Biostimulatory Solutions for PHC Contaminated Sites: Effects of C: N: P Ratios on Degrader Prevalence and Potential Activity

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By

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Abstract

Optimal aerobic nitrogen (N) and phosphorus (P) concentrations for in-situ biostimulation of petroleum hydrocarbon contaminated sites have been extensively investigated. However, it has not been assessed if C: N: P ratios may differ for anaerobic soils and if lower C: N: P ratios effect the microbial community structure. In addition, there are limited studies that explore if degradation can recover after eutrophic conditions are introduced. Before we were able to make these assessments, we created a bench scale microcosm design that mimics field conditions. Most laboratory studies on biostimulation have proven results in the lab that do not translate to successes in the field. We believe this disconnect is due to the alteration of soil that occurs in microcosm experiments. In most laboratory studies, soils are dried, sieved, and then spiked. This process alters the soil surface area coming into contact with biostimulatory solution, soil structure, fractured flow, microbial population and habitat, and hydrocarbon adsorption and desorption. The objectives were to: 1) design a microcosm experiment to stimulate field conditions; 2) determine how C: N: P ratios effect the microbial community and how soil properties influence C: N: P ratios; and 3) determine if degradation rates recover after high nutrient conditions have been introduced. To mimic field conditions, soil cores were sub-sampled using a 2 x 1.5 (OD) inch slotted polyvinyl chloride (PVC) pipe and each subsample was placed into a sealed 125 mL amber jar with a biostimulatory solution. We demonstrated that C: N: P ratios were not selective for hydrocarbon ratios, but higher P in solution at low contaminant concentrations enhanced benzene degradation more than the other chemicals in the F1 fraction. We also demonstrated that original site conditions and the amount of S and P in solution was more influential on degradation rate than the C: N: P ratio. Lastly, we demonstrated that the microbial community and degradation success was influenced the most by pre-excising site and within site conditions. These results suggest that sites may behave very different even when the same nutrient amendment is applied based on pre-existing site factors.

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Dedication

I dedicate this dissertation to my late Grandmother, Joyce V. Moehlman. Thank you for always supporting my dreams no matter where they took me.

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1.0 INTRODUCTION

1.1 General Introduction

Due to the oil and gas industry, there are thousands of petroleum hydrocarbon (PHC) contaminated sites across Canada that require remediation (Canadian Council of Ministers of the Environment, 2008). Federated Cooperative Ltd. (CO-OP) estimated that within the next 20 years 700 CO-OP sites in Western Canada may need remediation at a cost of approximately $350 million. Hydrocarbon contaminated sites are caused by improper disposal techniques and spills at bulk transfer stations, retail outlets, and other processing locations through surficial spills, pipeline ruptures, and leaky underground storage tanks (Sihota and Mayer, 2011). The current strategies for PHC remediation involves excavation which is invasive, expensive, disrupts business, and reduces revenue (April et al., 2000).

Bioremediation, on the other hand, is an in-situ remediation strategy that is cost-effective, sustainable, and socially acceptable (April et al., 2000). For this process to occur, there must be ample nutrient supplies (Chandran and Das, 2011; Korda et al., 1997). Optimal nutrient concentration plays a significant role regarding in situ bioremediation because it promotes degradation efficiency and effectiveness. Nutrients can be added to a site through biostimulatory solutions. In biostimulatory solutions macronutrients such as nitrogen (N) and phosphorus (P) are needed in large quantities. Nitrogen and P are also the most limiting nutrients in soil systems, particularly in calcareous Saskatchewan (SK) soils.

Optimal N and P concentrations for aerobic petroleum hydrocarbon degradation has been extensively investigated. However, it has not been assessed how C: N: P ratios may vary in anaerobic systems and are linked to degrader prevalence and activity. This thesis focuses on identifying how C: N: P ratios are linked to PHC degradative prevalence and potential activity and how microbial changes affect chemical patterns of hydrocarbon degradation and selectivity for certain hydrocarbon fractions. The findings from this study will be used to improve amendment solutions for PHC remediation projects across Canada.

1.2 Overall Objectives and Hypotheses

1. Improve current microcosm designs to better simulate the conditions in a cold region PHC contaminated site for laboratory experiments.

2. Determine the optimal anaerobic C: N: P ratio and how it is linked to hydrocarbon degrader prevalence and potential activity.

a) H_0**:** As the anaerobic C: N: P ratio decreases (higher N and P concentrations), percent degradation will increase.

   Ha: As the anaerobic C: N: P ratio decreases, percent degradation will reach an optimal amount and then decrease.

b) H_0: There will be no differences in microbial community composition and population with increases in the C: N: P ratio.

   Ha: The microbial community composition and population will differ as the C: N: P ratio is increased.

3. Determine if C: N: P ratios are selective for certain hydrocarbon fractions.

a) H_0: Different C: N: P ratios will not have a significant effect on degradation for different hydrocarbon fractions.
   Ha: Different C: N: P ratios will significantly affect the rate of degradation for different hydrocarbon fractions.

4. Determine if the degradation rates will be able to recover after a site is put under eutrophic conditions (high nitrogen and phosphorus concentrations).

c) H_0: After a system is under eutrophic conditions degradation rates will recover.

   Ha: Degradation rates will not recover after eutrophic conditions are introduced.

1.3 Organization of Thesis

This thesis is organized in manuscript style. Chapters 1 and 2 are an Introduction and a Literature Review that provides an overview for the thesis as a whole and relevant background. The studies presented in Chapters 3 through 5 are research chapters that address one or more of the above objectives. In Chapter 3, a microcosm design was developed for cold region PHC contaminated sites that is then used in Chapters 4 and 5. Chapter 4 determined how low C: N: P ratios are linked to hydrocarbon degrader prevalence and potential activity and how if C: N: P ratios are selective for certain hydrocarbon fractions. Chapter 5 determined if degradation rates

could recover after being exposed to eutrophic conditions using C: N: P ratios used in Chapter 4. Eutrophic conditions are defined as nutrient abundance and accumulation within a body of water that supports sense growth of organisms which when they decompose depletes oxygen. In the context of this thesis, we use eutrophic or eutrophic conditions to describe an amendment solution that is very high in nitrogen and phosphorus, thus promoting abundant microbial growth. These chapters are followed by a synthesis and overall conclusions for the study as a whole and recommendations for future work (Chapter 6). Chapter 7 is a compiled list of References cited throughout the thesis and Chapter 8 is the Appendices.

2.0. LITERATURE REVIEW

2.1 Product Chemistry and Environmental Regulations

Petroleum products such as gasoline and diesel are released at retail outlets and bulk transfer stations and are composed mostly of carbon and hydrogen. Gasoline and diesel are refined crude oil products which makes them complex and variable mixtures. Gasoline is predominantly in the C_4-C_{12} range, with C referring to the equivalent number of straight- chain hydrocarbons (IARC 1989). The typical composition of gasoline by percent hydrocarbon volume is: 4-8% alkanes, 2-5% alkenes, 25-40% isoalkanes, 3-7% cycloalkanes, l-4% cycloalkenes, and 20-50% total aromatics (0.5-2.5% benzene) (IARC, 1989). Diesel is predominantly in the C_{12}-C_{20} range and is, by volume, approximately 64-66% saturated hydrocarbons (linear and cycle chains), 30-35% total aromatics, and 1-4% alkenes (unsaturated hydrocarbons) (Martinez, 2016; ATSDR, 1995; IARC, 1989). Due to the variability of refined products, environmental regulations are based on hydrocarbon fractions. Hydrocarbon fractions refer to a defined range of elution times on a gas chromatography with each elution period corresponding to an average number of carbons in a hydrocarbon (Table 2.1). For each hydrocarbon fraction there are environmental soil and groundwater regulations for different soil types and land uses (Tables 2.2, 2.3 and 2.4).

Table 2.1: Petroleum hydrocarbon fractions and sub-fractions (adapted from CCME, 2008).

Fraction Number	Equivalent carbon number	Sub – fractions
Fraction 1	C_6 to C_{10}†	Aromatics: $C_{>7}$-C_8, C_8-C_{10} Aliphatics: C_6-C_8, $C_{>8}$-C_{10}
Fraction 2	$C_{>10}$ to C_{16}	Aromatics: C_{10}-C_{12}, $C_{>12}$-C_{16} Aliphatics: C_{10}-C_{12}, $C_{>12}$-C_{16}
Fraction 3	$C_{>16}$ to C_{34}	Aromatics: $C_{>16}$-C_{21}, $C_{>21}$-C_{34} Aliphatics: $C_{>16}$-C_{21}, $C_{>21}$-C_{34}
Fraction 4	$C_{>34}$ to C_{50}	Aromatics: $C_{>34}$ Aliphatics: $C_{>34}$

†C refers to the number of equivalent straight chain hydrocarbons.

Table 2.2. Saskatchewan criteria (mg kg^{-1}) for BTEX and F1 in surface (0-3 m deep) soils for different land uses (adapted from SEQS, 2016).

Soil Texture	Land Use	Benzene	Toluene	Ethyl-benzene	Xylene	F1 (C6-C10†)
Fine-grained	Agriculture	0.078	22.0	8.50	150	210
	Residential	0.078	22.0	8.50	150	210
	Commercial	0.12	82.0	13.0	560	320
	Industrial	0.12	NV	120.	NA	320
Coarse-grained	Agriculture	0.078	22.0	8.50	150	210
	Residential	0.078	22.0	8.50	150	210
	Commercial	0.12	82.0	13.0	560	320
	Industrial	0.12	NV	120	NA	320

†C refers to the number of equivalent straight chain hydrocarbons.

‡ NV- No value

§ NA- Mot applicable as value exceeds 1,000,000 mg/kg

Table 2.3. Saskatchewan potable and non-potable groundwater criteria (mg kg^{-1}) for BTEX (benzene, toluene, ethyl-benzene, and xylene) and F1 (adapted from SEQS, 2016)

Criteria	Benzene	Toluene	Ethylbenzene	Xylene	F1 (C6-C10†)
Potable	0.005‡	0.06	0.140	0.09	2.20
Non-Potable	900	258	76.0	9.00	NV

† C refers to the number of equivalent straight- chain hydrocarbons.

‡ Criteria does not differ based on land use and soil media.

§ NV- No value

2.2 Soil Microcosms

Prior to implementing new remediation strategies on sites, it is essential to conduct treatability studies to assess the performance and design (Driver et al., 2017) These treatability studies usually occur in laboratory settings at bench scale, where one can closely control and monitor environmental conditions (Driver et al., 2017). The most common laboratory bench scale studies use batch microcosms and continuous flow columns (Driver et al., 2017). Batch microcosms use glass bottles crushed soil and amendment where continuous flow column studies use glass cylinders with crushed soil inside (Fig. 2.1) (Driver et al., 2017). Amendment solution is constantly pumped through the cylinders and there are sampling ports at the inlet and outlet (Driver et al., 2017). However, these microcosm designs fail to mimic site conditions such as soil heterogeneity (Driver et al., 2017).

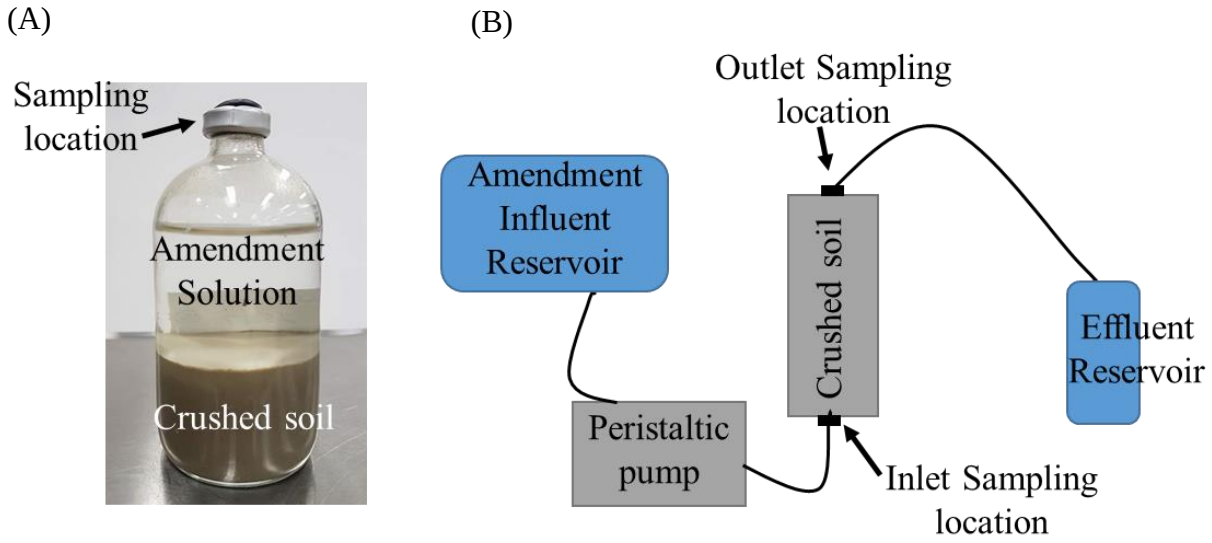


Fig. 2.1. Experimental set up of batch bottle (A) and continuous flow column studies (B) (adapted from Driver et al., 2017).

Most microcosm designs include variations of soil that is dried, sieved, and then spiked (Chaineau et al., 1995; Miles and Doucette, 2001; Shahi et al., 2016; Gao et al., 2014, Qin et al., 2013; Reddy et al., 2011). This process alters the soil surface area coming into contact with biostimulatory solution, the soil structure, the flow of solution through fractured clay minerals, heterogeneity of the contaminants (for example, the contaminants can be found as solids, liquid, gases, free or tightly bound to soil, minerals, or organic matter) (Balbla et al., 1998), hydrocarbon concentration distribution (presence of "hot spots"), hydrocarbon adsorption and desorption properties, and microbial population and habitat. To better replicate field conditions, intact soil columns can be used. This allows soil laboratory testing to be as close as possible to actual field conditions (Lewis and Sjostrom, 2010). Creating bench scale lab microcosms to mimic field conditions can reduce the risk of remediation failure at the field scale.

2.3 Nutrients and Hydrocarbon Degradation

Nitrogen and P are important macronutrients in biostimulatory solutions since they are required for metabolic activities and stimulate microorganism growth and reproduction (Cunningham et al., 2000; Chandran and Das, 2011). C: N and C: P ratios can be modelled off microbial C: N and C: P requirements (Xiong et al., 2012). Based on microbial needs, the

optimal aerobic C: N: P ratio is 100:10:1 (Xiong et al., 2012; Chaineau et al., 2004; Mill and Frankenberger, 1994). However, in literature, the optimal range varies greatly, for example the U.S. Environmental Protection Agency recommends ranges for C: N of 10:1-100:1 (US EPA, 1995). Additionally, the optimal ratio of 100:10:1 is based solely off microbial needs for biomass creation and does not consider additional N that may be needed in anaerobic systems where N is often the preferred electron acceptor. It is possible that more fixed N may be required for anaerobic systems for nitrate-reducing microorganisms (Eriksson et al., 2003). In Eriksson et al. (2003) they found that anaerobic degradation was heavily linked to denitrification. According to literature, highest denitrification rates occur from a C: N range of 1 to 4 (Zhang et al., 2016; Grebliunas and Perry, 2016). In Zhang et al. (2016) they found that the most denitrification took place at a C: N ratio of 1 and they found the highest concentration of nitrate reducers at a C: N of 1.5.

There are mixed reviews on the benefits of N in biostimulatory solutions (Walworth et al., 2007). Many studies indicate positive effects on degradation with increased N, but others indicate no effect or even negative effects with excess N (Rasiah et al., 1991; Ferguson et al., 2003; Watts et al., 1982; Brown et al., 1983; Genouw et al., 1994; Zhou and Crawford, 1995; Braddock et al, 1997, 1999; Walworth et al., 1997, 2007; Mohn et al., 2001). Research has shown that excess N increases lag phases and inhibits aromatic degradation, microbial respiration, and total hydrocarbon degradation (Walworth et al., 2007; Ferguson et al., 2003; Fayad and Overton, 1995).

This inhibitory effect is most likely due to osmotic stress (Walworth et al., 2007). Many N fertilizers have a large salt concentration that lowers the osmotic potential, inhibiting microbial activity and reducing the overall population (Walworth et al., 2007; Braddock et al., 1997). Nitrogen fertilizers can be over applied during hydrocarbon remediation since C: N ratios are based on the amount of N required to degrade the quantity of hydrocarbons on site (Walecka-Hutchison and Walworth, 2006; Walworth et al., 1997, 2007). The use of C: N ratios based solely on hydrocarbon concentration may lead to over fertilization and increased osmotic stress causing decreased degradation (Walecka-Hutchison and Walworth, 2006; Walworth et al., 1997, 2007).

The contribution of N fertilizer to osmotic potential can be estimated by calculating the N concentration in soil solution (N_{H2O}) (Walworth et al., 1997, 2007). This calculation is derived by the amount of N added and the soil moisture content (Walworth et al., 1997, 2007):

$$(\text{mg N})/(\text{kg soil}) \div (\text{kg } H_2O)/(\text{kg soil}) = (\text{mg N})/(\text{kg } H_2O) = N_{H2O}$$

Using N_{H2O}, the maximum N concentration is approximately 2000 mg N kg^{-1} H_2O (Walworth et al., 1997, 2007; Walecka-Hutchison and Walworth, 2006). Keeping the N in the C: N ratio below this amount as well as considering hydrocarbon concentration, osmotic potential, soil moisture content, and soil pore water nitrogen, we can optimize degradation and reduce the potential of osmotic stress.

There have also been inconsistent findings on the importance of P in biostimulatory solutions. Some studies show that adding P in addition to N can degrade up to 90% of diesel is soil (Gallego et al., 2001), where other studies show either no effect or lower degradation rates (Seklemova et al., 2001; Bento et al., 2005). In a study comparing biostimulation, natural attenuation, and bioaugmentation, adding N and P lowered the diesel degradation rate (Bento et al., 2005). The same study also examined N and P separately and found P addition had no effect on diesel degradation. Little degradation with P addition may be due to low nutrient bioavailability (Mills and Frankenberger, 1994).

Only a small fraction of total P is bioavailable for microbial uptake (Richardson and Simpson, 2011). Phosphorus addition is often difficult since P tends to bind to soil particles, form mineral complexes, adsorb on mineral surfaces, and form new secondary minerals. Phosphorus in soil exists mainly in inorganic fractions but is often unavailable since it adsorbs to soil mineral surfaces and precipitates into calcium, iron, or aluminum phosphates (Richardson and Simpson, 2011; Mills and Frankenberger, 1994). Organic phosphates are more mobile in soil and can be used by microorganisms; however, it must be mineralized to be available for microbes (Mills and Frankenberger, 1994; Richardson and Simpson, 2011).

Inorganic phosphate fixation is a particular problem in SK due to the high pH, calcium, and magnesium found in the soil. Soils with these conditions are particularly susceptible to P precipitating into secondary minerals such as calcium and magnesium phosphates and absorbing onto the surfaces of clay minerals and calcium carbonate ($CaCO_3$) (Havlin et al., 2014). The

mineral type and amount present in the soil alters the maximum amount of P in solution before it precipitates (Havlin et al., 2014). Absorption and precipitation reactions occur to some extent after fertilizer addition (Havlin et al., 2014). Adsorption will occur when the adsorption capacity is not saturated with P (Havlin et al., 2014). After maximum P adoption is reached, available P concentrations will increase (Havlin et al., 2014). However, with too much P, precipitation will occur as the solution P exceeds a specific mineral solubility (Havlin et al., 2014). Thus, in biostimulatory solutions, it is optimal to add enough P to reach adsorption capacity, but not too much that you are going to exceed your mineral solubility. Overall, the type of P added into biostimulatory solutions, as well as the type and amount of mineral present on site will largely affect microbial P bioavailability and, therefore, degradation.

There is evidence that different nutrient concentrations affect the degradation of different hydrocarbon fractions (Braddock et al., 1999; Chaineau et al., 2004). In multiple studies, increased fertilization has been reported to increase, inhibit, or have no effect on the mineralization of different hydrocarbon fractions (Braddock et al., 1999). Chaineau et al. (2004) concluded that different nutrient levels are needed for the optimum degradation of aliphatic and aromatic hydrocarbons. In a different study, they found increased nutrient concentrations were selective for linear alkanes compared to branched alkanes but had no effect on aromatic compounds (Braddock et al., 1999). There are few studies regarding if hydrocarbon selectivity, caused from different nutrient levels, is linked to changes in chemical processes, microbial community, and microbial functions.

2.4 Microbial Composition and Recovery

Mature hydrocarbon contaminated sites are dominated by microbes that are able to use and survive hydrocarbon toxicity (Zucchi et al., 2003). As a site is remediated through biostimulation and the hydrocarbon concentration decreases, other microbial populations may increase which enhances microbial diversity (Zucchi et al., 2003). There have been multiple studies showing increased microbial populations with higher nutrient concentrations through analyzing colony forming units and microbial respiration rates (Chaineau et al., 2005). Other studies showed decreased microbial populations and density with over fertilization and high C: N ratios (Walecka-Hutchison and Walworth, 2007). This suggests that fertilization only increases microbial growth and population to a certain point and then inhibits activity, therefore inhibiting

degradation. However, increases in colony forming units and activity cannot imply increased hydrocarbon degradation since it is unknown if the new microbial growth has the functional genes required for hydrocarbon degradation.

Microbial community composition (richness, diversity, and relative abundance), resilience (the rate at which microbial community composition return to its original composition after being disturbed), and functional pathways after disturbances, such as fertilization, have been assessed (Allison and Martiny, 2008). Microbial community composition is sensitive to disturbances; in 84% of studies, fertilization had a significant effect on microbial community composition (Allison and Martiny, 2008). Even though microbial community composition is likely to change with disturbances, such as fertilizer additions, soils are assumed to quickly return to the original microbial community composition since microbes have fast growth rates and a high degree of physiological flexibility (Allison and Martiny, 2008). However, in multiple fertilization studies, microbial community composition was not able to return to its original composition even years after the initial disturbance (Allison and Martiny, 2008). Disturbances often affect soil ecosystem processes and the rate they occur, thus, influencing the presence of functional genes (Allison and Martiny, 2008).

Overall, one could hypothesis that after a soil is introduced to high nutrient conditions that the microbial community composition will change and will not recover over time to the original microbial community composition. Therefore, after soil eutrophication, the microbial community may not return to the original composition, altering ecosystem functions and hydrocarbon degradation.

2.5 Tracking Hydrocarbon Degradation

Chemical parameters used to track hydrocarbon degradation overtime include tracking hydrocarbon and nutrient concentration. Tracking nutrients such as N, P, sulfur (S), and iron (Fe) over time can help determine what chemical and biological processes may be occurring since these nutrients are required for metabolic activities and stimulate microorganism growth and reproduction and/ or are commonly used as electron acceptors (Cunningham et al., 2000; Chandran and Das, 2011).

Biological parameters are measured by recording microbial presence and activity in soil. 16S rDNA analyses can be used to quantify and determine the types of microbes in a sample (Kuntze et al., 2011). Hydrocarbons are naturally found in soil; hence, there are a wide array of microorganisms that have evolved to degrade hydrocarbons (Devine, 2013; Robertson, 2007; Yergeay et al., 2009). Microorganisms can degrade hydrocarbons aerobically and anaerobically. Anaerobic microbial communities can degrade hydrocarbons under Mn (IV)-reducing, nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions (Edwards and Grbić-Galić, 1992, 1994; Lovley and Woodward, 1996; Lovley, 1997; Burland and Edwards, 1999). Microbial communities can also degrade aromatic and alkane petroleum hydrocarbons (Arvanitis et al., 2008; Fuentes et al., 2014; Das and Chandran, 2011). However, different communities are responsible for degradation of aromatic versus alkane compounds. For example, *Pseudomonas spp., Flavobacterium spp. Alcaligenes spp., and Sphingomonas spp.,* are common aromatic hydrocarbons degraders (Das and Chandran, 2011). The most common Phyla found to degrade aromatic hydrocarbons are Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes and Proteobacteria, Rhodococcus, Nocardia (Edwards, 2003; Robertson et al., 2007; Yergeau et al., 2009; Weelink et al., 2010; Zhang et al., 2012; van der Zaan et al., 2012; Kleinsteuber et al., 2012; Herbst et al., 2013; Fathepure, 2014; Luo et al., 2014, 2016; Zhang and Lo, 2015; Sheng et al., 2015; Quadros et al., 2016; Salanitro, 2000). On the other hand, common alkane degraders may include *Acinetobacter spp., Pseudomonas spp., Streptomyces spp., Arthrobacter spp., and Bacillus spp.* (Arvanitis et al., 2008; Fuentes et al., 2014). Many studies have found increases in hydrocarbon degraders after nutrient addition (Shahi et al, 2016). For example, TingTing et al. (2018) found increased abundance in the phyla Bacteroidetes, Chlorobi, Nitrospirae, OP11, Spirochaetes, Acidobacteria and Firmicute in soil and increased abundance the in orders Clostridiales, Rhizobiales, Rhodospirillales, Rhodocyclales, Desulfobacterales, Desulfovibrionales, and Desulfuromonadales in groundwater. All of these taxa are known to contain hydrocarbon degraders.

