ABSTRACT

ASSESSMENT OF TOLUENE BIODEGRADATION ACTIVITY IN GROUNDWATER FROM A SHALLOW BEDROCK AQUIFER UNDERLYING A PHYTOREMEDIATION SYSTEM

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Researchers have been striving to understand and model environmental groundwater remediation to facilitate case-specific decision-making and optimize remediation. This has proven difficult due to the highly site-specific nature of the processes affecting contamination fate and transport in natural environments. This study aims to contribute to the science for decision-making processes and improve our understanding of the role of microbes in groundwater remediation.

To achieve this, depth-discrete samples were obtained over three seasons from a toluene-contaminated, shallow, fractured bedrock aquifer underlying a phytoremediation system in Southwestern Ontario. Quantitative PCR was used to quantify abundance and potential activity of aerobic and anaerobic toluene degrading communities in groundwater. Active aerobic and anaerobic toluene degraders were present across the Site in all sampling events. Abundance and activity shifts were associated with both season and redox conditions, but not statistically by toluene concentration.
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ABBREVIATIONS

ATP = adenosine triphosphate
BP = British Petroleum
BSS = benzylsuccinate synthase
bssA = benzylsuccinate synthase gene
BTEX = benzene, toluene, ethylbenzene, and xylene
cDNA = complementary deoxyribonucleic acid
CL = confidence limit
CMT = continuous multichannel tubing
COC = contaminant of concern
Cq = quantification cycle
CSIA = compound specific isotope analysis
CSM = conceptual site model
DFN = discrete fracture network
DNA = deoxyribonucleic acid
DNQ = detectable but non-quantifiable
DO = dissolved oxygen
Eh = oxidation reduction potential
EPA = Environmental Protection Agency
GC-FID = gas chromatography with flame ionization detection
HGU = hydrogeologic unit
LNAPL = light nonaqueous phase liquid
LSCq = Cq values of the lowest dilution of the standard curve in a qPCR
m amsl = meters above mean sea level
m bgs = meters below ground surface
MLS = multilevel monitoring system
ND = non-detect
NSERC-CRD = Natural Sciences and Engineering Research Council of Canada-Collaborative Research and Development Grant
ORC = oxygen release compound
ORP = oxidation-reduction potential
PHE = phenol hydroxylase gene
QA = quality assurance
QC = quality control
qPCR = quantitative polymerase chain reaction
RMO = ring hydroxylating monooxygenase gene
RNA = ribonucleic acid
TCA = tricarboxylic acid
ToMO = toluene/o-xylene monooxygenase
T2MO = toluene 2-monooxygenase
T3MO = toluene 3-monooxygenase
T4MO = toluene 4-monooxygenase
VOA = volatile organics analysis
VOC = volatile organic compound
16S = bacterial rRNA
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CHAPTER 1.0 General Introduction

1.1 FOREWORD, SITE HISTORY AND CONTEXT

1.1.1 Foreword

This thesis represents a portion of the much larger scoped research collaboration funded by a Natural Sciences and Engineering Research Council of Canada- Collaborative Research and Development Grant (NSERC-CRD) with BP, the University of Guelph and the G360 Institute for Groundwater Research, and the University of Waterloo as industrial partners and collaborators. The focus of this collaboration was to produce a robust and comprehensive Conceptual Site Model (CSM). Multiple lines of evidence were used to confirm and assess the attenuation mechanisms at play within the Site of interest and assess factors influencing toluene plume behaviour in a shallow, fractured bedrock system within an urban environment. This system was located under a phytoremediation system where, below the reach of the trees for remediation in groundwater, intrinsic microbial activity was expected to be responsible for biodegradation of toluene. Groundwater flow and recharge, microbial activity, and activity of the phytoremediation system thus all influence contaminant remediation throughout the entire system. Multiple scientific approaches were therefore required for comprehensive site-characterization. A detailed analytical approach was taken to create a robust bank of data and use this to build a depth-discrete, high resolution CSM. The strength of this study lies in the multiple lines of evidence from a variety of disciplines in collaboration and allows us to achieve a more comprehensive understanding of the Site and Site characteristics. The information provided in this CSM is vital to risk assessment and management at the Site and the information obtained could potentially be applied to similar sites in the context of remediation management. The main goals of producing the CSM and assessing the efficacy of the phytoremediation system exceed the scope of focus.
for this thesis, although they may be referred to throughout. Analyses of total microbial community and toluene degrader abundances and activities which are the primary foci of this thesis constituted one line of evidence applied to accomplish the overarching project goals. The primary emphasis will be on groundwater and the bacterial degradation of toluene.

1.1.2 Site History and Context

This research focuses on a small site (approximately 25 m by 13 m) located in Southwestern Ontario. The overburden at the Site, consisting of sandy, cobbled soil, descends to a depth of approximately two meters (Fernandes, 2017). This is where the bedrock interface can be found as well as the groundwater surface which typically sits at or a little below (up to approximately 0.8 m lower) the bedrock interface. Annual fluctuations in groundwater level typically do not exceed two meters and conditions have previously found to be generally reducing (Fernandes, 2017). The fractured bedrock is comprised of Silurian Era dolostone possessing a porous matrix. The fracture network on the Site is dominated by primarily horizontal fractures that occur at highest frequency in the upper levels of bedrock down to approximately six meters below ground surface. Fracture intensity decreases with increasing depth (Figure 1.1) While vertical fractures are slightly more frequent in the upper bedrock, those found lower down between approximately 6 and 23 meters below ground surface (m bgs) in the subsurface profile are longer (Figure 1.1) (Fernandes, 2017). Frequency of vertical fractures may have been underestimated since only vertical boreholes were used for fracture characterization. The subsurface is divided into three defined hydrogeologic units (HGU’s) that are identified by contrasting vertical conductivity in groundwater (Figure 1.1). Characterizing HGU’s and conductivity was beyond the scope of this study, though was discussed by Fernandes (2017). A valuable freshwater aquifer contained within these bedrock formations supplies water to over 500,000 residents in Southwestern Ontario (Aqua Resource, 2010).
Figure 1.1 Bedrock cores from MW22 provide an example of the horizontal and vertical fracture distributions in bedrock at the Site. Hydrogeologic Units (HGU) are identified by contrasting vertical hydraulic conductivity (Fernandes, 2017). Red lines highlight the locations and lengths of the observed vertical fractures. Samples analyzed for this study spanned depths within only HGU 1 and the upper levels of HGU 2. The blue line indicates the last run of bedrock core that corresponded to depths sampled in this study. (Figure modified from Fernandes, 2017).
The Site is the historical location of a manufacturing facility that operated from 1952 until 1989. Toluene was used as an industrial solvent and was stored along with other industrial compounds including methanol and isopropyl alcohol on-site in partially buried storage tanks. The chemicals were fed directly into the building by a set of pipes buried in the overburden. Around the time the facility closed in the late 1980’s, toluene contamination was discovered in the storage tank and pipeline area. The storage tanks were removed and contaminated soils and the upper bedrock in the immediate vicinity of the tanks were excavated. To characterize the toluene plume in groundwater, consultants installed a suite of single-interval conventional monitoring wells with screen widths ranging from 1.5 – 3 m. These and other wells were later used from 1990 to 2000 in a pump-and-treat remediation effort to remove and treat the contaminated groundwater. This effort was eventually terminated because of decreasing returns and the concern that a proximal, unrelated contaminant plume may be affected. In 2003, an Oxygen Release Compound (ORC) pilot trial was initiated in the hopes that oxygen would stimulate aerobic contaminant-degrading bacterial activity and increase biodegradation. Though some reductions in toluene concentration were observed close to the well where the ORC was passively delivered, the method was only deemed somewhat effective and this approach too, was discontinued. By 2008 a phytoremediation system was implemented as an alternative means of contaminant containment and attenuation. Fifty-one hybrid poplar trees (Populus x canadensis) were planted within the historical location of the storage tanks and pipelines. In 2014, the poplar trees were deemed mature and hypothesized to be exerting maximal potential effects on the contaminated system, and a collaborative research program was established to examine the efficacy of phytoremediation to meet the site management objectives. Therefore, in 2015, 7 multi-level sampling wells with 7 short sampling intervals (ports) ranging from 0.6 to 1.3 m wide
were installed at the Site targeting areas surrounding the predicted toluene plume (Summary of sampling intervals of the wells studied for this thesis can be found in Table B.2 in Appendix B). Four additional multi-level wells were added in 2016.

1.2 LITERATURE REVIEW

1.2.1 BTEX in the Environment

In recent decades, research has focused on developing and optimizing efficient, cost-effective remediation techniques to counteract industrial pollution (Collins et al., 2002). Industrially-applied chemicals and organic compounds are significant pollutants impacting land productivity and the health of individuals and the environment. One such group of chemicals including benzene, toluene, ethylbenzene and xylene, is referred to as BTEX. These compounds are volatile and therefore are part of the larger group of volatile organic compounds (VOCs). BTEX are monocyclic aromatic hydrocarbons and can be found in fuels (particularly gasoline), solvents and other products including plastic, paint and adhesives (An, 2004; Collins et al., 2002). Burning fuel and volatilization emit contamination into the atmosphere while below-ground storage tanks, pipelines, waste sites and detention containers often become direct point-sources of subsurface contamination through leakage or dumping followed by seepage (Baehr et al., 1999; Do et al., 2011). In Canada alone, excluding accidental spills, more than 5900 tonnes of BTEX were released into the environment in 2013 according to the Government of Canada’s National Pollutant Release Inventory (NPRI, 2014).

Toluene has been frequently used in the past as a representative of BTEX (Barac et al., 2009; Kümmel et al., 2013; Reusser et al., 2002). The site of interest (the Site) for this study is subject to only toluene contamination and future discussion of this case study will focus on this specific contaminant of concern (COC) in groundwater. Toluene has a lower density than water
Figure 1.2 A 3-Dimensional model of the structure of a toluene molecule. Bonds between the carbon atoms (black) alternate between single and double bonds. The cyclical nature of the molecule leads to delocalization of electrons, indicated by the dotted line, and increases stability of the molecule so more energy is required for the degradation process. Hydrogen atoms are shown in white.

Figure 1.2 A 3-Dimensional model of the structure of a toluene molecule. Bonds between the carbon atoms (black) alternate between single and double bonds. The cyclical nature of the molecule leads to delocalization of electrons, indicated by the dotted line, and increases stability of the molecule so more energy is required for the degradation process. Hydrogen atoms are shown in white.

Although evidence is insufficient to characterize it as a carcinogen to humans, toluene exposure can cause irritation of the eyes and respiratory tract and is suspected to have toxic effects including structural and functional abnormalities in the brain, neuropsychological impairment, and potentially embryotoxic effects (Agency for Toxic Substances and Disease Registry, 2014; Do et al., 2013; WHO, 1986; Yücel et al., 2008). The hazards associated with environmental BTEX contamination necessitate development of effective techniques to monitor and attenuate contaminant plumes.

(specific density = 0.867 g cm\(^{-3}\), specific density of water = 1.0 g cm\(^{-3}\)) and tends to float on water. Although toluene has low solubility, it is more soluble than other hydrocarbons (solubility = 515 mg L\(^{-1}\) (Newell et al., 1995).

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Toxic effects are also possible in local microbial communities even in resilient toluene-degraders in groundwater at sufficiently high concentrations and may cause decreases in abundance and activity (Huertas et al., 1998; Huertas et al, 2000; Inoue & Horikoshi, 1989; Lueders, 2017; Sikkema et al., 1995). The hydrophobic nature of toluene molecules allows them to partition into bacterial membranes and disrupt membrane integrity producing toxic effects by disrupting osmotic equilibrium and destroying control on movement of compounds in and out of the cell (Inoue & Horikoshi, 1989; Sikkema et al., 1995). Toluene-tolerant bacteria utilize thick cell walls and lipopolysaccharide layers, efflux systems, and cis- to -trans isomerization of fatty acids in the membranes to prevent toxic effects, although even tolerant bacteria experience toxicity with varying concentrations (Huertas et al, 2000; Isken & de Bont, 1996; Sardessai & Bhosle, 2002; Weber et al., 1994).

1.2.2 Factors Affecting Contaminant Distribution and Remediation

BTEX are classified as light nonaqueous-phase liquids (LNAPLs). LNAPLs have a lower density than water and thus float on top when the liquids are mixed. Due to this difference in density, LNAPL contaminants tend to be retained at the capillary fringe and at the groundwater surface (Newell et al., 1995). These liquids can be completely immiscible or, like BTEX, they can have low levels of solubility (Newell et al., 1995). Toluene specifically has a water solubility of 515 mg L\(^{-1}\) at 20°C which is relatively high compared to other LNAPLs and allows toluene to be distributed more widely in a contaminant plume (Newell et al., 1995). In subsurface plumes, LNAPL distribution is affected by many factors including fracture networks and matrix porosity in bedrock, water table fluctuations, sorption, and phase transfers between liquid, aqueous, and gaseous phases (Hardisty et al., 1998; Hardisty et al., 2003; Lipson et al., 2005; Parker et al., 1994; Tomlinson et al., 2017). These factors may also make it difficult to monitor attenuation of
a plume by measuring contaminant concentration alone. Dilution or dissolution of the contaminant, as well as sorption, may produce misleading results.

In addition to attenuation of contaminant plumes exerted by factors like sorption and dilution, microbial communities also represent a significant mechanism of degradation. Factors affecting the viability, activity, abundance, and composition of microbial communities in contaminated groundwater thus also influence remediation. These include substrate availability, dissolved oxygen, redox conditions, temperature, salinity, pH and even competition amongst organisms.

Previous research has indicated that dissolved contaminants are often most bioavailable to bacteria since microbes typically take up substrate in the aqueous phase (Sikkema et al., 1995; Zhang et al., 1998). Early research even suggests a correlation between the rates of dissolution and degradation of cyclic hydrocarbons (Abbasnezhad et al., 2011; Sikkema et al., 1995; Zhang et al., 1998). Limited mass transfer from the NAPL phase to the aqueous phase due to low solubility can thus limit toluene bioavailability in groundwater systems (Sikkema et al., 1995). Degradation occurring at the interface between groundwater and residual NAPL stored in bedrock matrix at the Site may contribute to driving a chemical gradient and allowing dissolution of the contaminant (Abbanezhad et al., 2011; Fernandes, 2017).

Aerobic contaminant degradation rapidly consumes available oxygen in groundwater. In combination with the low solubility of oxygen, this often leads to a largely hypoxic environment within contaminated aquifers (Kleinsteuber et al., 2012; Larentis et al., 2013; McMahan & Chapelle, 2008). Dissolved oxygen (DO) in water, though originally assumed by many researchers to be extremely low in groundwater, can still be found even at depth in aquifers suggesting oxygen transport throughout subsurface groundwater environments (Rose & Long,
Such conditions would impact the redox conditions in the groundwater and likely influence the abundance and activity of aerobic and anaerobic toluene degraders. Aerobic toluene degraders have been previously detected in highly reduced groundwater (Hendrickx et al., 2005; Hendrickx et al., 2006; Larentis et al., 2013). This suggests that a proportion of aerobic degraders may also be facultative anaerobes, or they may simply be resistant to sub-optimal conditions.

Microbial degradation of dissolved LNAPLs affects, and is affected by redox conditions in groundwater. Historically, redox conditions within groundwater plumes were conceptualized with a longitudinal redox zonation whereby the plume core is dominated by the least energetically favourable (methanogenic) electron acceptors (eg. CO$_2$) and gradually transition to more thermodynamically favourable redox conditions (sulfate reducing followed by manganese (IV) and iron (III) reducing, then nitrate and finally oxygen reducing conditions) (Meckenstock et al., 2015). Recently, this concept was challenged by Meckenstock’s (2015) “plume fringe concept”. This suggested electron acceptors previously thought to only dominate the plume core also dominated downgradient since horizontal and limited transverse flow of groundwater prevent replenishment of thermodynamically favourable electron acceptors downgradient. More energetically favourable electron acceptors are used at the plume fringes where uncontaminated water mixing with the plume replenishes electron acceptors. Other factors of remediation characterization are also affected by the basis of this concept. Genes and transcripts from planktonic bacteria in groundwater are also affected by laminar flow and therefore may represent conditions from the groundwater source rather than the location where samples are acquired (Brow et al., 2013; Ginn et al., 2002).
Seasonal changes are accompanied by fluctuations in an array of other factors including precipitation, water table level and temperature which may also affect groundwater conditions and consequentially impact microbial communities involved in toluene degradation. Seasonal changes have been shown to influence contaminant degradation, however many studies focus on soil rather than groundwater (Blume et al., 2002; Yadav et al., 2012; Zeman et al., 2014). Studies investigating seasonal variation in remediation either simulate environmental conditions in microcosm studies (Roychoudhury & Merrett, 2006; Yadav et al., 2012), or study factors such as redox conditions, directly measured decreases in COC concentrations, or isotope analysis rather than directly linking degradation to microbial communities (Conrad et al., 1999; Landmeyer & Effinger, 2016; Roychoudhury & Merrett, 2006; Yadav et al., 2012). Given the broad range of factors that influence contaminant distribution and degradation, obtaining detailed knowledge of the contaminant, site characteristics, plume, and hydrogeophysical context is important for selecting an effective remediation strategy.

1.2.3 Remediation of BTEX Contamination

Several methods are available for treatment of terrestrial and aquatic BTEX-contaminated sites; however, methods vary in suitability depending on the contaminant, plume size, depth, contaminant distribution and the geology and composition of the site itself (Collins, 2007). For example, site excavation and air sparging (where oxygen is vented through the soil to stimulate microbial activity and pollutant degradation) are useful methods for treating shallow contamination of soils (Collins, 2007). However, excavation is expensive and a poor choice where site disturbance is an issue, and air sparging, though more cost-effective, may have little effect beyond soil overburden (Collin, 2007). Phytoremediation is an alternative for sites where invasiveness is an issue and contamination exceeds the boundaries of the soil to extend into
bedrock and groundwater (such as the urban setting for groundwater contamination studied for this thesis) (Collins, 2007).

Phytoremediation is the treatment of polluted soils, water, sediments and even rock using plants as a medium for on-site containment, removal, or neutralization of a COC (Barac et al., 2009; El-Gendy et al., 2009). This remediation method is considered long-term, non-invasive and cost-effective. Extensive research is available on which plants and organisms are effective for phytoremediation. For example, relevant to this study, phreatophytic trees like willow and poplar are better suited for attenuating deep subsurface contamination by organic compounds like toluene due to their high water-pumping capacity and potential for deep root penetration (Barac et al., 2009; Collins, 2007; Ferro et al., 2001). In addition to pollutant attenuation, trees help retard expansion of the contaminant plume by drawing polluted water up from the water table and preventing spread deeper into the system. This consequentially may allow oxygen to enter spaces where the contaminated water was drawn from and stimulate activity of aerobic toluene degrading bacteria associated with the tree (Barac et al., 2009; Collins, 2007). Trees also secrete exudates such as nutrient and carbon sources that improve conditions in the soil for microbial communities and encourage additional biodegradation (Oliveira et al., 2015).

The degradation performance of microorganisms and their interactions with plants and the environment remains less studied (Oliveira et al., 2015). Often research focusing on phytoremediation targets degradation occurring in close relation to the plants. In many phytoremediation studies, the focus tends to remain on the vadose zone and rhizosphere with respect to observing bacterial degradation. The containment effect of phreatophytic phytoremediation systems has also been examined, but often bacterial degradation activity occurring in the groundwater below the reach of the trees is overlooked (Barac et al., 2009; Ferro
et al, 2001; Ferro et al., 2013; Weishaar et al., 2009). The research completed for this thesis examines levels of bacterial degradation activity occurring in the groundwater beneath a phytoremediation system.

1.2.4 Bacterial Toluene Degradation Pathways

Bacteria can degrade toluene by both aerobic and anaerobic means. Degradation of pollutants like toluene often involves consortia of bacteria with varying metabolic abilities and syntrophy between these organisms to fully mineralize the compounds (Jindrová et al., 2002; Lueders, 2017). Six known types of aerobic degradation pathways exist; all of which are initiated by dioxygenase or monooxygenase enzymes and may be induced by the presence of toluene with the exception of the non-specific pathway, which is induced by degradation intermediates (Parales et al., 2008). The benzene-like structure of toluene provides the molecule with added stability and therefore the initial step of toluene biodegradation is the most difficult (Figure 1.2). Toluene must be activated for aerobic degradation by destabilizing the aromatic ring via oxidation. Enzyme-assisted hydroxylation at several positions initiates degradation followed by a series of oxido-reductase type reactions to transform toluene into less toxic, more bioavailable compounds (Jindrová et al., 2002; Weelink et al., 2010). A summary of the known aerobic and anaerobic bacterial degradation pathways can be seen in Figure 1.3.

One pathway uses a toluene dioxygenase to hydroxylate the aromatic ring at positions that are ortho and meta to the methylated carbon. Dehydrogenation yields 3-methylcatechol which proceeds via a meta cleavage pathway to produce acetyl-coA and enter the tricarboxylic acid (TCA) cycle to produce adenosine triphosphate (ATP) for cellular energy (Parales et al., 2008). Both ortho and meta cleavage pathways ultimately lead to the production of acetyl-coA. This is a common point of convergence that all toluene degradation pathways share.
Toluene 2-monoxygenase (T2MO), and toluene 4-monoxygenase (T4MO), hydroxylate at the ortho, and para positions of the ring respectively in two of the monooxygenase pathways. T2MO produces o-cresol by hydroxylating at the ortho position before performing a second oxidation to make 3-methylcatechol which is further metabolized via the meta cleavage pathway. The T4MO pathway, observed in *Pseudomonas mendocinca* KR1, begins when T4MO oxidizes toluene at the para position forming p-cresol. Further reactions lead to the production of protocatechuic acid which is the initial substrate of the ortho cleavage pathway. This is the only known toluene degradation pathway that uses the ortho cleavage pathway (Parales et al., 2008). Detection of an amplicon in toluene degraders *P. mendocina* KR1 and *Pseudomonas aeruginosa* J1104 that had high protein sequence similarity (95-99%) to the α-subunit of a known phenol hydroxylase suggests that they contain an enzyme that contributes to contaminant degradation in *P. mendocina* KR1 and possibly other organisms with T4MO pathway-utilization capabilities (Baldwin et al., 2003; Hendrickx et al., 2006).

