

Ulrich, A. C. (2004). “Characterization of Anaerobic Benzene-Degrading Cultures.” University of Toronto.

Ulrich, A. C., Beller, H. R., and Edwards, E. A. (2005). “Metabolites detected during biodegradation of 13C 6-benzene in nitrate-reducing and methanogenic enrichment cultures.” *Environmental Science and Technology*, 39(17), 6681–6691.

Ulrich, A. C., and Edwards, E. A. (2003). “Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures.” *Environmental Microbiology*, 5(2), 92–102.

Ulrich, A. C., Guigard, S. E., Foght, J. M., Semple, K. M., Pooley, K., Armstrong, J. E., and Biggar, K. W. (2009). “Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated groundwater.” *Biodegradation*, 20(1), 27–38.

Vallero, D. (2008). “Chapter 6 - Air Pollution Systems and Processes.” *Fundamentals of Air*

Pollution (4th Edition), 154–198.

Vieth, A., Kästner, M., Schirmer, M., Weiß, H., Gödeke, S., Meckenstock, R. U., and Richnow, H. H. (2005). "Monitoring In Situ Biodegradation of Benzene and Toluene by Stable Carbon Isotope Fractionation." *Environmental Toxicology and Chemistry*, 24(1), 51–60.

Vogel, T. M., and Grbić-Galić, D. (1986). "Incorporation of Oxygen from Water into Toluene and Benzene during Anaerobic Fermentative Transformation." *Applied and Environmental Microbiology*, 52(1), 200–202.

Vogt, C., Kleinsteuber, S., and Richnow, H. H. (2011). "Anaerobic benzene degradation by bacteria." *Microbial Biotechnology*, 4(6), 710–724.

van der Waals, M. J., Atashgahi, S., da Rocha, U. N., van der Zaan, B. M., Smidt, H., and Gerritse, J. (2017). "Benzene degradation in a denitrifying biofilm reactor: activity and microbial community composition." *Applied Microbiology and Biotechnology*, Applied Microbiology and Biotechnology, 101(12), 5175–5188.

Weiner, J. M., and Lovley, D. R. (1998). "Rapid Benzene Degradation in Methanogenic Sediments from a Petroleum-Contaminated Aquifer." *American Society for Microbiology*, 64(5), 1937–1939.

Westberg, H., Sexton, K., and Flyckt, D. (1981). "Hydrocarbon production and photochemical ozone formation in forest burn plumes." *Journal of the Air Pollution Control Association*, 31(6), 661–664.

Wilson, S., Card, G., and Haines, S. (2009). "Table 3.3 - Properties of Some Commonly Occurring Vapours." *Ground Gas Handbook*, Whittles Publishing.

Winfrey, M. R., Beck, E., Boehm, P., and Ward, D. M. (1982). "Impact of Crude Oil on Sulphate Reduction and Methane Production in Sediments Impacted by The Amoco Cadiz Oil Spill." *Marine Environmental Research*, 7(3), 175–194.

Yang, L., Lai, C.-T., and Shieh, W. K. (2000). "Biodegradation of Dispersed Diesel Fuel Under High Salinity Conditions." *Water Research*, 34(13), 3033–3314.

Yaws, C. L. (2014). "Table 118 - Henry's Law Constant for Compound in Water - Organis Compounds." *Yaws' Critical Property Data for Chemical Engineers and Chemists*.

van der Zaan, B. M., Saia, F. T., Stams, A. J. M., Plugge, C. M., de Vos, W. M., Smidt, H., Langenhoff, A. A. M., and Gerritse, J. (2012). "Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a

syntrophic process." *Environmental Microbiology*, 14(5), 1171–1181.

Zhang, T., Tremblay, P. L., Chaurasia, A. K., Smith, J. A., Bain, T. S., and Lovley, D. R. (2013). "Anaerobic benzene oxidation via phenol in Geobacter metallireducens." *Applied and Environmental Microbiology*, 79(24), 7800–7806.

Appendix A

Degradation Profiles of the Culture Enrichment Period

In efforts to keep Chapter 4 succinct, only three of the twelve treatments of the Culture Enrichment period were shown (one for each anaerobic condition). Those three treatments were chosen due to their greater degrees of degradation as well as clearly displaying the most salient features of a degradation profile. In Appendix A here, the degradation profiles of the remaining nine treatments will be displayed and their notable features and potential stalling points will be discussed.

Culture Enrichment: Treatment CNB[1] Results

Microcosm CNB[1] received an incubation period of 341 days throughout the Culture Enrichment period in which three benzene re-feeds occurred, resulting in four degradation cycles. The overall degradation profile is presented below as Figure A-1.

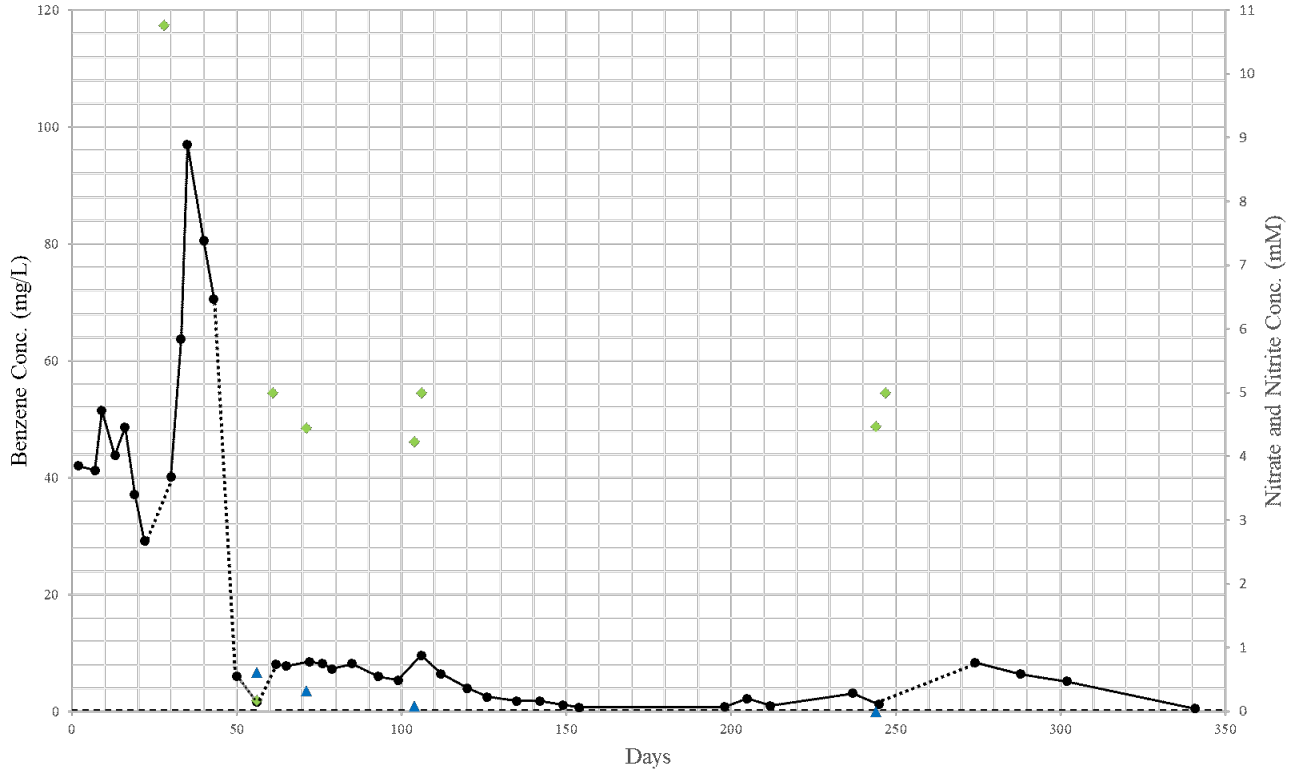

Figure A-1 – CNB[1] Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed (downwards for microcosm dilution). Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 350.

In the first degradation cycle, CNB[1] exhibited a relatively linear degradation rate involving moderate benzene content ranging between 29.2 and 52 mg/L. Benzene refeed occurred on Day 28 but did not fully re-equilibrate until Day 35, displaying in a sharp increase in benzene content

between these two times. The second degradation cycle began with a higher benzene content (96.96 mg/L) but was subsequently abandoned on Day 47 to dilute the microcosm with fresh anaerobic media mix (AMM) due to the above optimal levels of nitrite observed via anion analysis on Day 28. The third and fourth degradation cycles focused on lower benzene content (below 10 mg/L). The third cycle initiated at a concentration of 8.5 mg/L between Days 62 and 76 but had decreased to approximately 1.8 mg/L by Day 135. No significant degradation was believed to occur between Days 135 and 245 as benzene content remained relatively steady in conjunction to the non-depleting nitrate concentration. With the clarity of hindsight, this three-month period would have been more beneficial with a benzene refeed and nitrate re-amendment to generate an additional degradation cycle. The fourth degradation cycle resulted in relatively similar results with degradation of 8.4 to 0.5 mg/L between days 274 and 341. Following these four degradation cycles, microcosm CNB^1 was later incorporated into 0.0 g/L NaCl and sodium azide kill control microcosms of the Salinity Experiment.

Culture Enrichment: Treatment SNB[1] Results

Microcosm SNB[1] received an incubation period of 341 days throughout the Culture Enrichment period in which four benzene re-feeds occurred, resulting in five degradation cycles. The overall degradation profile is presented as Figure A-2.