3.0 A NEW MICROCOSM DESIGN FOR TREATABILITY ASSESSMENT IN COLD REGION PETROLEUM HYDROCARBON IMPACTED CLAYEY SITES

3.1 Preface

Many laboratory studies use microcosm designs that alter soil and hydrocarbon properties. To make our study as field relevant as possible and to accurately determine how C: N: P ratios effect microbial communities, degradation potential, and hydrocarbon selectivity, we must first develop a microcosm design that encompasses soil and hydrocarbon properties, especially those of cold region clayey sites that are seen in Western Canada. In this study, we created a microcosm design that better stimulates field conditions, and also allow us to monitor contaminant concentrations during the experiment.

3.2 Abstract

Numerous studies use soil microcosms to investigate new methods and techniques that improve in-situ remediation of petroleum hydrocarbon (PHC) impacted sites. However, many microcosm designs that have reported significant findings in the laboratory often produce different results on site, meaning there is a poor extrapolation from controlled studies to real world settings. A frequently-used step in microcosm creation is to mix, sieve, and re-spike the soil prior to starting the experiments. This alters soil characteristics such as surface area, structure, fractured flow, and the active microbial communities present as well as changing the soil's adsorption and desorption properties. We created a microcosm design that better suits the treatability assessment of cold region clayey soils. The soil was kept intact within the microcosm to better represent the soils' structure, PHC movement and microbial populations. Due to the heterogeneous nature of PHC contaminants in clayey soils, BTEX and F1 concentrations varied greatly within a small area. We demonstrated that a linear relationship exists between the concentration of BTEX/F1 present in PHC contaminated soil in the microcosm and bio-stimulatory solution. The objective of this experiment was to improve current microcosm designs to better convey the conditions of a cold region PHC contaminated site for laboratory experiments. Overall, we were able to design an intact core microcosm design that more accurately stimulates contaminate properties such as contaminate heterogeneity.

3.3 Introduction

Numerous studies use soil microcosms to investigate new methods and techniques to improve in-situ remediation of petroleum hydrocarbon (PHC) impacted sites. In many microcosm studies, researchers are unable to use intact column studies that use already contaminated soil because the soil contamination is too heterogeneous. Researchers need to homogenize the soil to get reprehensive samples and clearer results. However, many laboratory studies on biostimulation have proven results in the lab but were not as successful in the field. We believe this disconnect is due to soil alterations that occur in microcosm experiments. Most microcosm designs include variations of soil that is dried, sieved, and then spiked (Chaineau et al., 1995; Miles and Doucette, 2001; Shahi et al., 2016; Gao et al., 2014, Qin et al., 2013; Reddy et al., 2011). This process alters the soil surface area coming into contact with biostimulatory solution, the soil structure, the flow of solution through fractured clay minerals, heterogeneity of the contaminants (for example, the contaminants can be found as solids, liquid, gases, free or tightly bound to soil, minerals, or organic matter) (Balbla et al., 1998), hydrocarbon concentration distribution (presence of "hot spots"), hydrocarbon adsorption and desorption properties, and microbial communities and habitat.

Cold region clayey sites that are common in Western Canada (Alberta, Saskatchewan, and Manitoba) are particularly difficult to stimulate. These western sites are experience freeze thaw events, preferential flow paths, and have natural soil heterogeneity. These factors cause variability of hydrocarbon contamination throughout the soil profile (Berkowitz, 2002; Geller et al., 2000; Aislabie et al., 2004) that cannot be achieved in microcosm designs where soils are dried, sieved, and then spiked. Soils that undergo these modifications will have higher leaching rate and fewer macropores and preferential flow paths than what is seen in the field (Lewis and Sjostrom, 2010). To better replicate field conditions, intact soil columns can be used. This allows soil laboratory testing to more accurately reflect field soil conditions (Lewis and Sjostrom, 2010). Soil physical characteristics such as structure are only slightly altered with intact columns compared to other microcosm designs (Lewis and Sjostrom, 2010). These soil characteristics are essential when studying biodegradation since soil structures, such as fractured flow and macropores, significantly contribute to contaminant transport (Lewis and Sjostrom, 2010). In addition to contaminant transport, contaminant bioavailability including adsorption and

desorption properties are also altered in many microcosm designs during the sieving and re-spiking process (Liu et al., 2007; Hatzinger and Alexander, 1995; Ball and Roberts, 1991; Gonga et al., 1998).

When soils are dried and re-spiked with hydrophobic contaminants such as hydrocarbons, there is a weaker chemical bond and a faster release rate then contaminated site soils (Liu et al., 2007; Hatzinger and Alexander, 1995). The longer soil is in contact with contaminants, the slower the desorption rate. The contaminant becomes more bound to the soil, making the contaminant less bioavailable and more resistant to extraction (Liu et al., 2007; Hatzinger and Alexander, 1995). Multiple studies have found a negative correlation between biodegradation rates versus aging hydrophobic contaminates in soil (Hatzinger and Alexander, 1995; Ball and Roberts 1991; Gonga et al., 1998). Hence, when soils are re-spiked, remediation rates are significantly higher than matured contaminated site soil (Liu et al., 2007, Hatzinger and Alexander, 1995). In addition to the re-spiking process, sieving the soil can also increase remediation by decreasing the soil particle size (Liu et al., 2007). A decrease in soil particle size can reduce overall biodegradation inside a soil particle and decrease chemical removal rates because, the larger the soil particle the long the diffusion distance and the longer time it takes for contaminates to desorb (Liu et al., 2007). Mixing and sieving the soil can also cause hydrocarbon contamination to be more homogenous throughout the soil. For many sites, hydrocarbon contamination is variable, existing in high and low concentration pockets (Balbla et al., 1998). Overall, both the re-spiking and sieving process when making soil microcosms, can alter chemical desorption and bioavailability which in turn increases biodegradation rates.

Biodegradation rates can also be altered by influencing microbial population and habitat. Drying soil changes microbiological soil characteristics. Pesaro et al. (2004) found drying soil could decrease microbial biomass by 51% and delay degradation by multiple factors (1.5 to 5.4-fold). Sieving soil decreases particle size and increases surface area which can increase microbial population density and habitat. In addition, sieving and mixing will also homogenize the hydrocarbon contamination in the soil where naturally there may be high concentrations that are toxic or inhibit microorganisms, and low hydrocarbon concentrations that are not high enough to support microbial activities and promote hydrocarbon-degrading functional genes (Balbla et al., 1998). Microbial communities and functional gene prevalence can be negatively affected by

using microcosm designs involving spiking uncontaminated soil (Bakermans and Madsen, 2002; Hendrickx et al., 2005; Hendrickx et al., 2006; Guo et al., 1997; Hosada et al., 2005; Sutton et al., 2012). Contaminated site soil will have different microbial communities that already have established functional genes for hydrocarbon degradation and microbial populations that are known hydrocarbon degraders (Bakermans and Madsen, 2002; Hendrickx et al., 2005; Hendrickx et al., 2006; Guo et al., 1997; Hosada et al., 2005; Sutton et al., 2012).

Other investigators have long recognized the limitations of drying and spiking soils, however they have used spiked microcosms due to contaminant heterogeneity in the field, and the difficulty in assessing soil concentrations before and after a putative biostimulatory treatment. We hypothesized that we could create a microcosm design that better stimulates field conditions and would allow us to monitor contaminant concentrations during the experiment. Initially, we tested if F1 and BTEX degradation could be assessed by sampling the exterior portion of the borehole (soil not being used for the microcosm) for the initial concentration and sampling the interior soil (within the microcosm) for the final concentration. A second study was completed to assess limitations in hydraulic conductivity due to the high clay content and to determine how well biostimulation solutions penetrate the microcosm. Due to high spatial variability in contamination, a third experiment was completed to determine if the initial concentration within the microcosm could be correlated with the concentration in the biostimulatory solution. To follow up this study, two control experiments using this design, were completed to ensure F1 and BTEX losses were not due to unknown biotic and abiotic factors.

3.4 Materials and Methods

3.4.1 Sample Collection and Microcosm Creation

Soil samples were collected from multiple bulk transfer and gasoline stations with known spill and leak history within Saskatoon, Raymore, Brock, Feudal, and Davidson, Saskatchewan, Canada. During Phase II Environmental Site Assessment (ESA), consultants took duplicate boreholes within 0.5 meters (m) of known contaminated locations. Soil boreholes were collected from the surface to a maximum of 7.5 m below ground level using a direct push core drill with a Geoprobe® 7822DT (Salina, USA). The duplicate boreholes were collected with 1.5 m acrylic tube segments and were sealed with paraffin wax on site. Cores were kept on ice during field

sampling and then taken back to the University of Saskatchewan and stored at −20°C prior to microcosm creation.

To create the microcosms, borehole segments from 1.5-3 m below ground were cut open using a core cutter. The 1.5- 3 m borehole segments were chosen because across all of the sites there was contamination present at this depth range. Microcosms were filled with intact soil avoiding the outer smeared layer, by taking a 3-inch section from the borehole and using a PVC (2 x 1.25 (OD) inch, 1/16-inch slots) pipe as a coring device. The amount of soil inside the microcosm was weighed and two nylon mesh coverings (790 µm mesh opening, 54% open area, 24 mesh count) were placed at both ends of the microcosm. A nylon zip-tie was added to keep the soil intact inside the microcosm. Microcosms were then placed into a sealed 125 mL amber jar with approximately 70 mL of biostimulatory solution such that the soil was raised to 100% water holding capacity to ensure complete saturation and oxygen limiting conditions. An acceptable air pocket was the size of a nickel or smaller and the solution volume added to each microcosm was recorded.

All materials used to create the microcosms were acid washed (5% HCl) and rinsed with Milli-Q water to avoid nutrient contamination. Materials were also solvent rinsed using methanol and acetone to prevent hydrocarbon contamination. To avoid microbial contamination, all materials were autoclaved using a gravity 20 cycle (20 minutes at 121 °C) and the biostimulatory solutions were sterilized using a UV water purifier to mimic on site sterilization. To UV sterilize the solutions a SteriPEN Classic 3 was used within a sterile biosafety cabinet (sterilized with a 10% bleach and 10% ethanol solution). The solutions were re-sterilized when they were opened to the outer environment. The microcosms were constructed either in a sterile fume hood or in an anaerobic chamber. Microcosms and amendment solutions were stored in a 10ºC fridge without light to stimulate environmental soil conditions.

3.4.2 BTEX and F1 Concentrations within Heterogeneous Soil Microcosms

Since heterogeneous soil cores were used, there was a need to ensure degradation over time could be measured accurately by proving that the soil being sampled for the initial and final concentrations had similar starting concentrations. Soil samples for BTEX and F1 were taken during microcosm creation using the soil on the outer part of the borehole not being used for the

microcosm avoiding the outer smeared layer (initial concentration) and within the soil microcosm (final concentration). To determine if these locations had similar starting PHC concentrations, the two sampling locations were sampled at the same time and compared. Samples were taken using a 5 g plug (~3.175 x 1.905 cm) Terra Core™ soil sampler (Brewer et al., 2015). Samples were stored in sealed Teflon lined tubes with 10 mL of methanol.

3.4.3 Color Tracer Experiment

Due to the high clay content in the soils, a color tracer experiment was set up to test hydraulic conductivity limitations and if the soil was being saturated from the solution. Microcosms with varying soil textures, made from previously collected duplicate boreholes were constructed. Soil textures were split up into three groups including heavy clay, clay, and clay loam. Each texture group had two replicates and one control. All microcosms were filled with IRC solution (0.24 mM HNO_3 [3.4 mg $L^{-1}N$], 0.3 mM sodium tripolyphosphate ($Na_5O_{10}P_3$) [3.1 mg L^{-1} P], 0.24 mM Fe (III) NH4-citrate [13 mg L^{-1} Fe (III)], and 22 mM magnesium sulfate heptahydrate ($MgSO_4$ *$7H_2O$) [700 mg L^{-1} S] (made with tap water)) with 4 g L^{-1} of Brilliant

Blue For Coloring Food (Blue FCF) was added to the solution as a dye tracer (Flury and Fluhler, 1994, 1995). Controls had the IRC solution without the Brilliant Blue FCF. The microcosms were incubated for four weeks and the solution was replaced weekly by placing the microcosms into new jars with fresh dye and treatment solution. After four weeks, soil microcosms were cut into subsections, photographed, and visually inspected for color dye.

3.4.4 Analytical Methods

For BTEX and F1 analysis, soil samples were extracted using a 2:1 soil methanol ratio using CCME Guidelines (CCME, 2008). Approximately 5 grams of a soil sample was added to 10 mL of methanol in a Teflon-lined vial (CCME, 2008). Soil and methanol samples were shaken for 1 hour at 100 revolutions per minute and then the methanol was separated from the soil using a centrifuge at 1000 revolutions per minute for six minutes (CCME, 2008). One mL of the separated methanol was then added to a GC vial containing 9 mL of distilled deionized water for determination of volatile organics (CCME, 2008). To avoid volatile losses, the methanol extraction was carried out within 48 hours or sample collection and all methanol extracts were run for volatile organics within 7 days (CCME, 2008). All samples were run for head-space

analysis with gas chromatograph-flame ionization detection (GC-FID) on the Bruker Scion 436-GC (CCME, 2008). The Scion 436-GC has an FID detector and an HP-5MSUI column. The temperature ramp starts at 60°C, increase to 110°C at 15°C per minute, then increases to 120°C at 5° per minute, and then increases to 150°C at 60°C per minute and is held at this final temperature for 4.17 minutes. Each sample was replicated three times and run on the GC-FID. For the solution, BTEX and F1 was also tested on the GC-FID Bruker Scion 436-GC using the same detector, column, and temperature ramp. Samples were run by using 9 mL of solution and 1 mL of methanol. Each sample was replicated three times.

For quality control and assurance on the GC-FID blanks, spikes, external standards, and duplicates of each sample were included (CCME, 2008). The blanks included a method blank and a reagent blank. The method blank included Ottawa sand (uncontaminated soil) or the treatment solution (dependent of soil or solution analysis) and the reagent blank was the final solvent of the extraction (methanol for BTEX and F1). The two types of spikes used were matrix and blank spikes. The blank spike (performance sample) included Ottawa sand or a treatment solution and petroleum standards. The matrix spike included sample and petroleum. For all the spikes we used 0.5 mL of 200 mg L-1 commercial standard for F1, which included hexane, BTEX, and decane. The recovery for the spikes was from 70 to 130%. External standards were also used and included the final solvent of extraction and petroleum standard. The standards for BTEX and F1 contained nC6, nC10 and toluene (CCME, 2008). Toluene was used as a calibration standard (CCME, 2008). All area counts are integrated from the beginning of the nC6 peak to the apex of the nC10 peak to give F1 (CCME, 2008). All Chemical standards were purchased from Sigma-Aldrich.

3.4.5 Soil and Amendment Solution Correlation

From previous experiments, it was noted that hydrocarbons could vary by multiple degrees of magnitude within a small area, making it difficult to have reliable starting concentrations within microcosms. It was also noted that due to low hydraulic conductivity, that amendment solutions were not able to completely saturate the microcosm. With these limitations in mind, the microcosm design was slightly altered. Instead of sampling the soils during microcosm creation to determine the initial concentration within the soil, the amendment solution was analyzed for BTEX (benzene, toluene, ethyl-benzene, and xylene) and F1 (C6-C10)

after a one hour shaking period and weekly. In addition, all soil microcosms were pierced with needles to increase soil saturation. For all sites, microcosms were made as previously described in Section 3.2.3; however, after the soil was added to the microcosm, 48 (1.6 mm x 40 mm) holes were added. These holes were added by piercing the microcosm with a 16-gauge needle on the top and bottom of the soil microcosm where the area was divided into four quadrants. In each quadrant, the soil within the microcosm was pierced six times. After this step, soil microcosms were weighed, sterile IRC solution was added, and the microcosms were shaken for 60 minutes on a reciprocating shaker at 170 strokes per minute. Following the allotted time, the stale amendment was analyzed for BTEX and F1 and the soil microcosms were destructively sampled for BTEX and F1 after homogenizing the soil. The solution and soil results were then compared to determine if there was a correlation between the concentration within the stale amendment solution and the starting soil concentration.

To ensure hydrocarbon loss was based solely on microbial consumption in the soil, two quality control experiments were conducted. The purpose of the first quality control experiment was to ensure minimal degradation with no additional nutrients added and there was no washing effect from replacing the treatment solution weekly. The experiment was carried out using UV-sterilized tap water and distilled de-ionized water (DDI). Two treatments were used, sterile tap water and sterile DDI water, with five replicates each. Microcosms were constructed from a single borehole core using the methods as previously described in Section 3.2.1 in the anaerobic chamber. The experiment was carried out for four weeks during which, the stale amendment solution was assessed for BTEX and F1 concentrations each week and the stale solution was replaced with new solution. Microcosms were destructively sampled after the four-week incubation period for BTEX and F1 concentrations.

The purpose of the second quality control experiment was to evaluate if there were hydrocarbon losses from any abiotic factors. All soils were sterilized, after being constructed into a microcosm using gamma radiation. The gamma radiator used emits 2.40 GY min^{-1} with a Cobalt 60 source. Soils were irradiated for 21 days (70 KGY) (Aparecida and Aquino, 2012; Bachan et al., 2012; McNamara et al., 2003). UV sterilized DDI water was added to all microcosms in an anaerobic chamber. The soil microcosms were all constructed from the same borehole using the methods as previously described in Section 3.2.4. The microcosms were

incubated for four weeks and the solutions were replaced each week within the sterile anaerobic chamber. Stale solutions were sampled for BTEX and F1. After four weeks the soil microcosms were destructively sampled for soil BTEX and F1.

3.4.6 Statistical Techniques

Deming regression was used to determine if there was a link between the initial soil concentration and the concentration in solution. A deming regression differs from a linear regression because a deming regression accounts for errors in observations in both the x- and y-axis while a linear regression only allows the Y variable to be measured with error (Abdi and Williams, 2010). The standard errors of the regression coefficients and predicted values are calculated using the jackknife leave-one-out method (Abdi and Williams, 2010). This method involves leaving out each observation systematically from the data set and calculating the estimate and then averaging the calculations (Abdi and Williams, 2010). A weighted deming regression, using weights equal to the reciprocal of the square of the reference value, was used due to the heteroscedasticity within the data set (NCSS Statistical Software).

Statistical analyses were performed using R and graphs were made using R or SigmaPlot 10.0 (R Core Team, 2016). To improve linearity, all site data for soil and amendment solution data was log plus one transformed. Assumptions including: constancy of variance, assumption of normality of errors, and standardized residuals as a function of leverage were checked. A one-way analysis of variance (ANOVA) determined if there were any differences between weeks for PHC concentration in the control experiments. All tests were declared significant at $p < 0.05$.

3.5 Results

3.5.1 BTEX and F1 Concentrations within Heterogeneous Soil Microcosms

The exterior and interior BTEX and F1 concentrations for the soil microcosms varied greatly and there were no apparent trends (Table 3.1). Since the samples were tested at the same time and represent the same borehole location, the concentration should be similar. For example, microcosms 1-2 and 6-9 internal concentrations were two times greater than the exterior concentrations (Table 3.1). On the other hand, samples 11-14 had exterior concentrations that were two times greater than the internal concentration (Table 3.1). There were two samples (3

and 10) where the difference in initial and final concentration varied by an order of magnitude (Table 3.1).

Table 3.1. Exterior and interior BTEX and F1 concentrations.

Microcosm number	Soil Description[§]	Average[†] C6-C10 (mg kg^{-1}) Exterior	Average[†] C6-C10 (mg kg^{-1}) Interior
1	Clay	1700 (712[‡])	2800 (633)
2	Clay	770 (73)	2000 (858)
3	Clay	120 (4.8)	1800 (2039)
4	Clay	380 (117)	1500 (309)
5	Clay	2500 (365)	2100 (823)
6	Clay	690 (191)	1500 (1318)
7	Clay	2000 (905)	770 (767)
8	Clay	2200 (184)	3200 (650)
9	Silty Clay	83 (36)	140 (55)
10	Silty Clay	59 (6.1)	3500 (3527)
11	Sand	56 (8.8)	13 (1.6)
12	Sand	17 (2.7)	9.2 (1.8)
13	Sand	18 (4.4)	7.6 (0.7)
14	Sand	21 (1.4)	10 (3.5)
15	Sand	18 (3.7)	18 (4.0)
16	Sand	22 (3.8)	19 (5.4)

[†] Samples 1-10 were run in triplicate and averaged and samples 11-16 were run with five replicates then averaged.

[‡] Standard deviation

[§] Soil Description refers to the soil description used in the Phase II ESA.

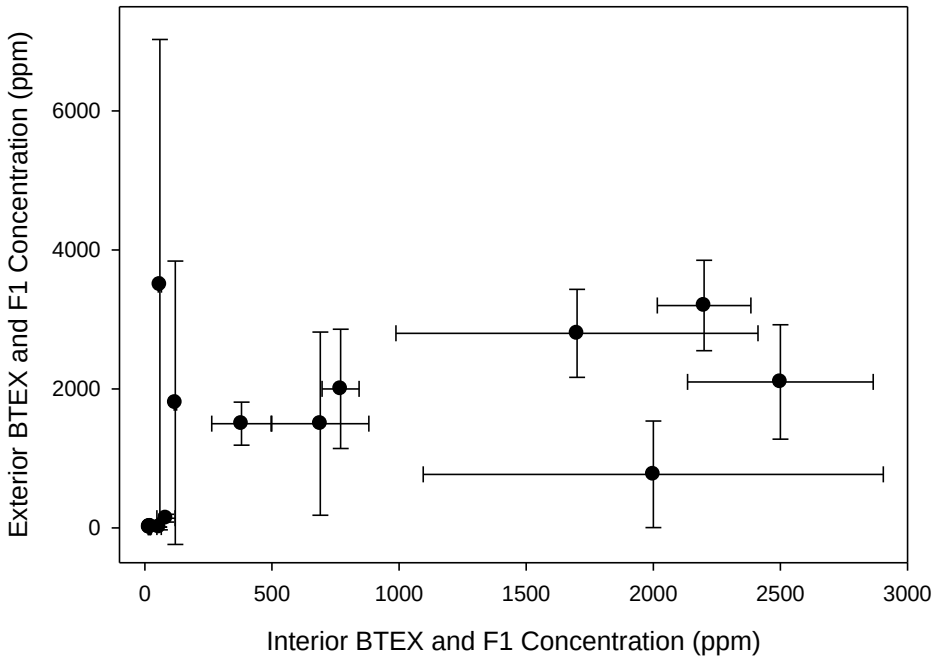


Fig. 3.1 Graphed exterior and interior BTEX and F1 concentrations, demonstrating that there are no apparent trends in the data.

3.5.2 Color Tracer Experiment

Hydraulic conductivity of amendment solutions was limited in the soil microcosms for all soil textures (Fig. 3.1). The amendment solution with the Brilliant Blue color tracer did not completely saturate or penetrate the soil (Fig. 3.1). For all soil types (heavy clay, clay loam and loam), the amendment completely saturated the outer soil on the top and bottom of the soil microcosm (Fig. 3.1). The saturation increased with coarser texture (Fig. 3.1). On the sides of the soil microcosm, amendment only saturated the soil where the slits in the PVC pipe were. Once again, for visual observation, permeation increased with coarser soil texture (Fig. 3.1).