The initial point of hydroxylation in the toluene 3-monoxygenase (T3MO) pathway remains controversial. Fishman et al. (2004) used gas chromatography and nuclear magnetic resonance analysis to show m- and p-cresol formed in a 1:9 ratio while Olsen et al. (1994) showed accumulation of m-cresol upon incubating toluene-degrading clones with toluene (Parales et al., 2008). The pathway used by the model organism *Ralstonia pickettii* PKO1 (previously *Pseudomonas pickettii* PKO1) examined in both these studies is still distinct from the T2MO and T4MO pathways since a monooxygenase hydroxylates either the meta or para positions and completes the metabolic process by means of meta cleavage (Parales et al., 2008). Phenol hydroxylase catalyzes a monooxygenation on the cresol substrate (either m- or p-cresol) produced by T3MO to produce the precursor to meta cleavage, 3-methylcatechol (Figure 1.3).
Xylene monooxygenase, the only non-ring-hydroxylating monooxygenase, is used in the TOL pathway and facilitates hydroxylation of the methyl group of toluene to form benzyl alcohol. Two dehydrogenation reactions convert benzyl alcohol to benzoate which is converted to catechol before entering the meta cleavage pathway (Parales et al., 2008).

A non-regiospecific monooxygenase catalyzes the first step of the final pathway. This enzyme, often referred to as toluene/o-xylene monooxygenase, or TOMO, is most comparable to T4MO in sequence and structure, however it allows the formation of \( o \)- and \( m \)-cresol in addition to \( p \)-cresol due to the lack of regiospecificity (Parales et al., 2008). Phenol hydroxylase subsequently catalyzes transformation to 3-methylcatechol or 4-methylcatechol depending on the cresol initially formed. The catechol product proceeds through the meta cleavage pathway to eventually enter the TCA cycle (Figure 1.3).

In addition to the aerobic oxygenase pathways discussed, some research has implicated the potential for other enzymes to initiate degradation. Upon cloning a phenol hydroxylase gene cluster into \( Escherichia coli \) BL21 and incubating the culture with toluene, Ma et al. (2013) found that the strain could produce \( o \)-, \( m \)-, and \( p \)-cresols due to a relaxed regiospecificity of the enzyme. Consecutive hydroxylations produced 3- and 4-methylcatechols. While this experiment was done \textit{in vitro}, these results could indicate the potential of phenol hydroxylases in initiation of environmental degradation of toluene.

Despite distribution in a range of metabolically diverse organisms, the only known anaerobic toluene degradation pathway is quite conserved (Parales et al., 2008). This pathway occurs in a variety of reducing conditions including manganese (IV)-reducing, denitrifying, iron-reducing, sulfate-reducing, and methanogenic conditions (Beller & Edwards, 2000; Heider et al., 1998; Parales et al., 2008; Weelink et al., 2010). The benzylsuccinate synthase (BSS) enzyme,
encoded by *bss* genes, catalyzes the addition of fumarate to the methyl group of toluene to initiate anaerobic degradation and produce benzylsuccinate (Biegert et al., 1996; Carmona et al., 2009; Funk *et al.*, 2015; Leuthner *et al.*, 1998; Parales *et al.*, 2008; Weelink *et al.*, 2010). Benzyol-CoA, a common intermediate in the anaerobic degradation of many aromatic hydrocarbons, is produced by the activity of benzylsuccinate CoA transferase and subsequent β-oxidation-like reactions (Harwood *et al.*, 1999; Heider & Fuchs, 1997; Heider *et al.*, 1998; Parales *et al.*, 2008; Weelink *et al.*, 2010,). Benzoyl-CoA reductase hydrolyzes ATP to facilitate electron transfer to benzyol-CoA which is ultimately reduced to acetyl-CoA and enters the TCA cycle (Heider & Fuchs, 1997; Parales *et al.*, 2008). The variety of toluene degradation pathways provides an ample array of genes that can be targeted to confirm bacterial degradation using molecular methods.

**1.2.5 Quantification of Microbial Communities: qPCR and Primer Selection**

In the past few decades, molecular genetic techniques have opened the door to vastly improved monitoring of microbial populations *in-situ* (Baldwin *et al.*, 2003). Quantitative polymerase chain reaction (qPCR) represents one such molecular tool that can provide valuable information on abundance and potential activity of target communities in environmental samples. This method allows for confirmation of potential microbial degradation of contaminants in a remediation context. It is important to note, that qPCR cannot account for multiple copies of a functional gene in a single bacterial genome or when a single organism expresses multiple functional gene transcripts. The ratio of bacterial cells with the functional gene to the number of genes quantified is not necessarily 1:1 when quantifying genes and transcripts.

To quantify total potential bacterial abundance and potential activity, universal primers targeting the gene encoding *16S* ribosomal RNA are often used. This gene contains regions of
high variability as well as very conserved areas. Areas of this gene that are specific only to certain groups of bacteria down to a species level may be targeted, while other fragments are highly conserved across most bacterial species and allow detection of almost all bacteria. The primers used in this research 338f and 518r target the variable V3-V4 regions of 16S rDNA (Muyzer et al., 1993). A summary of all the primer sets used for this thesis can be found in Table 2.2. Klammer et al. (2008) PCR amplified 16S rRNA genes using this primer set in comparison to other primer sets and performed denaturing gradient gel electrophoresis to compare coverage of diversity. They found that the 338f/518r primer set produced more bands possibly indicating greater species coverage. Mao et al. (2012) evaluated coverage of several universal bacterial primers with metagenomics data sets and found that 338F had a non-coverage rate (the percentage of sequences that cannot match a primer) of less than 5%.

Since intermediates found further down in the degradation pathway of toluene are often common in the degradation of other contaminants, \emph{bss} genes are typically targeted for detection of potential anaerobic degraders and activity. The enzyme catalyzing the first step of anaerobic toluene degradation would thus produce results most indicative of potential anaerobic microbial degradation of toluene (Beller et al., 2008; Kazy et al., 2010; Parales et al., 2008; Weelink et al., 2010; Winderl et al., 2007; Winderl et al., 2008).

Beller et al. (2002) first quantified \emph{bss} genes using qPCR to assess BTEX degradation. This group produced a primer set targeting \emph{bssA}, the gene encoding the large subunit of BSS, based only on sequences from denitrifying bacteria. Later, Winderl et al. (2007) recognized that degenerate primers may provide more comprehensive detection of \emph{bssA} since the single anaerobic toluene degradation pathway exists in a broad array of phylogenetic groups that are active in diverse redox conditions. Using publicly available \emph{bssA} sequence data, they produced
multiple degenerate primer sets and used a range of pure cultures to test specificity of the primers and establish reference sequences in a more extensive phylogenetic framework (Winderl et al., 2007). The degenerate bssA7772f/bssA856r primer set produced the best coverage and specificity despite amplification of a non-target amplicon and a bssA-like target in one of the strains (Desulfotomaculum sp. OX39). The bssA-like gene clearly exhibited bssA homology, despite having a more distant connection to other bssA genes, and the non-specific target was not due to targeted mispriming (Winderl et al., 2007). A previous study on this same strain (OX39) provided evidence to suggest that the organism degrades toluene by benzylsuccinate synthase action (Marasch et al., 2004). This may be the bss homologue detected by Winderl et al. (2007).

A more recent application of the bssA7772f/bssA8546r primers compared them to four other primer sets targeting bssA genes in a series of anaerobic toluene-degrading microcosms. The bssA772/bssA8546 primer set produced strong amplicons most successfully of all the primer sets tested (Sun et al., 2014). This primer set produced the best detection in methanogenic and nitrate-reducing conditions. It was not as reliable for sulfate-reducing toluene degraders, however, the only other primer that was more effective for detecting these genes did not allow detection of toluene-degrading methanogenic or nitrate reducing bacteria (Sun et al., 2014). Most other available primer sets targeting bssA focus on specific groups rather than attempting broader coverage of more groups, or include groups that are less specific to toluene degradation (Kleinsteuber et al., 2012). The bssA7772f/bssA8546r primer set is a good candidate for quantifying potential anaerobic toluene degrader abundance and activity for the above reasons as well as to cover a greater array of bssA genes with a minimal number of primers.

The enzymes involved in multiple aerobic pathways offer numerous gene targets, thus a greater diversity of primers is available for targeting aerobic toluene degraders. Baldwin et al.
(2003) targeted the large subunits of aromatic oxygenases of 52 known species using sequences from GenBank (Benson et al., 1999) to create a suite of primers targeting naphthalene dioxygenase, biphenyl dioxygenase, toluene dioxygenase, xylene monooxygenase, phenol monooxygenase (*PHE*), and ring-hydroxylating toluene monooxygenase genes (*RMO*). Alternative primer sets were designed later that mainly targeted monooxygenases and aromatic dioxygenases involved in the upper pathway (the initial stages of degradation), and catechol extradiol dioxygenases targeting the lower pathway (further along the pathways where common metabolites are observed between pathways) (Hendrickx et al., 2004 Hendrickx et al., 2006; Shinoda et al., 2004). Both primer sets used in this research, designed by Baldwin et al. (2003), target genes encoding the large subunits of ring-hydroxylating monooxygenases including phenol monooxygenases. The large subunit of oxygenase enzymes has been previously implicated in substrate specificity (Furukawa et al., 1993). This has important implications in ensuring the primers will detect potential degradation activity of the target of interest.

The first set, RMO-F/RMO-R was selected to target the initial ring hydroxylation step of the aerobic pathways. The bacterial strains aligned to generate the RMO-F/RMO-R primer set are known to degrade toluene via the T3MO, T4MO and non-specific (TOMO) pathways and possibly even T2MO (Baldwin et al., 2003; Parales et al., 2008). This primer set targets the group of ring-hydroxylating monooxygenases (*RMO*) designated by Baldwin et al. (2003) as the R.2 group which was found to be phylogenetically distinct from the R.3 group, though still related. Upon testing these primers using hybridization probes, however, Baldwin et al. (2003) discovered that this primer set also detects monooxygenases in the R.3 group thus broadening the ability of these primers to detect a greater range of potential aerobic toluene degraders and activity.
The other group of ring-hydroxylating monooxygenases that was targeted in this study was designated the R.1 group and is also phylogenetically related but distinct from the R.2 and R.3 groups (Baldwin et al., 2003). This group of monooxygenases specifically encompasses phenol hydroxylases (*PHE*) that catalyze oxidation of various phenol and cresol intermediates. While these enzymes do not catalyze the initial step in the known toluene degrading pathways, their role is either suspected or has been confirmed in half of the known aerobic toluene degradation pathways (Baldwin et al., 2003; Hendrickx et al. 2006; Ma et al., 2013; Parales et al., 2008). The T3MO and non-specific pathways have been confirmed to have phenol hydroxylase activity (Parales et al., 2008). There is also evidence to suggest that the genes coding for enzymes involved in the T4MO pathway harbor a phenol-hydroxylase encoding gene (Baldwin et al., 2003; Hendrickx et al. 2006; Ma et al., 2013). There also remains the possibility that phenol hydroxylases may be able to act directly on toluene due to relaxed regiospecificity of the active site (Ma et al., 2013). This offers a broad scope of aerobic toluene degradation activities that could potentially be detected with the PHE-F/PHE-R primers designed by Baldwin et al. (2003).

Baldwin et al. (2008) recommended the parallel use of the *PHE* and *RMO* primer sets since they previously allowed the highest numbers of genes to be detected in an *in-situ* context, when compared against other available primer sets. Further, detection of two separate stages of the degradation pathways provides additional evidence that degradation progresses beyond the initial activation stage. A combination of both primer sets discussed would therefore be expected to provide a strong basis for detection and quantification of potential aerobic toluene degrader abundance and activity at the Site.
A small number of studies have demonstrated the applicability of targeting toluene degradation functional genes in-situ in groundwater (Baldwin et al., 2003; Baldwin et al., 2009; Baldwin et al., 2010; Beller et al., 2008; Hendrickx et al., 2005; Kao et al., 2010; Key et al., 2014; Larentis et al., 2013; Nebe et al., 2009; Nolvak et al., 2012; Winderl et al., 2007). Our study examines the intrinsic bioremediation occurring in groundwater below the reach of the phytoremediation system and few of these studies have used functional gene quantification to study intrinsic remediation in groundwater (Hendrickx et al., 2005; Kao et al., 2010; Key et al., 2014; Larentis et al., 2013; Nebe et al., 2009; Winderl et al., 2007). Of the studies that have quantified functional degradation genes to evaluate potential biodegradation using qPCR, to the author’s knowledge, only one study successfully quantified transcripts of functional BTEX degradation genes, but this analysis was only performed in soil samples (Key et al., 2014). The quantification of transcripts allowed Key et al. (2014) to refine their CSM by determining where organisms where present and actively expressing degradation genes and where this activity was lacking. The lack of research into the relationships between abiotic site factors and the active expression of functional genes involved in degradation represents a large gap in our understanding of remediation systems and how to optimize remedial techniques with microbial communities in mind.

1.2.6 Challenges of Studying Remediation in Groundwater Systems

Groundwater represents a challenging setting in which to characterize microbial remediation. Sessile degrading communities and biofilms attached in the subsurface may provide a better representation of depth-dependent responses in abundance and activity of toluene degraders, however, sampling these communities is difficult in-situ (Hazen et al., 1991). Bedrock cores may only be removed and sampled once per location, and the anthropogenic impact of rock
removal may subsequently affect subsurface conditions and communities (Fernandes, 2017). This also removes opportunities to perform temporal studies of toluene-degrading bacteria. Study of planktonic bacteria in groundwater, too, has implications. Due to vertical and horizontal groundwater flow, groundwater samples may be indicative of upstream sources of groundwater and may not be as representative of the locations in which they were obtained (Brow et al., 2013; Ginn et al., 2002). In addition to this, fluxes in subsurface conditions and microbial community abundance and activity may occur at different times, thus making it challenging to ascertain related factors. Previous research has also found that aerobic degraders are notoriously detected in locations with reducing condition in subsurface contexts (Hendrickx et al., 2006; Larentis et al., (2013), Martinez-Lavanchy et al., 2015; Nebe et al., 2009) which makes predicting distribution of aerobic vs. anaerobic toluene degraders challenging.

Studying unique contaminants such as LNAPLs in a fractured bedrock context like that in this study provides additional challenges. The heterogeneity of the subsurface affects contaminant distribution and complicates groundwater flow patterns making it harder to determine the origin of groundwater that enters a specific location (Fernandes, 2017; Parker, 2007).

1.2.7 Conclusion

Examining the distribution, abundance and activity of microbial communities involved in the bioremediation of organic contaminants in groundwater, will, in turn, support research assessing how conditions can be modified to improve remediation efficiency in an intrinsic remediation setting below a phytoremediation system. Data collection from an extensive array of sites representing comprehensive combinations of pollutants, concentrations, site geologies, groundwater flow patterns, water chemistries and environments will provide the scientific
community with the tools to select truly appropriate and site-specific remediation methods. In addition to establishing some of the limits and benefits of phytoremediation and intrinsic bioremediation, this research will produce insights into the influence of abiotic factors on attenuation processes, particularly microbial degradation activity. Improved understanding of these remediation techniques and how they may be manipulated to maximize efficacy will make these options applicable in a broader variety of physiological and environmental circumstances.
Figure 1.3 A summary of the known aerobic and anaerobic bacterial toluene degradation pathways. Each aerobic pathway begins with the hydroxylation of the toluene molecule to activate it for further degradation. The anaerobic pathway commences with the addition of a fumarate molecule to toluene (Figure modified from Parales et al., 2008).
1.3 RESEARCH OBJECTIVES, HYPOTHESES AND THESIS FORMAT

1.3.1 Research Objectives:

The goal of this research was to investigate potential toluene degradation activity in groundwater across the Site. Clear evidence of bacterial degradation of toluene in groundwater at the Site has yet to be confirmed and is the goal of this study. Specifically, (i) to confirm abundance and activity of potential aerobic and anaerobic toluene degraders in groundwater across the site, across seasons, and in response to the level of toluene contamination; (ii) to quantify abundance and potential activity of aerobic and anaerobic toluene degraders in groundwater between depths and redox conditions with high resolution, depth-delineated sampling.

1.3.2 Hypotheses:

1. Abundance and activity of toluene degraders and total bacteria will fluctuate in response to varied toluene concentrations with a positive correlation in areas with low to moderate toluene concentrations.

2. Reduced abundance of total bacteria and toluene degraders in addition to reduced potential activity, due to toxic effects of toluene will be observed in the wells and sampling depths with the highest toluene concentrations near saturation.

3. Abundance and potential activity of toluene degraders will increase in the spring and summer months relative to the fall sampling events due to introduction of metabolites with spring snowmelt, and increased temperatures.

4. Anaerobic toluene degraders will dominate in the plume since conditions previously observed indicated generally reducing conditions, while more aerobic degrader abundance and potential activity will be found near the water-air interface.
5. Aerobic degraders have previously been detected in reducing environments in groundwater, thus detection of aerobic toluene degraders was expected throughout the site despite generally reducing conditions detected in previous characterization. However, anaerobic toluene degraders were expected to associate with more strongly reducing conditions than aerobic toluene degraders.

1.3.3 Thesis Format:

This thesis is partitioned into four chapters. Chapter One provides a general introduction describing BTEX and some of the issues surrounding their release into the environment. This provides key information about the context of the Site where all the samples for this research were obtained. A literature review of the relevant background information regarding toluene degradation in environmental groundwater systems is provided. Finally, this section outlines the objectives for the research at hand and presents the hypotheses for this thesis.

Chapter Two delves deeper into the molecular aspects of BTEX biodegradation and the monitoring techniques used. The approach and results for the microbial data are presented and discussed here as well as general microbial trends seen at four sampling events and between individual wells.

Chapter Three further investigates the data presented in Chapter Two in a high-resolution, depth-resolved manner. The patterns of potential degrader abundance and activity are investigated and compared with additional lines of evidence including additional microbial data, groundwater chemistry and Compound Specific Isotope Analysis (CSIA).

The final chapter provides discussion of the findings of the research and considers any implications that these findings may have for maximizing efficiency of groundwater bioremediation in the future.
CHAPTER 2.0 Seasonal Shifts in Activity and Abundance of Toluene Degrading Bacteria Across a Contaminant Plume in a Contaminated Fractured Bedrock Aquifer.

2.1 INTRODUCTION

Benzene, toluene, ethylbenzene and xylene, commonly referred to as BTEX are aromatic compounds that can be found in fuel and are commonly applied in an industrial context as solvents or additives in products like paint and adhesives (An, 2004; Collins et al., 2002). The transport, storage and use of BTEX provide many opportunities for these compounds to be released into the environment. Common sources of contamination include volatilization, waste, spills and leaking storage tanks and product lines (Baehr et al., 1999; Do et al., 2011). Toluene, is often used as a model for all BTEX in contamination studies and is the contaminant of concern (COC) at our Site of interest. Toluene will thus be the contaminant of focus of this study.

The study Site is a historical manufacturing facility located in an urban residential portion of Southwestern Ontario that used toluene as a solvent in their manufacturing process between 1952 and 1998 Fernandes, 2017). The toluene plume, discovered in the late 1980’s upon closure of the facility, is hypothesized to have originated from leakage of buried supply lines connecting the building to partially buried storage tanks located on-site. Toluene seeped through the two meters of the sandy, cobbled overburden of the Site to reach the fractured bedrock and underlying shallow aquifer system (Fernandes, 2017). The bedrock at the Site consists of a fractured network of Silurian-Era, porous dolostone.

To characterize the Site, high-resolution depth-discrete sampling was combined with CSIA and microbial analysis to quantify potential abundance and activity of anaerobic and aerobic toluene degraders in situ, over space and time. A discrete fracture network (DFN)
approach was implemented to characterize fracture network properties and toluene distribution at
the Site (Fernandes, 2017). Multilevel monitoring systems (MLS) were installed in the boreholes
which were advanced for the DFN approach. The strong influence of fracture networks on
contaminant distribution and groundwater flow necessitated application of fracture network
characterization and use of MLS. Combining this approach with CSIA and microbial analysis
serves to build a mechanistic, detailed CSM for toluene degradation, with multiple, diverse lines
of evidence to demonstrate efficacy of the remediation system. In this study of the Site
specifically, the lines of evidence discussed will contribute to determining the efficacy of
intrinsic toluene remediation below a phytoremediation system in the fractured bedrock aquifer.