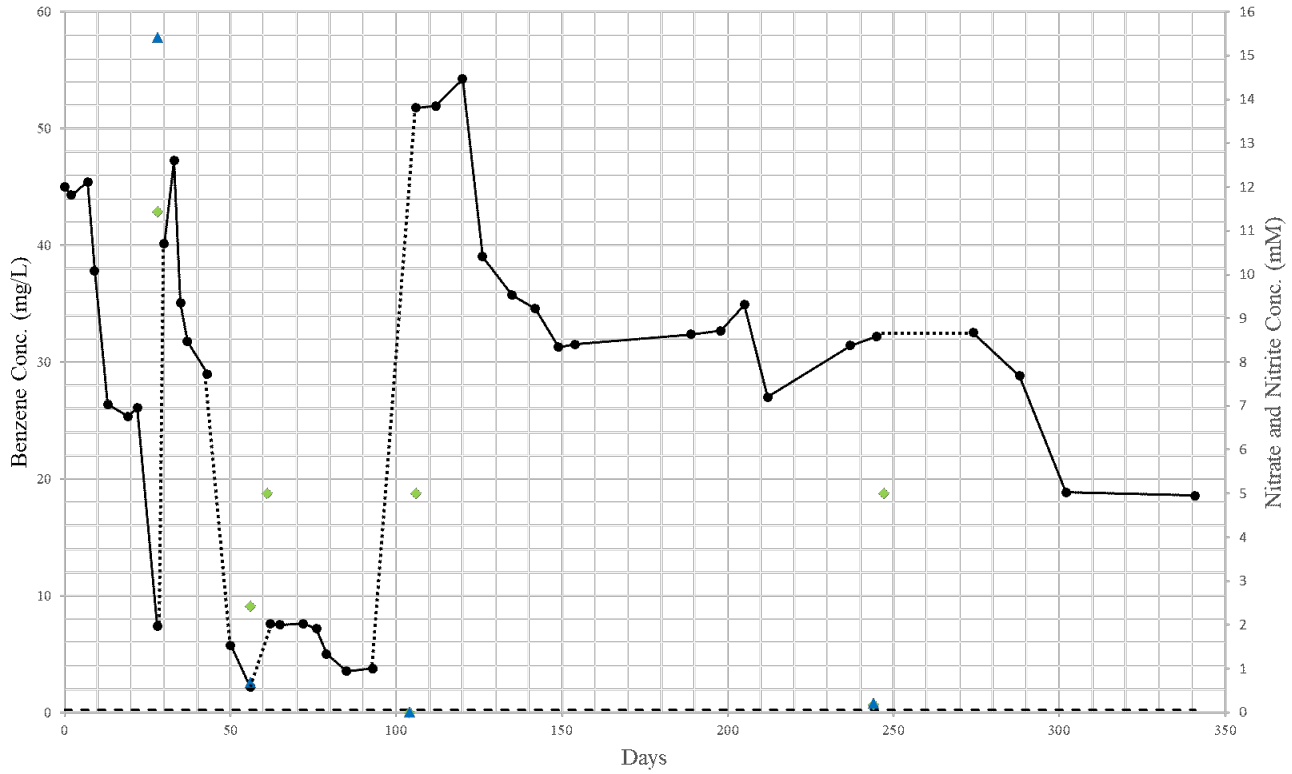

Figure A-2 – SNB[1] Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed (downwards for microcosm dilution). Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 341.

In the first and second degradation cycle, SNB[1] demonstrated similar capacity for benzene degradation (approximate initial concentration 45 mg/L) to 7.4 mg/L in 28 days for the first cycle and 29.0 mg/L in 24 days for the second cycle. The degradation observed in the second cycle appeared comparable but was prematurely abandoned dilute the microcosm with fresh AMM due to the elevated nitrite content observed on Day 28 (15.4 mM). No re-equilibrate period was observed between the first and second cycles following benzene refeed. In the third degradation cycle, SNB[1] encountered no difficulties in degrading a relatively lower benzene content (from 7.6 mg/L to 3.8 mg/L over 61 days). The fourth and fifth degradation cycles once again demonstrated similar degradation capacity in higher initial benzene concentrations (51.8 and 32.5 mg/L respectively). Due to the low nitrate content at the end of the fourth cycle and the lack of

degradation between days 154 and 245, degradation progress is most likely stalled due to insufficient terminal electron acceptor. Following a resupply of nitrate on Day 247, degradation recommenced for the fifth degradation cycle. Throughout cycles three through five, nitrate content was consistently near depletion, suggesting nitrate-reducing conditions to be linked with the observed benzene disappearance. However, it is noteworthy that nitrite levels remained negligible (0.014, 0.225, and 0.002 mM) after the dilution event and never replenished to significant amounts, suggesting the possibility of downstream microorganisms to metabolize nitrite as a similar rate in which it is formed from nitrate.

Culture Enrichment: Treatment SNB^2 Results

Microcosm SNB^2 received an incubation period of 198 days throughout the Culture Enrichment period in which two benzene re-amendments occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-3.

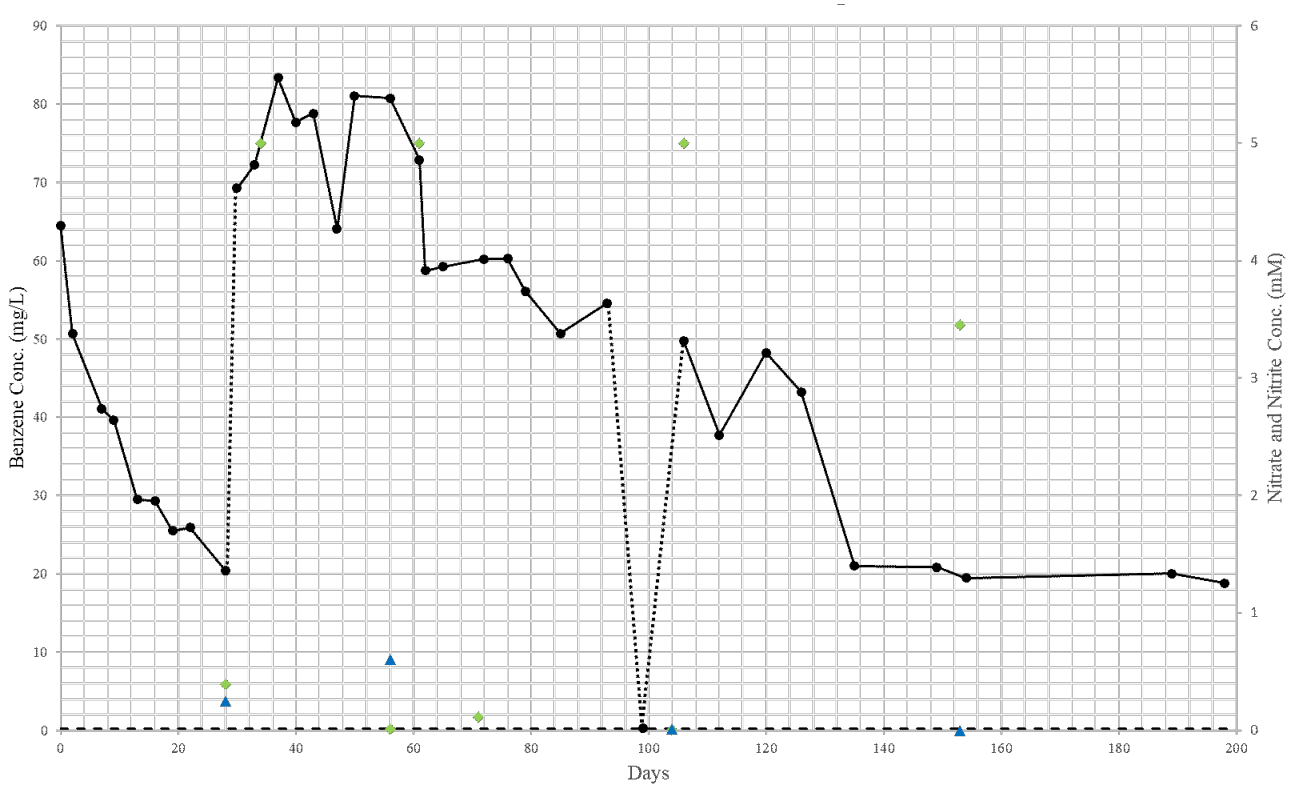

Figure A-3 – SNB^2 Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed (downwards for microcosm dilution). Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 198.

In the first degradation cycle, SNB^2 lowered the benzene content from 64.5 mg/L to 20.4 mg/L over a 28-day incubation period, concluding with a depleted nitrate content of 0.4 mM. Following nitrate re-amendment and benzene refeed without a re-equilibrate period, benzene content appeared to remain consistent at approximately 80 mg/L between days 29 and 59. Anion analysis on Day 57 indicated nitrate depletion and a second nitrate re-amendment to 5 mM occurred within the second degradation cycle on Day 61. Subsequently, benzene concentration decreased to 50.7 mg/L on Day 85. The lack of benzene degradation, depleted nitrate content on Day 57, and immediate resumption of benzene disappearance following nitrate resupply on Day 61 all suggest that the stalled degradation occurring between 29 and 59 was due to insufficient terminal electron acceptor in contrary to the previous nitrate re-amendment occurring on Day 34. The elevated nitrite content (6.4 mM) on Day 71 led to a dilution with fresh AMM on Day 97, concluding the second degradation cycle. The third and final degradation cycle lasted 92 days and degraded benzene content from 49.79 to 18.8 mg/L.

Culture Enrichment: Treatment CSB[1] Results

Microcosm CSB[1] received an incubation period of 198 days throughout the Culture Enrichment period in which two benzene refeeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-4.

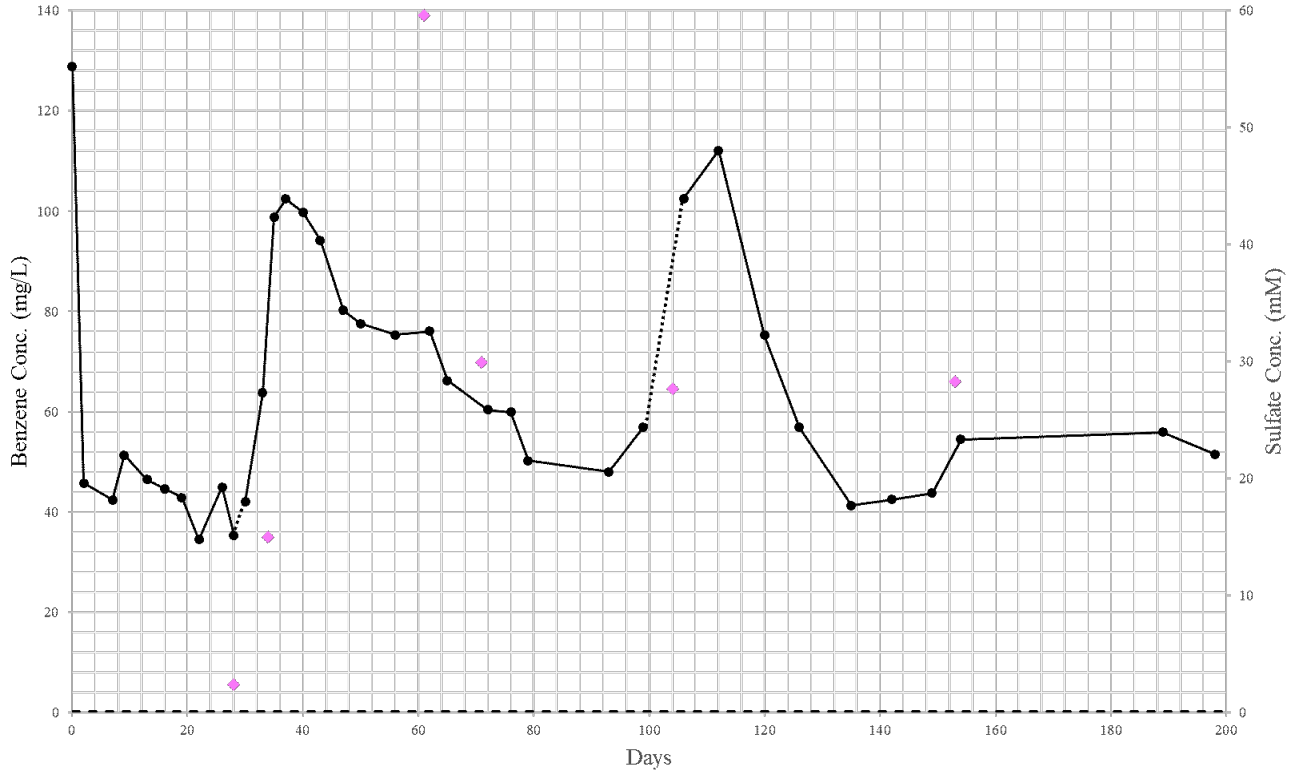

Figure A-4 – CSB[1] Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 198.