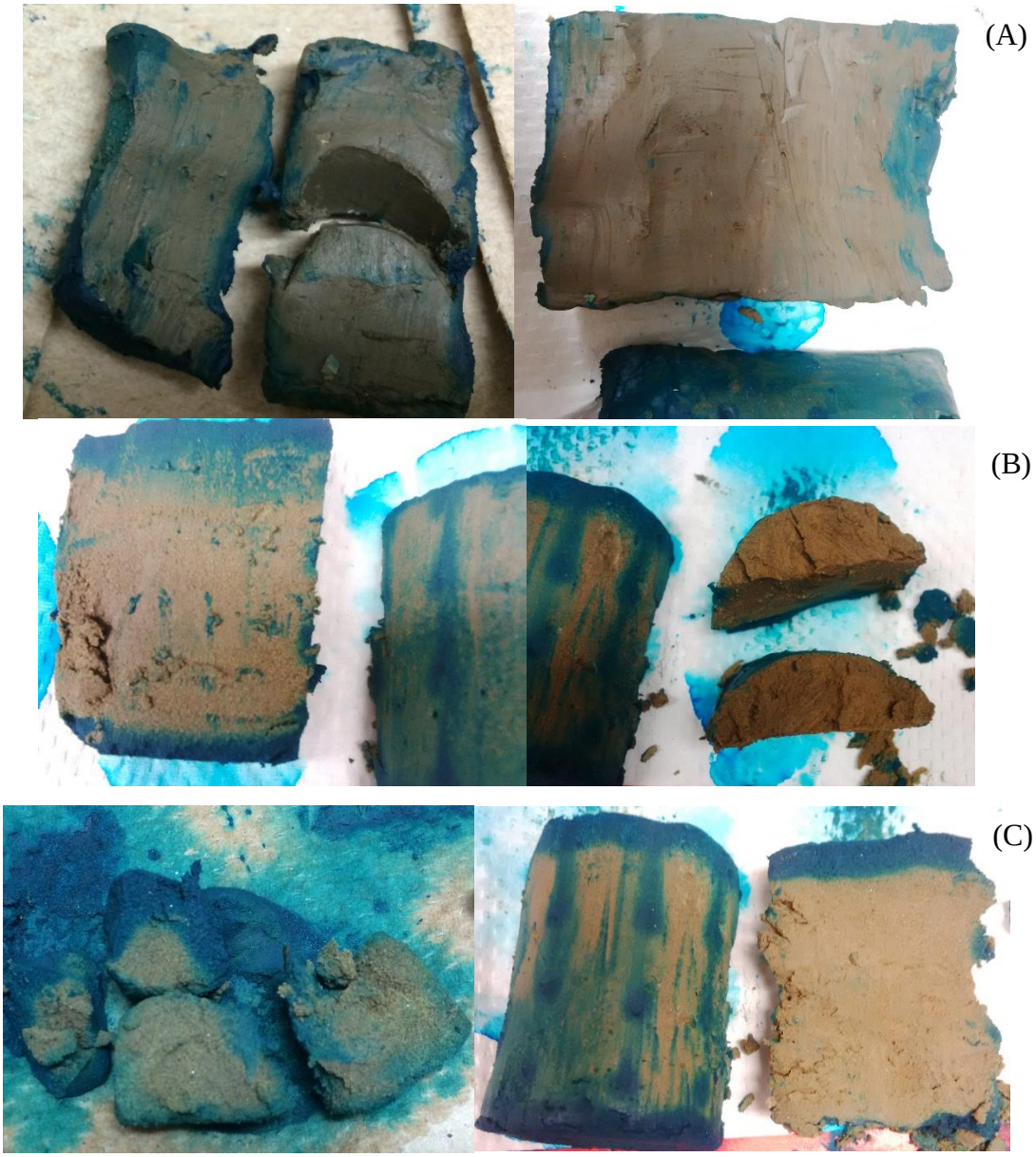


Fig. 3.2. Soil microcosms with different soil textures incubated with Brilliant Blue FCF color tracer. (A) Heavy clay, (B) clay loam, and (C) loam.

3.5.3 Soil and Amendment Solution Correlation

The amendment's BTEX and F1 concentrations and within the microcosm soil were linked in these heterogeneous clayey soils. A deming regression was used to account for errors in observations on both the x- and y- axis. The raw data was heavily left skewed for all the sites for both the concentration in amendment solution and soil (Fig. 3.2). To make the data unimodal and

to form a linear line we log transformed both the amendment solution and the soil concentration. Figure 3.3 demonstrates a strong correlation between BTEX and F1 in amendment solution and soil after one hour of incubation for three different impacted clayey sites. All sites had a Pearson's r above 0.75 (Table 3.2). However, one limitation was that the correlation is very accurate with the more soluble compounds such as benzene but does not work well for compounds that are less soluble, such as hexane (Fig. 3.4). To ensure this design would work over a four-week incubation (no losses due to weekly amendment changed, volatilization, etc.) two quality control experiments were completed.

Table 3.2. Deming regression results for concentration of BTEX and F1 in soil and in solution for each site.

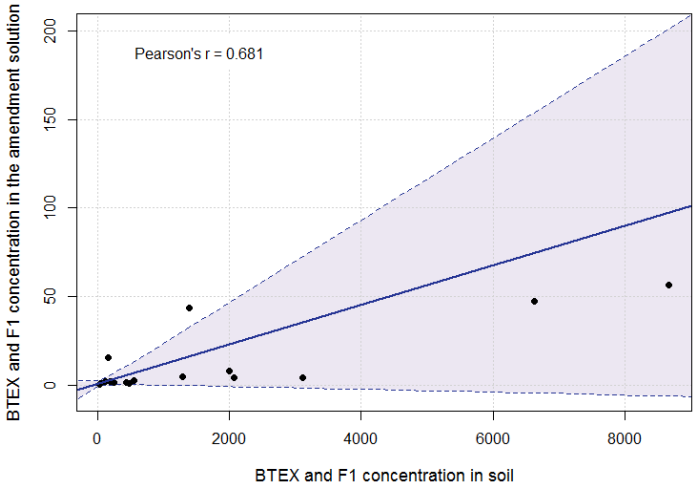
Site location	Line equation	SE of Intercept	SE of Slope	Pearson's r
Allan	$Y^{\dagger}$ =9.93x‡-6.78	3.81	2.47	0.77
Davidson	Y =0.73x-1.58	0.65	0.17	0.87
Outlook	Y=1.18x-2.46	0.63	0.18	0.85

† Log plus one BTEX and F1 concentration in the amendment solution

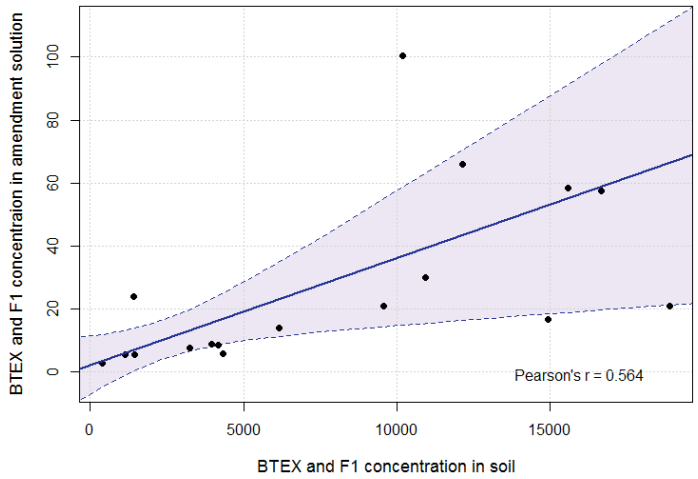
‡ Log plus one BTEX and F1 concentration in the soil

In the control experiments, no changes were noted in the sterile water and soil microcosms, suggesting no abiotic losses or hydrocarbon depletions from weekly amendment solution changes (Fig. 3.5, Table 3.3). There were no weekly differences for the gamma ray irradiated soil (Fig. 3.5, Table 3.4).

(A)



(B)



(C)

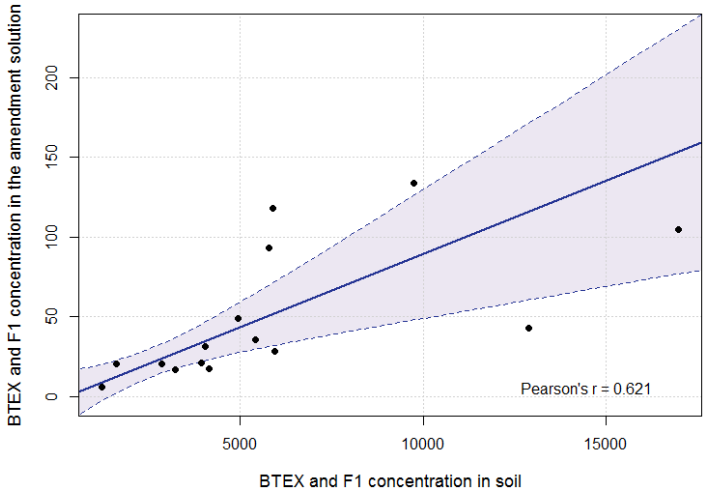
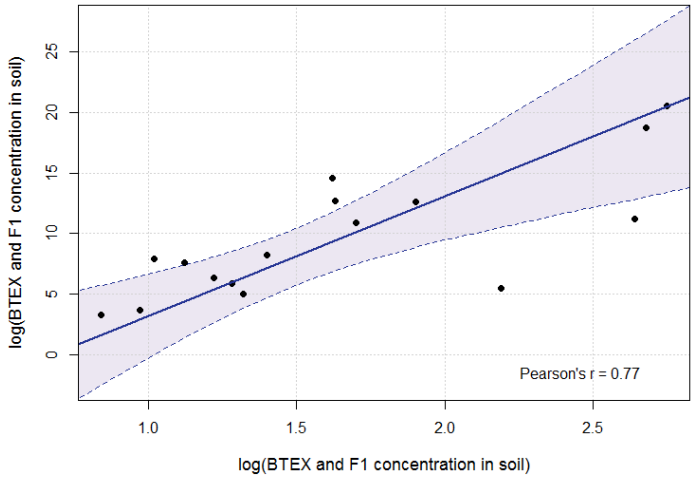
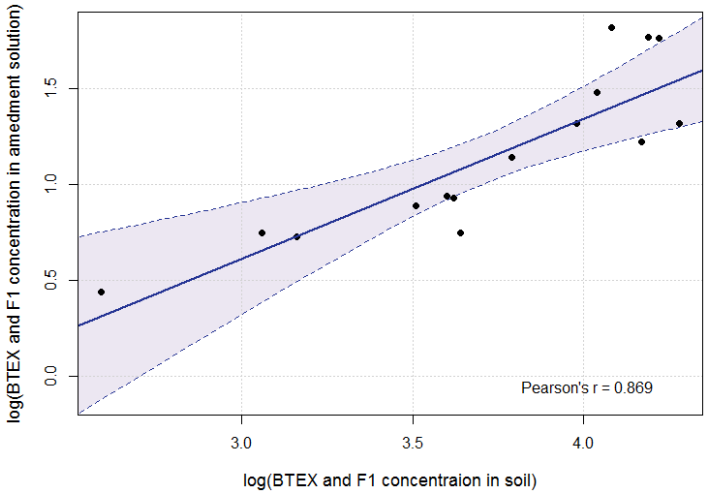


Fig. 3.3. BTEX and F1 concentrations in amendment solution versus concentrations in soil after a one-hour incubation for all three experimental sites. (A) Allan, (B) Davidson, (C) Outlook. Each point represents a microcosm and its associated average of three replicates of BTEX and F1 concentration in the biostimulation solution and soil. The shaded area represents the error in both soil and amendment solution concentration.

(A)



(B)



(C)

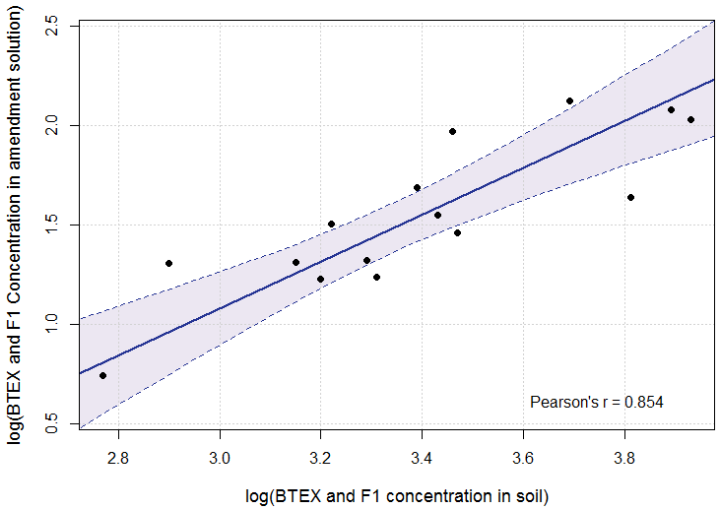


Fig. 3.4. BTEX and F1 concentrations (log transformed) in biostimulatory solution versus BTEX and F1 concentrations (log plus one transformed) for all three experimental sites. (A) Allan, (B) Davidson, (C) Outlook. The shaded area represents the error in both soil and amendment solution concentration.

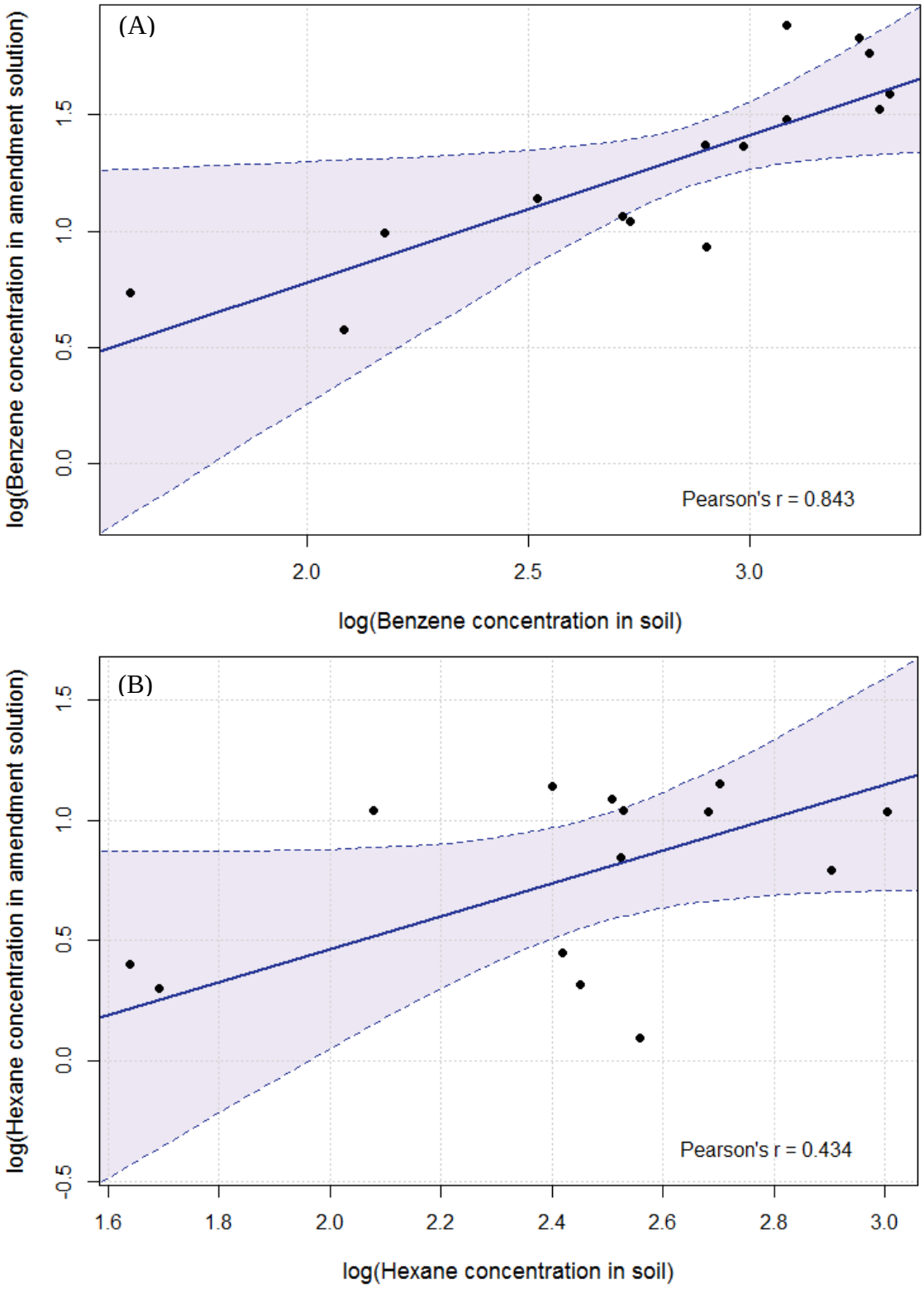


Fig. 3.5. Comparison of benzene (A) and hexane (B) concentration in amendment solution versus soil for the Davidson site. The shaded area represents the error in both soil and amendment solution concentration.

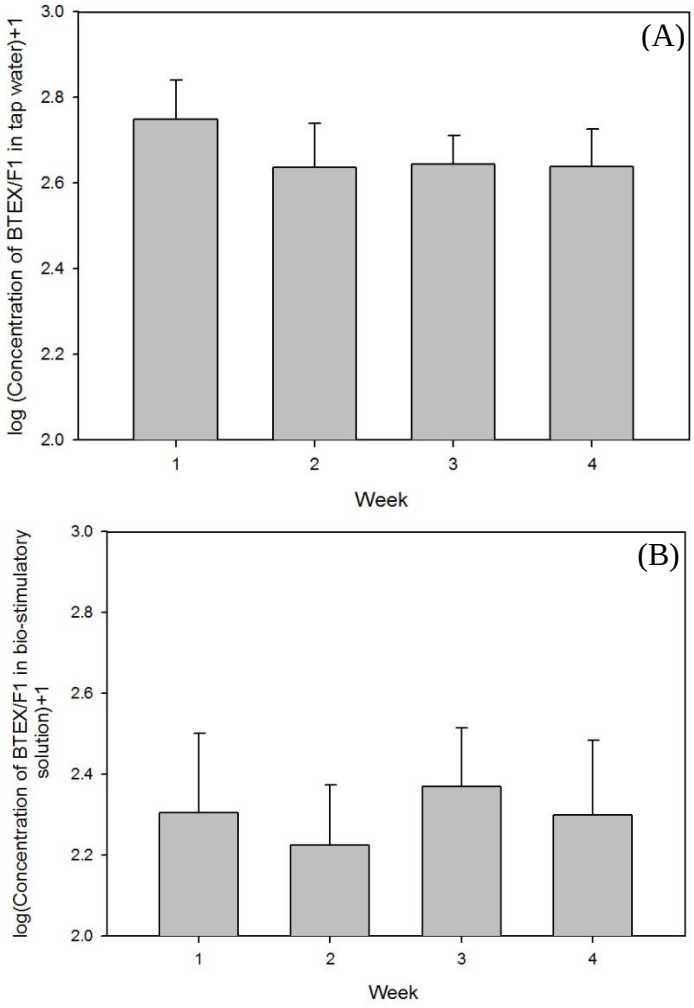


Fig. 3.6. Average log concentration C6-C10 (mg kg^{-1}) in amendment solution over four weeks of incubation. (A) Average log concentration of C6-C10 (mg kg^{-1}) with repeated solution changes in UV-filtered tap water. (B) Average log concentration of C6-C10 (mg kg^{-1}) with repeated solution changes following gamma-irradiation. Averages are taken from three replicates and error bars represent the standard error o f the estimate.

Table 3.3. Analysis of variance of change in log BTEX and F1 concentration (ppm) over four weeks with weekly solution changes.

Source	Degrees of freedom	Sum of squares	Mean square	F	P
Sterile Tap	1	0.026	0.26	0.58	0.46
Residuals	18	0.80	0.044		

Table 3.4. Analysis of variance of change in log BTEX and F1 concentration (ppm) over four weeks in gamma ray irradiated soil.

Source	Degrees of freedom	Sum of squares	Mean square	F	P
Gamma Ray	1	0.0041	0.0041	0.025	0.88
Residuals	18	2.97	0.16		

3.6 Discussion

Biostimulation presents a challenge to many environmental scientists and engineers because it is a site-specific process. Contamination characteristics such as contaminant heterogeneity, hydrocarbon concentration, adsorption, and maturity can vary by site and within site. Site environmental conditions such as soil microorganisms, soil structure (including macropores and preferential flow paths), temperature, redox potential, moisture content, and substrate bioavailability can also vary greatly by site (Balbla et al., 1998). These conditions can affect biodegradation via bioavailability, microbial population and growth, and available nutrients among many other factors (Balbla et al., 1998). Due to site-to-site and within site variations, it is vital to push microcosm designs to be more field relevant and to consider soil characteristics that may vary.

Microcosm designs can vary in complexity from simple static soil jars to highly sophisticated systems that allow the researcher to consider various environmental parameters seen on site (Balbla et al., 1998). Microcosms may be either closed or open, intact (little to no soil disturbance (i.e. soil core)), or homogeneous (soil is sieved and mixed to reduce natural

variability and repacked or placed into the microcosm container), and use contaminated site soil, re-spiked site soil (soil taken from contaminated site, dried, and re-spiked), or soil that has no previous contamination history that is spiked (Balbla et al., 1998). Homogenized microcosm designs may include packed soil or loose soil in a container. Amendment solution can be delivered by saturating the soil core or loose soil, incorporating fertilizer into the soil, adding fertilizer to the top of a column, or a pump system within the column to evenly distribute amendment solution. A more complex system that considers site characteristics is more likely to produce relevant results that are transferable to field studies (Balbla et al., 1998).

In many studies, contaminated site soil is air-dried, mixed thoroughly, sieved to 2 mm, and re-spiked (Chaineau et al., 1995; Miles and Doucette, 2001; Shahi et al., 2016; Gao et al., 2014, Qin et al., 2013; Reddy et al., 2011). Microcosm designs with these steps poorly represent on site conditions. These processes can largely effect: surface area, soil structure, contaminant heterogeneity, hydrocarbon concentration distribution, hydrocarbon adsorption and desorption properties, and microbial population and habitat. These restrictions are addressed in our design by using an unaltered intact soil column taken directly from the impacted site. With this design it does not substantially increase the surface area, it has little modifications on the natural soil structure and heterogeneity, the contaminant properties are unaltered, and there are little changes to the microbial community.

Another type of microcosm design that is common is using a soil column. For example, Gunther et al. (1996) used a design where they filled a glass column with contaminated soil mixed with sand (at a ratio of 2: l w/w). Soil was taken from an agriculture field with no previous contamination history, sieved to 2 mm, and spiked. A liquid amendment solution was delivered from the top of the column. Certain parameters of this experiment such as sieving the soil decrease the results variability. By adding sand to the soil, it allows the amendment solution to more freely flow through the soil column and for better saturation. However, with these modifications, it introduces aspects that are not as transferable to the field. By adding in sand, it disrupts the hydraulic conductivity, overall soil texture, structure, increases pore space and surface area. Sieving additionally alters soil physical properties such as structure. The soil was also not already contaminated and was spiked, this can alter the microbial community, contamination distribution, adsorption and desorption, and maturity. Our microcosm considers

these environmental site factors by using an intact soil core directly from the impacted site. This avoids mixing the soil with fillers and having to sieve and re-spike the soil. By skipping these steps, we ensure soil physical characteristics, contaminant properties, and microbial communities are representative of the field.

Another example of a column design is Boopathy (2004). In this study, contaminated soil taken from the impacted site was mixed thoroughly and then firmly packed into a column. This design used a multi-channel peristaltic pump, to force pore gases up for complete soil saturation. Enough water was added per day to fill all pore spaces. There was also an inlet and outlet pump for nutrient solutions and ports for sampling pore water and headspace. All columns were operated on a periodic cycle consisting of flooding with nutrient solutions followed by draining and dry cycle. During both the wet and dry periods, helium gas was pumped through to ensure anaerobic conditions.