The greatest fracture intensity occurs from bedrock surface down approximately 6 m
(~310 m amsl) reaching as many as 30 horizontal fractures per 1.52 m intervals of bedrock, but
fracture intensity decreases with depth. The fracture network consists of mainly horizontal
fractures (between over 30 and just over 10 fractures per 1.52 m in the depths sampled) (Figure
2.1). Vertical fractures occur most in the upper bedrock; however, below approximately 6 meters
below ground surface, vertical fractures are longer. The Site overlays a valuable aquifer that is a
freshwater source for over 500,000 locals in the area (Aqua Resource, 2010; Fernandes, 2017).
The contamination must therefore be monitored closely and attenuated to minimize risks
associated with contaminated drinking water.
Figure 2.1 An schematic representation of fracture frequency at the Site derived from bedrock cores in MW22 from Site characterization (adapted from Fernandes, 2017). Samples for this study were only taken from depths within HGU 1 and the top of HGU 2.
Phytoremediation with poplar trees (*Populus x canadensis* hybrids) was selected as an appropriate remediation technique since they are phreatophytic and able to contain the expansion of the plume within groundwater due to their high water-pumping capacity (Barac et al., 2009; Collins, 2007). The plants encourage contaminant attenuation through phytodegradation, phytovolatilization, endophytic activity, rhizodegradation and phytoextraction. Below the influence of the tree root system in the groundwater, however, intrinsic remediation of indigenous bacterial communities is responsible for contaminant degradation. This intrinsic remediation is largely impacted by abiotic factors including contaminant concentration, redox conditions, temperature and nutrient availability (Anneser et al., 2010; Azubuike et al., 2016; Cross et al., 2006). In a concurrent study assessing the efficacy of the phytoremediation system, molecular approaches paired with CSIA indicated toluene biodegradation in the vadose zone and suggested toluene degrader abundance increases with increased depth and toluene concentrations in the soil rhizosphere (Ben-Israel et al. 2018, submitted to Int. J. Phytoremediation) Assessment of the phytoremediation is beyond the scope of this study which will focus on the underlying groundwater in which intrinsic remediation is the main source of biological contaminant degradation.

Due to the fractured and porous nature of the bedrock at the Site, sorption factors into the distribution of the contaminant of concern (COC). Site characterization by collaborators confirmed that matrix diffusion due to the high porosity of the sedimentary rock comprising the bedrock along with sorption to the matrix most strongly influence contaminant distribution at the Site (Fernandes, 2017). These factors appear to be instrumental in maintaining approximately 80% of the toluene mass in the small area in and around the Source Zone (Table 2.1) (Fernandes, 2017).
Toluene, along with the other BTEX, is a light non-aqueous phase liquid (LNAPL) that will settle on top of water when the two are mixed due to its lower density (specific density of 0.867 g cm\(^{-3}\) at 20°C; specific density of water is 1.0 g cm\(^{-3}\)) and low solubility (515 mg L\(^{-1}\) at 20°C) (Newell et al., 1995). This means that in a contaminant plume such as the one under investigation, the toluene may sit at the surface of the groundwater, fluctuating with the water table, and extending into the capillary fringe at the surface of the water (Newell et al., 1995.). Characterization of toluene distribution in the bedrock matrix confirmed this with a shallow source zone horizon covering approximately 0.3 m where toluene concentrations were highest and even exceeded aqueous solubility. This horizon is proximal to the water table around 2 m bgs. Approximately 95% of the toluene mass in the matrix occurred between 2.3 m bgs to 3.5 m bgs which matches well with the fluctuating range of the water table (Fernandes, 2017). This indicates that fluctuations of the water table and phase transfers of the contaminant between liquid, aqueous and gaseous phases also factor into the contaminant distribution (Fernandes, 2017). While NAPL phase toluene will float on top of the water, the low solubility still allows toluene to enter the dissolved phase and extend the plume further into the groundwater system. This was observed at the Site in toluene concentrations deep in groundwater. Earlier characterization of the Site by Jeremy Fernandes (2017) indicated that the bulk of toluene in the groundwater was located above 10 m bgs though a broad range of concentrations were detected from approximately 500,000 µg L\(^{-1}\) to below detection limits (<0.2 µg L\(^{-1}\)) (Parsons, 2008). This indicates that in addition to sorption, back-diffusion and dilution of the contaminant also impact the plume.

Previous characterization at our Site has indicated that the contaminant plume is likely shrinking based on historical analysis of toluene contamination. More recent analysis produced
evidence that toluene within the Site continues to be attenuated by means of on-going dissolution and back diffusion from the bedrock matrix likely paired with biodegradation (Fernandes, 2017). Conclusive evidence of microbial degradation in the groundwater at the Site has yet to be confirmed and is the focus of this study.

Bacterial degradation of toluene may occur via one well-conserved anaerobic pathway, or by several possible aerobic degradation pathways (Parales et al., 2008). Anaerobic catabolism of toluene is initiated by the enzyme benzylsuccinate synthase (BSS), encoded by the \textit{bss} genes, which catalyzes the addition of fumarate to the toluene molecule (Biegert et al., 1996; Carmona et al., 2009; Funk \textit{et al.}, 2015; Leuthner \textit{et al.}, 1998; Parales \textit{et al.}, 2008; Weelink \textit{et al.}, 2010). Most of the aerobic pathways rely on monooxygenase activity to activate the toluene for subsequent degradation steps. Further oxidation steps depend on monooxygenase specificity and the position of initial hydroxylation on the toluene molecule (Jindrová \textit{et al.}, 2002; Parales \textit{et al.}, 2008; Weelink \textit{et al.}, 2010). Further down in the aerobic degradation pathways, phenol and cresol intermediates are frequently formed (Baldwin \textit{et al.}, 2003; Hendrickx \textit{et al.}, 2006; Parales \textit{et al.}, 2008). Phenol hydroxylase enzymes can act on both phenol and cresol substrates and are key facilitators of toluene degradation in multiple aerobic toluene degradation pathways (Baldwin \textit{et al.}, 2003; Hendrickx \textit{et al.}, 2006; Parales \textit{et al.}, 2008). In addition, there is some evidence indicating the ability of phenol hydroxylases to initiate toluene degradation themselves in some contexts (Ma \textit{et al.}, 2013).

Quantitative polymerase chain reaction (qPCR) can be used to target functional genes involved in the toluene degradation pathways from DNA and RNA extracted from environmental samples to confirm active potential bacterial degradation. The method provides more direct evidence of bacterial involvement in potential degradation than monitoring toluene
concentration, which may decrease due to dilution from groundwater recharge and sorption or may increase from dissolution (Beller et al., 2002; Key et al., 2014; Meckenstock et al., 2004). While dilution has the potential to produce misleading qPCR results by diluting the gene copies or transcripts present, sorption and increased dissolution of the contaminant will not affect qPCR results in this way and qPCR provides evidence of potential degradation despite these factors.

Primers have been designed to target both aerobic and anaerobic toluene degradation pathways (Baldwin et al., 2003; Beller et al, 2002; Beller, 2008; Brow et al, 2013; Hendrickx et al., 2004; Hendrickx et al., 2006; Winderl et al., 2007; Winderl et al, 2008;). Beller et al. (2002) developed primers based on alignments of genes encoding the large subunit of benzylsuccinate synthase in several denitrifying bacteria. Winderl et al. (2007) later designed a new primer set with greater diversity coverage. The 7772f/8546r primer set (Winderl et al., 2007) targeting bssA, the gene encoding the large α-subunit of BSS, was selected for quantifying potential anaerobic toluene degrader abundance and activity (Beller et al., 2008; Kazy et al., 2010; Parales et al., 2008; Weelink et al., 2010; Winderl et al., 2007; Winderl et al., 2008). Several other studies have successfully applied this primer set for qPCR on environmental and microcosm samples and found that it captured a broad range of bssA sequences in comparison to other available primers targeting bssA (Brow et al., 2013; Kleinsteuber et al., 2012; Lueders & von Netzer, 2014; Pilloni et al, 2011; Sun et al., 2014; Winderl et al., 2008).

Ring-hydroxylating monooxygenases are commonly targeted to quantify potential aerobic degraders and activity since most of the known aerobic toluene degradation pathways begin with a single hydroxylation of the aromatic ring. Targeting genes encoding these enzymes allows comprehensive detection of potential aerobic degradation by targeting multiple pathways at once using fewer primer sets. The dominant pathways at play at the Site were unknown at the
outset of the study therefore primers were selected to cover as many pathways as possible with few primer sets. This method, however, may underestimate the activity of organisms using the dioxygenase or TOL pathways.

In the current study, aerobic toluene degrader abundance and activity are quantified using the RMO and PHE primers developed by Baldwin et al. (2003). These primers target ring-hydroxylating monooxygenases that catalyze the first step of multiple aerobic degradation pathways and phenol hydroxylases that catalyze oxidation of phenol and cresol intermediates further down the pathways (Baldwin et al., 2003; Parales et al., 2008).

In-situ characterization of contaminated sites, though challenging, is critical for understanding ecological responses to remediation efforts. Multiple lines of evidence should be used to confirm degradation (Illman & Alvarez, 2009). A reduction in concentration of the COC should be demonstrated. This is often shown by monitoring COC concentrations over time; however, this method cannot distinguish concentration reductions stemming from abiotic factors like dilution, dispersion, or sorption from those seen due to microbial degradation (Beller et al., 2002; Key et al., 2014). In natural settings, active degradation may be ongoing but not apparent because of other processes contributing to the overall (i.e. measured) contaminant concentration in groundwater. These include dissolution of the contaminant from an effectively infinite source (e.g. presence of NAPL) and system transience (e.g. recharge), which serve to maintain steady COC concentrations, or may even result in short term concentration increases even where there is active contaminant consumption via biodegradation.

Other methods for monitoring BTEX-contaminated sites such as stable isotope fractionation analysis or applying deuterated BTEX to contaminated aquifers to confirm degradation have been done with success (Beller et al., 2008; Kümmel et al., 2013; Mancini et
al., 2003; Meckenstock et al., 2004; Reusser et al., 2002; Vogt et al., 2008). However, some of these studies cite sorption, back-diffusion, dilution and the broad variety of degradation enzymes resulting in large variations in fractionation as confounding factors or, as with adding deuterated BTEX to a site, may add to contamination levels which could be considered counterproductive (Meckenstock et al., 2004; Reusser et al., 2002).

While several studies have examined abundance of degraders in BTEX-contaminated groundwater (Baldwin et al., 2003; Baldwin et al., 2009; Baldwin et al., 2010; Beller et al., 2008; Hendrickx et al., 2005; Kao et al., 2010; Key et al., 2014; Larentis et al., 2013; Nebe et al., 2009; Nolvak et al., 2012; Winderl et al., 2007), representation of activity by quantifying the transcribed RNA transcripts of these microbes in-situ is lacking in groundwater. In addition, only one known study examines both aerobic and anaerobic degraders in groundwater (Larentis, et al., 2013), but data for each was collected at different times and transcripts were not quantified. Larentis et al. (2013) also acknowledged that the gene target used to quantify aerobic toluene degraders (tmoA) only covers one of the six possible aerobic degradation pathways.

There is also limited literature available on seasonal effects on degradation in groundwater contaminant plumes, particularly with reference to potential degrader abundance and activity. Many studies examine subsurface soils instead of groundwater (Blume et al., 2002; Yadav et al., 2012; Zeman et al., 2014). Other research analyzes metabolite consumption, reductions in toluene concentration, CSIA or general redox conditions rather than quantifying microbial activity and abundance to study seasonal effects (Conrad et al., 1999; Landmeyer & Effinger, 2016 Roychoudhury & Merrett, 2006; Yadav et al., 2012). In addition, some studies testing seasonal effects are not in-situ and instead use microcosms to represent environmental conditions (Roychoudhury & Merrett, 2006; Yadav et al., 2012).
This research aimed to confirm the active degradation of toluene by indigenous bacteria in a contaminated aquifer setting by detecting and quantifying actively transcribed functional genes involved in toluene degradation. The goal of this Chapter was to determine seasonal shifts associated with changes in total bacterial abundance and activity as well as abundance and activity of both aerobic and anaerobic toluene degraders at the Site. We then explored possible factors associated with changing seasons that may affect toluene degrader abundance and activity. Preliminary assessment of all the depth-resolved microbial data obtained indicated no clear trends in abundance or activity with depth; therefore, we focused on data representing entire wells (achieved by averaging data from the two ports in each well that were sampled in all sampling events) to determine if lateral plume location or seasons impact potential toluene degrader abundance and activity. A depth discrete analysis of an expanded chemical dataset along with molecular analysis was conducted for November 2015, and June 2016 sampling events and will be the focus of Chapter 3.

To assess intrinsic groundwater bioremediation in-situ seasonally and across the Site, we quantified both gene copies (DNA) and transcripts (RNA) of functional genes involved in both types of degradation pathways across three seasons. We used primers that capture multiple degradation pathways and sampled discrete depths (approximately 3.89 and 8.70 m bgs in the Proximal and Source Zone wells, and 4.19 and 8.76 m bgs in the Peripheral well. See Appendix B Table B.1) to fill some of these gaps in the literature. Characterizing bacterial activity and abundance of genes from both degradation pathway types provides a more complete representation of potential degradation activities at the Site in response to toluene concentrations and seasonal effects in monitoring wells than does targeting only one type, as some previous studies evaluating remediation systems such as that done by Key et al (2014) have done.
Transcript quantification represents a previously untapped information source that can clarify how site factors influence activity of degraders in the environment. The *in-situ* nature of our study will provide real-world data on responses of degrader activity in relation to contaminant concentrations and seasons that can be considered when implementing remediation techniques in similar plumes.

2.2 METHODS AND MATERIALS: Site Characterization and Groundwater Sampling/Analysis

2.2.1 Discrete Fracture Network and Volatile Organic Compound Profiles in Bedrock Core

Continuous cores were advanced at 11 locations across the Site. Locations selected were based on the understanding of the plume from historical groundwater monitoring (Figure 2.3) and targeted the suspected source zone as well as locations up- and downgradient of the source zone, and peripheral locations at the downgradient plume edge (Figure 2.3 & Table 2.1). Four cores were drilled with a PQ-3 sized (approximately 122 mm diameter) continuous coring method to a depth of approximately 22 m bgs (M20, M21, M22 and M23) (Figure 2.3). Six cores were retrieved from inside the tree stand in the phytoremediation zone (M24, M25, M26, M27, M28 and M29) and one across the street in the Peripheral Downgradient area (M30) (Figure 2.3). These were drilled using a Shaw Backpack Drill™ to produce cores with 51mm diameters. Detailed logging of lithology and fracture features was completed, followed by collection of rock core samples for analysis of toluene. Rock core sample collection and analysis is detailed in Fernandes (2017) Master’s thesis.
2.2.2 Borehole Characterization

Further DFN characterization was completed using an Acoustic Televiewer and an Optical Televiewer to improve visual characterization of lithology and fractures and natural gamma logs were run as per Fernandes (2017). Transient, depth-discrete fluid pressures were measured using a temporary, flexible, impermeable liner installed in the borehole to hold a line of pressure transducer sensors installed behind the lining in place (FLUTe™ liner) (Fernandes, 2017; Pehme et al., 2014). Transmissivity profiling in the four PQ boreholes was also completed during the time the FLUTe™ liners were in place. Further details regarding transient pressure data collection and transmissivity profiling can be found in the thesis of Jeremy Fernandes (2017). These data were used in characterizing the local flow systems and hydrogeology of the groundwater.

2.2.3 Multilevel Groundwater Monitoring Systems

Continuous Multichannel Tubing (CMT) systems each with seven sampling intervals (ports) separated by inflatable, rubber packers were outfitted into the seven 51 mm boreholes by members of the G360 Institute for Groundwater Research. Sampling intervals spanned an
average distance of 0.4 m and were concentrated in areas where the bulk of the toluene contamination resides based on previous plume delineation (Fernandes, 2017). The Waterloo Multilevel System (Cherry & Johnson, 1982; produced commercially by Solinst Canada) was implemented in the four PQ holes with inflatable rubber packers again isolating the sampling intervals. These systems spanned from the bedrock surface down to 22 m bgs and sampling intervals (ports) were an average of approximately 0.8 m in length. Pressure transducers in 15 of the monitoring intervals continuously collected head data on a data logger along with manual head measurements with a small water level meter (Fernandes, 2017).

2.2.4 Groundwater Sampling and Analysis

During the time this thesis research was performed (November 2015 to November 2016), groundwater monitoring occurred on an almost monthly basis. Groundwater sampling occurred in all 11 MLS systems, but the results presented in this thesis focus on results from three wells: M28, M29 and M30. These wells are located within the source zone (M29), downgradient but proximal to the source zone (M28), and off-Site at the downgradient edge of the plume (M30) (Figure 2.3).

Analyses performed included VOC analysis on an approximately monthly basis. Groundwater samples for microbial analysis were collected from a minimum of 2 ports on each of four time-points (November 2015, March 2016, June 2016 and November 2016) targeted to represent seasonal variation at the Site. The time points were selected to reflect high (spring/March), low (summer/June) and intermediate (late fall/November) groundwater table depths and the growth cycle of the trees in the phytoremediation system. The trees are in early growth stage in March, in full growth in June and entering dormancy in November. Ports sampled varied between sampling events and are summarized in Appendix B (Table B.1).
A Geotech Series II peristaltic pump was used to pump groundwater from sampling intervals through ethanol-sterilized ¼ inch polyethylene tubing. The lowest pump rate (approximately 100 mL min\(^{-1}\)) was used to reduce drawing groundwater from non-target areas. Small segments of silicon tubing were used as adaptors for the polyethylene tubing. Prior to sample collection, an initial volume of 3 times the volume of the polyethylene tubing running to the bottom of the sampling interval was purged. Upon sample collection, a YSI-556 multi-parameter probe and 200mL flow cell were used to measure pH, dissolved oxygen (DO), electrical conductivity, oxidation-reduction potential (Eh), and temperature when the values reached relative stability. Approximately 1 L of groundwater was collected into large 2 L pyrex media bottles containing 500 mL of lab-made RNALater\textsuperscript{®} solution (De Wit et al., 2012). Samples were stored in coolers on ice for transport to the lab where they were stored in the dark at 4°C until they were processed between one to five days after sampling.

Plastic containers were used to collect inorganic hydrochemistry samples; dissolved cation samples were filtered and acidified in the field. VOC samples were collected in glass vials and either Maxxam Analytics (using the EPA 8260 C method), or the University of Guelph (using the EPA 8260 B method) completed analysis. Both methods have a toluene detection limit of 0.2 µg L\(^{-1}\) (liquid). Dissolved gas samples were collected in glass VOA vials in November 2015, July 2016 and October 2016. These were analyzed by Maxxam Analytics with the RSKSOP-175m procedure and the headspace in the vial was analyzed with gas chromatography with flame ionization detection (GC-FID) (Fernandes, 2017). QA/QC procedures were carried out as stated by Fernandes (2017). It should be noted that this thesis focuses only on data pertinent to the sampling events in which microbiological samples were retrieved and analyzed.
Samples for CSIA analysis were collected in concert with VOC sample collection. A gas chromatograph coupled with a mass spectrometer was used to determine carbon and hydrogen isotope ratios in toluene as described in Wanner et al. (2016).

**Table 2.1.** Summary of toluene contamination at the Site. Ranges of toluene concentrations for each well presented are from the June 2016 sampling event. The highest concentrations observed were close to the solubility limit and occurred in the uppermost ports.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Well</th>
<th>Range of toluene concentrations (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source zone</td>
<td>29</td>
<td>25 - 571 200</td>
</tr>
<tr>
<td>Source zone</td>
<td>21</td>
<td>0.8 - 175 000</td>
</tr>
<tr>
<td>Source zone</td>
<td>27</td>
<td>&lt; 0.2 – 172</td>
</tr>
<tr>
<td>Proximal Downgradient</td>
<td>28</td>
<td>&lt; 0.2 - 395 000</td>
</tr>
<tr>
<td>Upgradient</td>
<td>24</td>
<td>&lt; 0.2 – 12</td>
</tr>
<tr>
<td>Upgradient</td>
<td>25</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Peripheral</td>
<td>30</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Peripheral</td>
<td>23</td>
<td>&lt;0.2 - 0.46</td>
</tr>
</tbody>
</table>
Figure 2.3. Site map detailing the locations of multilevel wells on the Site and the wells sampled for the current study (bolded). The perimeter of the planted tree stand (—) surrounds the location of the former toluene storage tanks (grey rectangles). The primary Source Zone (red) (M27, M29 & M21) at the Site occurs where the buried product lines historically were located; Downgradient (yellow) (M28 & M22) locations are downgradient of the Source Zone; and Peripheral (blue) (M30 & M23) locations are at the downgradient plume fringe. Upgradient (green) (not discussed further in this study) are upgradient of the Source Zone. Toluene has been detected intermittently at
2.2.5 Nucleic Acid Extraction and Reverse Transcription of RNA

Groundwater samples were split into two approximately 750 mL replicates (composed of approximately 500 mL of water and 250 mL RNALater) which were passed through separate 0.22 µm pore size membrane filters under vacuum to capture bacteria. Immediately after filtration, the membrane was loosely rolled using ethanol-sterilized forceps, placed in a 15mL conical tube and stored at -80°C until extraction. The exact volume of liquid passed through the membrane for each replicate was recorded and the percentage of RNAlater® in the total volume of both replicates combined was used to calculate the true volume of water sample in each replicate. Samples were filtered in random order.

After filtration, DNA and RNA were extracted using a MoBio PowerWater® DNA Isolation Kit following the prescribed protocol with the adjustment for DNA/RNA coextraction (MoBio Laboratories, Inc, Carlsbad, California.). Six random RNA samples were measured with a Qubit™ 4 Fluorometer (Thermofisher Scientific, Waltham, Massachusetts) using the Qubit™ RNA HS Assay Kit (Invitrogen, Thermofisher Scientific, Carlsbad, California) to determine an average of approximately 1.2 ng µL⁻¹. Six random DNA samples were measured using a NanoDrop™ 8000 (Thermofisher Scientific, Waltham, Massachusetts) to determine and average of approximately 12 ng µL⁻¹. Quadruplicate subsamples (10µL) of the extract were DNAsed to remove DNA using the Promega RQ1 RNase-Free DNase kit as per the supplied protocol. One of these reactions was set aside to test for contaminating DNA by targeting bacterial 16S in qPCR. RNA was reverse transcribed in triplicate using an Applied Biosystems® High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corp.) as per the protocol supplied. No-enzyme and no template controls were included in the reverse transcription to confirm DNA was not carried over. Once reverse transcriptions was completed, triplicate
samples were pooled to produce a larger volume and stored at -80°C until further analysis.

