2016

Sulfate-Reducing Bacteria Community Analysis: ISCO/ISB Coupled Remediation

Christopher K. Bartlett
Wilfrid Laurier University, bart8990@mylaurier.ca

Sulfate-Reducing Bacteria Community Analysis: ISCO/ISB Coupled Remediation

by

Christopher Bartlett

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfillment of the requirements for

the

Master of Science in Integrative Biology

Wilfrid Laurier University

2016

Acknowledgements:

First and foremost, I would like to extend my upmost gratitude to Dr. Robin Slawson, my supervisor, for her support and guidance through my undergraduate and graduate degrees. Dr. Slawson has always been there for me as an advisor through my academic career as well as provided support as a friend and mentor. She instilled confidence in me that I did not know I had by giving me a chance at resuming my career in microbiology and allowing me to pursue my dream in becoming a part of the scientific community. I will forever be grateful for that.

I would also like to thank my committee members, Dr. Neil Thomson and Dr. Joel Weadge, for providing their extended knowledge and positive attitude while I moved throughout my graduate studies. Without their continued support, I would not have been able to find my way past the 'bumps in the road' that entails being a Master's of Science candidate and for that, I will always be thankful.

Also, I would like to acknowledge my co-workers Felipe Solano, Wayne Noble, Shirley Chatten and Mahsa Shayan from the University of Waterloo. You provided your extended knowledge and skills to aid in the completion of my experiment and I will forever be indebted for that. Wayne, I will always be appreciative of the guidance you gave me while I learned and applied my skills in a field new to me.

Furthermore, I would also like to extend my appreciation to my fellow Master's candidates and friends that have supported me and will undoubtedly continue to support me through the different stages of my scientific career and life. Without you, I do not know where I would be today (I would probably still be at Canadian Tire emptying garbage cans!). Thank you!

I would also like to acknowledge the financial support for this investigation provided by the NSERC of Canada Collaborative Research and Development Grant entitled: *Evaluation and optimization of in situ chemical oxidation for groundwater remediation* (PI: N.R. Thomson) as well as the Southern Ontario Water Consortium (sowc) and Canada Foundation for Innovation (cfi).

Finally, I would like to thank my sister Robin, brothers Jay and Kyle, parents Ken and Trudy, as well as my girlfriend Taylor and her family for their continued support throughout my extended post-secondary career. Without your guidance, love and financial support I would not have been able to continue my passion for science. Taylor, without your support and caring efforts before and throughout my M.Sc., I would not have succeeded. I can never express how grateful I am. I love you guys and hope to make you proud as I pursue my future goals!

Dedication:

I would like to dedicate my thesis to my mother, Bonita Bartlett. You were the strongest and most caring person I will ever know. The strength you displayed on a daily basis and that you instilled in me as a single mother and nurse with two children and a farm while enduring the hardships of an 8-year battle with cancer will forever remind me that anything is possible if you put your mind to it. Without your support and confidence in me prior to my post-secondary career, I would have never come close to becoming the independent person I am or a M.Sc. Candidate in Biology. You were the one that saw potential in me and whom pushed me to go to university even though I had convinced myself that I did not have the knowledge or ability to succeed at that level. You are forever in my thoughts and I look forward to the day that we meet again so I can tell you what you inspired me to do and the person that you helped me become. Thank you, I love and miss you, mom.

Abstract:

Improved techniques for remediating groundwater systems are required for the more than 500,000 contaminated sites in North America. Many of these sites are the legacy of historical industrial operations, inappropriate disposal practices and accidental releases. The most widely observed contaminant at many of these sites is petroleum hydrocarbons (PHCs). Recently, remediation efforts that involve the sequential application of treatment technologies have gained widespread interest. One specific sequential technology application or treatment train employs the aggressive nature of a chemical oxidation followed by bioremediation for polishing. When persulfate is used as the chemical oxidant its natural degradation by-product is sulfate, an electron acceptor for sulfate-reducing bacteria. Hence in this thesis, the focus is on ways to optimize the mass removal behaviour of a treatment train that involves the use of PHC biodegrading sulfate-reducing bacteria as a bioremediation tool for the 'polishing' of a contaminated site. Persulfate was predicted to have a multitude of effects on microbial communities, both positive and negative. It was hypothesized that the production of sulfate would enhance the sulfate-reducing community and subsequently increase biodegradation potential following a persulfate treatment. However, the use of a strong oxidant like persulfate may also have detrimental effects on microbial communities. In order to test this hypothesis, a bench-scale system was implemented to gather data for the analyses of this remediation technique. Microbiological methods and chemical analyses of geochemical parameters were used to examine diversity, richness and abundance of sulfate-reducing communities following persulfate treatments. Initially, the successful generation of anaerobic bioreactors containing an indigenous sulfate-reducing microbial community from a freshwater aquifer was completed and confirmed using colony-PCR. Approximately 3 ppm total PHC was then introduced into the reactors and the microbial community was then allowed to acclimate to the conditions. PHC biodegradation was confirmed (~ 5.7 µg/L/hr). The community was then exposed to two types of oxidants, unactivated and alkaline-activated persulfate. Immediately following exposure, culture-based methods revealed almost complete reduction of the microbial community ($\leq 10^1$ CFU/mL and SRB cells/mL). qPCR on a gene conserved within the sulfate-reducing phylogeny confirmed this reduction. However, by the fourth week of the recovery phase, bacterial counts and target genes rose above pre-treatment levels, indicating enhancement of the sulfate-reducing community following the oxidant exposure(s). However, the recovered community displayed differences in structure and function, as revealed by microbial community fingerprint profiles and a lowered biodegradation potential (~2.7 µg/L/hr). Overall this research illustrated the successful application of a remediation treatment train at a bench-scale level.