Although this design is more accurately representing the field, there are still some aspects that may get lost in translation when scaling up to a field study. Once again, the soil was homogenized distorting environmental site conditions such as soil structure and contamination properties. It also seems unrealistic that flooding and drying effects would occur regularly on site especially in finer soil types. A multi-channel peristaltic pump can often stimulate injection systems similar to the site. However, in this scenario it would most likely, ensure that the entire column is receiving the amendment solution that is often not seen onsite. In addition, a multi-channel peristaltic pump in a microcosm design adds a degree of complexity and are not successful with the soil types in Western Canada because of high clay content and low hydraulic conductivity. For example, the hydraulic conductivity on the sites we are working with range from 10^{-5} to 10^{-9} cm s^{-1}. Our microcosm design addresses these issues by taking in intact soil cores and placing them into a jar with amendment solution. This keeps the soil intact within the microcosm to better represent soil structure, microbial populations, and PHC transport and contamination. It also allows the soil to be completely saturated with amendment solution, and for the soil to be exposed to new solution each week.

However, even though we have altered previous column designs to be more field relatable, there are still aspects in our design that do not represent field conditions. One aspect that our microcosm design is not able to account for is preferential flow paths where amendment

solution flows through fractured clay minerals. These features are common in Western Canada; however, are destroyed during the drilling process due to compaction. Since the flow paths are altered in sample collection we are unable to represent them in out microcosm design. A solution to this problem could be to mimic flow paths that are seen on site, by creating them within the intact soil column. Another limitation in our design is that the amendment solution may not reach certain portions of the intact soil microcosm due to the high clay content and low hydraulic conductivity. Another limitation in this design is dealing with compounds that are less water soluble. In this design, we correlated the concentration of F1 and BTEX in the soil with the concentration in the amendment solution. Although, this works well for total F1 and BTEX and for individual soluble compounds such as benzene, it does not work for individual compounds within F1 that are less soluble such as hexane. Due to this flaw, this design would not be feasible for highly hydrophobic compounds such as higher hydrocarbon fractions (such as F3, F4, and polycyclic aromatic hydrocarbons (PAHs)), polychlorinated biphenyl (PCBs), or dichlorodiphenyltrichloroethane (DDT). More research and design modifications would be needed to achieve reliable results for these hydrophobic contaminates. One other limitation is that since this design considers many environmental parameters, it increases the statistical complexity needed for analysis. This results in more complex statistical models and the time needed for analysis. However, with this limitation, we are also able to account for many environmental characteristics from site to site and within sites and determine how these parameters may be affecting degradation potential and rates.

In many microcosm studies, researchers are unable to use intact column studies that use already contaminated soil because the soil contamination is too heterogeneous. Researchers need to homogenize the soil to get reprehensive samples and clearer results. In the experiment using interior and exterior portions of the soil borehole we concluded that soil contaminant heterogeneity precluded destructively sampling un-sieved soil. When using a core with natural contamination we found it impossible to get a representative sample before and after the amendment solution was added due to the hydrocarbon spatial distribution. Therefore, many researchers resort to microcosm designs that involve sieving, mixing, and spiking the soil. This led us to find a way to avoiding sampling issues that arise from varied hydrocarbon concentration distribution when using an intact soil microcosm design. We were able to resolve this issue by finding a correlation between the initial concentration within the microcosm and the

concentration in the amendment solution. Although this design still has limitations, we were able to incorporate site specific properties specifically contaminate heterogeneity.

4.0 SOIL PARAMETERS THAT INFLUENCE C: N: P EFFECTIVENESS AND THE HOW RATIOS IMPACT HYDROCARBON CHEMICAL SELECTIVITY AND DEGRADER PREVALENCE

4.1 Preface

In Chapter 3, we developed a bench scale microcosm design that more accurately stimulates contaminate properties such as contaminate heterogeneity. Using this design, we were able to start analyzing how nutrients, specifically C: N: P ratios effect anaerobic degradation, the microbial community, and chemical selectivity of the F1 hydrocarbon fraction. To identify the effects of C: N: P ratios, we collected initial site soil and water chemistry data and then applied a low range of C: N: P ratios (0.7 – 2.5) to microcosms from these sites. We then performed laboratory based chemical analysis weekly on NO_3^-, PO_4^-, Fe (III), and SO_4^- in solution. Samples for microbial community and F1 in soil were also taken to further aid in how C: N: P was affecting degradation and the microbial community.

4.2 Abstract

Optimal nitrogen (N) and phosphorus (P) concentrations for aerobic in-situ biostimulation of petroleum hydrocarbon contaminated sites has been extensively investigated. However, it has not been assessed how C: N: P ratios effect microbial communities and are linked to degrader prevalence and chemical selectivity. Our overall objective was to determine how C: N: P ratios effect chemical selectivity and are linked to hydrocarbon degrader prevalence and potential activity in clay soils. We stimulated field conditions by using an intact core microcosm design with samples collected during Phase II Assessment. To assess how C: N: P ratios effected the microbial community we left the P ratio constant at a rate where formation of new P minerals, such a brushite was not predicted to occur. Nitrogen levels were kept below 2000 mg N kg^{-1} H_2O to eliminate any negative effects from osmotic stress. To determine how the solution effected the microbial community the C: N: P solutions were replaced and analyzed at the beginning of the incubation and weekly for nutrients, BTEX and F1. Soils were sampled before and after the four week incubation for microbial community composition. We demonstrated that C: N: P ratios were not selective for hydrocarbon ratios, but higher P in solution at low contaminant concentrations enhanced benzene degradation more than the other chemicals in the F1 fraction. We also demonstrated that original site conditions and the amount

of S and P in solution was more influential than the C: N: P ratio. Lastly, we demonstrated that the microbial community and degradation success was influenced the most by pre-existing site and within site conditions.

4.3 Introduction

A number of factors can limit or stall in situ bioremediation including low electron acceptors (nitrate, ferric iron, and sulfate), nutrients (particularly growth-limiting N and P), bioavailability, and microorganisms (Ron and Rosenberg, 2014; Powell et al., 2006; Walworth et al., 2007). Some of these limitations can be overcome by adding electron acceptors and nutrients via in-situ biostimulation to promote the indigenous microbial population to degrade hydrocarbons (Masakorala et al., 2013**)**. The addition of electron acceptors such as nitrate, sulfate, and iron (III) can also promote degradation. Nitrate is highly reactive and would likely be consumed first, then ferric iron, and lastly sulfate (Gray et al., 2010; Boren et al., 1995). Additionally, under sulfate-reducing conditions, researchers have found a significant lag period before remediation occurs (Edwards, et al., 1992, Morasch, et al., 2004, Morasch & Meckenstock, 2005). The addition of N and P can also increase degradation since these nutrients are often the most limited (Chang et al., 2010; Steliga et al., 2012). Nitrogen and P are important macronutrients in biostimulatory solutions since they are required for metabolic activities and stimulate microorganism growth and reproduction (Cunningham et al., 2000; Chandran and Das, 2011). One way of determining how much N and P to add, is a stoichiometric ratio of C: N: P based on the C content from the hydrocarbon contamination.

A stoichiometric ratio of C: N: P of 100:10:1 has been accepted as a common formula for aerobic biostimulation practice (Xiong et al., 2012; Ron and Rosenberg; Chaineau et al., 2004; Mill and Frankenberger, 1994). However, the optimal ratio of 100:10:1 is based solely off microbial needs and does not consider additional N that may be needed in anaerobic systems where N is often the preferred electron acceptor. Additionally, in literature, the ratio varies greatly. For instance, Zhu et al. (2001) reported a ratio of 100:5:1, Turgay et al. (2009) reported successful bioremediation at 100:15:1, and Qin et al. (2013) used a ratio of 100: 10: 1. While these ratios are similar, the U.S. Environmental Protection Agency recommends ranges for C: N of 10:1-100:1 (US EPA, 1995). Others have reported optimal C: N: P ratios of 50:10:1 (Yerushalmi et al., 2003), 120:10:1 (Thomas et al., 1992), 8.8:13:3 (Mohn and Stewart, 2000),

and 100:15:3 (Zitrides, 1983; Riser-Roberts, 1998). It is possible that more N may be required for anaerobic systems for nitrate-reducing microorganisms (Eriksson et al., 2003). In Eriksson et al. (2003) they found that anaerobic degradation was heavily linked to denitrification. According to literature, highest denitrification rates occur from a C: N rang of 1 to 4 (Zhang et al., 2016; Grebliunas and Perry, 2016). In Zhang et al. (2016) they found that the most denitrification took place at a C: N ratio of 1 and the highest concentration of nitrate reducers at a C: N of 1.5.

With a wide C: N: P variation in literature, Shahi et al. (2015) has stated that the optimal C: N: P ratio is site specific since each site has different petroleum properties and different treatment factors. Certain site characters such as initial impacts and initial soil and groundwater chemistry (e.g. initial Fe, S, N, Ca, and Mg concentration) can heavily influence degradation rate (Zhou and Crawford, 1994). The hydrocarbon concentration heavily influences degradation rate due to hydrocarbon toxicity to the microbial community (Margesin et al., 2006; Margesin et al., 2000; Nocentini et al., 2000). Higher impacts decrease degradation rate, for example, Margesin et al. (2006) found degradation rates in fertilized and unfertilized soils were much higher at 2500 mg kg^{-1} (70-80% TPH loss) compared to 10000 mg kg^{-1} (10- 46% TPH loss). Additionally, they also found that lower initial concentrations were less influenced by nutrient addition (Margesin et al., 2006). The higher levels of initial nutrients and electron accepters in soil and groundwater can increase degradation rate during biostimulation because there will already be a strong indigenous microbial community of hydrocarbon degraders.

Concentrations of Mg, Ca, and bicarbonate (HCO_{3-}) initially on site can also play a role in degradation rates. Degradation rates in groundwater can be inhibited by high levels of Mg and Ca (Li et al., 2014). Additionally, high concentrations of Mg, Ca, and $CaCO_3$ in soil may inhibit degradation since phosphate has a strong tendency to adsorb to Ca minerals resulting in low solution concentrations (Persson et al., 2012; Makie et al., 2013). It also may indicate the presence of phosphate minerals such as brushite and newberyite. Siciliano et al. (2016) demonstrated that these minerals can decrease degradation and can alter community composition.

There is evidence that different nutrient concentrations effect the degradation of different hydrocarbon fractions (Braddock et al., 1999; Chaineau et al., 2004). Chaineau et al. (2004) concluded that different nutrient levels are needed for the optimum degradation of aliphatic and

aromatic hydrocarbons. Zhou and Crawford (1994) found that nutrient addition enhanced aromatic more than aliphatic degradation. Additionally, different microbial communities are responsible for degradation of aromatic versus alkane compounds (Arvanitis et al 2008; Fuentes et al 2014; Das and Chandran, 2011; Edwards, 2003; Robertson et al., 2007; Yergeau et al., 2009; Weelink et al., 2010; Zhang et al., 2012; van der Zaan et al., 2012; Kleinsteuber et al., 2012; Herbst et al., 2013; Fathepure, 2014; Luo et al., 2014, 2016; Zhang and Lo, 2015; Sheng et al., 2015; Quadros et al., 2016; Salanitro, 2000; Arvanitis et al 2008; Fuentes et al 2014).

Diverse bacterial groups can degrade petroleum hydrocarbons under different electron acceptors. For aliphatic compounds there has been several anaerobic bacteria identified that use sulfate or nitrate as electron acceptors (Widdel and Grundmann, 2010; Grossi et al., 2008). These include bacteria mostly within the Proterobacteria phyum including the sulfate-reducers: *Desulfatibacillum alkenivoras* (Callaghan et al., 2008), *Desulfoglaeba alkanedexens* (Callaghan et al., 2010), and *Desulfococcus oleovorans* (So et al., 2003); and the nitrate reducer *Azoarcus sp.* (Grundmann et al., 2008). Many microorganisms that degrade BTEX compounds are also from the phylum Proterobacteria. Some examples of microorganisms that used different electron acceptors to degrade BTEX include: nitrate reduces such as *Dechloromonas*, *Azoarcus*, *Thauera*, *Magnetospirillum*, and *Aromatoleum* (Coates et al., 2001; Fries et al., 1994; Dolfing et al., 1990; Zhou et al., 1995; Song et al., 1999; Amders et al., 1995; Shinoda et al., 2004; Evens et al., 1991; Shinoda et al., 2005); iron-reducers such as *Geobacter* (Lovley et al., 1993; Coates et al., 2001) and sulfate-reducers such as *Desulfobacterium, Desulfobacula*, and *Desulfotomaculum* (Morasch et al., 2004; Harms et al., 1999; Rabus et al., 1993).

Two experiments were conducted to determine A) if a lower C: N ratio is needed for optimal anaerobic degradation and how it is linked to microbial activity and degrader prevalence and B) to determine how the C: N: P ratio is linked to chemical selectivity within the F1 hydrocarbon fraction and degrader prevalence. In the first experiment we had treatments with different C: N ratios and the P concentration was constant. The second experiment had treatments with varying C: P ratios and a constant N ratio based on the first experiment. Initial BTEX and F1, from all borehole horizons were analyzed identify the existing C to determine the maximum concentration of P that can be added to the solution that does not promote mineralization and elevated electric conductivity levels. The initial treatment solutions were analyzed for N, S and P. After each week in the four-week incubation, the microcosms were put

into new solution and the old solution was analyzed for BTEX and F1 and ferrous iron. After the fourth week, the amendment solution was also analyzed for N, S, and P. Through measuring BTEX and F1 in the solution each week we were able to determine the concentration in soil using the BTEX and F1 soil to water calibration curve as previously discussed. Sulfur and ferric iron were measured in the solution to keep track of chemical and biological processed occurring in the system. After four weeks the microcosms were destructively sampled using incremental sampling methodology for BTEX, F1, microbial community, N, and P.

4.4 Materials and Methods

4.4.1 Sample Collection and Microcosm Creation

Soil samples were collected from multiple bulk transfer and gasoline stations with known spill and leak history within Davidson, Outlook, and Allan Saskatchewan, Canada. These different sites have varying soil conditions. The Davidson site have glacial lacustrine-alluvial plain and glacio-fluvial plain. The soil lithology at Davidson consisted of fill material, silty clay with silt and sand pockets throughout, followed by clay till. The clay till layer at Davidson acted as an impermeable layer which stopped the contamination from continuing with depth. In the Outlook area there is typically glacial lacustrine-alluvial plain, Aeolian plain, and till plains. The stratigraphy at Outlook was sand and gravel fill, low plastic clay with sand layers, followed by a clay till. They clay till was at Outlook was dry, very stiff, was fractured. In the Allan area the regional geology is expected to consist of gravel fill underlain by silty lacustrine clay and glacial tills. At Allan there was coarse grained gravel fill followed by silt and medium plasticity clay, and under that was a firm plastic clay till. Throughout the boreholes at various depths there were sand lenses and pockets During Phase II Assessment additional F1 and BTEX samples were collected every 0.5 to 0.75 m to determine initial F1 and BTEX concentration concentrations. Sample collection and storage of duplicate boreholes and microcosm creation details can be found in Chapter 3.4.1 and 3.4.5. Different borehole samples were used for the C: N and the C: P experiment. Site maps with borehole locations are located in Appendix A. All microcosms were given a sample ID that corresponded to the site, borehole, and treatment number. For both experiments, microcosms were incubated in the dark for four weeks at 10°C. Each week the

amendment solution was replaced with new solution. Throughout the experiment the amendment solutions were also stored in the dark at 10°C.

4.4.2 Amendment Solutions C: N

The C: N: P was made using tap water with Nitric acid (HNO_3) as a nitrogen source and sodium tripolyphosphate (STPP) as a P source. In addition, 0.022 M of $MgSO_4 *7H_2O$ (700 mg L^{-1} S) was added as a sulfate source and a buffer. After the solutions were made, the pH was increased to a range of 6.5 to 7.5 by using sodium hydroxide (NaOH). All nutrient solutions were sterilized with UV light as seen in Chapter 3.4.1 after the pH increase and when the solution was opened to the outer atmosphere. Each microcosm was grouped by site and borehole and assigned the target ratio using a random number generator. For each C: N ratio there was three to five replicates and randomly selected treatment controls that have uncontaminated soil. There was also a control group with five replicates of contaminated soil with tap water.

To calculate C to N ratios we identified the existing C concentration by analyzing the initial BTEX and F1 concentration in the soil. We then took the mg kg^{-1} of each chemical in the F1 and BTEX range, converted it into grams, and then divided it by the molecular weight to get total C (mol kg^{-1}) (for F1 unlabeled we took the average of all the F1 and BTEX chemicals (102.46 g mol^{-1})). For each chemical we then converted mol kg^{-1} to mmol C kg^{-1} by multiplying by the number of carbons in the molecule. All chemicals were summed to achieve total C mmol kg^{-1}. We then converted to mmol C by multiplying by the average soil weight (0.080 kg) within the microcosms. An example calculation is demonstrated below using benzene.

$$\frac{\text{mg}}{\text{kg soil}}\ \text{Benzene}\times\ \frac{1\ \text{g}}{1000\ \text{mg}}\times\frac{1\ \text{mol Benzene}}{78.1\ \text{g}}\times\frac{6\ \text{mol carbon}}{1\ \text{mol Benzene}}\times\frac{1000\ \text{mmol}}{1\ \text{mol}}$$

$$\times 0.08\ \text{kg soil} = \text{mmol C}$$

We were then able to come up with our target ratios and calculate the correct concentration to add each week-based soil contamination. We had 11 different C: N ratios with the target C: N molar ratio ranging from 0.7 to 2.5 (0.7, 0.9, 1, 1.2, 1.3, 1.4, 1.5, 1.7, 2.0, 2.2, and 2.5). We decided on this range because according to literature, the highest denitrification occurs at a C: N range of 1 to 4. To determine the N concentration for each solution we calculated the total amount of N (mmol N) by multiplying the total C (mmol C) by the target ratio. We then divided that number by the total number of amendment replacements (four) to determine how much N is in the solution each week that we change out the solution. Next, we calculated mmol N Kg soil^{-1} to ensure it was under the 2000 mg N kg^{-1} H_2O maximum by dividing by the grams of water in the microcosm by using the soil moisture content. We then converted back to mg N needed for 1L of solution. An example calculation is demonstrated below.

Step one: Determine mmol N per amendment change.

$$\text{mmol C} \times \text{target ratio} \frac{\text{mmol N}}{\text{mmol C}} \div 4 \text{ amendment replacements} = \frac{\text{mmol N}}{\text{water replacement}}$$

Step two: Calculate mmols N g^{-1} soil water to ensure it is below 2000 mg N kg^{-1} H_2O.

$$\frac{\text{mmol N}}{\text{water replacement}} \div \text{g soil water}^* \frac{14 \text{ g}}{1 \text{ mmol N}} \times \frac{1000 \text{ mg}}{1 \text{ g}} = \frac{\text{mmol N}}{\text{g soil water}}$$

Step three: convert back to mg N L^{-1}.

$$\frac{\text{mmol N}}{\text{g soil water}} \times \frac{\text{per water change}}{0.067 \text{ L}} = \frac{\text{mg N}}{\text{L}} \text{ to add to solution}$$

Based on Walworth et al., (1997 & 2007) and Walecka-Hutchison and Walworth (200) we came up with a maximum N concentration of 2000 mg N kg^{-1} soil water to eliminate microbial stressed caused from high osmotic pressure. Samples that had higher than 2000 mg N kg^{-1} soil water were changed to a N level of 2000 mg N kg^{-1} soil water before and carried out through the rest of the calculation process. Based on the concentration in solution (mg N/ L) we came up with 35 amendment solutions. To make these solutions we added in HNO_3 for the mg N L^{-1} needed, 0.3 mM STPP [3.1 mg L^{-1} P], and 22 mM $MgSO_4$ *$7H_2O$ [700 mg L^{-1} S]. The concentrations of P and S used were based off what is currently being used in the amendment solutions at these sites.

4.4.3 Amendment Solutions C: P

To identify the initial amount of C to calculate the C: N: P ratios, initial BTEX and F1 was measured. The total amount of C was determined using the same calculations explained in Chapter 4.4.2. Based on preliminary degradation results from the C: N experiment, we decided to use a constant C: N ratio of 1.4. C: N ratios and the corresponding mg N L^{-1} were calculated as described in Chapter 4.4.2. We decided on a wide range of different C: P ratio's extending from 0.7 to 730. We decided the highest P of 0.7 P because at higher concentrations, the electrical conductivity of the biostimulatory solution would be greater than at the sites. To calculate the mg P L^{-1} we used the calculation Chapter 4.4.2 except using P. Briefly, we divided the target P ratio by the total C (mmol) to get mmol P and then converted it to mg L^{-1} P. Based on the N and P concentration needed for the C: N: P ratios we had 48 different amendment solution. Once again, all solutions also included 0.022M of $MgSO_4$ *$7H_2O$ (700 mg L^{-1} S). We increased all solutions pH to ~ 6.5 to decrease precipitation using NaOH. After the pH was adjusted, we measured the electrical conductivity to ensure it was not over site levels then UV sterilized the solutions as described in Chapter 3.4.1.

4.4.4 Chemical Analysis

F1 and BTEX concentrations were measured as described in Chapter 3.4.4. Stale amendment solutions were analyzed for nitrate and sulfate using the Ion Chromatograph (IC) (DIONEX IC2000, Thermo Fisher Scientific, Waltham, MA) and a Dionex AS-DV auto-sampler (Thermo Fisher Scientific, Waltham, MA). The type of column used was a Dionex IonPac AS18 (Thermo Fisher Scientific, Waltham, MA), 4 × 250 mm and the effluent source was Dionex

effluent generator cartage (EGC) III potassium hydroxide (KOH) (Thermo Fisher Scientific, Waltham, MA). The column temperature was 30℃ and the cell was 35℃. Other conditions included a 1mL/ min flow rate, 25 uL injection volume, a 3 loop overfill factor, a push full inject mode, an ASRS 3004 mm suppressor, and a 15-minute run time. For quality control nitrate and sulfate standards were run both separately and together ranging from 0 to 100 mg kg^{-1}. A set of standards was run at the beginning, end, and every 50 samples. A standard and blank was run every 10 samples. All solutions were collected in high-density polyethylene containers and were filtered using 0.45 um filters within 24 hours after collection. Samples were frozen in a -20℃ freezer prior to IC analysis. Before being run on the IC all samples were diluted 1:100 to be within the range of the standards.

Stale amendment solutions were also analyzed weekly for total Fe using a Spectroquant Colorimeter Move 100. All samples were filtered then analyzed immediately. The iron content was checked with the MQuant^{TM} Iron Test. To analyze total Fe 5 mL of filtered sample was added into a test tube and three drops of Reagent Fe-1 was added and mixed. The mixture was left standing for three minutes (reaction time) then measured in the photometer. Any samples containing more than 5.00 mg L^{-1} Fe were diluted with distilled water. For quality control a duplicate and a blank using distilled water, were run every ten samples.

Amendment solutions were analyzed for phosphate concentrations and selected soil samples were tested initially and after the four-week incubation for available nitrate and phosphate. Phosphate in water and soil extracts was measured using a Seal analytical auto-analyzer 3 (AA3) with a modified molybdenum blue method. Briefly, the ortho-phosphate reacts with molybdate and ascorbic acid to form a blue compound measured at 660nm or 880nm. Antimony potassium tartrate is used as a catalyst. For the soil P extracts the same method was used with adapted reagents. For measuring N in the soil extracts, we also used the Seal analytical auto-analyzer 3 (AA3) with a hybrid method. Briefly, the nitrate was reduced to nitrite by hydrazine in alkaline solution with a copper catalyst. The nitrate then reacts with sulphanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) to form a pink compound measured at 550 nm.

Initial treatment solutions and the stale microcosm solutions at week four were analyzed for P. Since the P source used in the amendment solution was STPP, STPP was converted to

phosphate (PO_4^-) via acid-hydrolysis before running the solutions on the AA3. For this conversion we used a modified method from the U.S. EPA 1979. Briefly, we subsampled 10 mL of the amendment solution and added 125 uL of concentrated sulfuric acid. After adding the acid, samples were heated for two hours at 75℃. The stale amendment solution samples were run before and after the conversion to determine PO_4^-, STPP, and total P. Samples out of range were diluted to 1:100 and re-run. For quality control and assurance standards were run at the beginning and end of each run and a baseline check and a duplicate sample was run every 10 samples.