At the onset of the first degradation cycle, benzene concentrated decreased from 128.8 mg/L to 45.7 mg/L within two days, reflecting a 531.5 µM/d degradation rate if these values are accurate. This cycle concluded with a substrate content between 35.3 and 42.9 mg/L. Benzene refeed occurred on Day 28 but did not fully re-equilibrate until Day 33, displaying in a sharp increase in benzene content between these two points. The second degradation cycle began with a higher benzene content (102.4 mg/L) but was gradually degraded to final concentration of 48.0 mg/L on Day 98. The third and fourth degradation cycles focused on lower benzene content (below 10 mg/L). The third degradation cycle peaked 112.1 mg/L on Day 112 and followed a relatively linear rate of degradation to 41.3 on Day 135. Little degradation is reported in the interval between Days 135 and 198 as benzene content remained steady. Following these three degradation cycles,

microcosm CSB[1] was later incorporated into the 0.0, 0.5, and 1.0 g/L NaCl of the Salinity Experiment.

Culture Enrichment: Treatment CSB[2] Results

Microcosm CSB[2] received an incubation period of 302 days throughout the Culture Enrichment period in which two benzene re-feeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-5.

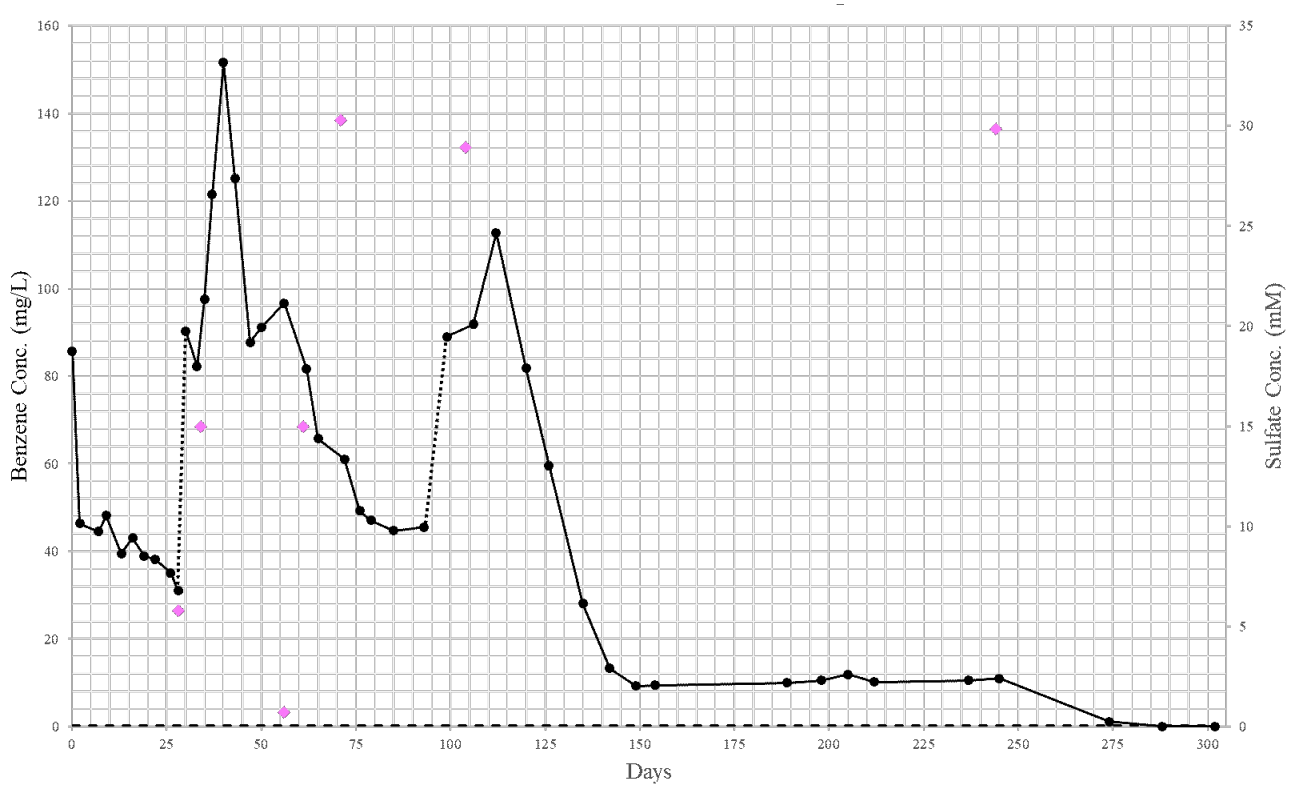

Figure A-5 – CSB[2] Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 302.

In the first degradation cycle, CSB[2] demonstrated significant benzene degradation (85.8 to 31.0 mg/L) in 28 days. Following sulfate re-amendment and benzene refeed which required approximately 10 days to re-equilibrate, benzene content decreased from 151.6 mg/L to 45.56 mg/L by Day 93 within the second cycle. Sulfate content was re-amended to 15 mM on Day 34, but anion analysis indicated only 0.7 mM remained on Day 56. Another sulfate re-amendment occurred on Day 61 to 15 mM. No significant degradation occurred between Days 149 and 302. Following these three degradation cycles, microcosm CSB[2] was later incorporated into the 0.0 g/L NaCl microcosm the sodium azide kill controls of the Salinity Experiment.

Culture Enrichment: Treatment SSB[1] Results

Microcosm SSB[1] received an incubation period of 198 days throughout the Culture Enrichment period in which two benzene re-feeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-6.

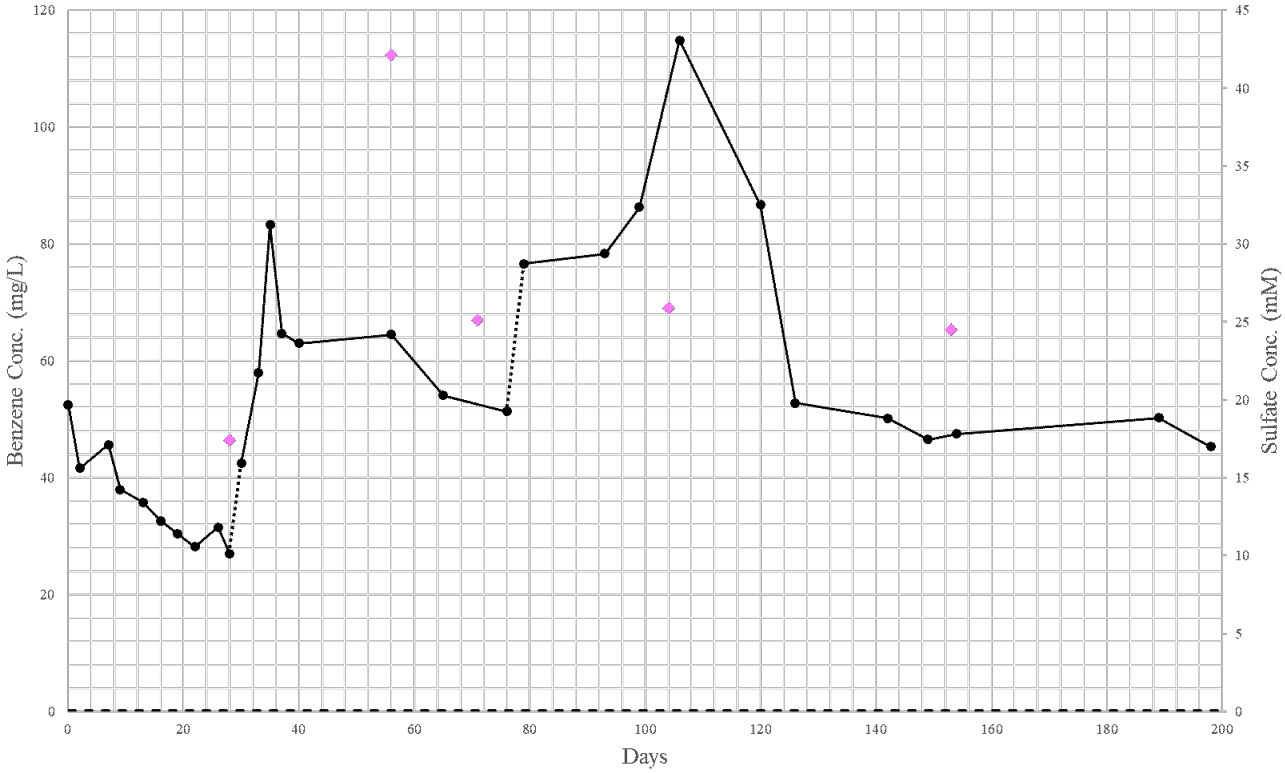

Figure A-6 – SSB[1] Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene refeed. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 198.

Benzene degradation followed a linear manner throughout the first degradation cycle and decreased from 52.4 to 27.0 mg/L over a 28 day incubation period. Only benzene refeed occurred before the onset of the second degradation cycle as sulfate content was observed to be above the target 15 mM level. The second degradation cycle required a 5 day re-equilibrate period and reached a peak concentration of 83.3 mg/L but was subsequently degraded to 51.3 mg/L by Day 76. In the third degradation cycle, benzene content re-equilibrated from 76.6 mg/L to a peak of 114.9 mg/L over a 27 day period. Degradation commenced afterwards and the substrate content dropped to 52.7 mg/L by Day 126. Minimal changes in benzene concentration was observed between Days 126 to 198. It is noteworthy that sulfate reamendment did not occur with microcosm SSB[1] as the terminal electron acceptor concentration was above the target amount during each

anion analysis monitoring session. Furthermore, not only did sulfate content remain above 15 mM, it had remained consistent (approximately 25 mM) throughout the entirety of the third degradation cycle in which benzene content decreased. Following these three degradation cycles, microcosm SSB[1] was later incorporated into the 0.0, 0.5, and 1.0 g/L NaCl of the Salinity Experiment.

Culture Enrichment: Treatment CMB[1] Results

Microcosm CMB[1] received an incubation period of 198 days throughout the Culture Enrichment period in which two benzene refeeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-7.