Inhibition tests were performed on DNA and cDNA. DNA was subsequently diluted 25 times for qPCR analysis, while cDNA was diluted either 10 times or 5 times since DNAs treatment and reverse transcription steps diluted samples previously.

Inhibition was tested by creating a dilution series of undiluted, 25, 50 and 100 times diluted environmental DNA from 6 samples with high, moderate, and low contamination from the Site. Reaction mixtures for qPCR targeting the M13 phagemid vector were prepared and 1 µL of environmental extract was added to the mixture, reducing the volume of Nanopure water used to properly adjust the reaction volume (Habtewold et al., 2017). A 1 µL aliquot of the 10^6 copies µL^{-1} dilution of an M13 plasmid was also added. The same amount of plasmid was added to a regular qPCR reaction mixture without environmental DNA extract. The reactions were run on qPCR to quantify the M13 phagemid and amplification curves were examined for inhibition which was characterized by amplification in later cycles (cycles with Cq values greater than 0.25 Cq more than those of uninhibited controls were considered inhibited). The lowest dilutions that effectively eliminated the effect of inhibition were selected (Habtewold et al., 2017).

2.2.6 Quantification of the Abundance and Potential Activity of Aerobic and Anaerobic Toluene Degraders by qPCR

Total bacterial abundance and 16S gene expression were quantified targeting bacteria 16S rRNA gene using the primer set 388f/518r (Muyzer et al., 1993). The 7772f/8546r primer set (Winderl et al., 2007) targeting bssA was selected as a gene target for quantifying potential anaerobic toluene degrader abundance and activity. Aerobic toluene degrader abundance and activity was quantified using the RMO and PHE primers developed by Baldwin et al. (2003). Primer sequences are provided in Table 2.2.
Quantitative PCR was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and analyzed with the Bio-Rad CFX Manager Version 3.1 software. All reactions contained 1X of Bio-Rad SsoFast™ Evagreen® Supermix mixed with 1 pmol of the forward and reverse primers respectively for gene targets. For 16S detection, 2µL of DNA (25 times diluted; average of approximately 1 ng based on average starting DNA concentrations) and cDNA (10 times diluted; average of approximately 0.2 ng based on average starting RNA concentrations), were added to the reaction mix. For bssA, 3µL and 5µL of template were added for DNA (25 times diluted, average of approximately 1.4 ng) and cDNA (5 times diluted, average of approximately 1 ng), respectively since cDNA concentrations were lower. In reactions for quantifying the aerobic genes, 2µL were added for DNA (approximately 1 ng) and 3µL (approximately 0.5 ng) were added for cDNA. The remainder of the 20µL reaction volume was made up with PCR-clean water. All qPCR plates were run with duplicated samples and a minimum of two no-template controls to ensure reagents were not contaminated. Standards came from reference strains *Thauera aromatica* and *Clostridium thermocellum* for bssA, and 16S respectively, and were cloned into the pCR™2.1-TOPO vector, while *RMO* and *PHE* standards came from environmental isolates cloned into pCR4-TOPO and pCR2.1-TOPO plasmids respectively. Sequences were checked using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Standard dilutions ranged from anywhere between $10^8$ copies to 1 copy depending on the gene and samples and the expected copy numbers. All standards had a limit of detection of 1 copy per reaction volume. A minimum of three orders of magnitude were covered by standards (three dilutions) for every qPCR and all standards were run in duplicate.

The qPCR reactions for 16S were carried out with the following conditions: 2 min. of preheating at 98°C, this was followed by 40 cycles of a 5 sec. melt at 98°C, 5 sec. of annealing at
55°C and a plate read (Muyzer et al., 1993). A melt curve analysis spanning 65°C -95°C by 0.5°C increments composed the final steps. The PHE protocol was carried out with 2 min. of preheating at 98°C, then 42 cycles of a 10 sec. melt at 98°C, 7 sec at 49°C for annealing followed by 3 sec. at 60°C to counteract any non-specific binding. The melt curve analysis for PHE spanned 65°C-95°C by 0.5°C increments. RMO reactions began with a preheat at 98°C for 2 min., followed by 42 cycles of 5 sec. at 98°C to melt and 30 sec. at 63°C annealing. The melt curve analysis was 58°C-95°C in 1°C increments (Baldwin et al., 2003). Finally, for bssA protocol had a preheat of 94°C for 3 min., followed up with 46 cycles of 15 sec. at 94°C to melt, and 58°C for 30 sec. for annealing. The melt curve analysis for this protocol ranged from 58°C - 95°C by 0.5°C increments (Winderl et al., 2007).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Target</th>
<th>Annealing Temp. (°C)</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>338F 518R</td>
<td>16S rDNA- Total Bacteria</td>
<td>55</td>
<td>ACTCCTACGGGAGGCAGCAG ATTACCGCG GCTGCTGG GACATGACCGACGCSATYCT TCGTCGTGRTTGGCCCAY TT</td>
<td>(Muyzer et al., 1993)</td>
</tr>
<tr>
<td>bssA7772f</td>
<td>a-subunit benzylsuccinate synthase</td>
<td>58</td>
<td></td>
<td>(Winderl et al., 2007)</td>
</tr>
<tr>
<td>bssA8546r</td>
<td>Phenol monooxygenases</td>
<td>49</td>
<td>GTGCTGACSAAYCTGYTGTC</td>
<td>(Baldwin et al., 2003)</td>
</tr>
<tr>
<td>PHE-F</td>
<td>Ring-hydroxylating monooxygenases</td>
<td>63</td>
<td>CGCCAGAAACCATYTTTTC TCTCVAGCATYCAACGVCACG TTKTCGATGATBACRTCCCA</td>
<td>(Baldwin et al., 2003)</td>
</tr>
<tr>
<td>PHE-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMO-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMO-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Oligonucleotide primers used for functional gene targeting and the temperatures used in qPCR.
Results were taken only from qPCR runs that produced an efficiency within the range of 95%-105% including a minimum of three standard curve points and had no template controls amplify at a lower Cq than the lowest standard dilution. Any runs that did not meet these criteria were repeated as necessary. Samples were considered quantifiable if the Cq value produced was less than the Cq produced by the lowest dilution (10^0 copies) of the standard curve (LSCq) plus one Cq since 1 Cq was considered close enough to the quantifiable range to generate useable results. Detectable, but non-quantifiable (DNQ) samples had Cq values that fell in the range LSCq + 1 < Cq ≤ LSCq + 2. Samples with a Cq value > LSCq + 2 were considered non-detects (ND) since two Cq values beyond the lowest dilution of standard was considered too far to be able to quantify reliable results. Periodically, 1% agarose gels were run on qPCR products to confirm that products of the correct size were being amplified.

2.2.7 Data and Statistical Analysis

All data were back-calculated to copies, either of gene copies (DNA) or transcripts (cDNA) for the gene of interest, per 100mL of groundwater. The data was subjected to analysis performed using Statistical Analysis Software for Windows version 9.4 (SAS Institute, Cary, NC, USA) to compare abundance and potential activity of toluene degraders between seasons and between the wells. For analysis of gene copies and transcripts, values that were considered DNQ were back-calculated and used in statistical analysis since they represent positive detection of functional genes, however values considered ND were substituted with a value of 0.01 to avoid undefined values for the statistical analysis (Boyer et al., 2013; Croghan & Egeghy, 2003). This analysis focused exclusively on monitoring wells M28, M29 and M30 since these were sampled in all four time-points. To represent each well, gene copies and transcripts of ports that were sampled in all four seasonal sampling events were averaged. In a single well, the first
replicates from all ports were averaged, and the second replicates from all ports were also averaged to produce replicated sample data for each well. Three data points in duplicate - two values for each well - were thus obtained for each sampling event.

The Shapiro-Wilks test was used to test for normality of residuals, and the homogeneity of error was tested by creating residual vs. predicted plots. The data was fit with a lognormal distribution with a few exceptions. When a lognormal distribution did not normalize residuals, a negative binomial distribution was applied. This occurred when analyzing both RMO gene copies, and PHE transcripts. The final exception was RMO transcripts which required a square root transformation to achieve normality of residuals. Outliers were retained in the analysis since often these values represented valuable data.

Gene copy ratios were obtained by dividing the replicated functional gene copy numbers for each well by the corresponding 16S gene copy numbers. This produced two replicate ratios for each well in each sampling event. Transcript ratios were similarly determined, however in this case, each replicate of transcript copies for each well were divided by the corresponding gene copy values of the same functional gene (e.g. 16S transcripts rep A / 16S gene copies rep A = 16S transcript ratio rep A).

To analyze the ratio data, first, all ratios were expressed as percentages with the exception of the RMO transcripts ratio. Samples where both replicates were considered ND resulting in a “zero” ratio were replaced with $1 \times 10^{-5}$ and included in the analysis. The exception was for RMO transcript to gene copy ratios, in which values that had both replicates showing as ND were excluded from the analysis. T-tests were performed to confirm that detectable values were significantly different from zero in this analysis. For all analyses, when only one replicate was ND, this was replaced with a value of zero. A lognormal distribution was used in all
analyses with three exceptions. The first exception was the *bssA* transcript to gene copy ratio which required a Poisson distribution for best fit, while a cube root transformation was used for the transcript to gene copy ratios for *PHE*. Finally, a negative binomial distribution was applied to the transcript to gene copy ratio for the *16S* gene. Outliers were again retained in the analysis.

All analyses applied a factorial analysis in which well effects were designated as fixed effects since the three wells were selected to represent areas that were in the Source Zone, Proximal Downgradient and Peripheral relative to the toluene plume. Similarly, since the sampling dates were selected to represent seasons with different water tables heights and points in the poplar tree annual growth cycle, they were also designated as fixed effects. A two-way ANOVA was performed to determine the significance of the well, sampling event (date) and interaction effects in all analyses. A Tukey’s adjustment was used in a multiple means comparison in analysis of ratios and well means. An error rate of less than 5% was used in all analyses.

Finally, a Spearman rank correlation was applied in SAS 9.4 (SAS Institute, Cary, NC, USA) to test correlations between average toluene concentration and average gene and transcript copies for wells.

**2.3 RESULTS**

**2.3.1 Site Characterization**

*Toluene contamination across the Site*

Toluene concentrations were consistently found to be the highest in the Source Zone (M29), with maximum concentrations reaching close to saturation (571 000 µg L\(^{-1}\) in June). Toluene concentrations were also elevated in the Proximal wells (M28), reaching values of 445
100 µg L\(^{-1}\) (Table 2.3). The Peripheral location (M30) contained much lower toluene that hovered close to the detection limit of 0.2 µg L\(^{-1}\) (Table 2.3).

**Toluene contamination across seasons**

Toluene concentrations fluctuated seasonally in the Source Zone; the lowest concentration measured in the deepest port (8.70 m bgs) occurred in March (339.50 µg L\(^{-1}\)) and the second shallowest port (2.90 m bgs) had the lowest toluene concentration in November 2016 (400,000 µg L\(^{-1}\)) (Table 2.3). Between the June and November 2016 samplings, while the maximum concentration in the Source Zone decreased, toluene concentrations at depth increased. In the Proximal well, the toluene concentrations gradually decreased in both ports studied from November 2015 and were lowest in June instead of March. In the Peripheral location, after the November 2015 sampling event, in which the toluene concentrations were 0.21 and 0.37 µg L\(^{-1}\) in the fourth port from the top (~4.19 m below ground surface) and deepest port, respectively, toluene concentrations were maintained at or below the detection limit.

**Groundwater levels**

Groundwater ranged from 313.08 meters above mean sea level (m amsl) to 313.47 m amsl in both fall sampling events. The water table was highest in March (313.79 - 313.82 m amsl) and lowest in June (312.93 - 313.18 m amsl) (Figure 2.4). Generally, the water table was encountered at a depth of approximately 2 meters below ground surface (m bgs).

**2.3.2 Abundance and Activity of Bacterial Communities Across the Site**

**Total bacteria**

Total bacterial abundance was relatively consistent between the wells for each sampling event, ranging between 1.25 x\(10^7\) and 8.34 x\(10^7\) 16S gene copies copies 100mL\(^{-1}\) groundwater. In
March and November 2016 total bacteria were more abundant in the Proximal well \( (8.34 \times 10^7 \) and \( 3.56 \times 10^7 \) \( 16S \) gene copies \( 100 \text{ mL}^{-1} \), respectively) than in the Source Zone \( (3.44 \times 10^7 \) and \( 1.57 \times 10^7 \) \( 16S \) gene copies \( 100 \text{ mL}^{-1} \) respectively). Peripheral well total abundance was also significantly lower than Proximal in March \( (2.66 \times 10^7 \) \( 16S \) gene copies \( 100 \text{ mL}^{-1} \)), but not in November 2016.

The \( 16S \) transcript copy numbers were more variable than gene copies ranging between \( 5.31 \times 10^6 \) to \( 3.77 \times 10^9 \) copies \( 100 \text{ mL}^{-1} \) groundwater, indicating that the bacterial population was active throughout this study. Transcripts were relatively consistent between the three locations in each sampling event with few observed differences. Similar to the gene copy data, the Source Zone yielded significantly fewer \( 16S \) transcripts \( (5.31 \times 10^6 \) \( 16S \) transcripts \( 100 \text{ mL}^{-1} \)) than the Proximal \( (2.72 \times 10^8 \) \( 16S \) transcripts \( 100 \text{ mL}^{-1} \)) and Peripheral wells \( (2.88 \times 10^8 \) \( 16S \) transcripts \( 100 \text{ mL}^{-1} \)) in November 2016.

Ratios of \( 16S \) transcripts to gene copies revealed the Source Zone had more potential activity per gene copy than the Peripheral well in November 2015, while the Peripheral well had significantly greater transcript to gene copy ratios than both other locations in March and November 2016 (Table 2.4) despite greater abundance observed in the Proximal well. No correlation was found between either \( 16S \) gene copies or transcripts and toluene concentration.

**Anaerobic Toluene Degraders**

Active anaerobic toluene degraders were detected throughout the Site with \( bssA \) quantification. Though differences were not all statistically significant, \( bssA \) gene copies were highest in the Proximal location with slightly lower average number of gene copies in the Source Zone. These locations indicated greater proportions of \( bssA \)-harbouring bacteria than in the
Peripheral location in all sampling events with significant differences observed in November 2015, and June 2016 (Figure 2.5). The Peripheral well, had the fewest \textit{bssA} gene copies in all sampling events except one (November 2016) (Figure 2.6) and a lower proportion of anaerobic degraders relative to total bacteria (Figure 2.5).

Despite greater average transcripts in the Source Zone in two events (November 2015 and June) (Figure 2.6) and having the lowest anaerobic degrader abundance, transcript to gene copy ratios indicated that the Peripheral well had the highest potential activity in all events (Table 2.4). Transcripts were most numerous in the Peripheral (March and November 2016) and Source Zone (November 2015 and June) locations although the difference was only significant in March (Figure 2.6). Although transcript ratios revealed the lowest activity in the Proximal well (except for November 2016 where there was no detection in the Source Zone), average transcripts were comparable to those in the Source Zone in all events except November 2015 where they were extremely low (1.50 copies 100 mL$^{-1}$) in the Proximal well (Figure 2.6). Gene copies of \textit{bssA} correlated positively to toluene concentration (R = 0.73, P < 0.05), although no correlation was observed for transcripts.

\textit{Aerobic Toluene Degraders}

Potential aerobic degrader abundance quantified by targeting the \textit{PHE} genes set was generally higher (ranging from 10$^4$ to 10$^6$ copies 100 mL$^{-1}$ groundwater) than abundance of \textit{RMO} genes which ranged from 10$^3$ to 10$^5$ copies 100 mL$^{-1}$ groundwater.

Though not consistently significant, aerobic degraders were most abundant in the Proximal well in all events except for November 2016 (Figure 2.6). The proportion of \textit{RMO}-harbouring bacteria supported this trend (Figure 2.5 C), however relative abundance of \textit{PHE}
diverged from this in June when a greater proportion of PHE genes was detected in the Peripheral well (Figure 2.5 B).

Transcripts of aerobic functional genes were much more variable than gene copies between wells. As with gene copies, transcripts were generally higher for PHE ranging from non-detect to the order of $10^7$ copies 100 mL$^{-1}$ groundwater while RMO transcripts ranged between non-detect and the order of $10^3$ copies 100 mL$^{-1}$ groundwater (Figure 2.6). Aerobic functional gene transcripts were not detected in the Proximal or Source Zone wells in November 2016 (Figure 2.6). Apart from this, either PHE, RMO or both were detected in all other wells and sampling events (Figure 2.6). Apart from PHE in June, transcripts for both aerobic genes were consistently detected in the Peripheral well which also yielded the most transcripts for both PHE and RMO in March and November 2016, while the Proximal well yielded the most in June (Figure 2.6). Detection of PHE transcripts was generally lower (though not always significantly different) in the Source Zone (Figure 2.6). RMO transcripts were less-consistently detected. They were only detected in the Proximal well in June while RMO transcripts in the Source Zone were only detected in November 2015 and March (Figure 2.6).

Transcript to gene copy ratios of PHE and RMO indicated more transcripts per gene copy usually occurred in the Peripheral well than the other two locations (Table 2.4). The only exceptions were a greater RMO transcript: gene ratio in the Source Zone in March, and in June where PHE transcript: gene ratios in the Source Zone and Proximal wells were higher than in the Peripheral well, though not significantly (Table 2.4). No correlation was found between either PHE or RMO gene copies or transcripts and toluene concentration.
2.3.3 Abundance and Activity of Bacterial Communities Between Seasons

*Total bacteria*

Total bacteria were generally most abundant in March in the Source Zone and Proximal wells (10$^7$ gene copies 100 mL$^{-1}$), however, in the Source Zone, 16S gene copies were not significantly different in March and June. The Peripheral well exhibited statistically similar 16S gene copies during all sampling events (Figure 2.6).

The most 16S transcripts were observed in March in all three locations (10$^8$ to 10$^9$ transcripts 100 mL$^{-1}$), suggesting the bacterial community was most active during the spring sampling event (Figure 2.6). In the Source Zone, significantly fewer 16S transcripts were observed in the November 2016 sampling (10$^6$ transcripts 100 mL$^{-1}$), while in the Proximal and Peripheral wells June had the fewest transcripts (both with 10$^7$ transcripts 100 mL$^{-1}$). Transcript to gene copy ratios indicated higher bacterial activity in the November 2015 and March 2016 samplings, particularly in the highly contaminated wells, while the transcript ratio of the Peripheral well was also highest in March (Table 2.4).

*Anaerobic Toluene Degraders*

Anaerobic toluene degraders were consistently most abundant in the June 2016 sampling event for all three locations (10$^5$ to 10$^6$ copies 100 mL$^{-1}$), though the difference was not consistently significant (Figure 2.6). Ratios of bssA to 16S gene copies also indicated an increase in the proportion of anaerobic degraders in June in all three wells (Figure 2.5). The lowest proportion of bssA containing bacteria was generally observed in March (Figure 2.5).

Transcripts of bssA generally followed the same trend as gene copies. Transcripts were highest in June in all three wells (all were 10$^3$ transcripts 100 mL$^{-1}$) (Figure 2.6). The March
sampling yielded the second-highest transcript copies in the Proximal ($10^2$ transcripts 100 mL $^{-1}$) and Peripheral ($10^3$ transcripts 100 mL $^{-1}$) wells, but in the Source Zone, $bssA$ transcripts were second highest in November 2015 ($10^3$ transcripts 100 mL $^{-1}$). No $bssA$ transcripts were detected in the Source Zone in November 2016 in the ports analyzed.

**Aerobic Toluene Degraders**

Trends between seasons for aerobic toluene degradation genes differed considerably and were much less consistent than for $bssA$. The highest $PHE$ gene copies typically occurred in March and June 2016 ($10^5$ to $10^6$ gene copies 100 mL $^{-1}$), although differences between seasons were not always significant. $RMO$ gene copies displayed the same trend as $PHE$ in the Proximal well with comparable copy numbers to March and June also observed in November 2015 (Figure 2.6). The Source Zone, however had the most average $RMO$ gene copies in the two November sampling events (both were $10^4$ copies 100 mL $^{-1}$). The Peripheral well also had the greatest $RMO$ gene copy number in November 2016 ($10^5$ copies 100 mL $^{-1}$). Interestingly, the proportion of $RMO$ to 16S gene copies in the Proximal well was slightly higher in November 2015 compared to March, in which average $RMO$ genes were most abundant (Figure 2.5 & Figure 2.6).

Although statistically the relative proportion of aerobic degrader genes ($RMO$ and $PHE$) in all wells between the seasons did not vary significantly, some observations could be made. The $PHE$ gene copy ratios for all wells were higher in March and peaked June, relative to the November sampling events. Relative proportions of $RMO$ followed an opposite pattern, with slightly higher ratios during the November sampling events relative to the spring and summer events (Figure 2.5 B and C). These results suggest that these two aerobic toluene degrading
genes may detect some different degrading species that are active under different environmental conditions.