Soil P was extracted using a 0.5 M sodium bicarbonate solution using the Olsen et al., 1954 method. We adjusted the soil P extracts pH with concentrated sulfuric acid and then converted from STPP to phosphate using the same method mentioned stated for the amendment solution conversion. The same quality controls and instrument conditions for the amendment solution were used to analyze the soil P extracts. Soil N was extracted using a 2 M potassium chloride (KCl) solution using methods from Keeney and Nelson (1982) and Haynes and Swift (1989).

4.4.5 Microbial Analysis

Based on degradation results, certain samples were selected for microbial analysis including DNA extraction, sequencing, and Most Probable Number (MPN). For the C: N experiment a list of samples and the associated site factors can be found in Appendix B; Table B.1. Samples were microbial analyzed initially and after the four-week incubation time. The selected samples were chosen based on the C: N range and site. We ensured the samples selected for each range contained all the sites. The C: N ranges chosen were a lower range, optimal, and upper range (2.2-2.5, 1.2-2 and 0.7-1.3). Additionally, to determine if hydrocarbon concentration influenced the microbial community and the degradation potential samples with a benzene concentration of 0-3 and 5 mg kg^{-1} were sampled for microbial analysis. For the C: P experiment, we selected samples based on C: P and made sure each site was represented in all the selected ratios (a table of the sample number and corresponding site information for the selected samples can be found in Appendix B; Table B.2). Samples were taken initially and after the four-week incubation, for the tap water controls and for C: P ratios at low, optimal, and high P concentrations.

Soil DNA was extracted using a PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). Bacterial community composition was assessed by high-throughput amplicon library sequencing as described in Lamb et al., 2016 by the . Samples were amplified with 926F/1392R primers and purified using Beckman Coulter AmPure XP Beads on a magnetic stand. The Illumina indexes/adapters were added in the next PCR and samples were purified again using the AmPure XP Beads. Following pooling, the samples were sequenced on an Illumina MiSeq using a V3 chemistry kit (600 cycle) and then analyzed using Dada2. The amount of SNV's (single nucleotide variants) were also calculated using Dada2. Sequences were trimmed to a length of 466 and the average quality score used was 20. The taxonomic data base used was SILVA. *Aliivibrio fischeri* DNA (0.1 ng: 2-3% w/w DNA extract) was used as an internal standard after DNA extraction for sequencing.

4.4.6 Statistical Techniques

All statistical analyses were performed using R v 3.4.4 (R Core Team, 2016). All tests were completed at the $p < 0.05$ significance level. A general additive model (GAM) was used to determine if C: N: P treatments for each of the BTEX and F1 compounds were significant. A GAM was used since there were non- linear relationships between the response variables and multiple explanatory variables. In addition, using a GAM, we were able to determine which chemical parameters were significant to degradation and if different borehole and sites were significant. To determine if there was hydrocarbon selectivity for the different C: N: P treatments we used a Mixed Liner Model (MLM) with an ANOVA. To determine which initial site factors influences borehole differences we used Principal Component Analysis (PCA). Lastly, a redundancy analysis (RDA) with a Hellinger transformation was used to determine how the microbial community was influenced by chemical parameters. Based on the RDA results, a general linear model was used with a Bonferroni correction for repeated testing to determine which genera were significantly different in the C: N and the C: P experiment.

4.5 Results

4.5.1 Contributing Factors to Hydrocarbon Degradation

Initial contaminant concentration (week one measurements in solution) is a key driver influencing degradation rate. In both experiments, benzene biodegradation rates peaked in the

medium initial concentration group that was approximately 5 to 10 mg L^{-1} (Fig. 4.1). Benzene degradation rates varied from 0.00 day^{-1} to 0.079 day^{-1}. Rates were calculated from amendment solution as this could be sampled non-destructively, but soil concentrations that correspond to the three groups, 0 to 5 mg L^{-1}, 5 to 10 mg L^{-1}, and >10 mg L^{-1} (low, medium and high initial concentration), were approximately, 210, 420, and 530 mg kg^{-1}. In contrast, for other chemicals, higher initial impacts enhanced biodegradation. For example, microcosms with higher F1-BTEX concentrations at week one increased F1-BTEX degradation rates (Appendix C; Fig. C.1.). In microcosms were the initial solution F1-BTEX concentration was 7 to 11 mg kg^{-1}, F1-BTEX degradation rates averaged 0.0036 day^{-1}; however, with concentrations above 15 mg kg^{-1} degradation rates averaged approximately 0.010 day^{-1}. This trend remained consistent among hexane, toluene, and xylene (Appendix C; Fig. C.1.).

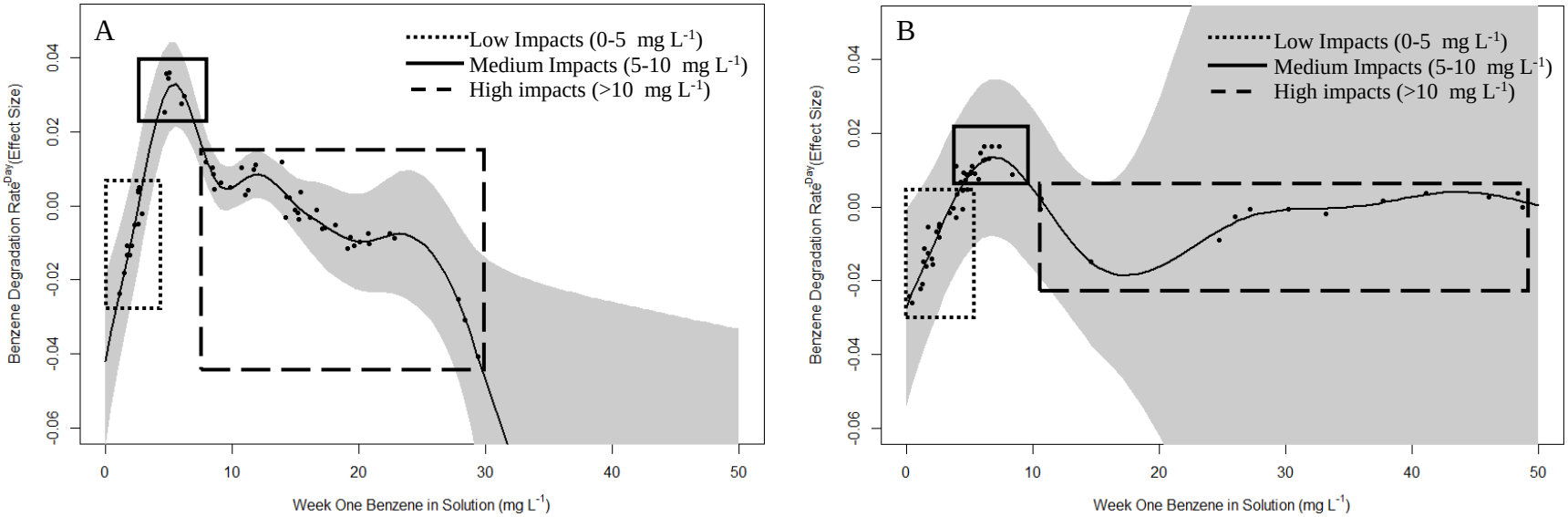


Fig. 4.1. A general additive model (GAM) demonstrating initial benzene concentration in week one solution effect on benzene degradation rate (effect size) in replicate experiments. A) Week one benzene concentration in solution effect on benzene degradation with different C: N treatments. B) Week one benzene concentration in solution effect on benzene degradation with different C: P treatments The smoothing term, Week one benzene in solution, had significant effects on benzene degradation ($P < 0.05$) in both experiments. The Y axis values are predicted values of the dependent variable as a function of the x axis centered around 0 (50/50 odds). Each point represents an observed value, the solid line is the trend after smoothing, and the grey shaded area is the standard error of the estimate. The boxes group low, medium, and high initial benzene concentrations.

Benzene biodegradation rates decreased with greater assimilated and/ or dissimilated sulfate (Fig. 4.2). For example, in both experiments, benzene degradation decreased starting at approximately 0.72 mg day^{-1} assimilated and/ or dissimilated sulfate that corresponds to 300 mg L^{-1} in solution. Rates were calculated from amendment solution by comparing the amount of S added to the amendment solutions to the concentration at week 4. The same amount of S was added to each microcosm. We did not measure sulfide production in solution, but on average, there was 10 times less total Fe from week one to week four in both experiments. Additionally, when compared to the controls, samples with added nutrients consumed twice less Fe then the controls. At Allan and Davidson field sites, initial groundwater iron (dissolved) concentrations were greater in the impacted areas. Initial sulfate concentration groundwater ranged from 2000 to 11000 mg L^{-1} but decreased in the plume. For example, at Davidson sulfate levels were lower in the plume ranging from 991 to 2900 mg L^{-1} S compared to locations located up gradient from the plume that ranged from 2620 to 7360 mg L^{-1} S and down gradient sites were in the 2000 mg L^{-1} range. Similar trends were observed at Allan, where locations with the greatest PHC impact only have sulfate levels of 1100 mg L^{-1} S, compared to areas with lower PHC impacts (4100 mg L^{-1} S), and uncontaminated areas (11,000 mg L^{-1}). At Outlook, there was limited initial groundwater data with only one location analyzed for groundwater nutrients outside of the plume and two locations inside the plume.

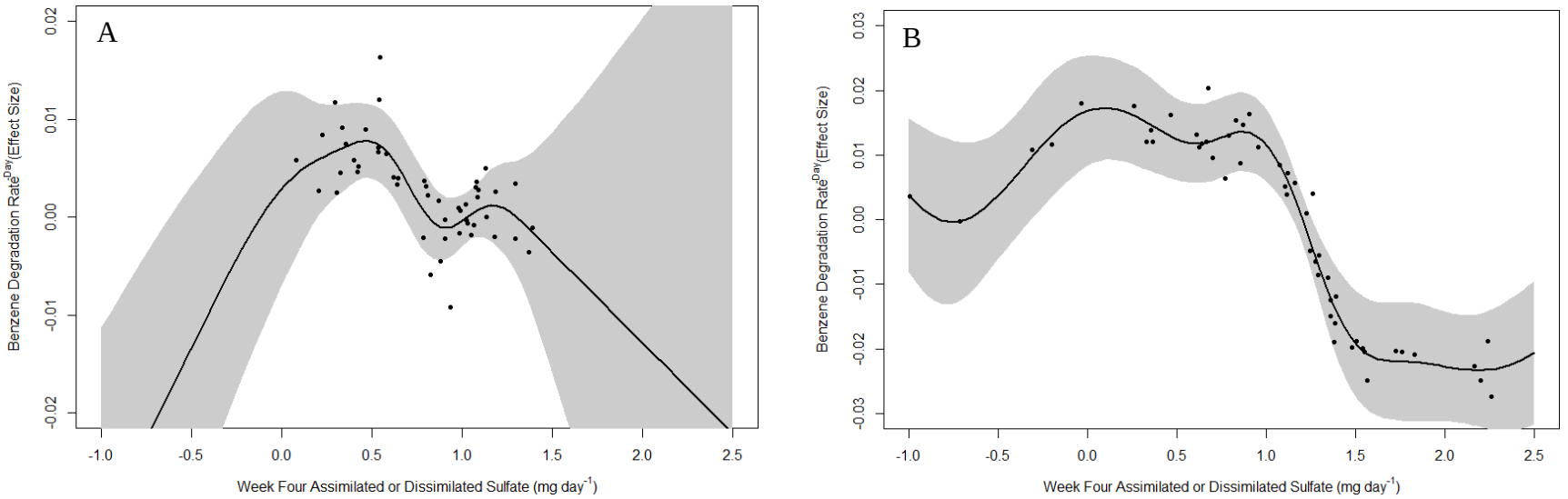


Fig. 4.2. Higher assimilated or dissimilated sulfate concentrations decreased benzene degradation rate in replicate experiments. A) Sulfate effect on benzene degradation during the C: N experiment. B) Sulfate effect on benzene degradation during the C: P experiment. The smoothing term, assimilated or dissimilated sulfate, had a significant effect on degradation ($P < 0.05$). The Y axis values are predicted values of the dependent variable as a function of the x axis centered around 0 (50/50 odds). Each point represents the observed value, the solid line is the trend after smoothing, and the grey shaded area is the standard error of the estimate.

Sodium triphosphate remaining in solution was a significant smoother for xylene and hexane degradation. In general, increased xylene and hexane degradation rates were linked to more triphosphate remaining in solution after four weeks (Fig. 4.3). This remained true at both low and high STPP concentrations in solution. At lower STPP concentrations in solution (0.00 to 2.00 mg L^{-1}), phosphate in solution ranged from 0.00 to 0.22 mg L^{-1}, with concentrations increasing with increased STPP in solution. The amount of immobilized or dissimilated P at lower concentrations (0.00 to 2.00 mg L^{-1} STPP in solution) decreased with higher concentrations of STPP in solution. For example, at 0.41 mg L^{-1} STPP in solution there was 2.42 mg L^{-1} of immobilized or assimilated P. In contrast, at 1.66 mg L^{-1} STPP in solution there was only 0.86 mg L^{-1} of immobilized P. At higher concentrations of STPP in solution (ranging from 0.00 to 88.00 mg L^{-1}), the amount of phosphate in solution ranged from 0.00 to 8.89 mg L^{-1}. With higher concentrations of STPP in solution, immobilized or assimilated P was also greater and ranged from 0.00 to 74.10 mg L^{-1}. However, unlike lower STPP concentration range, higher concentrations of STPP in solution increased immobilized and assimilated P.

Sodium triphosphate in solution had no overarching trend for benzene, F1-BTEX, F1, and toluene. In these studies, over 100 microcosms from 14 cores, and 3 sites were assessed in replicate experiments. We only considered trends ecologically significant if there were clear trends between replicate experiments. For example, the increase for benzene degradation shown in Appendix C; Fig. C.2., occurred for 50 microcosms out of a total of 100 microcosms and did not occur between experiments. Thus, we do not consider those findings ecologically robust.

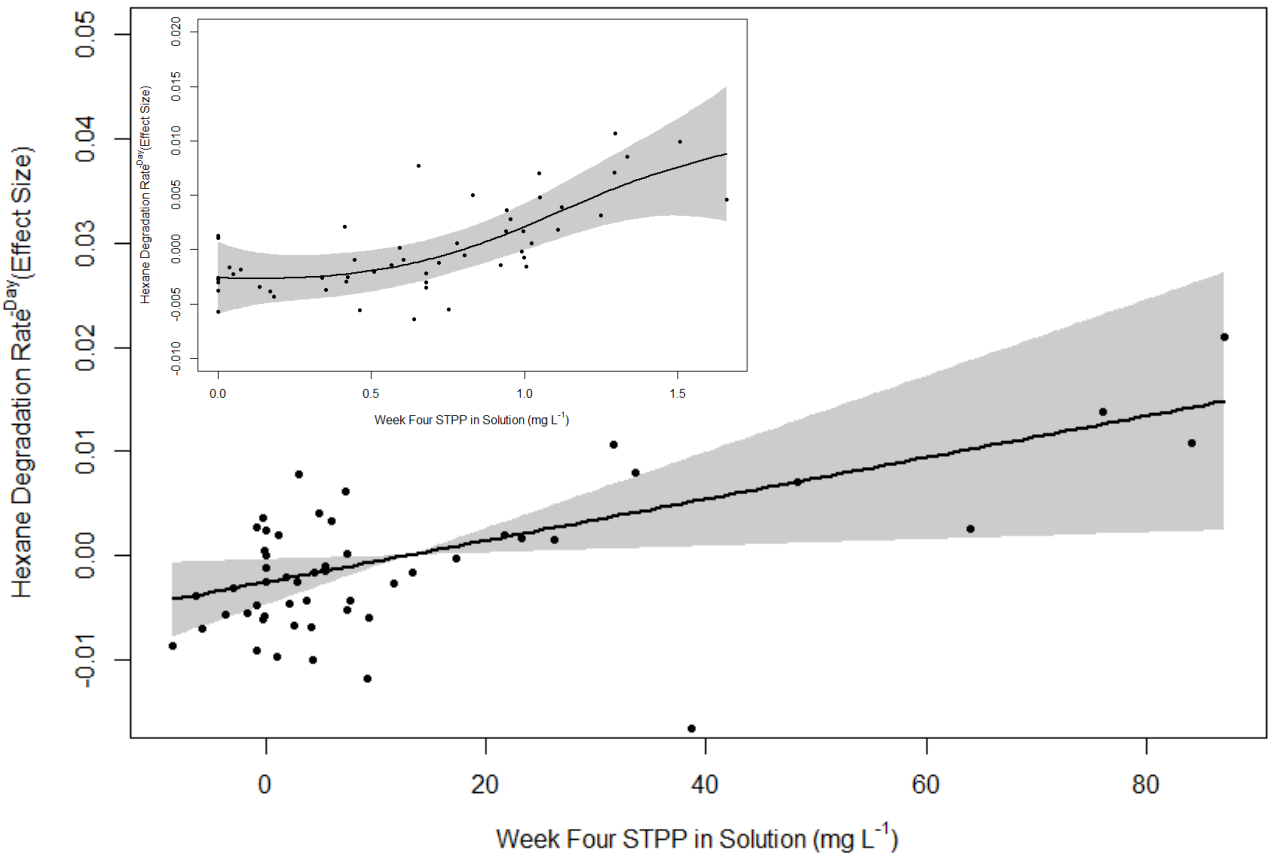


Fig. 4.3. Higher sodium triphosphate concentrations in solution increases hexane degradation rate. The insert shows STPP effect on hexane degradation at low STPP concentrations. The smoothing term, STPP in solution, had a significant effect on degradation ($P < 0.05$). The y-axis values are predicted values of the dependent variable as a function of the x-axis centered around 0 (50/50 odds). Each point represents the observed value; the solid line is the trend after four weeks.

There was a regional and borehole specific effect on degradation rate (Table 4.1). When comparing boreholes, initial benzene concentration in soil and initial Mg, S, and Fe in groundwater (Fig. 4.4) explained the majority of the variance in degradation rate. However, these chemical parameters were not as influential on the microbial community. Before amendment solution was added, bacterial composition was driven by site, borehole, and week one benzene concentration (Fig. 4.5 and Table 4.2). After the amendment solution was added, the community was structured by borehole, benzene degradation rate, experiment C: N or C: P (Fig. 4.5 and Table 4.3). The differences between the C: N and the C: P experiments was if the N or P were

left constant. Differences in the N and P experiment suggest that the microbes that are P limited are not the same microbes that are N limited. Our results suggested that some key hydrocarbon degraders such as *Geobacter*, *Desulfosporosinus*, and *Serratia* were P limited and *Acinetobacter*, *Pseudomonas*, *Desulfitobacterium*, and *Streptomyces* were N limited (Table 4.4). For example, *Geobacter* abundance increased by 20378 and *Pseudomonas* increased from 10085 to 18826 from the N to the P experiment. On the other hand, *Acinetobacter* abundance decreased from 9234 to 553 from the N to P experiment.

Table 4.1. Site and borehole significance for benzene in both (C: N and C: P) experiments.

Exp	Site	Bore	Sample depth (mbg)	Initial Soil Benzene (mg L^{-1})	Soil Lithology		Initial Groundwater Nutrients (mg L^{-1})				
					2.3- 3 mbg	Above 2.3 mbg	Nitrate	Sulfate	Phosphate (total)	Iron (total)	Magnesium
C: N	**Allan**	**1**†	2.3-3	273	Clay	Sand	1.4	4600	0.19	0.6	690
		19	2.3-3	666	Clay	Silt	3.5	7900	0.12	1.0	1400
	Outlook	**7**	1.5-2.3	444	Clay	Clay	<0.2	3600	0.53	1.74	716
		8	1.5-2.3	25	Clay	Clay	NA‡	NA	NA	NA	645
	Davidson	2	2.3-3	359	Clay Till	Clay	<0.2§	1550	0.22	14.4	283
		3	2.3-3	431	Clay Till	Clay	0.34	1570	0.69	13.5	307
		5	2.3-3	948	Clay Till	Clay	4.19	3140	0.11	10.3	675
C: P	Allan	2	2.3-3	416	Clay	Organic	0.41	2300	0.11	0.61	470
		4	2.3-3	109	Clay	Clay	<0.05	4100	0.040	0.6	710
	Outlook	**7**	2.3-3	444	Clay	Clay	<0.2	3600	0.53	1.74	716
		8	2.3-3	149	Clay	Clay	NA	NA	NA	NA	645
	Davidson	8	2.3-3	315	Clay Till	Clay	<0.2	1550	0.22	14.4	1050
		10	2.3-3	387	Clay Till	Clay	0.42	991	0.43	17.7	248
		11	2.3-3	83	Clay Till	Clay	<0.2	4170	0.08	0.25	505

† **Bold** indicates significant factors (P-value <0.05).

‡ NA indicates not applicable.

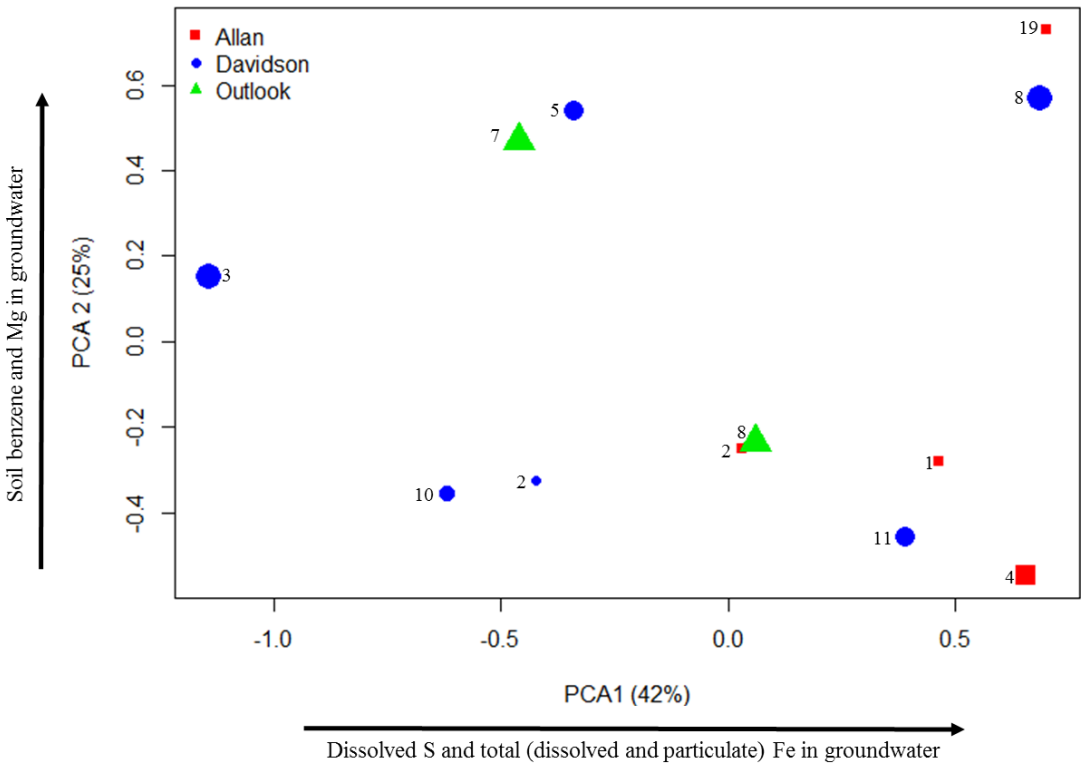


Fig. 4.4. Site scores of the first two PCAs demonstrating how initial soil and groundwater chemical characteristics differ among boreholes at each site and within sites (Scaling 1). Each point represents a borehole, different shapes and colors represent the site, and the number beside the point is the borehole number. All points are weighted on the average borehole benzene degradation rate, with larger points having greater benzene degradation. The labeling on X and Y axis demonstrates the percent variance explained by the PCA and the top two environmental factors that influence the dimensions.