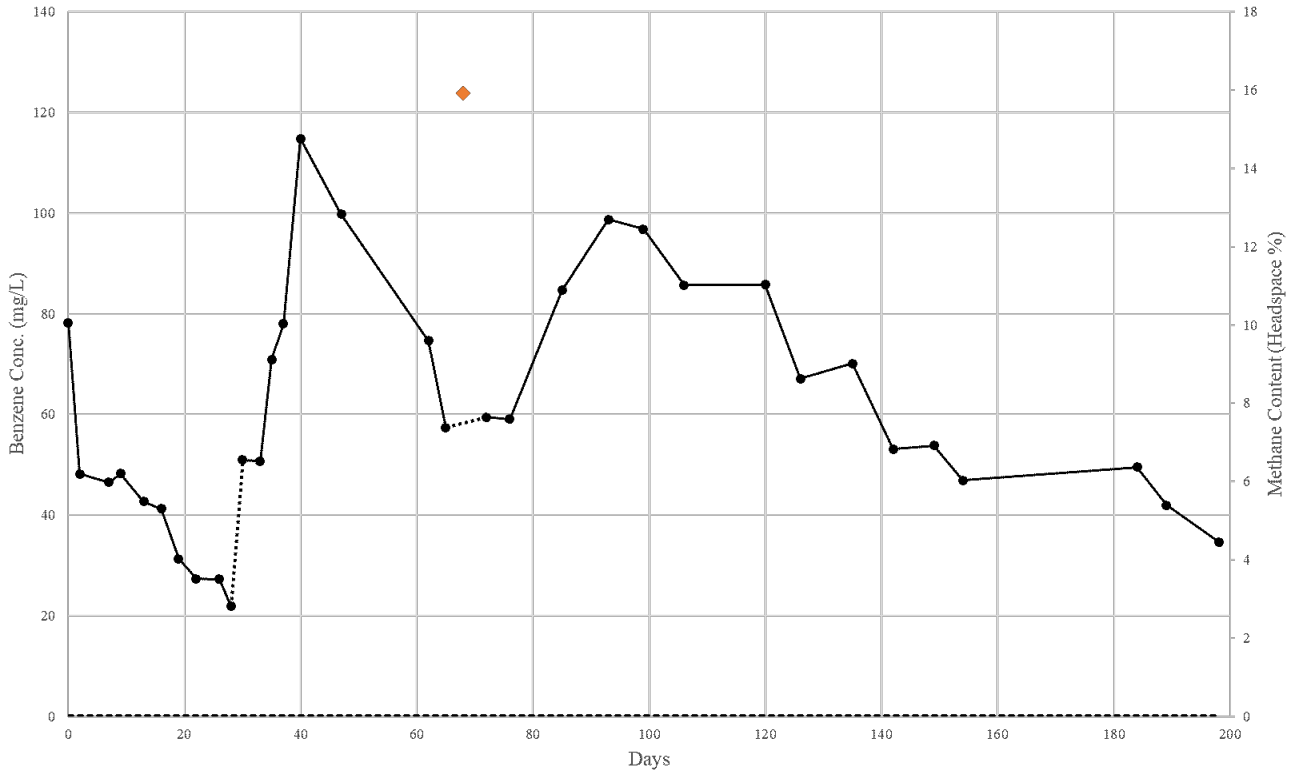

Figure A-7 – CMB[1] Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 198.

As mentioned in Section 4.2.3 of Chapter 4, the considerable benzene decrease between Day 0 (78.1 mg/L) and Day 2 (48.2 mg/L) is considered to be an erroneous due to the inflated reading on Day 0. By Day 28, benzene content has been degraded to 21.9 mg/L in the first degradation cycle. In the following cycle, a maximum concentration of 114.8 mg/L was reached after a 10 day re-equilibrate period. Substrate concentration then dropped to 57.3 mg/L within three monitoring periods 25 days later. In the third and last degradation cycle, benzene steadily decreased from 98.74 to 34.7 mg/L over a 105 day period. Only one headspace monitoring session for methane analysis was performed for this treatment: 15.9% on Day 68. Following these three degradation cycles, microcosm CMB[1] was later incorporated into the 0.0, 0.5, and 1.0 g/L NaCl of the Salinity Experiment.

Culture Enrichment: Treatment SMB[1] Results

Microcosm SMB[1] received an incubation period of 198 days throughout the Culture Enrichment period in which two benzene re-feeds occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-8.

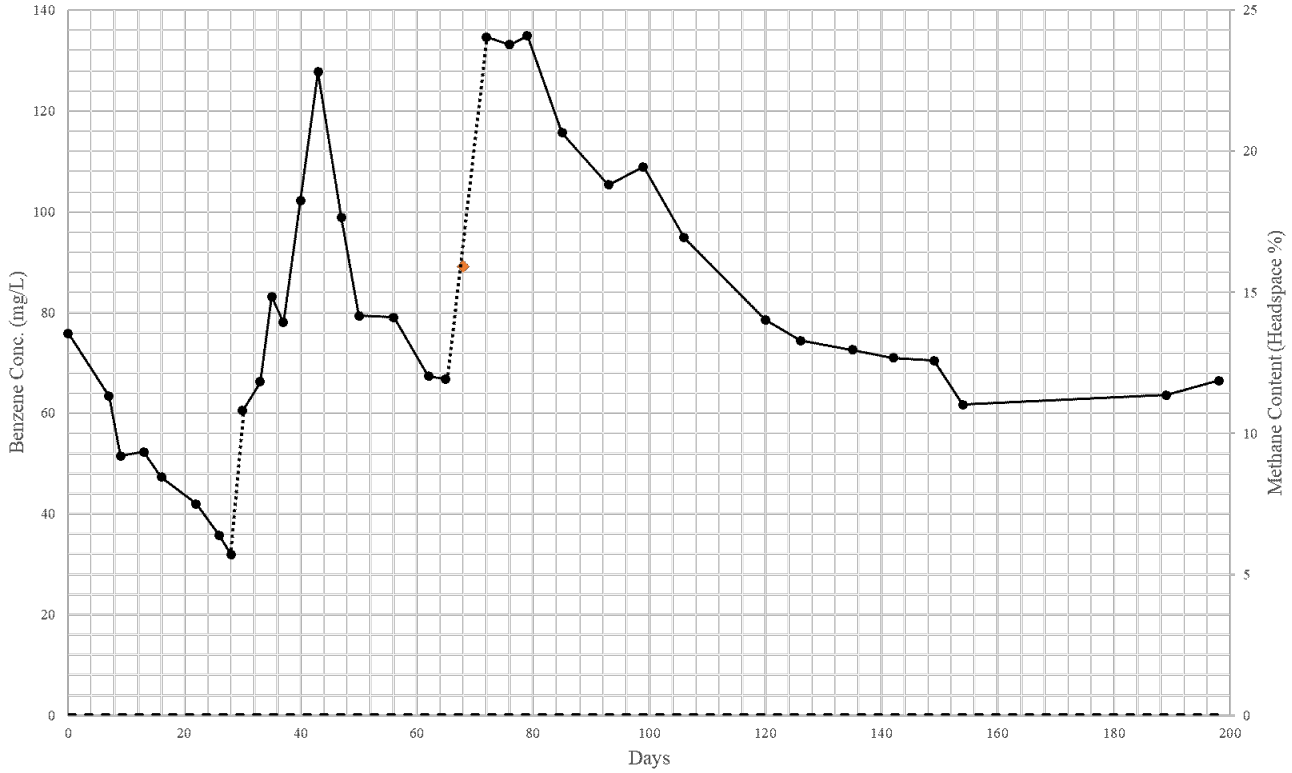

Figure A-8 – SMB[1] Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 198.

The three degradation cycles of SMB[1] each successively tested a greater benzene peak concentration. Benzene was steadily decreased from 75.8 to 32.0 mg/L over a 28 day incubation period of the first cycle. In the second cycle, a 13 day re-equilibrate period was required before a peak concentration of 127.8 mg/L was established. After 22 days, a concentration of 66.8 mg/L was observed and concluded the second degradation cycle. Prior to the third cycle, a methane content of 16.0% was observed in the headspace. In the third and last degradation cycle, benzene steadily decreased from 134.9 to 74.4 mg/L. Although residual degradation continued for another 72 day and ultimately lowered to 66.5 mg/L, degradation was most likely stalled during this period for unknown reasons. Following these three degradation cycles, microcosm SMB[1] was later incorporated into the 0.0, 0.5, and 1.0 g/L NaCl of the Salinity Experiment.

Culture Enrichment: Treatment SMB^2 Results

Microcosm SMB^2 received an incubation period of 341 days throughout the Culture Enrichment period in which two benzene re-amendments occurred, resulting in three degradation cycles. The overall degradation profile is presented below as Figure A-9.

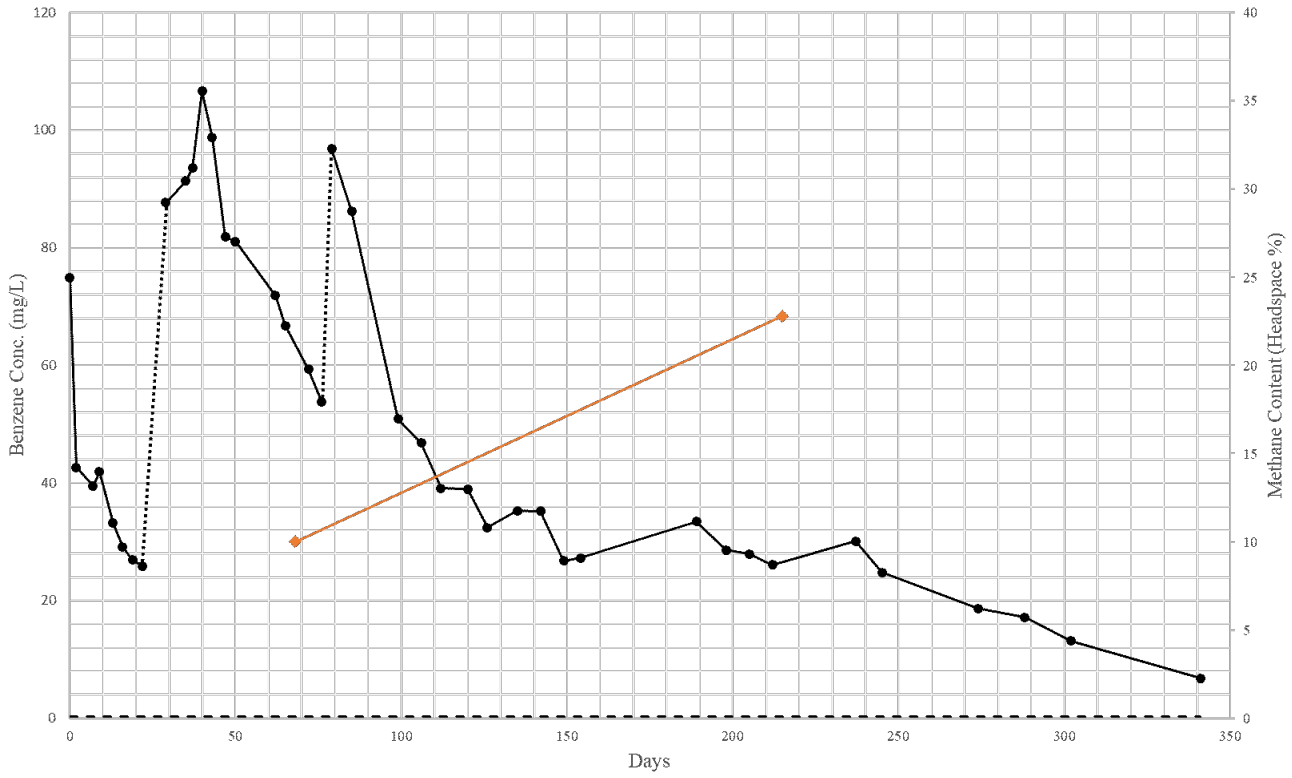

Figure A-9 – SMB^2 Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L. Day 0 to Day 341.