*RM* transcripts were detectable throughout the study. Results indicated that the greatest potential aerobic degradation activity occurred in March 2016 in the Peripheral well (10^3 transcripts 100 mL⁻¹) and Source Zone (10^2 transcripts 100 mL⁻¹). In the Proximal well, *RM* transcripts were only detected in June (Figure 2.6). In contrast, *PHE* were generally more actively transcribed throughout the study. The Source Zone and Proximal wells both had the most *PHE* transcripts in November 2015 (10^4 and 10^5 transcripts 100 mL⁻¹, respectively) (Figure 2.6, Table 2.4). The most transcripts per gene copy in the Peripheral well were observed in March (10^7 transcripts 100 mL⁻¹) followed by the November 2015 event (10^5 transcripts 100 mL⁻¹) (Table 2.4) which matched well with total transcripts (Figure 2.6).
Table 2.3 Summary of the range of toluene concentrations observed in Proximal (M28), Source Zone (M29) and Peripheral (M30) locations within the toluene plume.

<table>
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</thead>
<tbody>
<tr>
<td></td>
<td>Toluene (µg L⁻¹)</td>
<td>DO (mg L⁻¹)</td>
<td>Toluene (µg L⁻¹)</td>
<td>DO (mg L⁻¹)</td>
</tr>
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<td>123⁺ 200⁺</td>
<td>3.05 - 9.14</td>
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<td>&lt;0.2 – &lt;0.2</td>
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</table>

⁺ Indicates toluene concentration data came from samples obtained 13 days prior to the microbial sampling.
⁻ Indicates toluene concentration data came from samples obtained mid-October approximately 1 month prior to the November 2016 sampling event.
Figure 2.4 Depth of water table in meters above mean sea level (m amsl) for locations sampled in each sampling event. The dotted line indicates a depth of approximately 2 meters below ground surface (m bgs).
Figure 2.5 Proportions of toluene-degrading communities relative to total bacteria expressed as a percentage for (A) anaerobic degraders (*bss*A) (B) aerobic degraders (*PHE*) (C) aerobic degraders (*RMO*) between sampling events. Error bars represent 95% confidence intervals. • Represents ratios for the Proximal well. □ Represents ratios from the Source Zone and ▲ represents ratios from the Peripheral well. Letters in each graph represent significant differences between all wells in all sampling events. Y-axes are on a logarithmic scale to show the significant differences between wells at low values.
**Table 2.4** Ratios of functional gene transcripts to functional gene copies for total bacterial (16S), anaerobic degraders (bssA), and aerobic degraders (PHE and RMO). Letters represent significant differences between all wells in all sampling events, but are gene-specific.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Well</th>
<th>Sampling Event</th>
<th>Transcript Ratio</th>
<th>Upper 95% CL</th>
<th>Lower 95% CL</th>
<th>Tukey’s Compact Letter Display</th>
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Table 2.4 B  Transcript ratios expressed as percentages (except *RMO* which was analyzed and is expressed as a ratio) of functional gene transcripts to functional gene copies for total bacterial (*16S*), anaerobic degraders (*bssA*), and aerobic degraders (*PHE* and *RMO*). Letters represent significant differences between all wells in all sampling events, but are gene-specific.

<table>
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<th>Gene</th>
<th>Well</th>
<th>Sampling Event</th>
<th>Transcript Ratio</th>
<th>Upper 95% CL</th>
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* Indicates data is presented as a ratio and not as a percentage.
Figure 2.6. Average gene copy and transcript abundances for functional genes representing total bacteria (16S), anaerobic toluene degraders (bssA), and aerobic degraders (PHE and RMO) for wells Proximal to the Source Zone (M28) (light grey), in the Source Zone (M29) (medium grey) and on a Peripheral edge of the plume (M30) (dark grey). Each bar represents an average of all the ports sampled in each well respectively at the sampling event in question. Error bars represent 95 % confidence intervals. Letters represent significant differences between all wells in all sampling events, but are gene-specific.
2.4 DISCUSSION

2.4.1 Effects of Lateral Well Location Within the Plume

Lateral locations within the plume were associated varying levels of toluene contamination and it was thus expected that abundance and activity of toluene degraders would vary depending on the lateral position within the toluene plume.

The comparable ranges of transcript copies for both \textit{bssA} and \	extit{RMO} likely indicate that both aerobic and anaerobic toluene degradation activity is occurring in the Site, although the number of gene transcripts varies depending on season and subsurface conditions.

Several studies have found the number of toluene degrading genes to be positively correlated to toluene concentration (Baldwin et al., 2008; da Silva & Corseuil, 2012; Kazy et al., 2010; Key et al., 2013; Lovely, 2003; Sun & Cupples, 2012) although none recorded toluene concentrations as high as the highest concentrations observed at our Site. Toluene concentration did not correlate to transcripts for any of the functional genes and only \textit{bssA} exhibited a significant correlation between gene copies and toluene concentration. However, trends observed in functional gene copies suggest that toluene degrader abundance is influenced by the horizontal location along the plume-gradient. Most frequently, both aerobic and anaerobic toluene degrading communities were most abundant in the Proximal well (M28) downgradient of the Source Zone. We found that in 3 of 4 sampling dates, bacteria with \textit{RMO} genes were significantly more abundant relative to the total potential bacterial population in the Proximal well than in the Source Zone. Though significant differences were fewer, the trend also appeared for \textit{bssA} and \textit{PHE} with greater proportions of functional genes occurring in the Proximal well in half the sampling events.
Kao et al. (2010) saw similar results in their study monitoring the effectiveness of intrinsic remediation of a BTEX-contaminated groundwater plume. They detected \textit{PHE} only in the source zone and mid-plume (similar to our Proximal location), but gene copies were higher mid plume (Log 4.95 gene copies per liter of groundwater) than they were downgradient (4.63 gene copies per liter) or in the source zone (Log 3.49 gene copies per liter). They did not detect \textit{RMO}. Nebe et al. (2009) also recorded a similar trend in their baseline measurements of \textit{PHE} and \textit{RMO} gene copies prior to an oxygen release trial at another BTEX-contaminated site. Gene copies of \textit{PHE} were highest in the well downgradient of the source zone, while \textit{RMO} gene copies were highest even further downgradient. The Source Zone in this study also had the lowest copy numbers for both genes.

Surges in toluene concentration \textit{in-situ} can be caused by the back-diffusion of sorbed toluene or the dissolution of toluene from the non-aqueous phase (Fernandes 2017; Parker et al., 2010). This phenomenon has been hypothesized for this Site as sporadic increases in toluene concentration are often seen, leading to large ranges of toluene concentrations within wells (Table 2.1) (Fernandes, 2017). Fluctuating toluene concentrations, particularly in the broad range observed at the Site, are likely partially responsible for some of the shifts in toluene degrader populations and activities observed. In areas of very high concentration, toluene may reach levels sufficient become a detriment even to resistant organisms.

As observed in this study, bacteria capable of degrading and using toluene as a carbon and energy source are tolerant to a broad range of toluene concentrations. High levels of toluene; however, have been shown to be inhibitory or even toxic to some degraders. This was documented by Abuhamed et al. (2004) when they examined kinetics of the growth of toluene-degrading \textit{Pseudomonas putida F1} during degradation of toluene and determined inhibitory
effects occurred at high (60,000-180,000 µg L\(^{-1}\)) concentrations of toluene. Other research has indicated that increased degradation intermediates such as catechol and benzyl alcohol may induce loss of catabolic activity by inducing mutations although this is less likely to occur in-situ due to natural flow and dilution (Kim & Jaffe, 2007; Leddy, 1995; Murray et al., 1972). Jones et al. (1997) demonstrated negative correlations between fraction of viable cells and exposure time and toluene concentration respectively. Toluene concentrations in groundwater at the Site have been recorded to range from below detection to approximately saturation (Fernandes, 2017). It is possible that inhibitory or toxic effects may be observed in very highly contaminated areas within the Site (Lueders, 2017). For example, toluene concentrations close to saturation were recorded in the upper Source Zone port sampled (Table 2.1) and coincided with a significantly reduced proportion of RMO containing communities compared to the Proximal well in the first three sampling events. These concentrations (400,000 to 570,000 µg L\(^{-1}\)) exceeded the concentrations of toluene used previously (60,000 to 180,000 µg L\(^{-1}\)) that elicited toxic effects on degraders (Abuhamed et al., 2004) and may indicate some minimal toxicity to indigenous aerobic degraders. This trend was not observed for bssA, PHE, or 16S and suggesting that either microbes with RMO genes may be more susceptible to toluene toxicity at very high levels, or the effect observed is simply an artifact of other environmental factors.

Our results indicate that the resilience and adaptability of environmental bacteria should not be overlooked. Previous studies have also shown variability of bacterial responses to the presence of contaminants even between strains of the same species. In a soil microcosm study, Huertas et al. (1998) introduced 1% (v/w) toluene to three toluene-degrading P. putida strains in soil microcosms. Large decreases between 2 and 6 orders of magnitude in cell count were immediately observed in all strains tested, however, two strains eventually (between
approximately 10 and 14 days after) recovered to cell counts almost equivalent those observed before toluene was introduced. The experiment provided a glimpse of the variation in bacterial responses to harsh compounds in their environment even within a single species.

In uncontaminated sites, hydrocarbon degraders can constitute as low as 0.1% of bacterial populations or less, while in contaminated locations they can make up as much as 100% of a bacterial community (Atlas, 1981). One of the few studies that examined aerobic and anaerobic degraders in a toluene-contaminated aquifer found that when 16S genes ranged from $10^7$ to $10^9$ copies, gene copies of the aerobic gene quantified ($tmoA$) and $bssA$ both ranged from $10^6$ to $10^7$ (Larentis et al., 2014). While this study was performed on sediments, it illustrates the high proportions of toluene-tolerant degraders in a contaminated site which helps explain the small magnitude of differences in gene abundances between sampling locations at the Site. In comparison, 16S gene copies were all within $10^7$, while $bssA$ gene copies ranged from $10^4$ to $10^7$, $PHE$ from $10^4$ to $10^6$, and $RMO$ from $10^3$ to $10^5$.

Total bacterial gene abundances (16S) in the Peripheral well with minimal toluene were comparable to those in the highly contaminated Source Zone and Proximal wells. In most cases, 16S transcripts were also relatively consistent between wells. Elevated levels of toluene in the Source Zone and Proximal wells did not appear to affect the abundance of total bacteria despite fluctuating toluene concentrations, indicating a persisting, well-adapted community. However, the Peripheral well with very low toluene concentrations produced comparable functional gene copy numbers to those in areas of high contamination on most occasions, possibly suggesting that toluene in the Peripheral well is still actively being degraded by the microbial communities.

Lack of consistent correspondence between very high toluene concentrations and relatively reduced degrader abundance and activity indicated toxic effects could be occurring,
but they could be very localized if they were. This suggests that analysis of an averaged representation of an entire well may be insufficient to detect these influences and that greater resolution is required. Observations in the Peripheral well, however suggest that bacterial activity and populations likely respond to toluene fluctuations and its availability as a carbon and energy source at lower concentrations.

Though rarely significant statistically, it was interesting to note that functional gene abundance, especially that of PHE and bssA, was frequently slightly lower in the Peripheral well where toluene was very low or below detection. Ratios of bssA and PHE gene copies to 16S gene copies confirmed that generally, the proportion of functional genes was also frequently slightly lower in this well than in the highly contaminated wells. This could in-part be due to the limited toluene present in the Peripheral well providing further support that toluene degrading communities respond to toluene concentration. Low levels of contamination likely allow other less-tolerant or non-degrader species to colonize the area, thus the proportion of degraders could have been reduced in the Peripheral location. Hendrickx et al. (2005) detected only xylM/xylE genotypes in a location with very low toluene contamination (4.8 µg L⁻¹) adjacent to a BTEX plume while both tmoA and xylM/xylE genes were detected in the contaminated zone possibly suggesting fewer toluene degrading organisms, or at the very least a reduced diversity of degraders colonized the peripheral location.

In the Peripheral well, toluene concentrations were very low in November 2015 (0.21-0.37 µg L⁻¹) then dropped below detection limit from March to November 2016. Transcript copies of RMO, PHE and bssA indicating potential active degradation were frequently high in the March 2016 sampling event where toluene first dropped below the detection limit. Transcripts of PHE and bssA were particularly high in March in the Peripheral well suggesting
greater potential degradation activity compared to the other locations. This pattern was also visible for RMO, though less drastic. General reduction in toluene concentration in the other wells coinciding with detection of functional gene transcripts suggests microbial degradation was at least partially responsible for the observed reduction in toluene. After March, bssA and RMO transcript copies remained generally stable in the Peripheral well.

The generally consistent detection transcripts in the Peripheral well indicates degraders were actively degrading toluene and likely maintaining toluene concentrations at or below 0.2 µg L⁻¹. Although the Peripheral well is removed perpendicularly to the general flow path of groundwater, toluene likely still extends to that well and the local bacterial communities appear to be playing a role in attenuating further extension at the fringes of the plume.

The increase in bssA transcripts in the Peripheral well in March which was maintained to November 2016 and the frequent detection of PHE and RMO transcripts in the Peripheral well support the “plume fringe theory”. The theory postulates that increased degradation activity occurs at plume fringes due to overlapping gradients of electron donors and acceptors that are refreshed through mixing from uncontaminated water (Anneser et al., 2008; Carmona et al., 2009; Lueders, 2017; Meckenstock et al., 2015). Numerous studies have reported increased degradation activity for toluene and other contaminants localized to plume fringes (Anneser et al., 2008; Carmona et al., 2009; Herzyk et al., 2017; Pilloni et al., 2011; Winderl et al., 2008). Particularly for bssA and PHE genes, transcript to gene copy ratios frequently indicated slightly more transcripts per gene copy in the Peripheral well than in the other wells. Although the difference was not always statistically significant, biologically, the trend further supports the plume fringe theory.
In November 2015 and between March and June, toluene concentrations generally increased suggesting back-diffusion of toluene, likely from bedrock. BssA transcripts were high in all wells in June and were relatively high in the Source Zone in the November 2015 sampling event, both with relatively high toluene concentrations. This could suggest anaerobic degrader activity increased in response to increased toluene in the Source Zone at the Site.

Increased dissolution of toluene beginning in March would also induce expression of toluene degradation genes in the Peripheral location. The significantly increased potential aerobic degrader activity in that well in the March 2016 sampling event supports this (Figure 2.6), however no toluene increase was observed to indicate back-diffusion of toluene at this location. As mentioned previously, it is possible that the toluene was so low in this area that degradation activity maintained the levels of toluene below our detection limit and masked back-diffusion evidence. Consistent detection of functional gene transcripts in the Peripheral location when toluene was close to, or below detection limit indicated an active anaerobic community was potentially degrading toluene in that well. Real-time and depth-delineated monitoring of potential bacterial abundance and activity at the Site may produce more detailed data in which fluctuations of degrader abundance and activity in response to toluene may be seen. Our observations seem to hint that seasonality may play a role in biodegradation activities occurring on Site.

2.4.2 Seasonal Effects

The general net reductions in toluene concentration (with a few exceptions) over all the sampling events suggests increased degradation over the spring and summer months. These sampling events most often coincided with increased degrader genes and transcripts indicating bacterial toluene degrader activity was responsible. Anaerobic degraders most consistently
displayed seasonal effects in all three sampling locations with increased gene copies and transcripts in June. Aerobic degraders were more variable, though frequently most gene copies and transcripts were recorded in March and June. However, gene copies and transcripts copies for both PHE and RMO also were often high in the November sampling events.

Spatiotemporal trends in total bacterial abundance was also found to be higher in the summer and autumn months in a sandy aquifer system (Ayuso et al., 2009). Ayuso et al. (2009) associated seasonal changes in bacterial abundance with temperature change (ranging from 14.9°C to 22.53 ± 0.15 °C). Fluctuation in historical groundwater temperatures in our Site is limited (8°C- 12°C) (Water Wells & Ground Water Supplies in Ontario, 1994). It is therefore, unlikely that temperature change associated with seasonal variation would have a strong effect on degrader abundance and activity, although it could be a small contributor to the trends observed at our Site.

Fluctuations in water table levels throughout our study also may have contributed to the seasonal changes in the toluene degrader populations. At the Site, toluene is mainly sorbed to the matrix around the mean water table between 2.3 and 3.5 m bgs. An elevated water table at approximately 313.8 m amsl reached this zone in bedrock and distinguished the March 2016 sampling event. This suggests the higher water table in March likely increased back-diffusion and contributed to the higher toluene concentrations observed in June (Table 2.3).

The highest water table in March (approximately 313.8 m amsl) contrasts to the subsequent June 2016 sampling in which the water table was lowest among sampling events (approximately 313.0 m amsl). Similarly, the water table was only slightly lower in November 2015 at approximately 313.4 m amsl, while in November 2016 it had dropped to approximately 313.1 m amsl. The main source of oxygen in most groundwater is the atmosphere (Rose & Long,
When water tables rise, the same oxygen source must penetrate greater depths by simple diffusion and advection, therefore reduced oxygen caused by higher levels of groundwater seems intuitive. One might thus expect increased abundance and activity of anaerobic degraders to coincide with increased water table levels. However, often in groundwater this is not the case and subsurface water reservoirs are often found to contain appreciable DO levels making it difficult to predict where anaerobic degradation is more likely to occur (Rose & Long, 1988).

For aquifers located in temperate zones such as ours, springtime is associated with increased rainfall, as well as snowmelt that causes recharge in groundwater levels (Treidel et al., 2012). As the surface water percolates through the overburden to reach the saturated zone it entraps oxygen bubbles and dissolves other organic nutrients from the soil (Danczak et al., 2016; Landmeyer & Effinger, 2016; Treiden et al., 2012; Yabusaki et al., 2017). Although low-oxygen conditions typically dominate the aquifer in the area, this influx of oxygen rich water to the system is likely instrumental in stimulating the potential aerobic degradation activity observed in the spring (Fernandes, 2017; Zanini et al., 2000). A vertical downward gradient in the Source Zone between approximately 2.0 and 6.5 mbgs encourages further mixing of the oxygen-rich water and toluene deeper into the system (Fernandes, 2017). Furthermore, previous records indicate that aerobic degraders are frequently detected in highly reducing environments that are expected to be more suitable for anaerobic degraders (Hendrickx et al., 2005; Hendrickx et al., 2006; Larentis et al., 2013). The dissolved oxygen introduced into this largely low-oxygen environment is likely quickly depleted by aerobic toluene degraders that are well-adapted to intermittent fluxes in oxygen (Carmona et al., 2009; Danczak et al., 2016; Fernandes, 2017; Kümmel et al., 2013; Landmeyer & Effinger, 2016; Vogt et al., 2008; Yabusaki et al., 2017). The increased aerobic activity observed in the November 2015 sampling event, particularly evident
from *PHE* transcript to gene copy ratios, could be a product of such a flux in oxygen concentration. It seems likely that the somewhat sporadic trends and increased variability in aerobic degrader abundance and activity is linked to this apparent robustness of aerobic degraders as well as transient redox and contamination conditions. Increased resolution of sample analysis would probably provide better information to explain the patterns in the potential abundance and activity of aerobic degraders.

The rapid consumption of DO often leads to an oxygen-depleted environment conducive to the proliferation and activity of the intrinsic anaerobic toluene-degrading communities in environmental systems (Carmona et al., 2009; Danczak et al., 2016; Kümmel et al., 2013; Landmeyer & Effinger, 2016; Vogt et al., 2008; Yabusaki et al., 2017). The increase in abundance and activity of anaerobic toluene degraders in June 2016 matches well with this since the likely influx of oxygen from spring meltwater would be consumed leaving conditions more suitable for anaerobes.

It should be noted that the aerobic genes targeted in this study do not capture genes involved in all the possible toluene degradation pathways and may under represent sulfate reducers and possibly methanogens due to primer design and limited sequence availability. Future studies on seasonal analysis should target genes involved in all the aerobic degradation pathways to determine if seasonal differences in expression of genes involved in the various aerobic pathways exist.
2.5 CONCLUSION

Quantification of potential abundance and activity of aerobic and anaerobic toluene degraders in wells located in the Source Zone, Proximal and Peripheral locations indicated that both aerobic and anaerobic toluene degradation is actively occurring across the Site.

Analysis indicated that, although limited, some differences may be observed in locations with varying contamination levels. It was an interesting result that potential degradation activity occurring in the minimally-contaminated Peripheral location was comparable to that occurring in the severely contaminated wells in most cases, occasionally exceeding them in potential activity. Apart from *bssA*, lack of correlation of functional genes to toluene concentrations seems to indicate that these differences between wells seem to be associated with seasons and the effects of water table fluctuations and influx of oxygen-rich water from snow melt rather than from the intrinsic severity of toluene contamination within the entire well. There were two instances, however, in the spring and late fall (November 2015) sampling events that exhibited increased degrader activity in the Peripheral location with minimal toluene (>0.2 µg L\(^{-1}\)) while activity in the highly contaminated wells remained lower. It was hypothesized that increases in toluene could potentially have an inhibitory or even toxic effect on degrader catabolic activity. While the data does provide some support to this, the trend is inconsistent and cannot be attributed to toluene contamination only.

Our data indicated that seasonal trends did exist in the temporal sampling campaign at the Site. Anaerobic degraders indicated a particularly consistent seasonal effect, favouring the summer sampling event with a low water table. This sampling succeeded the spring sampling which was distinguished by a higher water table; a result of oxygen-rich groundwater recharge.
from snowmelt. It was in this sampling that aerobic degraders most frequently exhibited increased abundance and activity. However, this pattern was much less consistent than that of \textit{bssA} and increased aerobic abundance and activity were also observed in June and both November events.