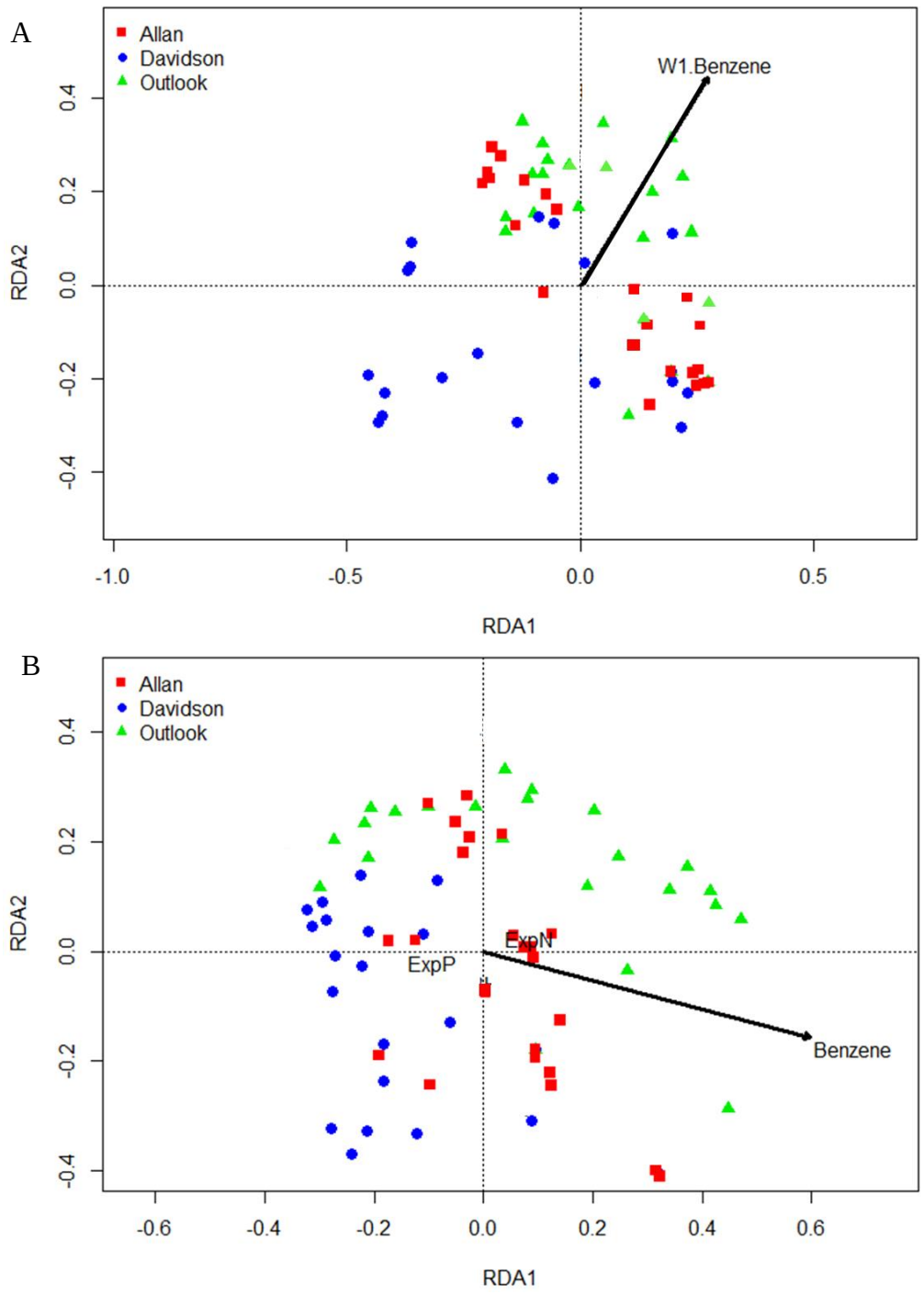


Fig. 4.5. Redundancy analysis (RDA) ordination of the bacterial community composition and other environmental properties before amendment solution (A) and after four weeks in amendment solution (B). Red square symbols represent samples from Allan, blue circle symbols represent samples from Davidson, and green triangle symbols represent samples from Outlook. The week one benzene concentration in solution is represented by W1.Benzene arrow and benzene degradation rate is represented by Benzene arrow. Experiment N and P are represented by ExpN and ExpP.

Table 4.2. Analysis of variance for week zero (before amendment solution) redundancy analysis on how environmental parameters influence the microbial community structure. [1]

	Degrees of freedom	Variance	F-value	P-value
Site	2	0.05	2.91	0.001
BH	9	0.14	1.60	0.001
W1.Benzene	1	0.02	1.69	0.038
Residual	54	0.51		

Table 4.3. Analysis of variance after four weeks of amendment addition redundancy analysis on how environmental parameters influence the microbial community structure.

	Degrees of Freedom	Variance	F-value	P-value
BH	11	0.24	2.76	0.001
Benzene degradation rate	1	0.02	2.24	0.004
Experiment	1	0.01	4.66	0.049
Residual	54	0.43		

[1] For week zero, Site, borehole and W1.Benzene had the lowest AIC's (-22.8, -20.2, and -20.26) and P-value and highest F-value as shown above. On the other hand, PCA1 and PCA2 and AIC's of -20.5 and -20.8; F-values of 1.03 and 1.41; and P-values of 0.385 and 0.125. These differences in values indicate that PCA1 and PCA2 were not trivial factors in the RDA model. Similar results were seen for week four data.

Table 4.4. List of genera that were significantly different between the C: N experiment and the C: P experiment. Negative slope values suggest genera are N limited and positive values suggest genera are P limited.

Genera	Slope (N to P)	Bonferroni Corrected P-value
Acinetobacter	-3.24	<0.01
Desulfitobacterium	-0.81	<0.01
Streptomyces	-0.81	<0.01
Pseudomonas	-0.70	<0.01
Petrimonas	-0.58	<0.01
Enterovibrio	-0.54	<0.01
Desulfosporosinus	0.57	<0.01
Geothermobacter	0.87	<0.01
Massilia	0.98	<0.01
Caenimonas	1.04	<0.01
Proteiniphilum	1.48	<0.01
Lentimicrobium	1.48	<0.01
Ercella	1.54	<0.01
Pseudoxanthomonas	1.96	<0.01
Desulfocapsa	2.58	<0.01
Geobacter	2.59	<0.01
Polaromonas	3.24	<0.01
Serratia	3.92	<0.01

The C: N and C: P ratio of the amendment solution did not alter benzene degradation rates, suggesting that fertilizer addition is not as critical for benzene degradation in comparison to other site factors. For example, in both studies for benzene the C: N and the C: P ratio was not used in the GAM as a smoothing term due to poor model fit and higher Akaike Information Criterion (AIC) values. C: N and C: P was excluded for all chemicals.

4.5.2 Chemical Selectivity within F1 Hydrocarbon Fraction

There was no chemical selectivity within F1 hydrocarbon fraction with varying C: N: P ratios. For most of the individual chemicals C: N: P was not a significant smoothing term. In addition, the nitrogen concentration in solution also did not show selective degradation of individual chemicals. In Figure 4.6 we can see in both experiments that chemicals had different degradation rates. The benzene degradation slope averaged -0.013 between both experiments that was significantly lower than remaining BTEX chemicals (0.014) which was expected since benzene is known to be harder to degrade because of the stable aromatic ring structure without any reactive substituents (Fig. 4.6).

In the C: N experiment, where lower concentrations of P were used (0 to 1.5 mg L^{-1}), N was not significant to degradation. Phosphorus and week one concentration in solution was significant to degradation and there was also a three-way interaction suggesting at low P concentration, the amount of P in solution is selective for individual compounds. At lower concentrations, we saw a positive trend in benzene degradation as we increased P in solution. The P concentration in solution seems to influence Benzene the most (0.064 degradation slope) compared to the reminder of the BTEX (- 0.065 average degradation slope) compounds and F1-BTEX (-0.012 degradation slope). In addition, P also significantly increased toluene, ethylbenzene, xylene, and hexane degradation compared to F1-BTEX degradation. For the representative chemicals, degradation rate was the greatest at high initial concentrations and higher STPP concentrations. For example, at 2 mg L^{-1} and 0.04 mg L^{-1} STPP in solution degradation raters were 0.008 $^{-Day}$. However, when initial impacts were 38 mg L^{-1} and STPP concentrations in solution were doubled (1.03 mg L^{-1}) degradation rates also doubled. On the other hand, F1- BTEX and benzene degradation increased in higher STPP concentrations in solution at both high and low initial concentrations. For example, when STPP in solution was greater than 1 mg L^{-1}, benzene degradation rates were approximately 0.04 $^{-Day}$ when the week one concentrations were 2.53 and 28.79 mg L^{-1}. Similar to the C: N experiment, in the C: P experiment, nitrogen was also not significant to overall degradation and phosphorus was significant. However, there was no difference in the degradation rate of individual chemicals based on the amount of STPP in solution.

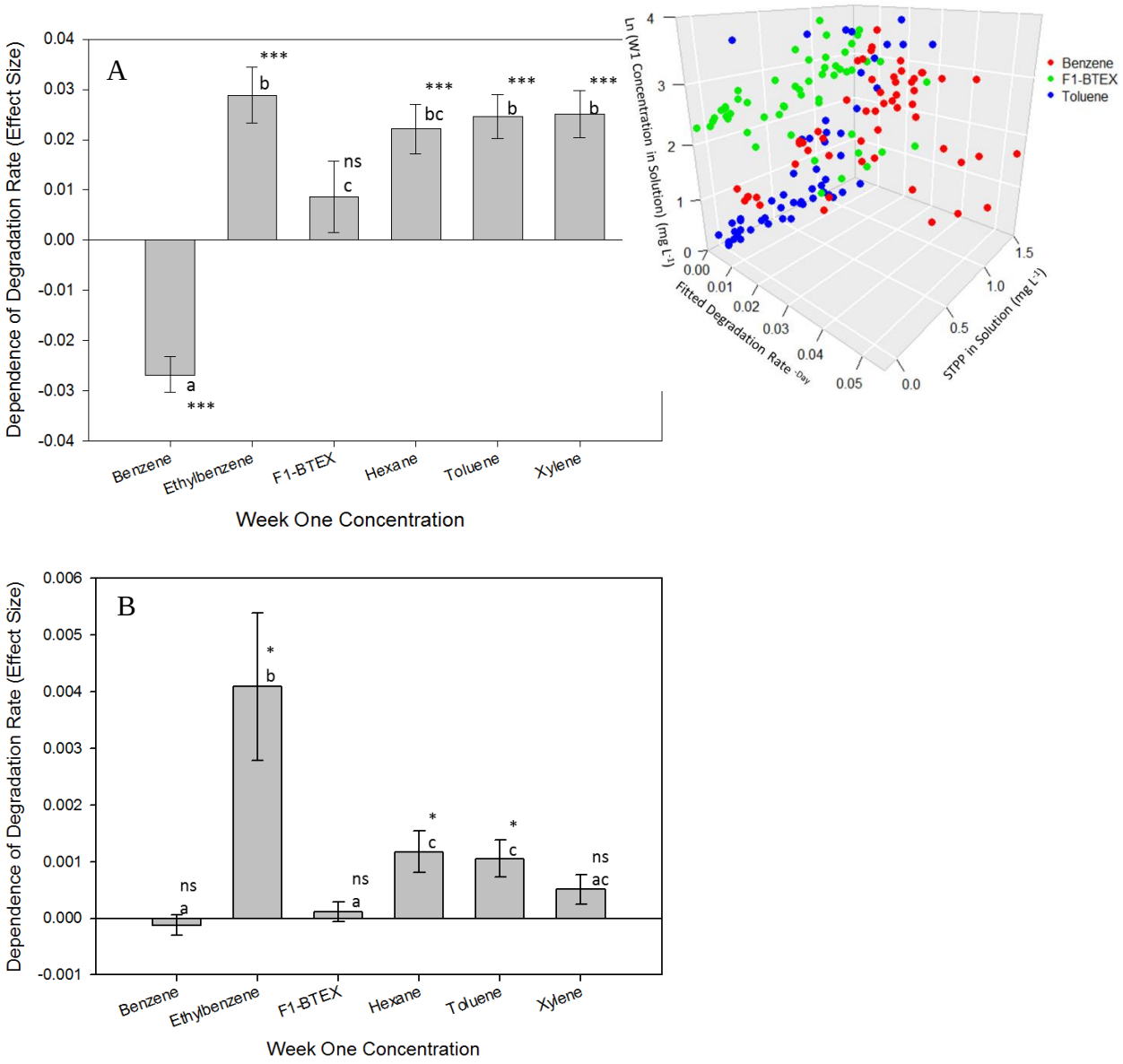


Fig. 4.6. Degradation of individual chemicals. Degradation slope for specific chemicals that make up the F1 hydrocarbon fraction for the C: N experiment (A) and the C: P experiment (B). Mean values are presented with standard error bars. Different letters indicate significant differences among groups ($p < 0.05$). The (A) inset shows the interaction of degradation rate, week 1 concentration, and Sodium triphosphate (STPP) in solution. Red circles are benzene, green circles are F1-BTEX, and toluene in blue, was used as a representative chemical for toluene, hexane, xylene and ethylbenzene. The *, **, *** at p= 0.05, 0.01 and < 0.001 indicate significant differences from zero; ns is not significant.

4.6 Discussion

For both experiments and for all of the F1 and BTEX chemicals, C: N: P ratios were not significant for degradation rate. In the general additive models, C: N and C: P ratio were often not used in the model based off of a high AIC value. In the cases were the ratio was used as a factor, it was not significant. There have been many laboratory and field studies that dertemined the nutrient concentration for biostimulation of hydrocarbon contaminated sites based on the C: N: P value (Xiong et al., 2012; Ron and Rosenberg; Chaineau et al., 2004; Mill and Frankenberger, 1994; Turgay et al., 2009; Qin et al., 2013; Zhu et al., 2001; Yerushalmi et al., 2003; Thomas et al., 1992; Zitrides, 1983; Riser-Roberts, 1998). In multiple studies, they found a ratio around 100:10:1 to be optimal for degradation (Xiong et al., 2012; Ron and Rosenberg; Chaineau et al., 2004; Mill and Frankenberger, 1994). However, within the literature there has been a wide range of reported optimal C: N ratios (10:1- 100:1) (US EPA, 1995). With this large variation, Shahi et al. (2015) as well as Walecka-Hutchison and Walworth (2006) both suggested that C: N: P ratios may not be as relevent and nutrient additioneffectiveness is dependant on site physical and chemical factors. In Margesin et al. (2007), they had two fertilizer treatments with different C: N: P ratios (62:7.4:0.7; and C: N ratio of 20:1.) the first using inorganic NPK fertilizer (containing 9.5% NH_3-N, 5.5% NO_3-N, 6.6% P_2O_5-P, and 12.2% K_2O-K) and the second using N in the form of urea emulsified with oleic acid and P in the form of lauryl phosphate; and found no significant differences between the treatments. This suggests that as long as some nutrients are added, the concentration may not be as critical as previously thought. This study, is in agreement that site factors are more influential than nutrient concentration and that as long as some nutrients are added degradation will occur. The enviromental factors that were measured in this study that influenced degradation were initial concentration, sulfate in solution, and STPP in solution.

Initial contaminant concentration was a key driver influencing degradation rate. Previous studies have also found that initial concentration influences degradation rate due to hydrocarbon toxicity to the microbial community (Margesin et al., 2006; Margesin et al., 2000; Nocentini et al., 2000). In this study we found decreased benzene degradation, suggesting hydrocarbon toxicity at concentrations above 10 mg L^{-1} benzene. However, unlike other literature, we found that as F1-BTEX, hexane, toluene, and xylene concentrations increased, degradation rate also increased. The differences in degradation rate with increased concentration is likely due to the

difference in magnitude of the impacts. In this study, we separated and treated the chemicals within the F1 fraction as separate entities. In most literature that analyzes hydrocarbon toxicity, they only report total petroleum hydrocarbons (TPH) which includes all of the F1 fraction and F2-F4. Additionally, most literature analyzed much higher concentration rages compared to this study. For example, Margesin et al. (2007) analyzed a range of 2500 to 20000 mg L^{-1} compared to our study where the highest individual chemical concentration was 50 mg L^{-1}. Margesin et al. (2007) also found that lower initial concentrations (2500 mg kg^{-1}) were less influenced by nutrient addition and lower TPH removal compared to samples with higher initial concentrations (20000 mg kg^{-1}) (Margesin et al., 2007). We also found that nutrient addition was more important when hydrocarbon contamination was higher.

Higher STPP in solution increased xylene and hexane degradation in the C: N and the C: P experiments but did not influence the other F1 and BTEX chemicals. This increase in degradation with higher STPP concentration in solution may be due to the amount of P staying in solution vs being precipitated or absorbed. In Hamilton et al. (2018), when adding STPP as an amendment, labile P precipitated especially at lower concentrations (~15-20 mg P L^{-1}). Once absorbed, STPP is not readily desorbed, but microbes can hydrolyze and cleave P from linear and poly-P, making P available to the microbial community (George et al., 2007). Even though STPP does absorb, it does not form CaP minerals until after hydrolysis (Hamilton et al., 2018). A greater amount of absorbed P compared to CaP minerals is significant because for biostimulation, absorbed P is the preferred species and since the formation of CaP minerals can significantly lower P availability to microbes (Siciliano et al., 2016; Hamilton et al., 2018). A greater amount of STPP in solution after four weeks suggests that STPP has already saturated mineral surface absorption sites and more STPP in solution P resulted in more available P to the microbial community compared to the only absorbed P.

In this study, we also found that the initial concentration of sulfate groundwater and the sulfate concentration remaining in the amendment solution significantly influenced degradation. Sulfate-reducing bacteria are well-known anaerobic gasoline degraders (Phelps and Young, 1999; Philp et al., 2002; Townsend et al., 2004) but typically degrade gasoline at lower rates compared to nitrate or iron-reducing bacteria. In boreholes where sulfate was higher in the groundwater, there were lower degradation rates. For example, Allan 19 and Allan 1 had sulfate

concentration of 7900 and 4600 mg L^{-1} and average benzene degradation rates of 0.024 and 0.021 day^{-1}, while Davidson 3 and 8 had groundwater sulfate concentrations of 1570 and 1550 mg L^{-1} and had average degradation rates of 0.042 and 0.034 day^{-1}. These results suggest that higher initial sulfate concentrations in groundwater can negatively influence degradation. The concentration remaining of sulfate remaining in amendment solution also follows this pattern. We found as more sulfate assimilated or dissimulated that degradation decreased. This might suggest that in samples were more the microbes are using sulfate as the main electron acceptor that degradation rate is lower. This might be a simple explication since degradation is known to be slower under sulfate-reducing conditions compared to aerobic, nitrate- reducing, iron-reducing, and Mn (IV) - reducing conditions. Additionally, other researchers have also found lower degradation rates under sulfate-reducing conditions, due to a significant lag period before remediation occurs (Edwards, et al., 1992, Morasch, et al., 2004, Morasch & Meckenstock, 2005).

Throughout statistical analysis we found site and borehole to be significant factors. This suggests that site and within site characterizes are influencing degradation. As mentioned previously, there has been multiple studies stating that biostimulation is site dependent (Shahi et al., 2015; Walecka-Hutchison and Walworth, 2006). Knowing that site and borehole location was significant, we ran a PCA that included borehole chemical and physical properties. The characteristics that drove the differences in boreholes were initial soil benzene concentration and S, Fe, and Mg in groundwater. As mentioned previously, the initial contaminant concentration and S in groundwater has been shown in this study and others to influence degradation rate.

Concentrations of Mg initially on site can also play a role in degradation rates. High concentrations of Mg in soil may inhibit degradation since phosphate has a strong tendency to adsorb to Mg minerals resulting in low solution concentrations (Persson et al., 2012; Makie et al., 2013). Manimel Wadu et al. (2013) stated that the Ca to Mg ratio could largely influence the availability of P in soils that in turn influences degradation. The types of CaP minerals formed, and the solubility are largely influenced by the presence of Mg and Mg- bearing carbonate minerals (Hamilton et al., 2018). High concentration of Mg may also indicate the presence of phosphate minerals such as newberyite. Siciliano et al. (2016) demonstrated that these minerals can decrease degradation and can alter community composition.

The bacteria community composition before and after amendment solution was largely structured by differences in boreholes. Before the amendment solution was added, the environmental parameters that influenced the microbial community structure included site, borehole, and week one benzene concentration in solution. From the previous PCA results, we discovered that the main differences in boreholes were initial sulfur and iron in groundwater (PCA1) and Mg in groundwater and initial soil benzene concentration (PCA2). However, the RDA suggested that both PCA1 and PCA2 were not significant in structuring the microbial community. It is likely that there is a mineralogy effect and a benzene or TPH effect structuring the microbial communities. Previous literature suggested that mineral thermodynamic stability influences soil genotypes (Carson et al., 2009; Hemkemeyer et al., 2014; Siciliano et al., 2014) and the overall mineralogy largely influences genotypes and phenotypes (Siciliano et al., 2016).

The total concentration of hydrocarbons, particularly benzene as our results suggest, may also be influencing the microbial community. Contaminant concentration influences microbial composition (MacNaughton et al., 1999; Margesin et al. 2007). Hydrocarbon contaminated soils have a significantly different microbial community composition compared to soils that have low or no impacts due to general toxicity (Shi et al., 2002; Bundy et al., 2004). The soils used in this study have a wide variation of starting benzene concentration ranging from 25 to 948 mg L^{-1}. The bacteria community within higher impacted areas tend to be less diverse then lower impacted areas because the organisms present in the impacted area need to be able to use and survive toxic contamination (MacNaughton et al., 1999). In Margesin et al. (2007) they found when comparing initial TPH concentrations of 2500 and 10000 mg kg^{-1} soil, that initial concentration significantly influenced the microbial community and was more influential compared to fertilization treatment and incubation time. This study showed similar results, suggesting that initial impacts significantly affect the microbial community composition and that amendment solution was not a determining factor.

After four weeks of amendment solution the community was structured based on borehole, benzene degradation rate, and if the N or P was constant (experiment C: N or C: P). The difference in microbial community composition based on benzene degradation rate may suggest that in areas where more benzene is being degraded, that it is lowering hydrocarbon toxicity and allowing a more diverse community. On the other hand, it may suggest that in

samples that had more benzene degradation vs less that there are more aromatic degraders present. As previously discussed, previous literature has stated contaminant concentration largely impacts microbial community composition (Shi et al., 2002; Bundy et al., 2004; MacNaughton et al., 1999; Margesin et al., 2007). Additionally, some literature has linked hydrocarbon concentration with known hydrocarbon genera (Margesin et al., 2003). For example, Margesin et al. (2003) found a positive correlation with increasing contamination and the abundance of *Acinetobacter* and *Pseudomonads*.

Structuring based on C: N vs C: P study suggests that the genera that are N limited are not the same as the genera that are P limited. Our results suggested that some key hydrocarbon degraders such as *Geobacter*, *Desulfosporosinus*, and *Serratia* were P limited and *Acinetobacter*, *Pseudomonas*, *Desulfitobacterium*, and *Streptomyces* were N limited. *Acinetobacter* and *Pseudomonads* are genera that tend to do well in nutrient rich environments and genera such as *Streptomycetes* tend to do well in resource-limited environments (Margesin et al., 2003). Although, research has showed that the addition of nutrients, particularly N and P, can help increase and stimulate microbial communities, there is limited research on N and P limitations of known hydrocarbon degrading genera.

In this study, we found the individual F1 fraction chemicals degraded at different rates based on the initial contaminant concentration and STPP concentration in solution. There was no chemical selectivity within F1 hydrocarbon fraction with varying C: N: P ratios. In multiple other studies, there had been evidence that different nutrient concentrations are needed for the different hydrocarbon fractions (Braddock et al., 1999; Chaineau et al., 2004). Chaineau et al., 2004 suggested that C: N: P ratios should be calculated based on saturated hydrocarbon concentration since that are the most sensitive to nutrients. In our study, we found no difference in degradation of benzene, toluene, xylene, ethylbenzene, hexane and F1-BTEX with different C: N: P ratios. However, we found at lower STPP concentration that P concentration added was more influential on benzene > toluene, ethylbenzene, xylene, and hexane > F1-BTEX. The chemical specific influence of P suggests that aromatic chemicals may be more sensitive to P concentrations then saturated compounds. Fayad and Overton (1995) found similar results, suggesting preferential aromatic degradation with higher nutrient addition. On the other hand, Chaineau et al., 2005 found that higher nutrients limit the assimilation of aromatics. Further

research is needed to assess if the microbial community is behind the differing nutrient concentrations needed for optimal degradation.