The three degradation cycles of SMB^2 each demonstrated continual degradation and resilience to elevated benzene feed concentrations (upwards of 106.6 mg/L) throughout the Culture Enrichment period. Akin to first monitoring session previously discussed for CMB^1, the first data point is most likely erroneous and elevated within the context of the degradation cycle. A 49.0 mg/L decrease over 22 days established the first degradation cycle. In the following cycle, significant degradation occurred following a 11 day re-equilibration period in which benzene was degraded from 106.6 to 53.8 mg/L between Days 40 and 76. The third and final degradation cycle lasted 262 days and saw prolonged degradation from 96.8 to 6.8 mg/L. Two methane monitoring sessions occurred for SMB^2 and indicated more than doubling of 10% to 22.8% headspace between Days 68 and 215. Following these three degradation cycles, microcosm SSB^2 was later

incorporated into the 0.0 g/L NaCl microcosm the sodium azide kill controls of the Salinity Experiment.

Appendix B

Control Treatments of the Salinity Experiment

In order to keep Chapter 5 succinct, only two of the nine benzene profiles of the control treatments were shown. In Appendix B here, the remaining seven profiles will be displayed below as Figures B-1 through B-7. While no significant benzene degradation occurred for any sodium azide kill and media controls, a downward trend in benzene content is demonstrated for these treatments. This gradual decrease in benzene is most likely due to the sum of abiotic losses including sorption to sediments within the anaerobic media mix (such as iron (II) sulfide), sorption to the inner glass walls of the bottle, sorption to the rubber stopper, and chemical oxidation.

The sodium azide kill controls were developed from active benzene degrading treatments, amended to a sodium azide concentration of 1 g/L, and autoclaved once per day for a total of three consecutive days to eliminate the microbial culture within a treatment bottle. In parallel, media controls were also developed. These controls do not contain a benzene degrading culture but instead, consist solely of fresh anaerobic media mix (AMM) amended with the same concentration of terminal electron acceptor as the live degrading treatments (5 mM sodium nitrate or 15 mM sodium sulfate). The methanogenic media controls contained only AMM. These media controls were also autoclaved once per day for a duration of three days.

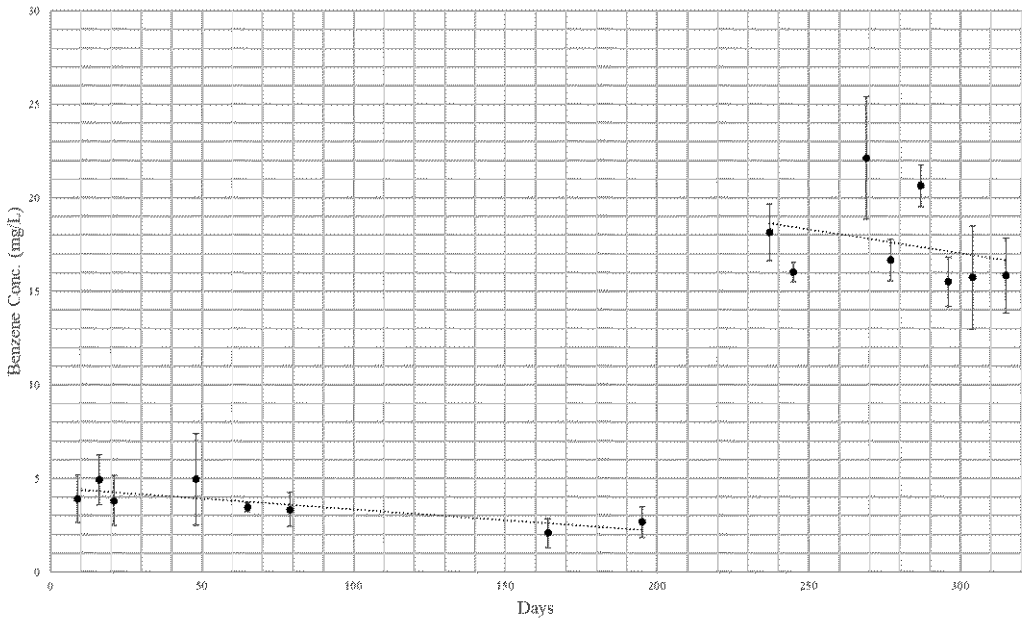

Figure B-1 – Salinity Experiment: Nitrate-Reducing, 1.0 g/L NaCl, Sodium Azide Kill Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

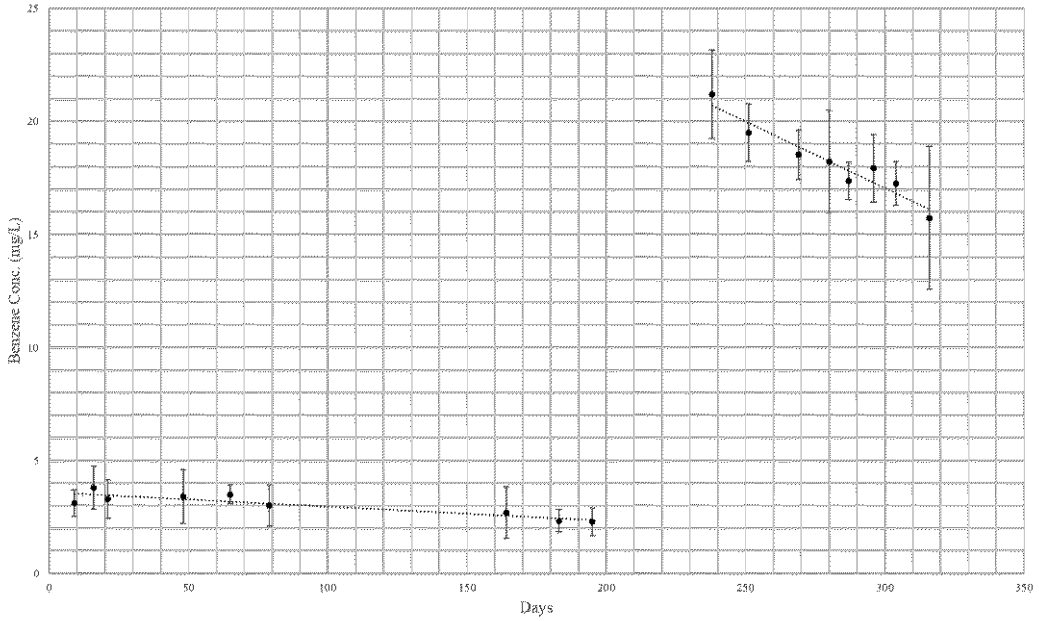

Figure B-2 – Salinity Experiment: Sulfate-Reducing, 0.0 g/L NaCl, Sodium Azide Kill Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

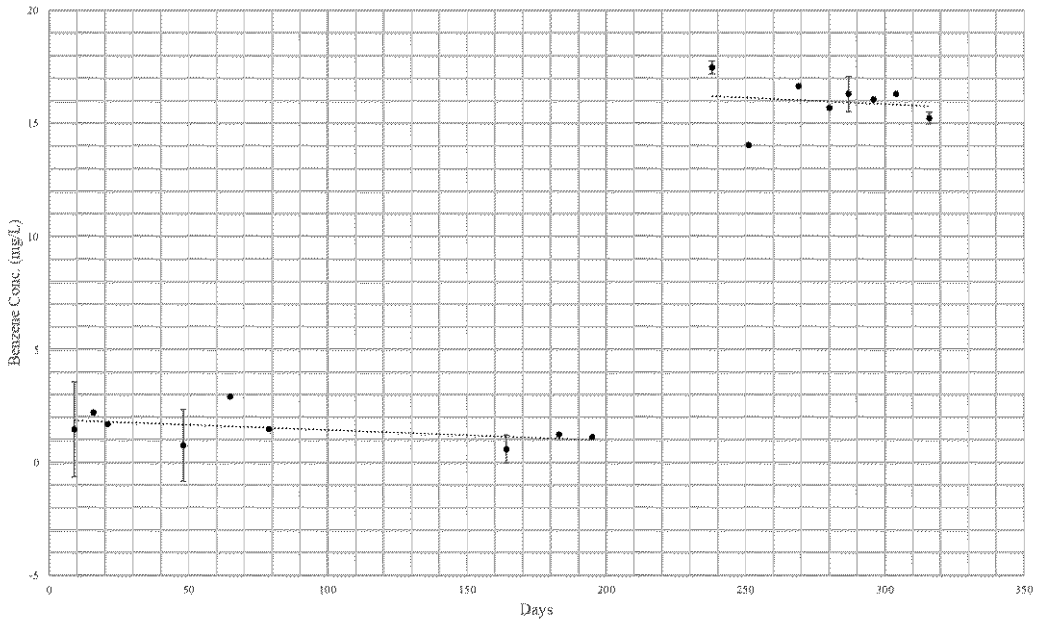

Figure B-3 – Salinity Experiment: Sulfate-Reducing, 1.0 g/L NaCl, Sodium Azide Kill Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

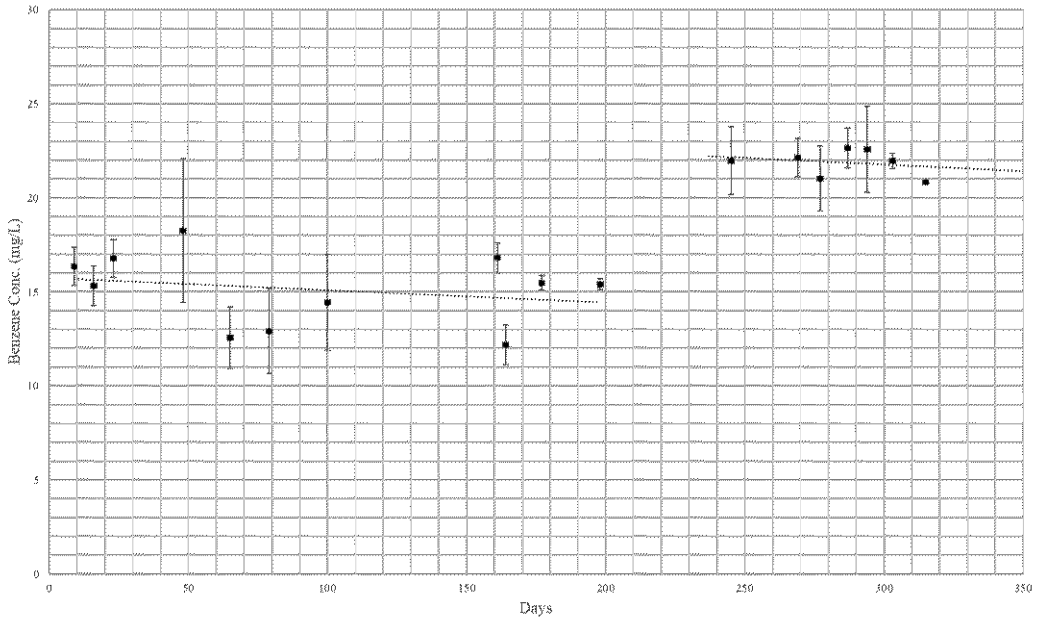

Figure B-4 – Salinity Experiment: Sulfate-Reducing, 0.0 g/L NaCl, Media Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