While these data proved useful for examining broad seasonal trends across the locations of varying contamination, some results are difficult to explain when considering an overall average of the monitoring wells. The fractured bedrock network of the Site offers a particularly complex scenario in which to study the effects of toluene concentration. The asynchronous toluene fluctuations recorded within single wells substantiates the necessity of our highly resolved data for gathering information about the trends in toluene concentration and how they may relate to the abundance and activity of aerobic and anaerobic toluene degraders at the Site.

Future research on \textit{in-situ} contamination plumes should use additional primer sets to more accurately capture all the aerobic pathways in addition to the anaerobic pathway. Although primers targeting all aerobic pathways have been developed, (Baldwin et al., 2003; Hendrickx et al., 2004; Hendrickx et al., 2006; Shinoda et al., 2004), to our knowledge broad aerobic pathway coverage has not been done in combination with the anaerobic pathway on the same samples to study the effects of contamination effects and seasons on degrader abundance and activity \textit{in-situ}.
CHAPTER 3.0 Depth-discrete analysis of bacterial toluene degradation genes in a toluene contaminated aquifer

Author Contributions:
A. Roebuck was the primary author and responsible for the microbial characterization in groundwater samples. Installation of the monitoring network and site characterization is credited to Jeremy Fernandes and Beth Parker in association with the G360 Institute for Groundwater Research and Compound-Specific Isotope analysis is credited to our collaborators, Philipp Wanner with the University of Guelph, and Ramon Aravena, with the University of Waterloo.

3.1 INTRODUCTION

Benzene, toluene, ethylbenzene and xylene, individually or as a group (BTEX), are broadly applied in an industrial context as solvents, constituents in products like adhesives and paint, and are often found in fuels and other petroleum-based mixtures (An, 2004; Collins et al., 2002). Use of BTEX compounds in such a variety of everyday and industrial contexts increases the likelihood of environmental contamination due to spillage, improper waste disposal and even simple application. These compounds are classified as light non-aqueous phase liquids (LNAPL) with low solubility and a specific density less than that of water (Newell et al., 1995). Though only a few studies have examined LNAPL in the context of a fractured bedrock setting (e.g. Fernandes, 2017), the characteristics of LNAPLs elicit unique mobility and dispersion patterns in contamination plumes in groundwater and the subsurface (Hardisty et al., 1998; Hardisty et al., 2004; Schwille, 1981; Newell et al., 1995; Morrison, 1999). Water table fluctuation, matrix diffusion and sorption of the LNAPL to subsurface media all influence immiscible and aqueous phase contaminant distribution in the subsurface. LNAPLs tend to remain in the upper areas of the plume within the capillary fringe (Newell et al., 1995; Schwille, 1981). The low solubility of LNAPLs can create an exclusion barrier within the capillary fringe at the water interface.
Smaller pore sizes retain water and may make it difficult for LNAPLs to penetrate the capillary barrier in the absence of additional solvents. This can cause the immiscible phase of LNAPL to extend the plume laterally (Fernandes, 2017; Schwille, 1975). Depending on their physicochemical characteristics, however, LNAPLs may also partition into the aqueous and gaseous phases via dissolution and volatilization respectively (Meckenstock et al., 2004).

Microbial degradation of dissolved LNAPLs has a feedback relationship with redox conditions in groundwater; each impacting the other. Historical approaches to conceptualize redox conditions affected by degradation processes assumed the formation of a longitudinal redox zonation due to rapid consumption of electron acceptors where the least energetically favourable (methanogenic) dominate the plume center and gradually transition to more thermodynamically favourable redox conditions (sulfate reducing followed by manganese (IV) and iron (III) reducing, then nitrate and finally oxygen reducing conditions) (Meckenstock et al., 2015). However, Meckenstock et al. (2015) observed electron acceptors used upgradient are not easily replenished downgradient due to laminar flow and limited transverse flow of groundwater. Electron acceptors previously thought to only be used in the plume core also dominate redox conditions downgradient. Meckenstock et al. (2015) called this the “plume fringe concept”. The basis of this concept may also be extended to quantification of functional genes from planktonic bacteria in groundwater. Genes and transcripts in groundwater are affected by laminar flow and thus may represent conditions upgradient of the location where a sample is taken (Brow et al., 2013; Ginn et al., 2002). Although biofilms may provide a better representation of degrader abundance and activity at a particular location, they are difficult to sample in the subsurface (Hazen et al., 1991).
Our Site of interest is a former manufacturing facility that operated from the 1950’s to late 1980’s. The operation used toluene as a solvent and stored it on-Site, external to the building, in partially buried storage tanks that supplied the adjacent building directly through a set of buried pipes/product lines. Toluene contamination was discovered upon decommissioning the storage tanks in the area of the tanks and product lines. Impacted overburden and shallow bedrock material were removed in the vicinity of the tanks. Several additional methods were used subsequently to remediate impacts measured in groundwater, including conventional pump-and-treat using single-interval wells, and an Oxygen Release Compound (ORC) trial. Each method was either applied to the point of decreasing returns in attenuation or discontinued due to limited efficacy. In 2008, a phytoremediation system was implemented. The hypothesis was that dense planting of *Populus* × *canadensis* hybrid poplar trees in the historic location of the toluene storage tanks and pipe system would bring about enhanced bioattenuation of residual toluene in a number of ways. This included growth of the tree roots into the same fractures in the upper bedrock in which residual toluene occurs, which would provide both direct uptake and volatilization of the toluene by the trees and indirect promotion of biodegradation through creation of favourable localized conditions (chemical and microbiological). Shallower tree roots could promote biodegradation in the overburden, and the root system could also exert hydraulic containment in the area to prevent further spread of the contaminant plume. By 2014 the tree stand was considered mature and stable enough to be producing effects within the remediation system and a collaborative effort to characterize and study the Site in detail commenced. The remediation system is best described as a phytoremediation system with intrinsic remediation in the underlying groundwater aquifer. The Site provides an ideal scenario for *in-situ* study of pure-
phase contaminant (toluene) attenuation by bacteria in groundwater within a porous fractured bedrock context.

This research aimed to confirm the active degradation of toluene by indigenous bacteria in a contaminated aquifer setting by detecting and quantifying genes and actively-transcribed functional genes involved in toluene degradation. Multiple lines of evidence were applied to mechanistically characterize natural toluene biodegradation processes in groundwater at the Site.

The first line of evidence for monitored natural attenuation is typically a decrease in the dissolved concentrations of the contaminant of concern (COC), and the mechanisms of degradation are inferred through analysis of geochemical indicator ions. However, this method cannot be used to distinguish between concentration reductions stemming from abiotic factors like dilution, dispersion, sorption or volatilization from those seen due to microbial degradation (Key et al., 2014; Beller et al., 2002). At the Site, toluene concentrations have been measured since 1989, and concentrations support a slow retraction in the lateral plume extent over time (Fernandes, 2017). However, highly variable concentrations of toluene within, and proximal to, the source zone even at high vertical sampling resolution, speak to multiple processes affecting the overall measured concentration. A second line of evidence is compound specific isotope analysis (CSIA). CSIA, which involves using spectrometry to measure the ratios of heavy and light isotopes in a specific compound (such as toluene) to determine what attenuation mechanisms may be at play, was applied as well (Meckenstock et al., 2004). This method allows differentiation between some attenuation mechanisms. Light isotopes in a compound tend to be bound in a molecule more weakly than heavy isotopes. Consequently, molecules of a compound containing light isotopes are preferentially degraded by indigenous bacteria due to a lower
activation energy requirement and resulting in enrichment of heavy isotopes (Meckenstock et al., 2004).

Research has shown that when sorption is involved in plume containment, lighter isotopes may experience higher retardation, but this is typically only at the front and back edges of the plume in an initial contamination pulse which is a rare occurrence since contaminant release is typically continuous (Meckenstock et al., 2004). Evaporation has also been shown to produce small isotopic enrichment effects of heavy isotopes, however this process tends to only occur in the upper plume levels at the capillary fringe and may not be relevant in the bulk of the plume (Harrington et al., 1999; Meckenstock et al., 2004). Therefore, enrichment of heavy isotopes in remaining toluene when mass reduction is observed is likely indicative of biological degradation, while other mechanisms of attenuation such as volatilization or sorption will not produce heavy isotope enrichment. Not only this, but in anoxic environments, distinct isotopic enrichment signatures have been shown as a function of redox conditions. This is attributed to the use of different electron acceptors for the biological degradation processes (Mancini et al., 2003; Meckenstock et al., 2004).

Together, monitoring of COC concentrations, geochemical indicator ions, and CSIA can provides key lines of evidence for the nature and extent of biodegradation in a system. However, results of these methods can be difficult to interpret in groundwater systems, where there are number of physical and chemical processes affecting flow conditions and chemical concentrations. Factors like recharge events may have a transient diluting effect, and could make it seem that COCs are being attenuated faster than they truly are. On the other hand, processes including dissolution from LNAPL phase or back diffusion of sorbed toluene may make it seem like contaminants are persisting or even increasing when degradation is actively occurring. Even
CSIA may not be able to accurately detect degradation in these scenarios since signatures will fluctuate. By combining CSIA results with data from molecular biological tools such as qPCR, COC degradation may be more accurately detected.

Quantitative polymerase chain reaction provides a means to quantify the degradation potential of bacterial communities. Multiple primer sets, typically targeting enzyme subunits involved in substrate specificity, must be used to quantify potential degradation in the multiple toluene degradation pathways. However, some primer sets, such as those targeting phenol hydroxylases which act on phenol and cresol intermediates of multiple monooxygenase pathways, may target downstream steps.

The number of studies targeting DNA and RNA in intrinsically remediated groundwater in-situ is limited, and fewer still have also targeted both aerobic and anaerobic pathways within the same samples (da Silva & Couseuil, 2012; Larentis et al., 2013). Larentis et al. (2013) and Winderl et al. (2008) quantified genes involved in aerobic and anaerobic pathways in the same site, as we do in this study, however anaerobic genes were quantified from samples obtained at a different time than aerobic genes. They found a surprising maxima of aerobic toluene degradation genes in the strongly reduced plume core (Larentis et al., 2013), while anaerobic degraders were found to correlate strongly with areas of increased degradation activity (Winderl et al., 2008). While that study did not target activity (RNA) of the degrader populations, a high-resolution approach was applied for vertical sampling of the plume. The abundance of potential degraders was thus quantified at a fine scale with depth. Depth discrete sampling can significantly increase the robustness of a Conceptual Site Model (CSM) and the conclusions that may be drawn therefrom. Therefore depth-discrete sampling of groundwater was implemented at the Site to create a detailed CSM.
At our Site, we have combined high-resolution depth-discrete sampling with CSIA and microbial analysis to quantify potential abundance and activity of anaerobic and aerobic toluene degraders \textit{in situ}, over space and time. To produce a high-resolution CSM, a discrete fracture network (DFN) approach was implemented to target fracture network properties and the distribution of toluene mass at the Site (Fernandes, 2017). Boreholes advanced for the DFN approach were completed as multilevel monitoring systems (MLS). Fracture network characterization and use of MLS is particularly important when studying groundwater in a fractured bedrock setting since fractures exert strong influence on contaminant distribution and groundwater flow. MLS’s allow a depth-delineated view of the Site which is otherwise lost when using conventional single interval wells. The addition of CSIA and microbial analysis to this high-resolution approach serves to build a mechanistic CSM for toluene degradation, with a high level of detail and multiple, diverse lines of evidence to demonstrate efficacy of the remediation system. At this Site specifically, the lines of evidence discussed will contribute to determining the efficacy of toluene remediation below a phytoremediation system in the fractured bedrock aquifer. To our knowledge a study with this level of detail examining both types of pathways and quantifying transcripts for degradation of a single LNAPL in this context has not previously been done.

3.2 METHODS AND MATERIALS

3.2.1 Site Characterization

Methods for the DFN approach to characterizing bedrock at the Site and data collection for VOC profiles in bedrock cores as well as installation of the monitoring well network were carried out as described in Chapter 2 (See sections 2.2.1 to 2.2.3).
3.2.2 Groundwater Sampling

Groundwater sampling for microbial analysis targeted various points in the annual tree growth cycle and the varying groundwater levels typically associated with autumn, spring and summer seasons (November 2015, March 2016, June 2016 and November 2016). A minimum of 2 ports were sampled in wells in the Source Zone (M29), Proximal (M28) to the Source Zone in the direction of groundwater flow, and at a more distal downgradient location, referred to as the “Peripheral” location (M30) (Figure 2.3) on each sampling date. Ports sampled varied between sampling events and are summarized in Appendix B (Table B.1).

In each sampling event, ports that were sampled in the multilevel monitoring wells (additional details provided in Chapter 3) were purged three times the volume of the ¼ inch polyethylene sampling tube (See Appendix B Table B.2 for details regarding depths of the sampling ports of the wells studied for this thesis). Approximately 1 L of groundwater was sampled by pumping using a Geotech Series II peristaltic pump in line with a YSI 556 Multiprobe Water Quality Meter. To minimize drawing water from non-target areas the pump was operated at the lowest setting (approximately 100 mL min$^{-1}$). Once stabilized, pH, specific conductivity, temperature, dissolved oxygen (DO), and oxidative reductive potential (ORP) were recorded for each sample. Groundwater samples were added to 2 L pyrex media bottles containing 500mL aliquots of a lab-made RNALater® stabilization solution to preserve RNA (De Wit et al., 2012). Samples were stored on ice for transport back to the lab where they were kept in the dark at 4°C until further processing. Dedicated tubing was used for sampling each port.

3.2.3 Groundwater Analysis

Samples were collected for analysis of nitrate, iron, sulfate, sodium and chloride ions by Maxxam Analytics (Mississauga, ON) to characterize the terminal electron acceptors being used for microbial toluene degradation. VOC samples were collected in glass vials and analysis
completed either by Maxxam Analytics using the EPA 8260 C method, or at the University of Guelph using the EPA 8260 B method of analysis. Both methods have a toluene detection limit of 0.2 µg L\(^{-1}\) (liquid). Glass VOA vials were used to collect dissolved gas samples in November 2015, July 2016 and October 2016. These were analyzed by Maxxam Analytics using the RSKSOP-175m procedure and the headspace in the vial was analyzed with gas chromatography with flame ionization detection (GC-FID) (Fernandes, 2017). Quality Assurance/Quality Control (QA/QC) procedures were carried out as detailed in Fernandes (2017).

Samples for CSIA analysis were collected in concert with VOC sample collection. A gas chromatograph coupled with a mass spectrometer was used to determine carbon and hydrogen isotope ratios in toluene as described in Wanner et al. (2016).

### 3.2.4 Microbial Sample Processing

Each 1.5L sample including RNALater\textsuperscript{®} was split approximately in half (approximately 750 mL each composed of approximately 500 mL of groundwater and 250 mL of RNALater\textsuperscript{®}). Each half was filtered independently through separate 0.22 µm pore size membrane filters by vacuum filtration. Exact filter volumes were recorded. Membranes were removed from the filtration unit with ethanol-sterilized forceps and rolled into 15 mL conical tubes to be stored at -80°C until DNA and RNA were extracted between one and five days after filtration.

MoBio PowerWater\textsuperscript{®} DNA Isolation Kits were used to co-extract DNA and RNA from the samples (MoBio Laboratories Inc.). Six random RNA samples were measured with a Qubit\textsuperscript{TM} 4 Fluorometer (Thermofisher Scientific, Waltham, Massachusetts) using the Qubit\textsuperscript{TM} RNA HS Assay Kit (Invitrogen, Thermofisher Scientific, Carlsbad, California) to determine an average of approximately 1.2 ng µL\(^{-1}\). Six random DNA samples were measured using a NanoDrop\textsuperscript{TM} 8000 (Thermofisher Scientific, Waltham, Massachusetts) to determine and average
of approximately 12 ng µL\(^{-1}\). A Promega RQ1 RNase-Free DNase kit was used following the prescribed protocol to remove DNA from a 10 µL sub-sample (in quadruplicate) of the extract to prepare for reverse-transcription of the RNA. Following DNase treatment, three of the four subsamples of RNA were reverse-transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit following the protocol provided in the kit. These sub samples were pooled post-reverse transcription to achieve a larger volume of cDNA. DNA and cDNA were both stored at -80°C.

The extra sub-sample of RNA was tested post-DNase treatment for contaminating DNA. This test involved running qPCR with 16S primers designed to capture total bacteria (338F/518R). Controls with no reverse transcriptase added to an additional random RNA sample were used along with no-template controls to provide additional tests for cleanliness of the reagents and again to ensure no DNA carry-over occurred during reverse transcription. These controls were also tested using the same 16S primers in qPCR.

3.2.5 qPCR Analysis

Total bacteria, potential anaerobic toluene degraders, potential aerobic degraders and their potential activities were all quantified to confirm bacterial biodegradation of toluene was occurring at the Site. For potential anaerobic degraders, the gene encoding the enzyme catalyzing the first step of the only known anaerobic toluene degradation pathway, benzylsuccinate synthase, was targeted (Winderl et al., 2007). Two primer sets were used to detect bacterial toluene degradation occurring through the aerobic degradation pathways. One set targeted ring-hydroxylating monooxygenases commonly involved in the first step of multiple aerobic toluene degradation pathways (\textit{RMO}), while the other targeted phenol monooxygenases involved in later
steps of some of the aerobic toluene degradation pathways (PHE) (Baldwin et al., 2003). The
primers used for qPCR are summarized in Table 2.2.

Inhibition testing as described in Section 2.2.5 was performed for DNA and cDNA prior to qPCR to test dilutions that would reduce the effect of inhibitors, but still maintain quantifiable levels of target genes should they be present in the samples. After inhibition testing was performed, qPCRs using each of the primer sets were completed for all samples from M28, M29 and M30 since these wells were most consistently sampled throughout the microbial sampling campaign. All qPCRs were completed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using Bio-Rad CFX Manager Version 3.1 software. Detailed thermocycler protocols and reaction mixture components for all genes tested as well as criteria for sample quantification found in Section 2.2.6.

3.3 RESULTS

3.3.1 Groundwater Toluene and Geochemical Indicator Concentrations

Toluene concentrations in groundwater at the Site were highly variable in the Source Zone (M29) and Proximal (M28) locations, however in the Peripheral well (M30), concentrations were consistently < 1µg L\(^{-1}\) in all sampling events and dropped below the detection limit of 0.2 µg L\(^{-1}\) in all ports for all events after November 2015. The sampling interval centered around 2.9 m bgs consistently yielded the highest toluene concentrations, ranging between 400 000 µg L\(^{-1}\) to 571 200 µg L\(^{-1}\) in the Source Zone and 172 625 µg L\(^{-1}\) to 445 000 µg L\(^{-1}\) in the Proximal location (Figure 3.2).

Redox conditions in the Source Zone and Proximal locations were generally similar with depth during the November 2015 and June 2016 sampling events. At both locations, results suggest that oxygen may be present (Figure 3.4), but that conditions creating strongly reducing
conditions can also be present (Figures 3.5 & 3.6). In November 2015, conditions in the Source Zone and Proximal wells appeared to be most strongly reducing in the upper ports (methane ranging between approximately 6 and 8 mg L\(^{-1}\)), although it appeared multiple electron acceptors were being used. Dissolved manganese and iron concentrations were also relatively increased (approximately 0.4 mg L\(^{-1}\) and 3 mg L\(^{-1}\) respectively) while sulfate was moderately low (up to only approximately 10 mg L\(^{-1}\)) and nitrate was very low (below detection), suggesting active or previously active use of these alternate electron acceptors. With increasing depth, conditions became gradually less reducing in the Source Zone and Proximal wells as methane concentrations decreased and sulfate concentrations decreased to almost zero mid-well suggesting a shift to sulfate-reducing conditions (Figure 3.5). Increased sulfate (over 20 mg L\(^{-1}\)) in the deepest port in both the Proximal and Source Zone wells in November 2015 indicated sulfate was likely not used as an electron acceptor in the deepest port.

In June, the strongest reducing conditions were also observed in the uppermost ports of the Source Zone and Proximal wells, indicated by much higher manganese concentrations and slightly higher methane (<1 mg L\(^{-1}\)), though conditions were not as strongly reducing as in November 2015 (Figures 3.5 & 3.6). Stronger reducing conditions (slightly increased methane to approximately 1 mg L\(^{-1}\) and low sulfate near detection limits of 1 mg L\(^{-1}\)) also appeared between depths of approximately 4.95 and 6.45 m bgs in the Source Zone where a relative increase in iron concentrations to 2.1 mg L\(^{-1}\) was observed along with a marginal increase in methane (Figure 3.6). It was evident that the Source Zone typically had stronger reducing conditions than the Proximal location in both November 2015 and June 2016 as indicated by higher dissolved methane concentrations (Figure 3.5 and Figure 3.6). Stronger reducing conditions in the Source
Zone were possibly related to the higher levels of toluene in that location and possibly greater degradation activity consuming electron acceptors and creating a stronger reducing environment.

Redox conditions observed with depth in the Peripheral well were different from those in the Source Zone and Proximal locations. In the Peripheral well, conditions became increasingly reducing with depth. Methane concentrations of approximately 9.5 mg L\(^{-1}\), comparable to those observed where toluene concentrations were highest in the Source Zone, were measured in the deepest ports of the Peripheral well (between 6.45 and 8.69 m bgs). In the mid-depth ports of the Peripheral well, groundwater conditions were less strongly reducing, with sulfate reduction dominating up to 4.19 m bgs in November 2015 (Figures 3.5). Above this depth, sulfate and nitrate concentrations increased suggesting weakly reducing or even oxidising conditions in the top port (2.13 m bgs) of the Peripheral well in November 2015 (Figure 3.5). In June, conditions were similar with depth to those in November 2015, however; generally lower methane concentrations in June compared to November 2015 suggested conditions were more reducing in the fall than summer (Figures 3.5 & 3.6). It seems in all wells, that nitrate may have been used preferentially at our Site as an electron acceptor for anaerobic toluene degradation due to the very low concentrations observed at all locations in both sampling events (Figures 3.5 & 3.6).