5.0 DEGRADATION RECOVERY AFTER EUTROPHIC CONDITIONS DURING BIOSTIMULATION AT HYDROCARBON IMPACTED SITES

5.1 Preface

In Chapter 3, we developed a bench scale microcosm design that more accurately stimulates contaminate properties such as contaminate heterogeneity. Using this design, in Chapter 4 we were able to start analyzing how nutrients, specifically C: N: P ratios affect degradation, the microbial community, and chemical selectivity of the F1 hydrocarbon fraction. In Chapter 4, we found that initial site and borehole factors were more significant than the C: N: P ratio. We also found the C: N: P ratio added was not a driving factor for why individual BTEX and F1 chemicals degraded at different rates. This chapter focuses on the individual BTEX and F1 chemicals and how high nutrient rates effect degradation and if rates recover after high nutrient additions.

5.2 Abstract

High nutrient concentration can negatively impact the degradation rates by increasing the prevalence of non-degrading microbes in the microbial community. By understanding how degradation responds to eutrophic conditions, we can make recommendations on how to continue site remediation on sites where excess nutrients are added. Our overall objective was to determine if degradation rates can recover after a site has been exposed to eutrophic conditions. We created a microcosm design that mimicked field conditions at each field site. Microcosms were prepared in groups of two and then randomly assigned for destructive sampling at four or eight weeks. Five treatments were then applied to each group of two. The treatments were: 1) eutrophic conditions for four weeks, followed by four weeks in optimal concentration, 2) eutrophic conditions for four weeks, followed by four weeks in un-amended water, 3) uncontaminated soil in eutrophic conditions for four weeks, followed by four weeks in optimal conditions 4) un-amended water for eight weeks, 5) optimal nutrient conditions for eight weeks. The un-amended water was city tap water that was UV sterilized and dechlorinated. Soils were incubated under nitrate and sulfate-reducing conditions for four weeks at 10°C and amendment solutions were replaced weekly. Each week the amendment solution was analyzed for BTEX and F1. In the first four weeks, there was lower BTEX degradation in eutrophic conditions compared to the optimal treatment. After week four, when solutions were changed to optimal nutrient

concentrations, samples that were previously exposed to eutrophic conditions continued to have a lower degradation rates in comparison to samples in the optimal nutrient amendment for the full eight weeks. However, samples that had excess nutrients then were put in un-amended water had the highest degradation rates. Our results suggest that increasing nitrate and phosphate additions to sites will inhibit degradation. In situations, where this has occurred, injection systems should be reset with background water concentrations to allow remediation rates to recover.

5.3 Introduction

The form and concentration of N and P during biostimulation can present a challenge to environmental consultants and industry partners. The fertilizer concentration and form that works on one site may not work on another site, presenting room for error (Shahi et al., 2016). One error that can take place is adding too high of nutrient concentration based on a site's soil water content, texture, mineralogy, and electrical conductivity (Mohn and Stewart, 2000; Peltola et al., 2006; Walecka-Hutchison and Walworth, 2007; Siciliano et al., 2016). Other scenarios where over fertilization may occur are errors where excess concentrations were added due to mechanical failure or logistical mistakes, limited site knowledge of groundwater and soil chemistry leading to an over-estimation of fertilizer requirements, or purposefully adding excess nutrients in a vain attempt to re-start a site where remediation has stalled.

Over fertilization can lead to multiple outcomes depending on the severity. In the worst-case scenario, over application of N can lead to nitrate plumes. The high solubility of nitrate in combination with high application rates can lead to leaching into groundwater. Nitrate in drinking water is important from a human risk point of view since nitrate levels above 10 mg L^{-1} can cause methemoglobinmea (also known as blue baby syndrome) (Knobeloch et al., 2000). Excess N can also reduce microbial populations and lower activity of hydrocarbon degraders due to osmotic stress (Walecka-Hutchison and Walworth, 2007, Mohn and Stewart, 2000; Peltola et al., 2006; Walworth et al., 2007; Braddock et al., 1997). According to Walecka-Hutchison and Walworth (2006) the optimal N concentration is below 1950 mg N kg^{-1} soil H_2O. Above 1800 mg N kg^{-1} soil H_2O concentration they found up to a 50% decrease in microbial respiration. Over application can decrease or stall degradation rates (Siciliano et al., 2016; Braddock et al., 1997; Ferguson et al., 2003; Fayad and Overton, 1995; Walecka-Hutchison and Walworth, 2007;

Mohn and Stewart, 2000; Peltola et al., 2006; Walworth et al., 2007). For example, Walecka-Hutchison and Walworth (2006) found hydrocarbon degradation two times less in samples with 1000 mg N kg^{-1} soil (2540 mg kg^{-1} diesel degraded) compared to samples with 250 mg N kg^{-1} soil (5130 mg kg^{-1} diesel degraded). Siciliano et al. (2016) reported that excess P decreases the amount of hydrocarbon degradative genes due to the formation of inhibitory minerals. They found that excess P increase precipitation of Ca and/ or Mg phosphate minerals such as brushite and newberyite.

Although many studies have focused on degradation rates and over nutrient application, few studies have focused on if degradation rates are able to recover after high nutrient conditions are applied. According to literature, the microbial community can be severally altered by high nutrient conditions that in turn influences degradation rates (Shahi et al., 2016; Chaineau et al., 2005; Smith et al., 1998; Margesin and Schinner, 2001; Rubertoa et al., 2003; Lee et al., 2007; Liu et al., 2011). Shahi et al. (2016) stated that bacteria are able to adapt to high nutrient conditions, but it can cause significant changes to the pre-existing bacterial community. For example, in multiple fertilization studies, the microbial composition was not able to return to its original composition even years after the initial nutrient addition (Allison and Martiny, 2008). It is important to remedial efforts to determine if degradation rates and microbial communities can recover and how to move forward with site remediation after these events.

In this experiment, we elevated nutrient levels to a "eutrophic rate". Eutrophic conditions are defined as nutrient abundance and accumulation within a body of water that supports sense growth of organisms which when they decompose depletes oxygen. In the context of this thesis, we use eutrophic or eutrophic conditions to describe an amendment solution that is very high in nitrogen and phosphorus, thus promoting abundant microbial growth. We applied a eutrophic amendment for four weeks to determine effects on degradation rate. For the nitrogen concentration we used 3000 mg N kg^{-1} soil H_2O since previous literature recommended maintaining N_{H2O} levels below 1800 mg N kg^{-1} soil H_2O (Walworth et al., 2007). Additionally, throughout literature the lowest level that microbial inhibition was observed was at 2500 mg N kg^{-1} soil H_2O (Walworth et al., 1997; Braddock et al., 1997; Braddock et al., 1999; Mohn and Stewart, 2000; Ferguson et al., 2000). When creating the eutrophic amendment solution, we ensured the solution had a similar electrical conductivity compared to the site groundwater to

decrease degradation effects from osmotic stress since other soil salts can contribute to osmotic stress (Haines et al., 1994; Rhykerd et al., 1995). After four weeks we replaced the eutrophic amendment solution with either tap water or optimal amendment solution (C: N: P = 12: 8.4: 1) to determine if the degradation rates were able to recover after being exposed to high nutrient conditions. To determine how degradation rates changed after each week, the microcosms were put into new solution and the old solution was analyzed for BTEX and F1.

5.4 Materials and Methods

5.4.1 Sample Collection and Microcosm Creation

Soil samples were collected from multiple bulk transfer and gasoline stations with known spill and leak history within Davidson, Outlook, and Allan Saskatchewan, Canada. During Phase II Assessment, additional F1 and BTEX samples were collected every 0.5 to 0.75 m to determine initial F1 and BTEX concentrations. Sample collection and storage of duplicate boreholes can be found in Chapter 3.4.1. Microcosms were assembled as described in Chapters 3.4.1 and 3.4.5. For each sample, two soil microcosms were taken within very close proximity and treated as the same sample. Both microcosms were given the same amendment solution and were sampled weekly for F1 and BTEX in solution. After four weeks one of the microcosms were destructively sampled for soil microbiology and the other microcosm continued to receive an amendment solution for an additional four week until it was also destructively sampled at week eight. All microcosms were given a sample identification number that corresponded to the site, borehole, and treatment number (associated identification numbers and site information can be found in Appendix D; Table D.1). The treatments included:

- Contaminated soil in eutrophic conditions for four weeks, followed by four weeks in the optimal C: N: P ratio.
- Contaminated soil in eutrophic conditions for four weeks, followed by four weeks in tap water.
- Uncontaminated soil in eutrophic conditions for four weeks, followed by four weeks in the optimal C: N: P.
- Contaminated soil in tap water for eight weeks.

- Contaminated soil in optimal C: N: P for eight weeks.

There were five replicates per treatment. Time one and two microcosms received the initial eutrophic conditions or tap water treatment and incubated in the same conditions as the previous experiments for four weeks. The treatment solution was replaced weekly by putting the microcosm into new solution and the stale solution was analyzed for F1 and BTEX. The time two microcosms after the four-week incubation received optimal C: N: P ratio or tap water and be incubated for another four weeks. The incubation occurred under the same conditions and the treatment solutions were replenished weekly and analyzed for F1 and BTEX.

5.4.2 Amendment Solutions

For the optimal C: N: P ratio we used 12: 8.4: 1. For the high concentrations/ eutrophic conditions we used 15 mM P and 3000 mg N kg^{-1} (a C: N: P of approximately 0.8: 1.1: 1). These concentrations were chosen for the eutrophic conditions because at concentrations higher than 15 mM P solutions were precipitating and the EC in solution was greater than the groundwater on site. For eutrophic N conditions 3000 mg N kg^{-1} was used because according to Walworth et al., 1997, 2007 and Walecka-Hutchison and Walworth, 2006, after 2500 mg N kg^{-1} there is evidence of microbial inhibition.

5.4.3 Analytical Methods

F1 and BTEX concentrations were measured as described in Chapter 3.4.4.

5.4.4 Statistical Techniques

A mixed linear model was used to determine differences between treatments and for the individual BTEX and F1 chemicals. Within the model, site and borehole were treated as random effects and the amendment solution treatment was the fixed effect. All tests were declared significant at $p < 0.05$. Statistical analyses were performed using R (R Core Team, 2016).

5.5 Results

Eutrophic (high for full eight weeks) amendment solutions significantly hindered degradation for all the BTEX chemicals compared to the optimal treatment. For example, the average toluene and xylene degradation rates were twice as high, and benzene and ethylbenzene degradation rates were 1.5 times greater in the optimal treatment compared to the eutrophic

treatment. For the aliphatic chemicals, there was no significance in degradation rate between the high and optimal amendment solutions. The significant difference in aromatic compounds may suggest that microbial community responsible for aromatic degradation is more sensitive to higher nutrient concentrations than the community that degrades aliphatic compounds.

When the eutrophic amendment solutions were switched to either tap water or optimal conditions only ethylbenzene, xylene, and hexane were able to recover to rates similar to the degradation rates seen with optimal nutrient conditions (Fig 5.1.). For ethylbenzene and xylene, only samples that were switched to tap water were able to recover (Fig 5.1.). For example, the average degradation rates in the high to tap treatment were twice as high compared to the high to optimal treatment. For toluene, even after eutrophic amendment solution was switched to either tap water or optimal conditions, the degradation rates were not able to recover (Fig 5.1.). The inability to recover to optimal degradation may suggest a change in the microbial community or nutrient toxicity. Benzene degradation rates in the eutrophic amendment solution were also unable to recover after switching to tap water or optimal conditions (Fig 5.1.). However, when continuing to add nutrients to the system (high to optimal) degradation rates were significantly lower than the eutrophic amendment and the eutrophic to tap water treatments (Fig 5.1.).

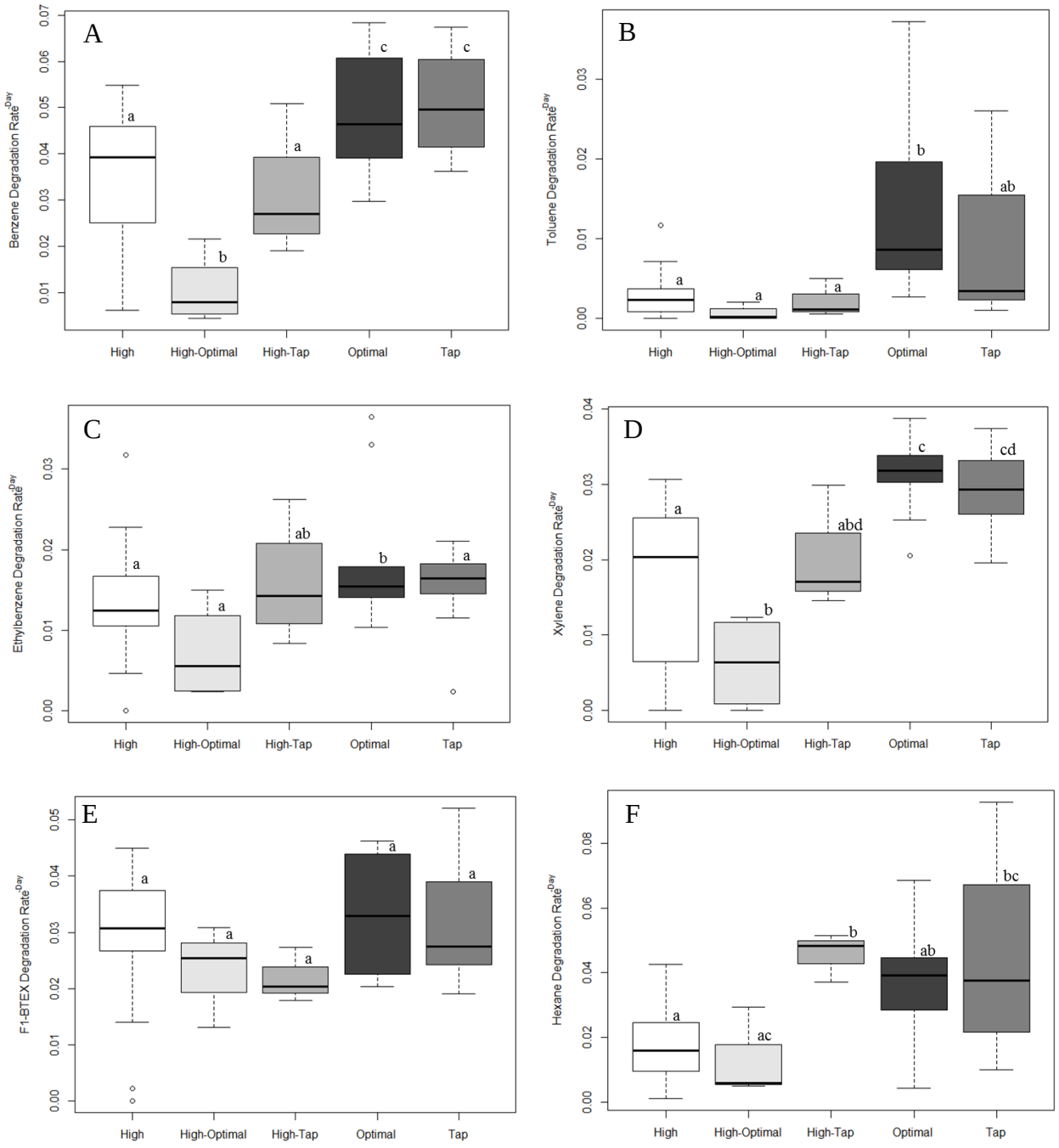


Fig 5.1. Degradation rates for different treatments. A) benzene, B) toluene, C) ethylbenzene, D) xylene, E) F1-BTEX, and F) hexane. Different letters indicate significant differences ($p < 0.05$). The horizontal line is the median and the bottom and top of each box shows the 25th and 75th percentiles. Outliers (points considered to be 1.5 times the interquartile range) are plotted individually. When there are no outliers present, the whiskers show the maximum and minimum values. The optimal, tap and high treatment degradation rates are from week one to eight and the high-optimal and high- tap are from weeks five to eight with the first four weeks of high taken into account. The corresponding C: N: P ranges for the optimal and high treatments were: 12: 8.4: 1 and 0.8: 1.1: 1.

5.6 Discussion

Over the first four-week period, where samples were placed in a eutrophic amendment solution, all BTEX chemicals had significantly lower degradation rates compared to samples in the optimal amendment solution. For instance, the benzene half-life for the high treatment was 32.3 days and the optimal treatment was only 18.6 days. In microcosms and field trials, degradation decreased under high nutrient conditions. (Braddock et al., 1997; Walworth et al., 2007; Ferguson et al., 2003; Fayad and Overton, 1995; Mohn and Stewart, 2000; Peltola et al., 2006; Walecka-Hutchison and Walworth, 2007; Atagana et al., 2003; Margesin and Schinner, 2001; Rubertoa et al., 2003; Lee et al., 2007). For example, aromatic degradation was decreased by 20% under elevated nutrients compared to the low nutrient amendment (Chaineau et al., 2005), similar to the decrease we saw here of 16%.

Different nutrient concentrations are needed for aliphatic and aromatic compounds based on the microbial communities that are responsible (Carmichael and Pfaender, 1997; Chaineau et al., 2005; Bell et al., 2011). For example, Chaineau et al. (2005) suggested different nutrient levels are required for optimal aliphatic and aromatic chemical degradation. They specifically found that higher nutrients limit the assimilation of aromatics. However, Fayad and Overton (1995) found the opposite, suggesting preferential aromatic degradation with higher nutrient additions. In our previous results (Chapter 4.5.2), we determined that phosphorus concentrations in solution were a determining factor for hydrocarbon selectivity (different degradation rates among the BTEX and F1 chemicals). For example, we found that, P significantly increased toluene, ethylbenzene, xylene, and hexane degradation compared to F1-BTEX degradation. We also found that the concentration of P influenced benzene degradation the most compared to the other chemicals.

The differences in the individual chemical degradation and recovery may be driven by the sensitivity of the bacterial community that degrades each chemical. Here, after high nutrient conditions were introduced, only ethylbenzene and xylene degradation recovered after solutions were switched to tap water, but not when samples were switched to the optimal amendment. Additionally, even though rates were not able to recover for benzene and toluene, samples that were switched from eutrophic conditions to tap water had better degradation rates then samples that were switched the optimal treatment. The degradation recovery when switching to tap water

is likely driven by nutrient dilution and in turn, lower osmotic stress. For instance, P concentrations were dilution by a factor of 2,600,000 when switching from high to tap water, but when switching to the optimal treatment the dilution was only 11 to 83 (range is due to the different concentrations used for the C: N: P ratio). In contrast to the tap water, switching to optimal conditions seemed to hinder degradation rates even further. For example, at Allan, the average benzene degradation rate in the high treatment after four weeks was 0.030 $^{-Day}$ and after switching to the optimal treatment, the average degradation rate decreased to 0.018 $^{-Day}$. The continued decrease might be because switching to the optimal treatment continues to add nutrients to the system causing even further osmotic stress and super-saturation of different phosphate minerals that in turn, negatively effects the hydrocarbon degrading microbial community and genotype (Siciliano et al., 2016).

In this study, there were large differences in initial BTEX and F1 chemical concentrations. For example, the average concentration across all the sites for F1-BTEX was 19.33 mg L^{-1} and for ethylbenzene, it was 2.66 mg L^{-1}. There were also large variations of starting concentration among the different sites and boreholes. For example, the maximum starting benzene concentration across all the samples was 72.32 mg L^{-1} (Allan Borehole 2) while the lowest concentration was only 0.15 mg L^{-1} (Allan Borehole 4). Different initial concentration of individual chemicals can affect the overall degradation rate. We saw in Chapter 4.6 (Fig. 4.6 and Appendix C; Fig. C.1) that higher concentrations (up to 50 mg $^{L-1}$) of hexane, toluene and F1-BTEX can increase degradation rates. We also found benzene degradation rates increased until 10 mg L^{-1}, and at higher concentrations, there was evidence of toxicity. In the statistical analysis, we accounted for differences in starting concentration in the boreholes by adding in sites and boreholes as a random factor in the mixed linear model. Both site and borehole were significant factors in all the chemical models ($p < 0.05$).

Overall, the nutrient concentration altered degradation and degradation recovery in different manners for the BTEX and F1 chemicals. We found that high nutrient concentrations hindered degradation in only the BTEX compounds. We also found that when switching from high treatments to optimal conditions that degradation stalled and continued to decrease. In high samples switched to tap water, we found degradation rates were only able to recover for ethylbenzene and xylene, which may be due to the soil microbial communities responding

differently to the biostimulatory treatments. It can also be noted that the tap water treatment had similar degradation rates to the optimal treatment for all of the chemicals. Future directions will include exploration on why tap water seems to have such a positive effect on degradation.

6.0 SYNTHESIS AND CONCLUSIONS

6.1 Summary of findings and conclusions

Approximately 600,000 tons of crude oil is released into the environment per year, making petroleum hydrocarbons some of the most widespread contaminants in the world (Rohrnacher and St-Arnaud, 2016). Federated Cooperative Ltd. (CO-OP) estimated that within the next 20 years that 700 CO-OP sites in Western Canada may need remediation at a cost of approximately $350 million. Just within a 200 km radius of Saskatoon, there are over 49 bulk fuel plants CO-OP is remediating. Bioremediation is an attractive remediation technique because it is cost effective, environmentally friendly, has a low carbon foot print, requires less labor, and reduces site disruption allowing sites to retain their social and business functions. However, there are multiple factors that can limit bioremediation such as nutrients (particularly N and P), PHC degrading microorganisms, and hydrocarbon bioavailability (McGuinnes and Dowling, 2009; Schwitzguebel et al., 2011) There is desire to come up with a biostimulatory solution that can be used across sites.

The use of C: N: P ratios when making amendment solutions not only adds in essential nutrients, but also considers the hydrocarbon concentration (C amount) at the site. Being able to adjust the N and P concentration based on the impact level makes it universal. However, in literature there is a very wide range on what is considered the optimal ratio. The research presented in thesis helps to fill some of these knowledge gaps. The general goal of this research was to assess how C: N: P ratios effect degradation and degrader prevalence and how this varies at and within sites with a new laboratory microcosm design that more accurately stimulates field conditions in cold clayey soils. In addition, we also assessed if degradation was able to recover after eutrophic conditions were added as the amendment solution.

In Chapter 3 we developed a bench scale microcosm design that more accurately stimulates contaminate properties such as contaminate heterogeneity. Soil duplicate boreholes using a direct push rig were collected at each impacted site. From these duplicate cores we were able to take sub-samples using PVC pipe to keep the soil intact and not disrupt any contaminant properties. The use of an intact soil core taken from the impacted site is a good option to test amendment solutions before field trials and can mimic field conditions more closely than other bench scale microcosms. Additionally, using this design we were able to sample hydrocarbons in

solution every week instead of using destructive sampling methods. We were also able to correlate the concentration in solution to the soil hydrocarbon concentration. We previously found when using samples directly from the field, that the hydrocarbon impacts were extremely heterogeneous, which limited us in achieving an initial hydrocarbon concentration. By sampling the hydrocarbons in solution each week, we found a correlation (using a log transformation) to determine the starting concentration in soil. We were able to apply this microcosm design to analyze C: N: P ratios (Chapter 4) and to determine if degradation rates can recover to optimal rates after eutrophic conditions are applied.