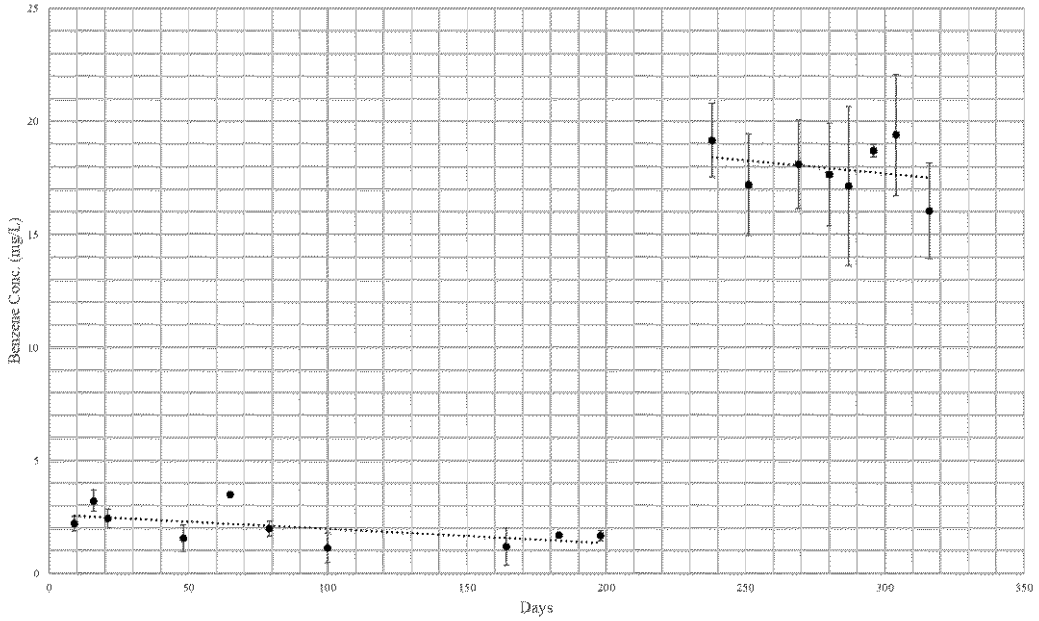

Figure B-5 – Salinity Experiment: Methanogenic, 0.0 g/L NaCl, Sodium Azide Kill Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

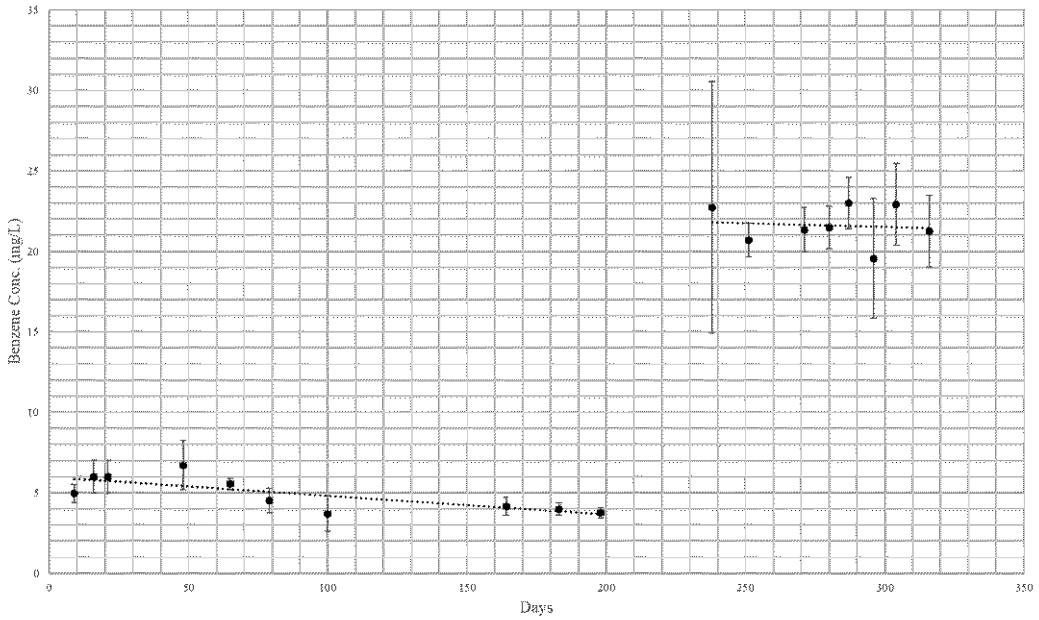

Figure B-6 – Salinity Experiment: Methanogenic, 1.0 g/L NaCl, Sodium Azide Kill Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

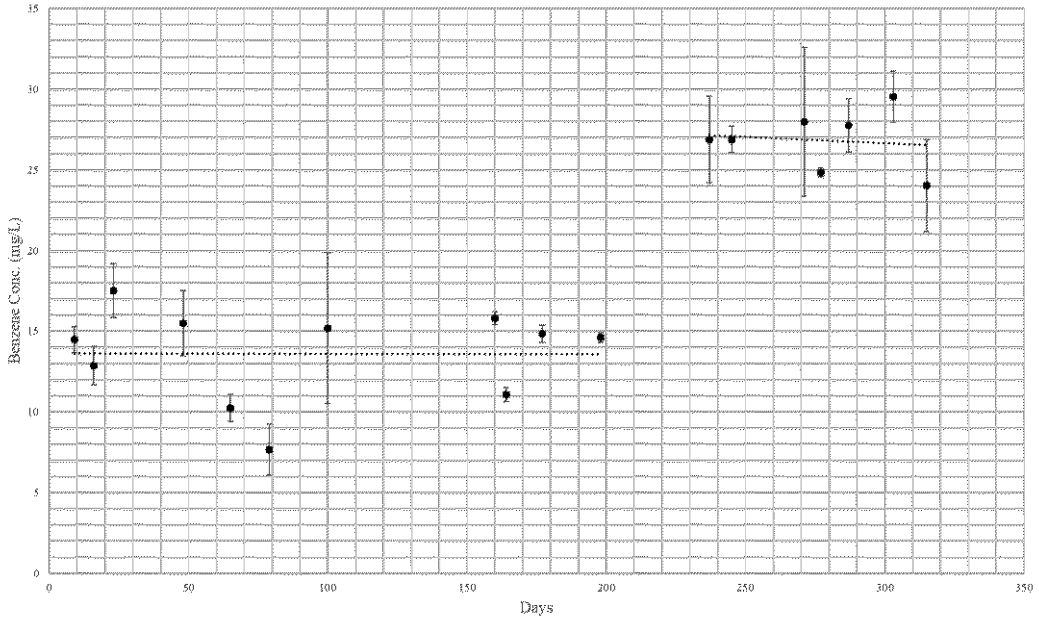

Figure B-7 – Salinity Experiment: Methanogenic, 0.0 g/L NaCl, Media Control: Abiotic benzene losses. Dotted black downward trendline represents the gradual abiotic benzene losses. Error bars depict one standard of deviation. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 325.

Appendix C

Degradation Profiles of the Salinity Experiment

In Chapter 5, Figures 5-6 through 5-8 each compile all the degradation profiles of the salinity experiment. For ease of comparison, degradation profiles of the same redox condition are placed in a side-by-side manner with the vertical and horizontal axis' set to the same scale for ease of comparison between salinity levels. For this reason, some data sets were not displayed in the most visually optimal fashion. In Appendix C here, each of the 12 degradation profiles are shown individually and enlarged for a detailed view of each treatment. Figures C-1 through C-4 display those for the nitrate-reducing treatments. Likewise Figures C-5 through C-8 are for sulfate-reducing treatments while Figures C-9 through C-12 correspond to the methanogenic treatments.

Salinity Experiment: Nitrate-Reducing – 0.0 g/L NaCl

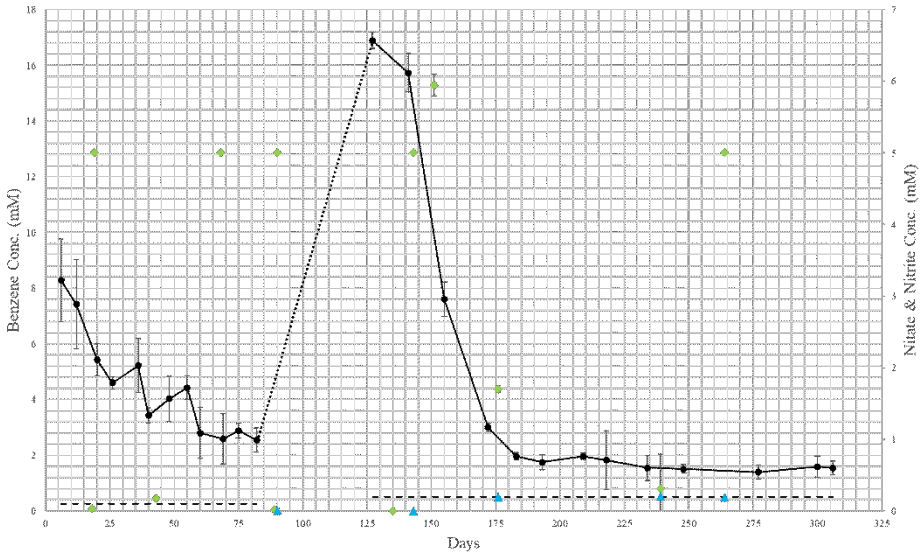

Figure C-1 – Salinity Experiment: Nitrate-Reducing, 0.0 g/L NaCl Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 306.