3.3.2 Evidence of Degradation

Due to limitations in CSIA analysis typically requiring approximately 30 µg L\(^{-1}\) of toluene, CSIA was not always possible on all groundwater samples. The November 2015 sampling event had the most comprehensive sampling in terms of data collection for CSIA, redox conditions, and microbial analysis. Interestingly, in November 2015, greater enrichment of the \(^{13}\)C isotope was generally observed in the Proximal well (\(\delta^{13}\)C values ranging from -28.60‰ to -27.74‰) than in the Source Zone (\(\delta^{13}\)C values ranging from -29.24‰ to -28.59‰), while
enrichment of the heavy hydrogen isotope ($^2$H) was greater in the Source Zone ($\delta^2$H values ranging from -94.73‰ to -66.58‰) than in the Proximal well ($\delta^2$H values ranging from -104.20‰ to -90.80‰).

In both the Proximal and Source Zone wells, enrichment of the $^{13}$C and $^2$H isotopes in toluene did not vary greatly between the November 2015 and June sampling events. Exceptions to this included: greater $^{13}$C enrichment in toluene in the upper port (2.9 m bgs) of the Proximal well in November 2015 ($\delta^{13}$C = -27.74‰) versus June ($\delta^{13}$C = -29.09‰) (Figure 3.1). Although this is likely indicative of relatively more degradation in that location in, or slightly prior to, November 2015, only one depth was measured with CSIA in June, thus making it difficult to ascertain if the difference was due to season, or sampling depth. The other exception was observed in the bottom port of the Source Zone well where enrichment of $^2$H in toluene was greater in June (-61.64‰) than in November 2015 (-77.39‰) possibly indicating either increased degradation of toluene, between these sampling events in the Source Zone or increased of toluene concentration that masked degradation signals (Figure 3.1). Also in the Source Zone, $\delta^{13}$C values in toluene observed in November 2015 was higher than June in the central sampling depths (4.95 and 6.45 m bgs) by approximately 0.3‰, however the difference was not large and likely does not indicate a very large change in degradation (Figure 3.1).

The $\delta^{13}$C values in November 2015 in the Proximal well indicate $^{13}$C was more depleted in the deepest port (8.73 m bgs) relative to other depths suggesting less degradation occurred there. In the Source Zone, however, the greatest enrichment of $^{13}$C isotopes ($\delta^{13}$C = -28.59‰) compared to other depths was observed at the same depth (8.73 m bgs) in both November 2015 and June. The greatest enrichment of $^{13}$C was observed in the spring (March 2016) in the Source Zone in the deepest port ($\delta^{13}$C = -27.77‰), then at 4.95 m bgs ($\delta^{13}$C = -28.54‰) (Figure 3.1).
Greater enrichment of $^2$H in toluene typically occurred in the June and November 2015 events than in the other sampling events (Figure 3.1). However, in both November 2015 and June, depletion of the heavy hydrogen isotope in the uppermost port of the Source Zone well (at approximately 2.90 m bgs) indicated less degradation. The deeper ports all exhibited greater enrichment spanning values between -77.39‰ to -66.58‰ (Figure 3.1). Toluene concentrations in the Peripheral well were too low to perform CSIA.

### 3.3.3 Potential Toluene Degrader Abundance and Activity with Depth

For all wells, total bacterial abundance ranged between the orders of $10^7$ to $10^8$ copies 100 mL $^{-1}$ groundwater. Total bacterial gene copies ($16S$ DNA) in the Proximal and Source Zone wells (M28 and M29, respectively) in all sampling events revealed that generally bacteria were most abundant in the uppermost ports sampled, and the deepest port (Figure 3.2). Bacterial abundance was more consistently similar between depths in the Peripheral location (M30) (Figure 3.2).

Transcript copies of $16S$ were more variable than gene copies, ranging between $10^5$ to $10^{10}$ copies 100 mL $^{-1}$ groundwater. In general, when considering all sampling events, transcript copies were often higher in the upper ports than the bottom ports. Gene expression was greatest in the bottom port for two of the three wells in the November 2015 ($10^9$ copies 100mL$^{-1}$ Proximal and Source Zone), March 2016 (Proximal and Peripheral $10^9$ to $10^{10}$ copies 100mL$^{-1}$), and June 2016 (Source Zone and Peripheral $10^7$ to $10^8$ copies 100mL$^{-1}$) sampling events. In each case, the remaining well had the largest number of $16S$ transcripts in the uppermost port that was sampled. That is, in June 2016, in the Proximal location, the most transcripts were detected at 3.88 mbgs (Figure 3.3). For the November 2015 and March 2016 events in the Peripheral and Source Zone locations respectively, the most transcripts were detected at 2.13 m bgs. Ratios of
16S transcripts to gene copy numbers consistently confirmed that at depths where transcripts were most abundant for each sampling event and well, the increased transcripts were indicative of increased potential activity rather than being an artifact of increased abundance (Appendix B Figure B.1).

Gene copies of bssA targeted to quantify potential anaerobic toluene degrader abundance covered a broad range between $10^3$ to $10^{10}$ gene copies 100 mL$^{-1}$ of groundwater (Figure 3.2). Gene copies of bssA typically followed a similar pattern to that observed for total bacterial abundance with greatest abundance of anaerobic degraders in the shallowest and deepest ports (Figure 3.2).

Relative to other depths within all three wells, anaerobic degraders constituted a larger portion (up to approximately 0.18) of the bacterial population most often in the deepest and upper ports. Abundance of anaerobic degraders relative to total bacteria was low (close to zero) in all three wells in November 2015 except for the deepest port where the proportion of anaerobic degraders was greater (up to 0.02) in the Proximal (M28) and Source Zone (M29) wells (Figure 3.7 and Figure 3.8). Proportions of anaerobic degraders were larger by up to an order of magnitude in several instances in June compared to November 2015 (Figure 3.7 and Figure 3.8). Gene copy ratios of bssA in June were somewhat lower in the Source Zone (M29) (between approximately 0.02 and 0.17) than in the Proximal well (between approximately 0.05 and 0.26), but still increased relative to November 2015. In the June event, the greatest proportion of anaerobic degraders were observed at 6.45 m bgs (approximately 0.26) and the bottom port in the Proximal well, and in the upper port and at 6.45 m bgs in the Source Zone well. The Peripheral well (M30) had relatively fewer anaerobic degraders compared to the other
wells and did not appear to be greatly increased at any depth between November 2015 and June (Figure 3.7 and Figure 3.8).

Transcripts of \textit{bssA} were detected in all wells in every season, however detection did not occur at all depths (Figure 3.3). These transcripts were also variable, however; patterns between depths in each season were not as pronounced as they were for \textit{bssA} gene abundance. Within each season where \textit{bssA} transcripts were detected, transcript copies did not vary by more than one order of magnitude between depths in any of the wells (Figure 3.3). The highest transcript copy numbers occurred most frequently in the June 2016 sampling event.

\textit{PHE} gene copies ranged between orders of $10^3$ to $10^6$ copies 100 mL$^{-1}$ groundwater in all samples while \textit{RMO} gene copies were generally lower and ranged between orders of $10^2$ and $10^5$. The highest aerobic functional gene abundance seemed to occur most frequently at depths between 2.13 and 4.19 mbgs in all wells for each season; however, the deepest port also occasionally reached comparable levels of aerobic degraders (Figure 3.2).

Generally, \textit{PHE} and \textit{RMO} gene copies were highest at the same depths with a few exceptions. When this did not hold (in the Proximal well and Source Zone in November 2015 and June 2016, respectively), \textit{RMO} genes were most abundant at the depth where \textit{PHE} genes were second-most abundant and gene copies were similar between the two depths (Figure 3.2). The other exceptions occurred in the Peripheral well in the March and June events. In these cases, \textit{RMO} gene copies were lowest at the depth where \textit{PHE} copies were highest, although differences between depths were not large (no more than one order of magnitude). Although proportions of \textit{RMO} genes relative to total bacteria were similar between November 2015 and June, proportions of \textit{PHE} were larger in June (up to approximately 0.08) than November 2015 (up to approximately 0.02) (Figure 3.7 and Figure 3.8).
In November 2015, gene copy ratios for \textit{RMO} and \textit{PHE} in the Proximal well (M28) were relatively low at approximately 6.45 m bgs compared to the upper ports sampled (2.90 to 4.95 m bgs) and the deepest port where relative abundance of aerobic degraders was highest (Figure 3.7). In the Source Zone (M29) on the other hand, relative abundance of aerobic degraders was highest at this depth (6.45 m bgs) and lower at the other depths in November 2015. In June however, the Proximal well (M28) depths between 3.88 and 6.45 m bgs had the highest proportion of aerobic toluene degrader communities while the deepest port had the lowest ratio (Figure 3.8). In the Source Zone in June the greatest proportion of aerobic degraders also occurred in the shallower depth range (3.88 to 4.95 m bgs). In the Peripheral well (M30) in November 2015, aerobic degraders were relatively lower in proportion to total bacteria except at 4.95 m bgs, but in June aerobic degraders appeared to constitute a larger proportion of the total bacterial community, particularly at shallower depths (2.90 to 4.19 m bgs) (Figure 3.7 and Figure 3.8).

Generally, transcripts for \textit{PHE} and \textit{RMO} followed similar patterns between depths (Figure 3.3). As with gene abundance, transcripts were usually most abundant in each season between 2.13 m bgs and approximately 6.45 m bgs. Apart from \textit{PHE} detection in November 2015, in both the Proximal (M28) and Source Zone (M29) wells, potential aerobic degradation activity was only detected in the deepest sampling port in the Peripheral well (M30) (Figure 3.3). While \textit{RMO} transcripts ranged anywhere from non-detect to $10^3$ transcripts 100$^{-1}$ mL groundwater, \textit{PHE} transcripts were again higher and ranged from non-detect to $10^7$ copies 100$^{-1}$ mL groundwater. Only weak negative correlations were found between toluene concentration and \textit{PHE} ($R=-0.27$, $P<0.05$) and \textit{16S} ($R=-0.30$, $P<0.05$) gene copies.
Figure 3.1. CSIA results that coincided with the microbial samplings in November 2015 (●), March 2016 (▲), and June 2016 (■). Results from an earlier October sampling event (▼) were added in lieu of the November 2016 sampling since no CSIA was performed on the samples obtained November 2016. Signature heavy isotope δ values of the source toluene are indicated by the red lines. Shifts to the right of these lines represent enrichment of heavy isotopes and therefore degradation. All δ values are expressed in permil (‰). Toluene concentrations were not high enough in the Peripheral well to perform CSIA.
Figure 3.2 Gene copies at each sampling depth in the Proximal (M28), Source Zone (M29) and Peripheral (M30) wells sampled in November 2015 (●), March 2016 (△), June 2016 (■), and November 2016 (◆). Error bars represent standard error between duplicate samples at each depth. Toluene concentrations below the detection limit are displayed as ◊ with the colour of the corresponding sampling event in the white half of the box.
Figure 3.3 Transcript copies at each sampling depth in the Proximal (M28), Source Zone (M29) and Peripheral (M30) wells sampled in November 2015 (●), March 2016 (▲), June 2016 (■), and November 2016 (●). Non-detects (⦁) are displayed on the y-axis as placeholders. Error bars represent standard error between duplicate samples at each depth. Toluene concentrations below the detection limit are displayed as □ with the colour of the corresponding sampling event in the white half of the box.
Figure 3.4 Dissolved oxygen (DO) concentrations from groundwater in the Proximal (M28), Source Zone (M29), and Peripheral (M30) locations sampled in November 2015 (●), March 2016 (▲), June 2016 (■), and November 2016 (♦).
Figure 3.5. Redox indicators for November 2015. Samples for analysis of redox indicators were obtained in the same consecutive days as microbial samples from all sampling intervals in the Proximal (M28), Source Zone (M29) and Peripheral (M30) wells. Colours overlaid indicate general reducing conditions where pink represents areas of methanogenic conditions, orange indicates sulfate reducing conditions and green indicates areas where iron, manganese and nitrate reduction dominates.
Figure 3.6. Redox indicators for June 2016. Samples for analysis of redox indicators were obtained a week prior to sampling groundwater for microbial analysis in the Proximal (M28), Source Zone (M29) and Peripheral (M30) wells. Values below the detection limit (0.1 mg L\(^{-1}\) and 1 mg L\(^{-1}\) for nitrate and sulfate, respectively) include all nitrate values except those in the deepest ports of the Proximal and Source Zone wells, and all sulfate values indicated on the y-axis. Colours overlaid indicate general reducing conditions where pink represents areas of methanogenic conditions, orange indicates sulfate reducing conditions and green indicates areas where iron, manganese and nitrate reduction dominates.
Figure 3.7 Ratios of functional genes to 16S gene copies in the Proximal (top), Source Zone (middle) and Peripheral (bottom) sampling locations in the November 2015 sampling event. Colours overlaid indicate general reducing conditions (from Figure 3.5) where pink represents areas of methanogenic conditions, orange indicates sulfate reducing conditions and green indicates areas where iron, manganese and nitrate reduction dominates.
Figure 3.8 Ratios of functional genes to 16S gene copies in the Proximal (top), Source Zone (middle) and Peripheral (bottom) sampling locations in the June 2016 sampling event. Colours overlaid indicate general reducing conditions (from Figure 3.6) where pink represents areas of methanogenic conditions, orange indicates sulfate reducing conditions and green indicates areas where iron, manganese and nitrate reduction dominates.
3.4 DISCUSSION

Historically, plumes were thought to be laterally delineated with longitudinal redox zonation where conditions are expected to be least thermodynamically favourable in the plume core, gradually becoming more favourable with increased distance from the core. However, the “plume fringe concept” proposed by Meckenstock et al. (2015) suggests mixing of redox conditions mainly occurs on the plume fringes, while the electron acceptors previously thought to be used in the core exclusively, are also used further down gradient due to laminar groundwater flow. Meckenstock et al. (2015) also suggests that in highly contaminated aquifers electron donors (in our case, toluene) are not limited and are too high for there to be competition for the electron donor. The oxidation capacity of all electron acceptors is exceeded and therefore simultaneous respiration processes may be observed. In these cases, bacterial degradation is limited by availability of specific electron acceptors rather than thermodynamics (Meckenstock et al., 2015). This seems to be the case in this study.

Concentrations of geochemical/ redox indicators at the Site, generally support the “plume fringe concept” proposed by Meckenstock et al. (2015) which suggests the possibility of simultaneous methane, iron and manganese reducing conditions. This better explains the patterns of redox indicators observed at the Site than the “longitudinal redox zonation concept” whereby redox processes form a longitudinal gradient due to preferential consumption of the most energy-efficient electron acceptors (Meckenstock 2015; Roychoudhury & Merrett, 2006). Less reducing conditions, when iron, manganese, and nitrate reduction occurred, were observed somewhat sporadically with depth at the Site, particularly in the Proximal and Source Zone locations. The dynamic redox conditions and mixed use of electron acceptors observed at our relatively small Site may also be an example of, continuous mixing of groundwater which replenishes some of
the more energetically favourable electron acceptors, thereby allowing multiple metabolic pathways to occur simultaneously which has been suggested previously (Lueders, 2017; Roychoudhury and Merrett, 2006).

The sporadic reducing conditions observed did not necessarily match the plume fringe concept whereby the greatest degradation activity is typically found on the outskirts of the plume. It did, however, coincide with the theory because the plume edges are typically hypothesized to host less reducing conditions relative to the plume core and our increased activity was observed in moderate reducing conditions, specifically in non-methanogenic conditions, though they were more sporadically distributed throughout the Site.

In both the November 2015 and June sampling events the highly contaminated Source Zone (M29) and Proximal (M28) locations closest to the center of the plume had the strongest reducing conditions. The Source Zone well located very close to the suspected point source of contamination (a leaking supply line buried in the overburden above the bedrock) exhibited the strongest reducing conditions. In addition, the strongest reducing conditions in both wells were observed in the uppermost ports coinciding with the highest toluene levels in groundwater (up to 388 367 µg L\(^{-1}\) and 530 000 µg L\(^{-1}\) in the Proximal and Source Zone wells, respectively, in November 2015) as well as the greatest mass of sorbed toluene in the bedrock matrix (Fernandes, 2017). These data are supportive of both the classical “longitudinal redox zonation concept” and Meckenstock’s “plume fringe concept” since both hypothesize strong reducing conditions at the plume core, however; the “plume fringe concept” better explains conditions observed across the entire Site.

In the Peripheral well (M30), the increasingly reducing redox conditions with depth may have reflected dissolution of atmospheric oxygen failing to penetrate deeply in the groundwater
as assumed by previous researchers (Rose & Long, 1988) and could be explained by the much lower contamination levels. Due to low toluene concentrations at this location, supply of electron acceptors was likely not depleted as quickly and conditions did not become as strongly reducing in the upper ports. The observed patterns in redox conditions in the Peripheral well is complex and may also be related to vertical transport of water from the Source Zone to the Peripheral well, however; it can only be concluded from our data that the bottom of the Peripheral well receives water depleted in most alternate electron acceptors.

Potentially stronger reducing conditions observed in June than November 2015 in the upper ports of the Peripheral well demonstrated the effect of the supply of toluene on redox conditions. Spring snow melt prior to the June sampling would likely replenish electron acceptors as the water table rose in March, also effectively dissolving toluene that was previously sorbed to bedrock above the water table (Danczak et al., 2016; Fernandes et al., 2017; Landmeyer & Effinger, 2016; Treiden et al., 2012; Yabusaki et al., 2017). Functional gene transcripts were detected throughout the sampling campaigns despite toluene concentrations being below detection limits. These factors collectively suggest active bacteria consumed the available electron acceptors to degrade toluene and maintain concentrations below detection thus contributing to the stronger reducing environment in the upper ports of the Peripheral well in June compared to November 2015.

A recurring pattern observed relatively consistently in all functional genes studied was the apparent increase in both gene and transcript abundance between approximately 2.13 and 4.23 m bgs and in the deepest port of all wells (Figure 3.2 & Figure 3.3). This was partially linked to the trends observed for 16S gene abundance, which was highest in both the shallowest and deepest ports of the wells, and highlights the importance of considering gene copy ratios. Consideration
of relative abundance indicated that, particularly for \textit{bssA}, proportions of toluene degraders relative to total bacteria were still higher in the shallowest or deepest locations compared to other depths.

The data suggest a link between less reducing to moderately reducing conditions and relatively increased toluene degrading populations. Both aerobic and anaerobic toluene degrading communities were frequently most abundant and most active in areas of less reducing conditions where methanogenesis appeared to be less dominant. The consistently low concentration of nitrate in the groundwater is consistent with preferential use of nitrate as a more energy-efficient electron acceptor. Interestingly, points where the \textit{bssA} to \textit{16S} gene copy ratio was greatest always coincided with relatively increased ORP values (Appendix B Figure B.2).

Detection of \textit{bssA} was frequent either in the shallowest or deepest ports sampled (Figure 3.2 & Figure 3.3) and mostly coincided with less reducing conditions (ie. typically iron and manganese reducing conditions) (Figure 3.5 & Figure 3.6). In November 2015, both the Source Zone and Proximal wells had the greatest proportion of anaerobic degraders in the deepest port where redox indicators suggested manganese and possibly iron reducing conditions dominated (Figure 3.5). In June, a greater proportion of \textit{bssA} was observed in the Proximal well which exhibited less reducing conditions than the Source Zone. The Proximal well in June again had most anaerobic degraders in the deepest port where, based on available data, manganese and iron reducing conditions were most probable suggesting the anaerobic degraders at the Site may prefer moderate or less reducing conditions. In the Source Zone in June, however, the greatest proportion of anaerobic degraders was observed in the shallowest port (2.90 m bgs) which coincided with the most reducing conditions in that well in June. While this may not seem to fit the pattern, it must be noted that the groundwater in the June sampling event exhibited less
reducing conditions in general. The strongest reducing conditions in June indicated iron and manganese-reducing conditions, and were comparable to the least reducing conditions observed in November 2015. The general, increased abundance of anaerobic degraders in June relative to November 2015 in all wells also supported the idea that anaerobic toluene degraders favour less reducing conditions. CSIA results did not perfectly match these results suggesting degradation on the Site is due to the activities of both aerobic and anaerobic degraders in combination.

Relative abundance of bssA genes in the Peripheral well was low and did not vary greatly, however this may be due to the very low toluene concentrations that were maintained consistently throughout that well after November 2015.

Other studies have also found evidence of increased anaerobic degraders in less-reducing conditions. In a sandy aquifer in South Africa that exhibited redox conditions similar to those observed at our Site (mixture of nitrate, manganese, iron and sulfate reducing conditions) sulfate and iron reducing seemed to be the dominant metabolic pathways (Roychoudhury & Merrett, 2006). Winderl et al. (2008) observed the highest bssA to 16S gene copy ratios and “hot spots” of anaerobic degradation in sulfate and iron reducing gradient zones in a BTEX contaminated aquifer. One study testing bssA primer sets in anaerobic microcosms with varying reducing conditions found that nitrate followed by sulfate produced the greatest bssA gene copies while methanogenic microcosms had the least (Sun et al., 2014). However, they suggested that sequence information available for methanogenic toluene degraders is limited and therefore, primer sets available for their detection may be limited. This limits conclusions that may be drawn from interpreting field data of this type (Sun et al., 2014).