Chapter 4 identified that initial soil characteristics are more influential than the concentration of N and P added in the amendment solution. Soil characteristics that influenced degradation included initial contaminant concentration and concentration of STPP remaining in solution. However, trend and the extent that these factors influenced degradation rates were chemical dependent. We also found that degradation was largely dependent on borehole location. The initial site properties that drove these differences in degradation were soil benzene concentration and Mg, S, and total Fe in groundwater. The microbial community before and after four weeks in the amendment solution was also driven by borehole location. However, the initial site properties that drove the chemical differences between boreholes did not also drive the microbial composition differences. Before the amendment solution was added, the RDA suggests microbial community is structured based by site, borehole, and initial soil benzene concentration. There are likely other soil chemistry factors such as mineralogy that was outside the scope of this project. After the soils were given amendment solution, the communities were structured by borehole, benzene degradation rate, and experiment (N or P). The differences in N and P experiments suggest that there were some genera that were P and N limited. Our results suggest that some key hydrocarbon degraders such as *Geobacter*, *Desulfosporosinus*, and *Serratia* were P limited and *Acinetobacter*, *Pseudomonas*, and *Streptomyces* were N limited. Chapter 4 also addressed if there was hydrocarbon selectivity occurring with different C: N: P ratios. We found that C: N: P ratios did not cause chemical selectivity within the F1 fraction. Instead, we found selectivity was caused from initial concentration of each chemical and the amount of STPP in solution.

Chapter 5 addressed the ability of degradation rates to recover to optimal after eutrophic conditions were used for four weeks. The results suggest that the ability to recover after eutrophic conditions are introduced are chemical dependent. Similar to other research, we found adding high nutrient concentrations significantly hindered degradation when compared to the optimal nutrient ratio, this was especially prevalent in the BTEX chemicals. After four weeks in eutrophic conditions, the high nutrient amendment solution was replaced with either tap water or the optimal solution for an additional four weeks. At the end of eight weeks, we were able to compare the high- to –optimal and the high- to –tap water treatment to samples that were in optimal conditions for the full eight weeks. Samples that were switched to the optimal solution after eutrophic conditions had the lowest degradation rates compared to all the treatments. Furthermore, switching to optimal continued to decrease the degradation rate. When switching to tap water from high nutrient conditions hexane, xylene and ethylbenzene degradation rates were able to recover to degradation rates similar to rates seen in optimal or tap water four the full experiment duration. However, degradation rates were not able to recover for benzene and toluene. The inability for benzene and toluene being unable to recover may be related to the microbial community. It is well known that different microorganisms are responsible for the different chemical degradation of the F1 compounds (Widdel and Grundmann, 2010; Grossi et al., 2008; Callaghan et al., 2008; So et al., 2003; Grundmann et al., 2008; Coates et al., 2001; Fries et al., 1994; Dolfing et al., 1990; Zhou et al., 1995; Song et al., 1999; Amders et al., 1995; Shinoda et al., 2004; Evens et al., 1991; Shinoda et al., 2005; Lovley et al., 1993; Coates et al., 2001; Morasch et al., 2004; Harms et al., 1999; Rabus et al., 1993). The effect of high nutrients could be more influential on the communities that degrade toluene and benzene. Additionally, higher toluene and benzene may be more toxic to the microbial population then the other F1 chemicals.

The results of these bench scale laboratory studies highlight that there is no silver bullet when it comes to adding a biostimulatory solution for site remediation. More specifically, we have found that as long as nutrients are being added, but not too much, the site will have degradation occurring, and the rate is more dependent on-site characteristics. In conclusion, I believe this study contributes a substantial progress in site-specific biostimulation. The bench scale microcosm approach developed in Chapter 3 also aids in improving research tools for those

attempting to test innovative biostimulation amendments prior to field trials and is already being used in multiple laboratory studies at the University of Saskatchewan.

6.2 Future research

This body of research provided a first look at how optimal amendment solutions vary by site and even within the site. The Canadian prairies are known for glacial till with varying soil properties and each spill has different chemical characteristics such as concentration, mixture ratio, and how long the contaminant has been there. These factors affect how efficient the amendment solution is on degradation rate. Although this study highlights how environmental characteristics may be more important than the amount of nutrients added, understanding how the optimal amendment solution changes for each site based on soil chemical, physical, and biological properties is crucial for deciding if biostimulation is the right remediation choice for a particular site.

Multiple site studies with similar and dissimilar soil chemical and physical characteristics are needed to examine how optimal nutrients may change by site and how to factor these characteristics in while making site remediation decisions. In the current study, we included three sites from southern Saskatchewan that had varying soil characteristics. It would be ideal to expand the number of sites to include more of the Canadian prairies, for example sites within the Alberta and Manitoba prairie region. When selecting sites, it would also be ideal to have sites that have similar and dissimilar soil chemical, physical, and biological characteristics within each region. Additionally, in future studies, the C: N ratio would need to be expanded.

Site characterization during Phase I and II Environmental Site Assessment may be a valuable tool in deciding if biostimulation is the best remedial option and to determine how effective an amendment solution will be for a site. However, it is time consuming and expensive to do a thorough site characterization for each site. Completing detailed site investigations in the end may save time and money because environmental consultants and industry partners will be able to determine if biostimulation will be successful on a particular site and to get a better estimate on site closure.

Another objective from this chapter was to determine if C: N: P ratios are selective for certain hydrocarbon fractions. We found the C: N: P ratio were not selective, but the initial

concentration of the chemical and P in solution effected degradation rate differently for the F1 chemicals. Previous literature has stated that different P concentrations are needed for aromatic vs aliphatic compounds and that hydrocarbon toxicity can vary depending on the chemical. Expanding the study to include more of the hydrocarbon fractions such as F2 – F4 would increase the scope of work and help determine if these trends are also consistent in the heavier hydrocarbon fractions.

Additionally, this body of research also provided a look at how high nutrient concentrations effect degradation rate and if degradation rates can recover after high nutrient levels are added. In Chapter 5 we found that recovery was different for the F1 compounds; however, we do not have a complete understanding of why these differences may be occurring. Future studies should focus on why degradation was able to recover and why were there differences among the F1 chemicals. Literature suggests that there are different microbial communities that degrade the F1 chemicals. By analyzing the microbial community through time, one may be able to get a fuller understanding of why the chemicals differ in recovery.

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8.0 APPENDICES

Appendix A. Site Maps Indicating Location of Boreholes

Fig. A.1. Allan site map with borehole locations (adapted from Stantec Consulting Ltd.). Impacted borehole locations used for experiments are outlined in red.

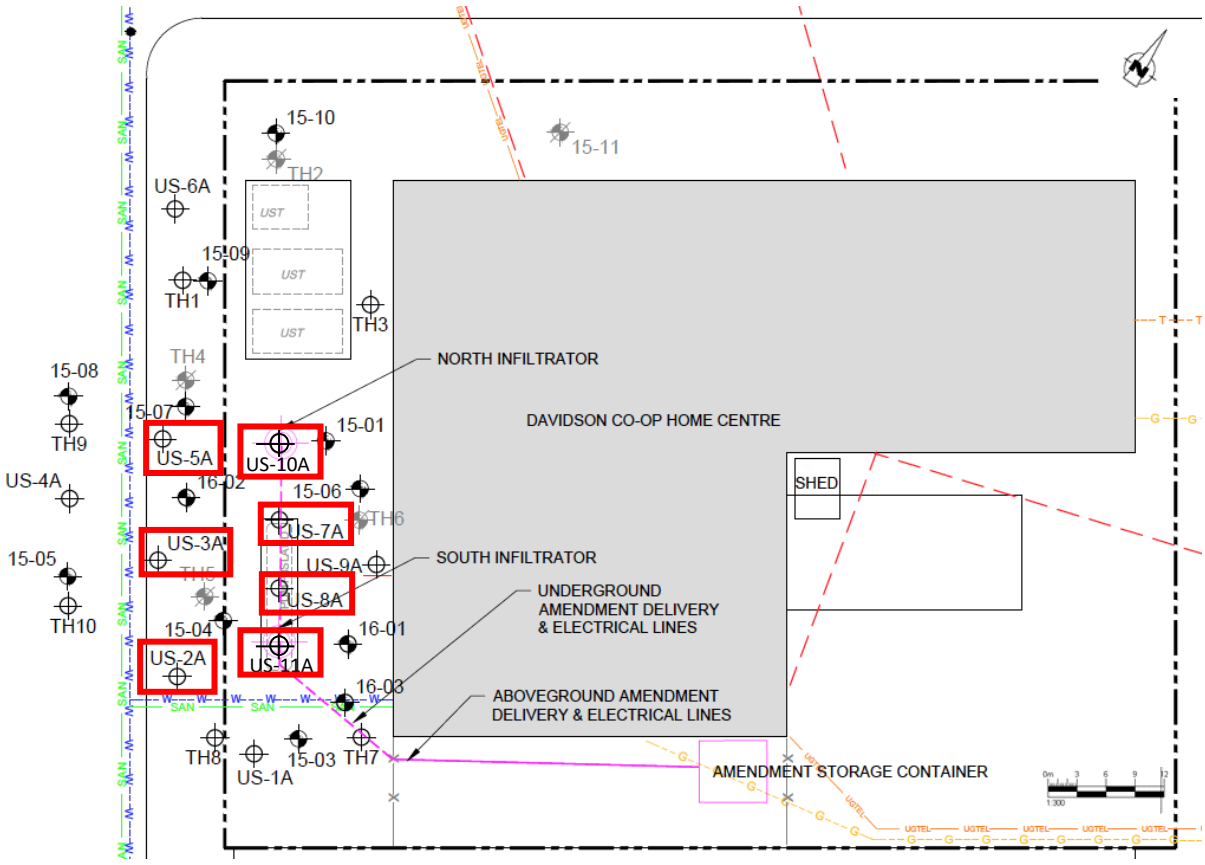

Fig A.2. Davidson site map with borehole locations (adapted from Wood plc). Impacted borehole locations used for experiments are outlined in red.

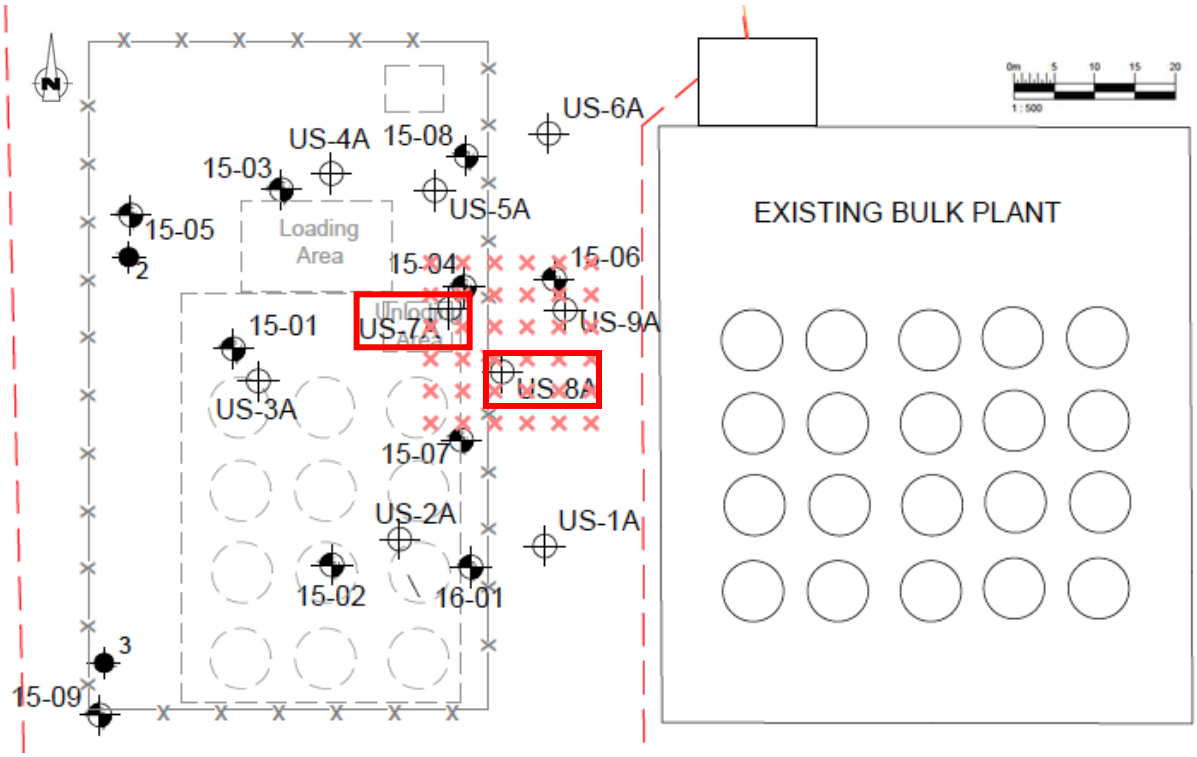

Fig A.3. Outlook site map with borehole locations (adapted from Wood plc). Impacted borehole locations used for experiments are outlined in red.

Appendix B. Amendment Solutions and Samples for Microbial Analysis

Table B.1. C: N samples chosen for microbial analysis and associated site factors.

Sample	C: N	Site	Borehole	W1 Benzene Concentration (mg L-1)
31	1.2-2	Outlook	7	10
32	1.2-2	Outlook	7	22
38	1.2-2	Outlook	7	14
39	1.2-2	Outlook	7	19
55	1.2-2	Davidson	5	6
56	1.2-2	Davidson	5	14
77	1.2-2	Allan	19	9
78	1.2-2	Allan	19	33
81	1.2-2	Allan	19	28
83	1.2-2	Allan	19	28
84	1.2-2	Allan	19	21
33	2.2-2.5	Outlook	7	15
34	2.2-2.5	Outlook	7	21
37	2.2-2.5	Outlook	7	17
57	2.2-2.5	Davidson	5	15
58	2.2-2.5	Davidson	5	15
59	2.2-2.5	Davidson	5	11
79	2.2-2.5	Allan	19	16
80	2.2-2.5	Allan	19	29
82	2.2-2.5	Allan	19	19
74	1.3	Allan	1	5
40	1	Outlook	8	5
45	0.7	Outlook	8	5
43	0.7	Outlook	8	18
46	1	Outlook	8	6
47	1	Outlook	8	15
49	0.7	Davidson	3	16
50	0.7	Davidson	3	8
53	1	Davidson	3	14
54	0.7	Davidson	3	11
68	0.8	Allan	1	11
75	0.8	Allan	1	1
61	1.3	Davidson	2	2
63	1.3	Davidson	2	2
70	1.4	Allan	1	3
76	0	Allan	1	3

Table B.2. C: P samples chosen for microbial analysis and associated site factors.

Sample	C: P	Site	Borehole
48	0	Outlook	7
60	0	Davidson	10
66	0	Davidson	11
76	0	Allan	2
85	0	Allan	2
83	0.7	Allan	4
38	7	Outlook	7
47	73	Outlook	8
49	3	Davidson	8
77	3	Allan	4
36	20	Outlook	7
40	221	Outlook	8
55	9	Davidson	10
70	9	Allan	2
81	2	Allan	4
41	295	Outlook	8
50	12	Davidson	8
65	12	Davidson	11
72	13	Allan	2
80	13	Allan	4
35	39	Outlook	7
39	39	Outlook	7
54	18	Davidson	8
61	19	Davidson	11
82	4	Allan	4
45	738	Outlook	8
34	65	Outlook	7
62	29	Davidson	11
63	29	Davidson	11
67	29	Allan	2

Appendix C. Chemical Factors Influencing Degradation

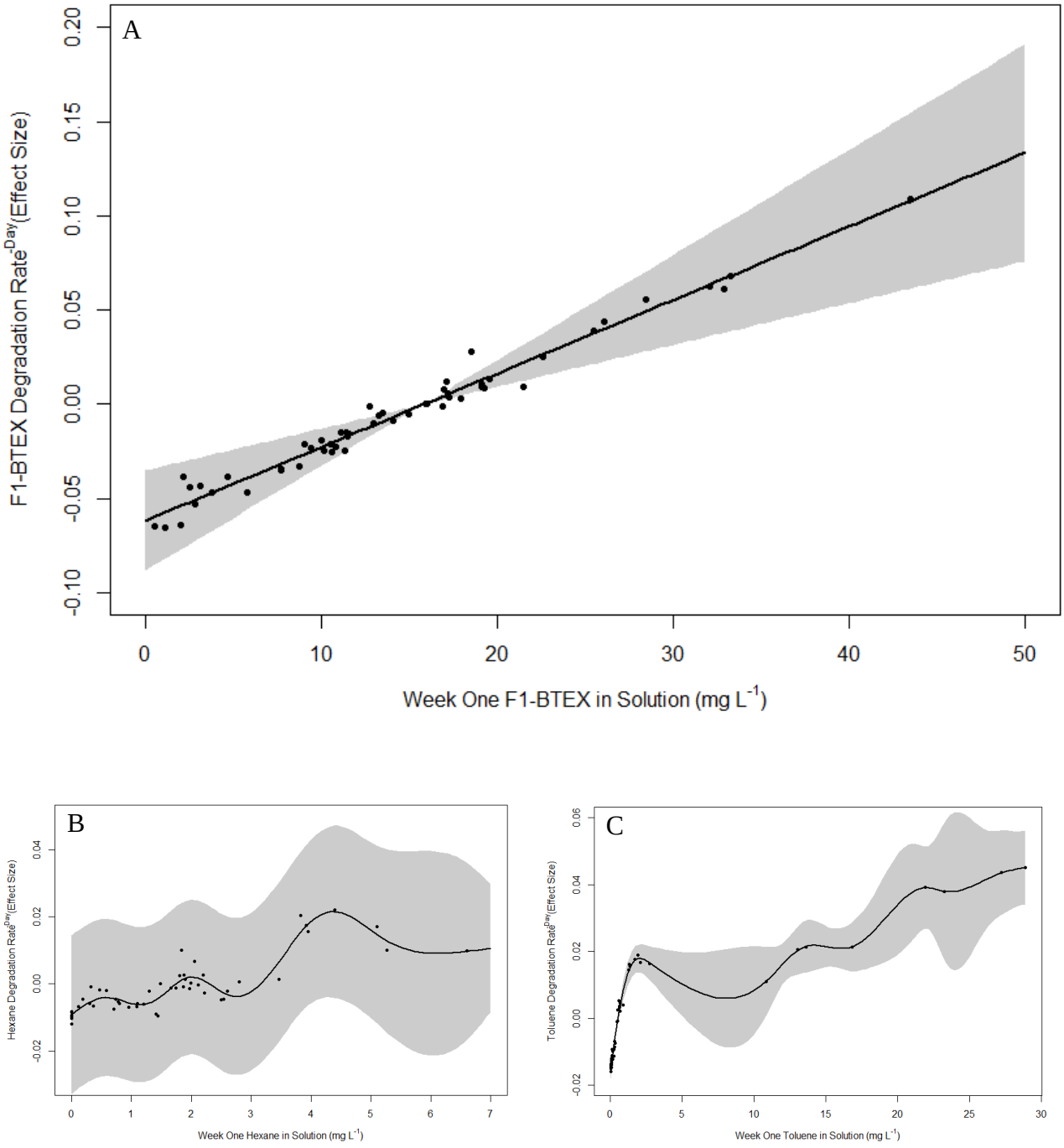

Fig. C.1. Initial concentration influence on degradation (effect size). A) Initial F1-BTEX concentration effect on degradation. B) Initial hexane concentration effect on degradation. C) Initial toluene concentration effect on degration. The smoothing term, initial impacts (F1-BTEX, hexane, and toluene), had significant effects on degradation ($P < 0.05$).The Y axis values are predicted values of the dependent variable as a function of the x axis centered around 0 (50/50 odds). Each point represents an observed value, the solid line is the trend after smoothing, and the grey shaded area is the standard error of the estimate.

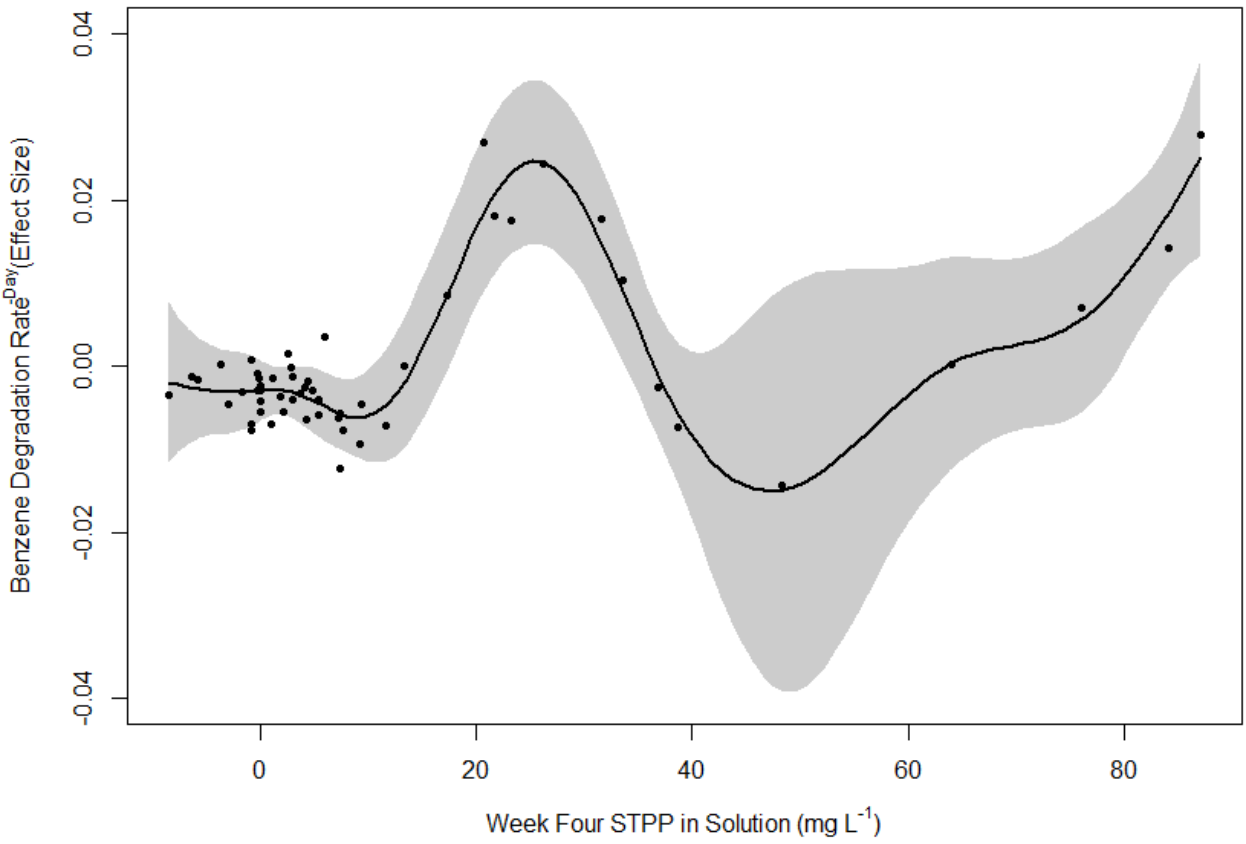

Fig. C.2. Higher sodium triphosphate concentrations in solution alters benzene degradation rate. The smoothing term, STPP in solution, had a significant effect on degradation ($P < 0.05$). The Y axis values are predicted values of the dependent variable as a function of the x axis centered around 0 (50/50 odds). Each point represents the observed value, the solid line is the trend after smoothing, and the grey shaded area is the standard error of the estimate.

Appendix D. EUTROPHICATION SAMPLES

Table D.1. Associated site, borehole, and treatment for each microcosm.

Samples	Site	Borehole	Treatment
1, 2†	Allan	2	2
3, 4	Allan	2	4
5, 6	Allan	2	5
7, 8	Allan	4	1
9, 10	Allan	4	2
11, 12	Allan	4	1
13, 14	Allan	18	5
14, 16	Allan	18	4
17, 18	Allan	18	2
19, 20	Allan	18	1
21, 22	Allan	18	4
23, 24	Davidson	7	1
25, 26	Davidson	7	5
27, 28	Davidson	7	5
29, 30	Davidson	7	2
31, 32	Davidson	8	1
33, 34	Davidson	8	4
35, 36	Davidson	8	5
37, 38	Davidson	10	2
39, 40	Davidson	10	4
41, 42	Allan	1	3
43, 44	Allan	1	3
45, 46	Allan	1	3
47, 48	Davidson	4	3
49, 50	Davidson	4	3

†Sample are grouped into pairs; all odd numbered samples were destructively sampled after week four and all even samples were destructively sampled after week eight.