Salinity Experiment: Nitrate-Reducing 0.5 g/L NaCl

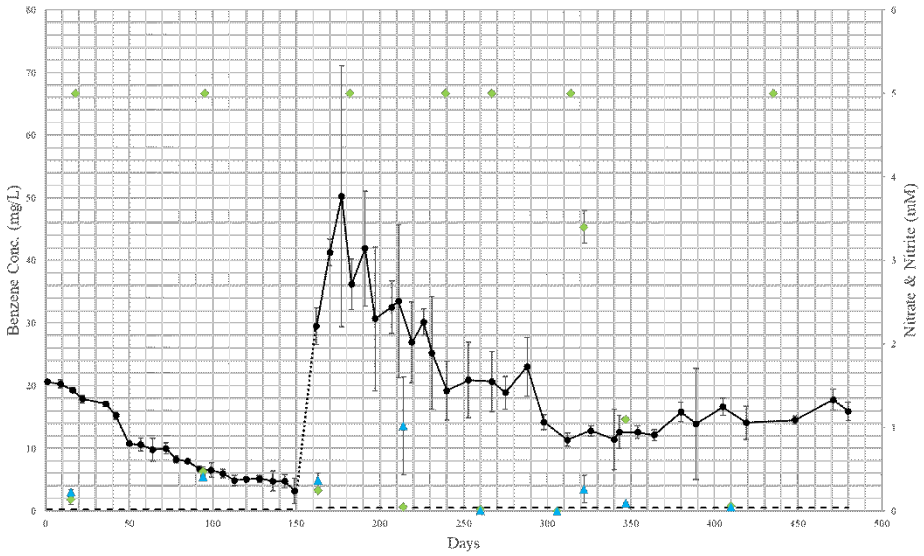

Figure C-2 – Salinity Experiment: Nitrate-Reducing, 0.5 g/L NaCl Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 480.

Salinity Experiment: Nitrate-Reducing 1.0 g/L NaCl

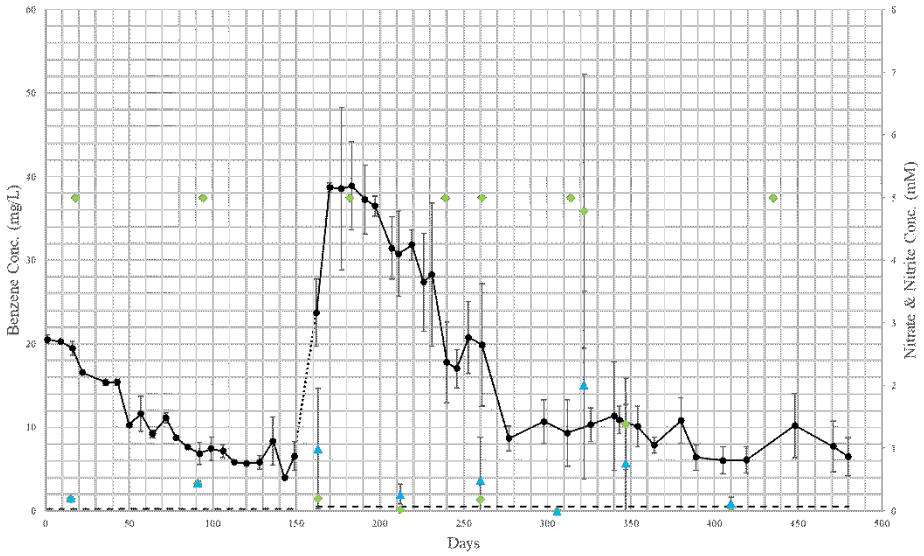

Figure C-3 – Salinity Experiment: Nitrate-Reducing, 1.0 g/L NaCl Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 480.

Salinity Experiment: Nitrate-Reducing 2.0 g/L NaCl

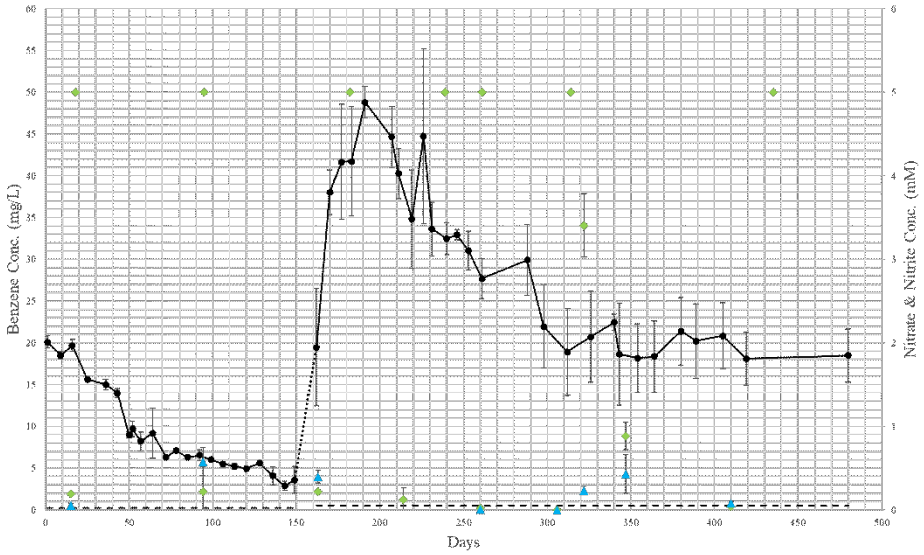

Figure C-4 – Salinity Experiment: Nitrate-Reducing, 2.0 g/L NaCl Overall Degradation: Nitrate depletion (◆), nitrite generation (▲), and benzene biodegradation (●). Nitrate (◆) concentrations at 5mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 480.

Salinity Experiment: Sulfate-Reducing 0.0 g/L NaCl

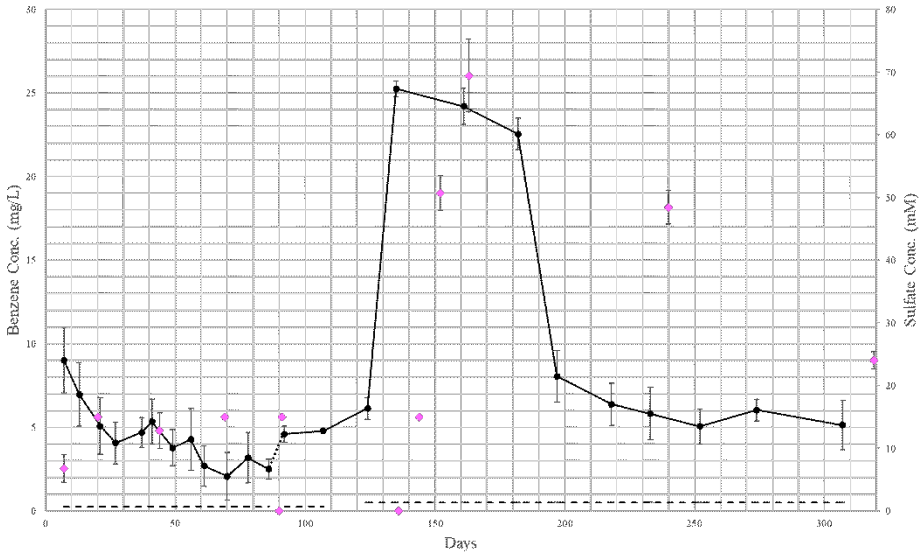

Figure C-5 – Salinity Experiment: Sulfate-Reducing, 0.0 g/L NaCl Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 307.

Salinity Experiment: Sulfate-Reducing 0.5 g/L NaCl

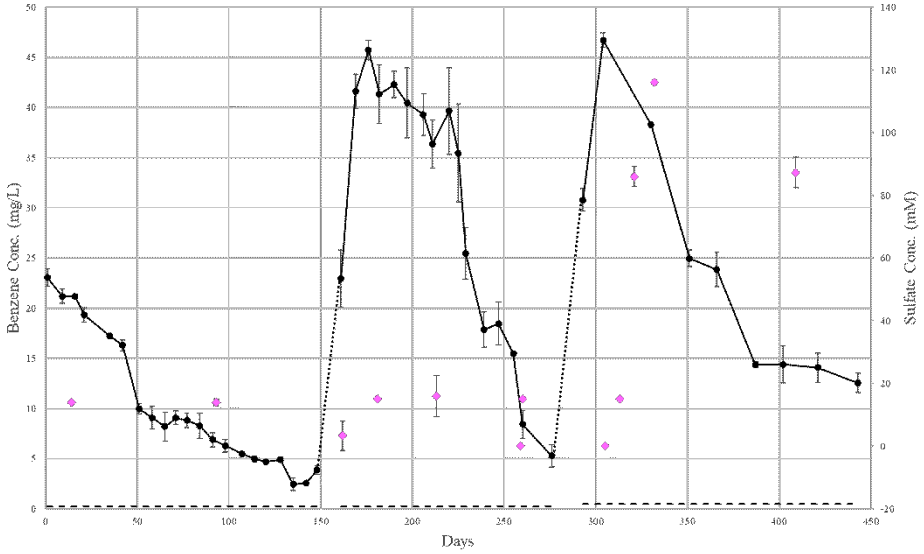

Figure C-6 – Salinity Experiment: Sulfate-Reducing, 0.5 g/L NaCl Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 443.

Salinity Experiment: Sulfate-Reducing 1.0 g/L NaCl

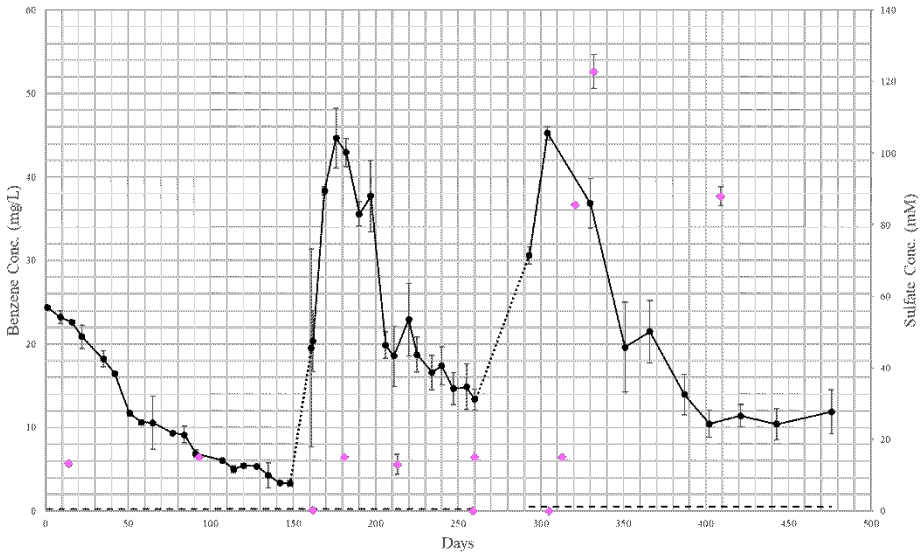

Figure C-7 – Salinity Experiment: Sulfate-Reducing, 1.0 g/L NaCl Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 476.