Results from our study suggest that the community of anaerobic toluene degraders at the Site is likely dominated by a combination of iron, manganese sulfate and possibly nitrate-
reducers. Kümmel et al. (2013) discussed a clustering of bssA amino acid sequences into two groups: obligate anaerobes and facultative anaerobes. The facultative anaerobes consisted of organisms including various nitrate-reducing Thauera and Azoarcus species, while obligate anaerobes included Geobacter and Desulfosarcina species which tend to be sulfate- or iron-reducing organisms (Kümmel et al., 2013). While dissolved oxygen is very low at the Site, it was consistently detected, indicating that while conditions were reducing on the Site, they were not anoxic. This provides additional evidence that anaerobic degraders colonizing the Site were likely facultative anaerobes which, as observed by Kümmel, tend to fare well in less strongly-reducing conditions. Fluctuating water tables like those found at the Site contribute to entrapment of air below the water table and fluctuating DO (Zhou et al., 2015) and are advantageous to facultative over obligate anaerobes, increasing the likelihood that more facultative anaerobes exist at the Site. In addition, sulfate reducing and methanogenic bacteria typically are active only at redox potentials below approximately -200 mV (Hendrickx et al., 2005; Widdel & Rabus, 2001). ORP measurements obtained from the Source Zone and Proximal wells rarely dropped below -200 mV in the four sampling campaigns (Appendix A Figure B.2). In the Peripheral well, however, redox potentials were mostly below -200 mV except for in the June sampling event where DO was much higher. Oxygen is the most energetically favourable electron acceptor available for biodegradation of toluene and thus is rapidly consumed in degradation processes, resulting in more hypoxic conditions (Herzyk et al., 2017; Vogt et al., 2008; Yabusaki et al., 2017). It is possible that the June sampling event may have captured a time point where most of the bioavailable dissolved toluene was degraded at the Peripheral of the plume. This would diminish degradation activity and allow the observed resurgence in oxygen concentrations such as that seen throughout the Peripheral well in June. This along with the
generally reducing conditions in the Peripheral well suggest a larger proportion of facultative anaerobes may have colonized that well. Herzyk et al. (2017) noted that communities can respond to changes in contamination and redox conditions within days, thus subsequent detection of \textit{bssA} after June suggests resurgence of toluene, although it was still below detection limits. Since toluene was persisting in the Proximal and Source Zone wells, oxygen available was likely continuously being utilized in aerobic degradation processes and thus DO remained low.

The similar patterns between depths observed for both relative abundance and transcripts of the aerobic functional genes indicated degradation by aerobic pathways was actively occurring and proceeded beyond the initial activation steps of degradation. CSIA results indicated no larger differences in $^2$H and $^{13}$C isotopes in toluene between the November 2015 and June sampling events, suggesting degradation was likely not very different in the two events. This was supported because \textit{RMO} relative gene abundance remained approximately similar between the two events despite an increase in the relative abundance of \textit{PHE} in June. The \textit{RMO} gene encodes part of the enzyme responsible for activation of toluene molecules (Baldwin et al., 2003; Parales et al., 2008) while \textit{PHE} encodes part of an enzyme that acts on degradation intermediates. Since CSIA was performed only on toluene it would be expected that degradation of toluene indicated by CSIA would match better with \textit{RMO} gene abundance as it did. In addition to the increase in \textit{PHE} between November 2015 and June, the much higher abundance of \textit{PHE} was observed consistently at the Site. These results are supported by studies by Baldwin et al. (2008) and Kao et al. (2010) which also found that \textit{PHE} was more abundant and more commonly detected than \textit{RMO}. Nebe et al. (2009) suggested that the \textit{RMO} primer set may not amplify more divergent ring-hydroxylating monooxygenases, thus the presence of these genes in the samples would be missed. Additionally, Nebe et al. (2009) suggested that the overlapping substrate specificities of
phenol hydroxylases and toluene monooxygenases may factor in to the larger numbers of $PHE$ gene copies since $PHE$ can target more substrates. $RMO$ is meant to target monooxygenases that initiate toluene oxidation, while phenol hydroxylases act later in the degradation pathway. However, some enzymes, such as the monooxygenase that activates degradation in the $Burkholderia$ sp. strain JS150 (Nebe et al., 2009) perform both steps and may have increased homology to phenol hydroxylases, so the $PHE$ primer set was designed to capture these homologous enzymes. It is therefore possible that the values for $PHE$ gene abundance include some toluene monooxygenases as well (Nebe et al., 2009). It should be noted that the toluene dioxygenase and TOL pathways were not quantified in this study, and that our primers may under-represent sulfate reducers.

Generally, abundance of aerobic degraders was higher between 2.03 and 4.19 m bgs. This occurred despite coinciding with an array of strongly reducing and less reducing redox conditions as well as a few slight increases in the dissolved oxygen in groundwater. In addition to this, the spike in relative abundance of aerobic degraders in the Peripheral well in November 2015 at 5.26 m bgs coincided with a spike in dissolved oxygen to 5.26 mg L$^{-1}$ when oxygen at all other depths in this well were very close to 0 mg L$^{-1}$. These data may suggest that aerobic toluene degraders were more abundant in areas with increased dissolved oxygen which is logical, however, these trends were inconsistent and were not completely representative of the usual distribution of aerobic degraders which was somewhat erratic. It was very interesting to note that $PHE$ transcript copies often were very synchronous with $bssA$ transcripts between depths in all three wells in the November 2015 sampling event (Figure 3.3). Although not as consistently observed in the other sampling events, it was still frequently observed that $bssA$ and the aerobic degrader functional genes would be relatively higher or lower at the same depths. This was
particularly evident for gene copies in the Proximal well in November 2015 (Figure 3.2). These results may indicate that, in addition to possibly responding to DO, aerobic toluene degraders are likely also more abundant and active in areas with somewhat less reducing conditions. Distribution of aerobic toluene degraders is therefore difficult to predict based on the data collected for this study. It is well documented in the literature that aerobic toluene degraders exhibit resilience to conditions that stray from the expected optima for these organisms since genes associated with aerobic toluene degradation are notoriously detected in reducing environments. It has been suggested that organisms harbouring these genes may be well suited to degrading toluene in conditions with low and fluctuating DO such as those at the Site (Hendrickx et al., 2006; Larentis et al., 2013, Martínez-Lavanchy et al., 2015; Nebe et al., 2009). The commonalities in trends of aerobic and anaerobic functional gene copies and transcripts between depths suggest a robust community that is resilient to the generally hypoxic conditions found in the groundwater at the Site. This further supports the hypothesis that many toluene degraders in the groundwater are facultative anaerobes and capable of degrading toluene in the variety of conditions observed.

In the three wells examined in this thesis, most of the sampling ports were in a hydrogeologic unit which spanned approximately 2 m bgs to approximately 6.5 m bgs (HGU 1). The remaining two were in another hydrogeologic unit which spans approximately 6.5 to 14.5 m bgs (HGU 2) (Figure 1.1 and Figure 2.1) (Fernandes, 2017). HGU 1 was found by Fernandes (2017) to have a strong vertical gradient between ports, indicating low vertical connection in this unit. This was particularly evident in the Source Zone and Proximal wells; strong vertical gradients were not observed at the Peripheral location. This could also help explain why aerobic toluene degradation activity was observed deep into the groundwater horizon. Some vertical
mixing of groundwater occurs between HG1 and HG2, but overall groundwater quality in HG2 with depth will be influenced by upgradient water quality in that unit. Vertical connections were increased in HGU 2 with increased occurrence of longer vertical fractures in the bedrock. These fractures, combined with evidence indicating the probable presence of facultative anaerobes may explain how aerobic activity was still detectable even in the deepest sampling ports. Another explanation of why \textit{PHE} and \textit{RMO} transcripts were detected in reducing conditions is that most of the aerobic toluene degradation pathways are inducible by the presence of toluene, thus expression of the transcripts may still occur in the absence of oxygen or oxidizing conditions (Parales et al., 2008). In this case, gene expression would likely not be strongly associated with degradation of toluene in the absence of adequate DO concentrations.

The observable variability in gene copy and transcript abundance over the depths studied suggested that features associated with specific depths, such as fracture patterns influencing groundwater flow and mixing, also had some impact on degrader abundance and activity. There is evidence to suggest that other factors associated with depth, such as toluene concentration, oxygen concentration, redox conditions, or the direction of flowing groundwater can impact the abundance and activity of the degraders on the Site and likely other remediation sites. Predicting these impacts and estimating degradation rates, however remains a challenge for the scientific community to meet.

This research fills a very specific niche in the field of remediation not previously studied. While depth-resolved analysis of microbial communities has been done in the past \textit{in-situ} (Larentis et al., 2013; Stapleton et al., 2000; Winderl et al., 2008) these studies did not quantify functional gene transcripts involved in toluene degradation.
Transcripts, when they are quantified, are typically used to assess applied remediation techniques such as the in-situ bioreactor tested by Key et al. (2013) or the oxygen infusion system implemented by Baldwin et al. (2010), rather than intrinsic remediation such as that occurring below our phytoremediation system. Quantification of transcripts has also not been used in combination with depth-discrete sampling of groundwater specifically (Kazy et al., 2010; Táncsics et al., 2012). Other studies quantifying transcripts of genes involved in BTEX degradation did not quantify transcripts in groundwater, but soil samples (Key et al., 2014). Quantification of transcripts involved in both aerobic and anaerobic toluene degradation from the in-situ groundwater of a BTEX-contaminated, fractured bedrock remediation system underlying phytoremediation is a previously unexplored scenario in remediation research.

3.5 CONCLUSION

Supported by CSIA data, quantification of both gene copies and transcripts of aerobic and anaerobic toluene degrading bacteria successfully confirmed the potential for active bacterial degradation of toluene throughout the Site horizontally and vertically over the year.

Depth-delineated sampling permits data collection on a highly-resolved scale that can provide greater insights into microbial communities and activities, redox conditions, and even degradation patterns in contaminated groundwater systems. It is particularly useful when applied to complex systems such as the fractured bedrock aquifer on which this research was conducted, however, bacterial communities in the natural environment can vary on a small scale spatially (within centimeters). Microbial communities are also prompt responders to environmental stimuli including varying contaminant and substrate concentrations, fluctuating oxygen levels, and fluxes in redox conditions and abundance and activity may change within days (Herzyk, 2017). Inferences from seasonal sampling therefore can be limited. In some cases, increasing the
resolution of sampling will no longer produce meaningful results since the media of sampling, groundwater, is constantly moving and mixing. Resolution on small scales is thus better suited to sampling sediments and soils rather than groundwater. Valuable conclusions may still be drawn from depth-delineated sampling techniques on a scale larger than centimeters.

Redox data and bacterial activity indicated that the Site generally fit Meckenstock’s plume fringe concept. Aerobic degraders seemed to be slightly influenced by fluctuations in oxygen, although the trend was neither consistent or reliable for predicting abundance and activity of aerobic toluene degrading communities.

Gene detection at our Site may be somewhat influenced by toluene concentration, although factors including redox conditions, sampling events, and groundwater mixing were more strongly related to abundance and potential activity of toluene degraders and likely have a more direct influence. The abundance and activity of anaerobic, and to a lesser extent, aerobic toluene degraders were found to vary over the study period and suggested a seasonal pattern of higher values occurring in Spring/Summer versus Fall. Results also indicated higher abundance and activity were coupled with moderately reducing (specifically non-methanogenic) redox conditions. This research has provided additional support to the recently devised “plume fringe concept” and will provide a valuable resource to researchers or remediation organizations in decision-making for bioremediation and monitoring approaches in the context of BTEX contaminated groundwater. Future research should focus on highly resolved sampling as was performed here, but a repeated measures experimental construct, in which all parameters are measured consistently in the same locations and depths over time, may be useful in applying statistical models to better understand the factors affecting microbial abundance and activity and ultimately, biodegradation.
Environmental systems and the physical, chemical and biological processes that influence them can be represented by a Conceptual Site Model (CSM) using visual or written tools, or a combination of both (Kresic & Mikszewski, 2012). CSMs are typically applied in a hydrogeologic context, specifically when groundwater contamination is a concern, and strive to characterize factors including the media through which groundwater flows, groundwater flow and recharge patterns, contaminant distribution, and processes that may influence contaminant transport and persistence (Kresic & Mikszewski, 2012). The goal of producing a CSM is to provide an accessible representation of the site of interest that encompasses all pertinent information and provides a valuable reference for decision-making with regards to remediation and projects involving the site (Kresic & Mikszewski, 2012). CSMs vary in detail, but perhaps the greatest information is provided when they draw from multiple lines of evidence and integrate a variety of analytical practices to produce a Site Model with comprehensive data. A CSM-based approach was thus appropriate for characterizing toluene fate and transport at our study site, located in Southwestern Ontario. A phytoremediation system is in place at the Site, and the overall multidisciplinary research objective was to develop a comprehensive CSM as the basis for assessing the efficacy of that system. Processes of toluene attenuation in all relevant media (i.e. within the saturated and unsaturated zones and within the trees) have been, and continue to be characterized (Ben-Israel et al., 2018 and Fernandes, 2017). With respect to the saturated zone, the development of the CSM has involved characterization of toluene concentrations, distribution and phase, and hydrochemistry, hydrogeology, and water flow patterns (Fernandes, 2017 and ongoing research). A preliminary fate and transport model for the Site has also been developed to describe plume behaviour on the basis of the physical, chemical
and biological processes that affect toluene distribution and attenuation (Fernandes, 2017). The work presented in this thesis builds upon the existing saturated-zone CSM by characterizing the community of toluene-degrading bacteria in groundwater spatially within the plume and on a seasonal time scale. While it is acknowledged that the penetration of tree roots into the upper groundwater zone may affect the microbial community, the effect of the phytoremediation system was not examined explicitly in this research.

The research conducted for this thesis applied quantitative polymerase chain reaction (qPCR) to enumerate genes and transcripts of the functional genes *PHE, RMO*, and *bssA* which are involved in toluene degradation via three aerobic pathways and the anaerobic pathway respectively. Depth-delineated sampling was performed in three wells targeting areas in the Source Zone, where the highest toluene concentrations are measured, slightly downgradient Proximal to the Source Zone, where intermediate toluene concentrations are measured, and the downgradient edge (Periphery) of the plume, where low or non-detect toluene concentrations are measured. Sampling was also conducted on a seasonal basis. Thus, investigated were the potential effects that season (represented by the different sampling events), and lateral location within the toluene plume (represented by the three individual wells and associated toluene concentrations) - had on the potential abundance and potential activity of aerobic and anaerobic toluene degrading communities in the groundwater at the Site.

The molecular approach described above, in concert with Compound Specific Isotope Analysis (CSIA), provide direct evidence for active toluene degradation by bacterial communities in the saturated zone. Although both aerobic and anaerobic toluene degraders were detected temporally and spatially, detection of potential anaerobic degrader activity was more
consistent between locations than detection of aerobic degrader activity. This was expected given the generally reducing conditions of the groundwater at the Site.

Location within the plume and toluene concentrations were, overall, not identified as being statistically significant for abundance and activity of toluene degraders; active degraders were detected along the plume from the Source Zone to the Downgradient edge. When analyzed in relation to measured toluene concentrations, the results did identify some weak correlations. A weak negative correlation was found between toluene and concentrations of PHE and 16S gene copies when examined on a depth-discrete basis (Chapter 3). A weak positive correlation was found between bssA transcripts and toluene when considering concentrations of these two parameters on a well-averaged basis (Chapter 2). Since correlations were so weak, it was hypothesized that other Site factors and seasons may play more integral roles in influencing toluene degrading populations and differences observed between wells.

Temporally, the data indicated a seasonal effect which was particularly strong for quantification of the bssA gene. Anaerobic degrading communities were most abundant and active in the June sampling event. We suggest that this seasonal effect may be due to the replenishment of more thermodynamically favourable electron acceptors as a result of spring recharge conditions arising from snow meltwater (shown during the March sampling event by the highest seasonal water table). Seasonal patterns for aerobic degrader abundance and activity were not as strong as those observed for the anaerobic pathway. Activity of the aerobic degraders was most frequently increased in March despite increases also observed in the other sampling events. Again, this was attributed to spring recharge conditions, including entrapped oxygen and replenished nutrient levels in groundwater early in the year.
Also evaluated in this thesis was whether or not there was any evidence for a toxic effect of toluene to the microbial communities. Dissolved toluene concentrations in the Source and Proximal zones were recorded at or near the solubility limit of pure-phase toluene in water. A toxic effect was not clearly shown by the results of this thesis, in either the well-averaged or depth-discrete results (Chapters 2 and 3, respectively). For example, around 2.90 m bgs in the Source Zone and Proximal wells where the maximum toluene concentrations were observed, degrader potential abundance and activity was frequently comparable to other areas of minimal contamination. Indications of potential toxicity were insufficient to support any claim that toxic effects were observed on the Site.

Interpretation of the depth-discrete patterns of redox conditions indicated that the Site resembled the plume fringe concept proposed by Meckenstock (2015) which suggests multiple electron acceptors may be used simultaneously in the same location due to the complex mixing conditions of groundwater. Microbial abundance and activity data indicated that both aerobic and anaerobic degraders exhibited a tendency to be most abundant and active in areas of moderately reducing conditions. Aerobic degraders also showed signs of responding to areas of relatively elevated oxygen, although this pattern was more difficult to distinguish and is considered unreliable as a predictor or aerobic degrader abundance and activity. The data suggest that toluene concentration does seem to play a minimal role in influencing degrader abundance and activity as was suggested in Chapter 3, however, the depth discrete data again confirmed that other factors such as redox and the availability of electron acceptors and season appeared to be more strongly related to trends in abundance and potential activity of toluene degraders.

This research has contributed to fill a gap within the broadening topic of groundwater remediation, particularly with respect to studying microbial communities. Few studies have
quantified functional genes associated with degradation in an intrinsic remediation context in groundwater underlying a phytoremediation system. No other studies have quantified transcripts of genes involved in *in-situ* toluene degradation in groundwater across seasons with depth-delineated sampling targeting the anaerobic and multiple aerobic degraders within the same samples. The results of this study will provide a valuable addition to the current research available on groundwater remediation systems. It will supply as an additional reference point for the remediation endeavors of researchers and companies alike and may become a tool to refer to in the decision-making process. The research has helped to further our understanding of microbial communities in groundwater and how their distribution and activity may change in response to site conditions, location and seasons.

Future research at the Site should attempt to use primer sets that capture all the aerobic pathways at play within the Site. While this study examined most of the known pathways, the remaining pathways still represent a potential reservoir of untapped information. Metagenomics and metatranscriptomics using Next Generation Sequencing would allow more detailed analysis of the types of communities in groundwater. Not only would these methods illuminate species diversity, but it would also allow the parsing of which aerobic degradation pathways dominate in certain conditions and seasons. This information would be valuable for this research Site and may help decide if alternate approaches could expedite the remediation techniques already implemented at the Site.
LITERATURE CITED


Danczak, R. E., Yabusaki, S. B., Williams, K. H., Fang, Y., Hobson, C., & Wilkins, M. J. (2016). snowmelt induced hydrologic perturbations drive dynamic microbiological and


APPENDIX

Appendix A: Formulas
A.1 Formulas for back-calculating gene copies (DNA) and transcripts (cDNA) from qPCR outputs to copies 100 mL$^{-1}$ groundwater

**DNA Back Calculation:**

Copies 100 mL$^{-1} = \frac{[x / l + d \times e]}{f} \times 100 mL$

**cDNA Back Calculation:**

Copies 100 mL$^{-1} = \frac{[x / l + d1 \times d2 \times e]}{f} \times 100 mL$

Where $x$ represents the copies in the reaction volume, $l$ is the volume of sample loaded, $d$ is the dilution factor used to counteract inhibition, $d1$ and $d2$ are the dilution factors resulting from mixing samples with reagents in the DNase treatment and reverse transcription steps respectively (pertains to cDNA back calculations only), $e$ represents the total volume that was extracted from filters (100 µL for all calculations in this study), and $f$ is the exact volume of water that was filtered through the membrane from which DNA and RNA were extracted.
### Appendix B: Supplementary Data

**Table B.1** Sampling summary of depths and wells that were sampled in each of the sampling events. Each “X” represents a duplicated set of samples taken from the corresponding sampling event and location.

<table>
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<th>Port</th>
<th>Depth of Port Center (m bgs)</th>
<th>November 16-20, 2015</th>
<th>March 23, 2016</th>
<th>June 16-17, 2016</th>
<th>November 17, 2016</th>
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<td>X</td>
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### Table B.2 Summary of the depths and spans of sampling intervals for the wells sampled.

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<td>m bgs</td>
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Figure B.1 Ratios of cDNA to DNA for 16S DNA for the Proximal (left), Source Zone (middle) and Peripheral (right) locations in each of the seasonal sampling events. Error bars represent standard error for duplicated samples taken at each depth.
Figure B.2 Oxidative reductive potential for the Proximal (M28), Source Zone (M29) and Peripheral (M30) wells with depth sampled in November 2015 (●), March 2016 (▲), June 2016 (■), and November 2016 (◇).
Figure B.3 Scatterplots of toluene concentration (x-axis) vs. gene copies (top) and toluene concentration vs. transcript copies (bottom) with regression lines for 16S (left), bssA (right).
Figure B.4 Scatterplots of toluene concentration vs. gene copies (top) and toluene concentration vs. transcript copies (bottom) with regression lines for PHE (left), RMO (right).