Salinity Experiment: Sulfate-Reducing 2.0 g/L NaCl

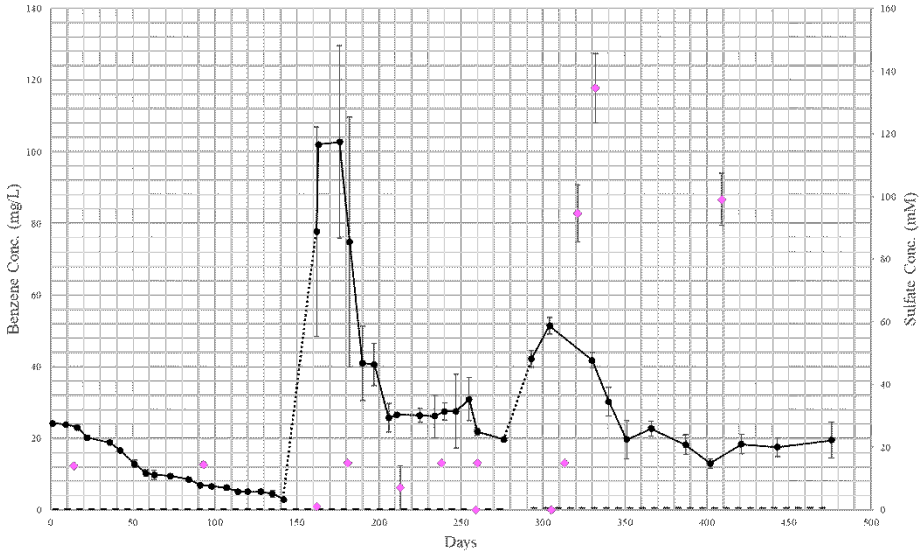

Figure C-8 – Salinity Experiment: Sulfate-Reducing, 2.0 g/L NaCl Overall Degradation: Sulfate depletion (◆) and benzene biodegradation (●). Sulfate (◆) concentrations at 15 mM represent re-amendment. Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 476.

Salinity Experiment: Methanogenic 0.0 g/L NaCl

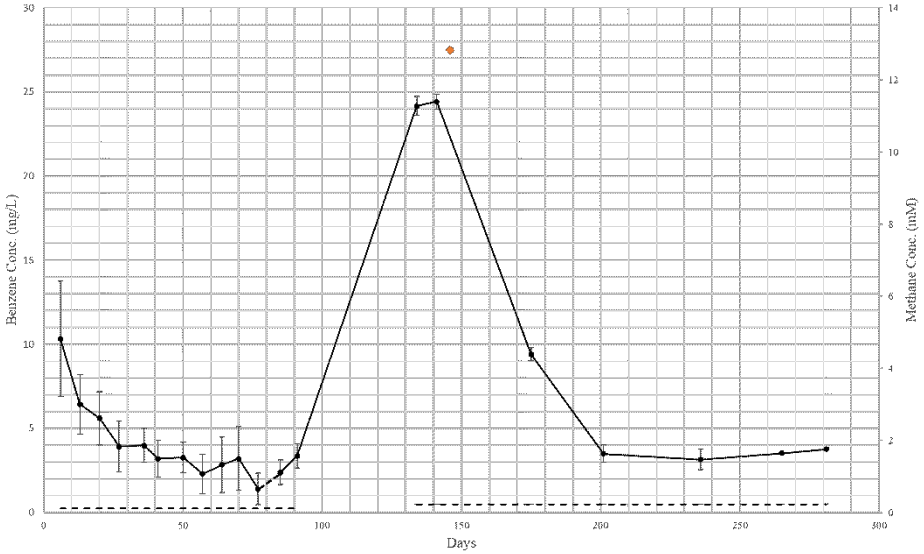

Figure C-9 – Salinity Experiment: Methanogenic, 0.0 g/L NaCl Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 281.

Salinity Experiment: Methanogenic 0.5 g/L NaCl

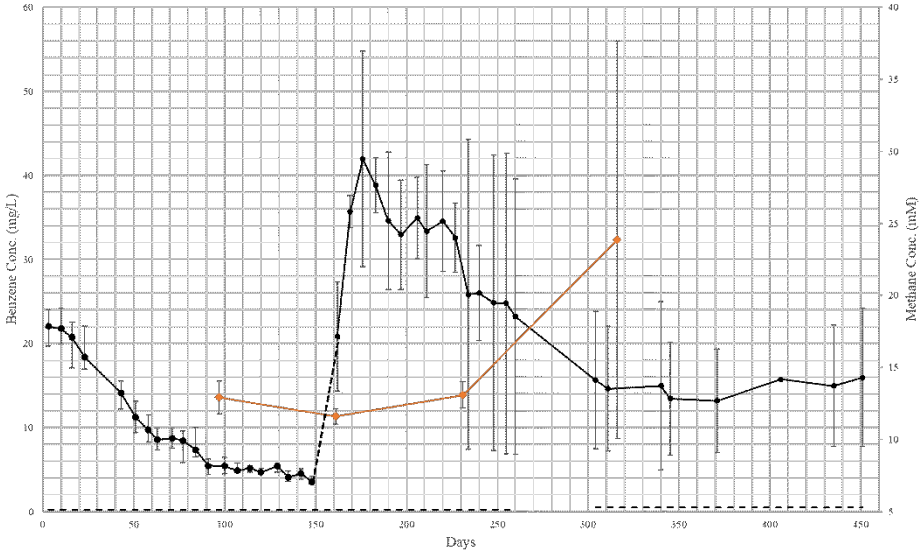

Figure C-10 – Salinity Experiment: Methanogenic, 0.5 g/L NaCl Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 451.

Salinity Experiment: Methanogenic 1.0 g/L NaCl

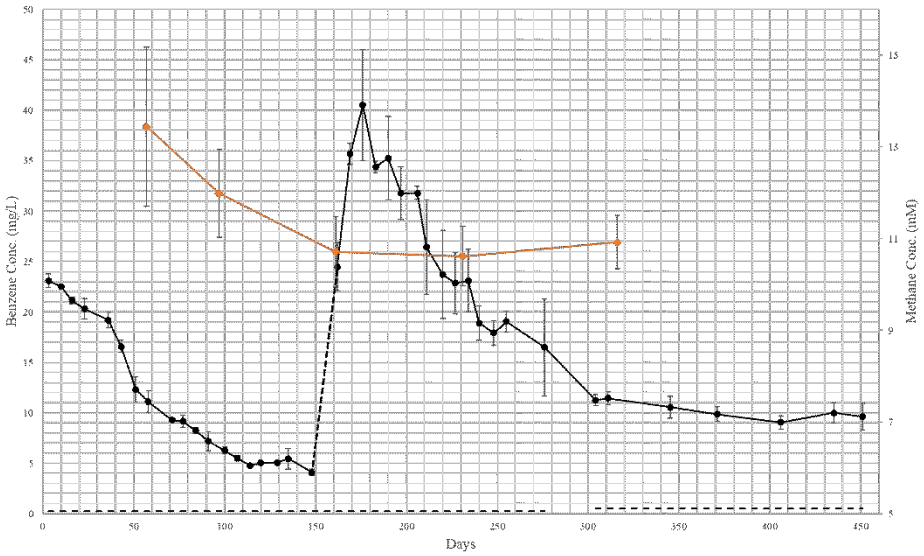

Figure C-11 – Salinity Experiment: Methanogenic, 1.0 g/L NaCl Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 451.

Salinity Experiment: Methanogenic 2.0 g/L NaCl

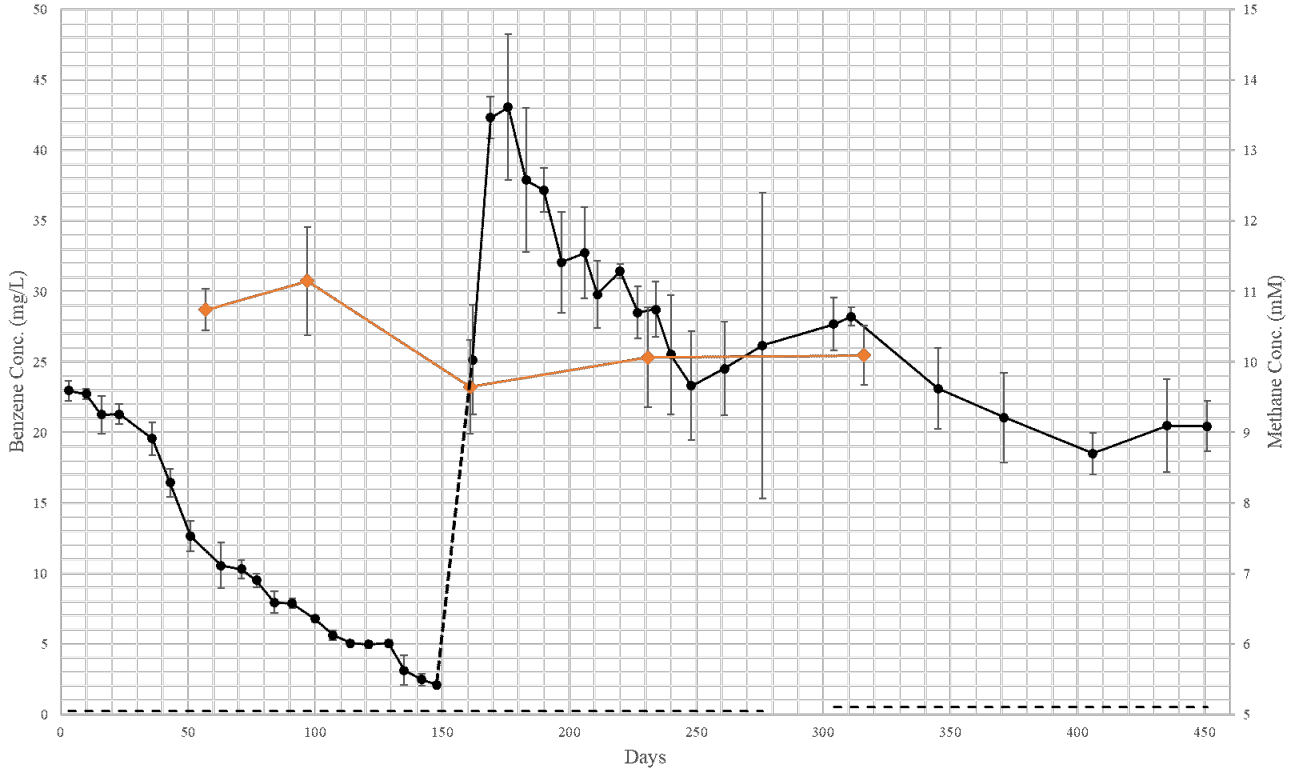

Figure C-12 – Salinity Experiment: Methanogenic, 2.0 g/L NaCl Overall Degradation: Methane content (◆) and benzene biodegradation (●). Vertical upwards black dotted lines represent benzene re-amendment. Horizontal black dashed line represents a lower detection limit of 0.25 mg/L with the GC-FID and 0.5 mg/L with the P+T GC-FID. Error bars represent one standard of deviation associated with experimental set up in triplicates. Instances where error bars do not appear visible indicates a small standard of deviation between triplicates. Day 0 to Day 451.