TABLE OF CONTENTS

Acknowledgements: ii

Dedication: iii

Abstract: iv

TABLE OF CONTENTS v

List of Tables: ix

List of Figures: x

Chapter 1. Introduction 1

1.1 General Background 1

1.2 Microbial Bioremediation: 4

1.3 Microbial Communities in Freshwater Aquifers (Borden, ON, Canada) 7

1.4 Sulfate-reducing Bacteria: 9

1.5 Persulfate-based *in situ* Chemical Oxidation (ISCO): 14

1.6 Summary and Study Description 18

1.7 Research Needs: 20

1.8 Research Objectives 22

Chapter 2. Experimental Approach and Methodologies 24

2.1 Column Design and Development 24

2.2 Culture-Based Methods 27

2.2.1 SRB Enumeration Medium and the Most Probable Number Method for Sulfate-reducers:..... 28

2.2.2 Monitoring Fluctuations in the Anaerobic Supporting Microbial Community using a Spread Plating Technique: Colony-Forming Units per Milliliter (CFU/mL) on R_2A 29

2.2.3 Community-Level Physiological Profiling Analysis using BIOLOG Ecoplates:..... 30

2.3 Molecular-based Methods.....31

2.3.1 Colony Polymerase Chain Reaction Confirmation..... 32

2.3.2 Real-time Quantitative Polymerase Chain Reaction..... 34

2.3.3 Denaturing Gradient Gel Electrophoresis (DGGE) 38

2.4 Chemical Analyses.....42

Chapter 3: Results 45

3.1 The Creation and Development of Anaerobic Bioreactors.....45

3.1.1Colony-Polymerase Chain Reaction for SRB confirmation at Borden and in the Bioreactors 45

3.1.2 Structural Fingerprints of Microbial Communities Prior to Treatment Phase 48

3.1.3 Structural Fingerprints of the Sulfate-reducing Consortia in the Bioreactors Prior to Treatment Phase 51

3.1.4 Supporting Parameters 53

3.2 Treatment Phase 56

3.2.1 Culture-Based Results: 56

3.2.1.1 Colony-forming-units per millilitre (CFU/mL) observed in all bioreactors 56

3.2.1.2 Analysis of the Most-Probable-Number method for Sulfate-reducing Bacteria 61

3.2.2 Molecular-Analysis 64

3.2.2.1 Quantitative Detection of Changes in Sulfate-reducing Bacterial Loads using Quantitative Real-time PCR (qPCR) 64

3.2.2.2 Structural Fingerprinting of SRB Communities within Bioreactors 71

3.2.2.3 Structural Fingerprinting of Supporting Microbial Communities within Bioreactors 88

3.2.3 Chemical Analyses 105

3.2.3.1 Total Petroleum Hydrocarbon concentration throughout Experimental Trial 105

3.2.3.2 Dissolved Hydrogen Sulfide (H_2S) 108

3.2.3.3 Dissolved Methane (CH_4) 109

3.2.3.4 Monitoring Sulfate 110

3.2.3.5 Monitoring pH 113

3.2.3.6 Monitoring ORP..... 114

Chapter 4. Discussion 116

4.1 The Development of Anoxic Bioreactors Containing Indigenous Microbial Communities from a Freshwater Aquifer 117

4.2 Treatment Phase.....121

4.2.1 Unactivated Persulfate Treatment..... 122

4.2.2 Alkaline-Activated Persulfate..... 130

4.2.3 Alkaline-Only Bioreactors and SRB-Only Bioreactors..... 138

5.1 Summary.....144

5.2 Future Directions and Recommendations.....150

Chapter 6. Integrative Nature of this Research.....154

REFERENCES 155

Appendix-A.....166

List of Tables:

Table 2.1: The target genes and primers explored in this study for colony PCR and qPCR……..38

Table 3.1: Most-Probable-Number (MPN) analysis observed for each treatment, pre-injection (18/09/2015) and post-injection (Sept. 27th → Nov. 22nd) samples. The numbers shown are representative of SRB cells per milliliter …..……..63

List of Figures:

Figure 1.1 Persulfate/EBR treatment train schematic (Shayan, 2015) ……19

Figure 2.1 Schematic of the bioreactor design for the different treatments ……25

Figure 2.2: qPCR results illustrating the relationship between gene copy numbers/reaction (or DNA amount) and cycle threshold …..…….36

Figure 3.1: Colony-PCR confirmation for ~1.9kb dsrAB gene from the aquifer at Borden ……...46

Figure 3.2: Colony-PCR confirmation for the ~1.9kb dsrAB gene in the bioreactors …47

Figure 3.3: Universally-based DGGE analysis for the development of UP1 (A), UP2 (B), and UP3 (C) bioreactors ……....49

Figure 3.4: Universally-based DGGE analysis for the development of AP1 (A), AP2 (B), and AP3 (C) bioreactors ….……...49

Figure 3.5: Universally-based DGGE analysis for the development of AO1 (A), AO2 (B), and AO3 (C) bioreactors ….……...50

Figure 3.6: Universally-based DGGE analysis for the development of Con1 (A), Con2 (B), and Con3 (C) bioreactors ……..50

Figure 3.7: SRB-based DGGE analysis for the development of UP1-2 (A), UP3/AP1 (B), and AP2-3 (C) ……..52

Figure 3.8: SRB-based DGGE analysis for the development of AO1-2 (A), AO1/Con1 (B), and Con2-3 (C) …..……...…….52

Figure 3.9: The average [sulfate] and [lactate] present in the reservoirs (res.) and bioreactor effluents …….53

Figure 3.10: Average percent reduction in BTEX throughout July and August in the unactivated persulfate (UP1-3) ...……..54

Figure 3.11: The bioreactor weights, in grams ....……55

Figure 3.12: Colony-forming-units per mL (CFU/mL) observed in the unactivated persulfate column replicates ……57

Figure 3.13: Colony-forming-units per mL (CFU/mL) observed in the alkaline-activated persulfate column replicates …….58

Figure 3.14: Colony-forming-units per mL (CFU/mL) observed in the alkaline-only bioreactor replicates ……..59

Figure 3.15: Colony-forming-units per mL (CFU/mL) observed in the control bioreactor replicates ……...60

Figure 3.16: Average colony-forming-units per mL (CFU/mL) observed for each treatment, pre-injection (18/09/2015) and post-injection (Sept. 27th → Nov. 22nd) …….61

Figure 3.17: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the unactivated persulfate bioreactors throughout the experiment ……65

Figure 3.18: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the alkaline-activated persulfate bioreactors throughout the experiment ……...66

Figure 3.19: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the alkaline-only bioreactors throughout the experiment …….....68

Figure 3.20: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the control bioreactors throughout the experiment ……..……...69

Figure 3.21: Average Relative abundance (target gene copies/mL) fluctuations observed for each treatment, pre-injection ……..……..70

Figure 3.22: SRB DGGE-oriented analysis of the SRB consortia(s) present in the unactivated persulfate bioreactor replicates …… 72

Figure 3.23: SRB DGGE-based averaged banding patterns ……74

Figure 3.24: SRB DGGE-based analysis of the SRB consortia(s) present in the alkaline-activated persulfate bioreactor replicates ……..76

Figure 3.25: SRB DGGE-based averaged banding patterns **..**……..79

Figure 3.26: SRB DGGE-based analysis of the SRB consortia(s) present in the alkaline-only bioreactor replicates ……..81

Figure 3.27: SRB DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-only persulfate bioreactor replicates **..**……83

Figure 3.28: SRB DGGE-based analysis of the SRB consortia(s) present in the control bioreactor replicates …..……85

Figure 3.29: SRB DGGE-based averaged banding patterns ……87

Figure 3.30: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the unactivated persulfate bioreactor replicates ……89

Figure 3.31: Universal DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the unactivated persulfate bioreactors ..……91

Figure 3.32: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the alkaline-activated persulfate bioreactor replicates ……...93

Figure 3.33: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-activated persulfate bioreactors: A) AP1; B) AP2; C) AP3 ……96

Figure 3.34: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the alkaline-only bioreactor replicates……..….98

Figure 3.35: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-only persulfate bioreactor replicates: A) AO1; B) AO2; C) AO3 ……100

Figure 3.36: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the control bioreactor replicates ……..102

Figure 3.37: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the control bioreactor replicates ……...104

Figure 3.38: Average percent reduction in BTEX September to November in the unactivated persulfate (UP1-3), alkaline-activated persulfate (AP1-3), alkaline-only (AO1-3) and control (Con1-3) bioreactors ……107

Figure 3.39: Average dissolved hydrogen sulfide (H_2S) present in the effluent samples of all treatment and control bioreactors throughout the experiment ……....109

Figure 3.40: Average dissolved methane (CH_4) present in the effluent samples of all treatment and control bioreactors throughout the experiment .……110

Figure 3.41: Sulfate concentration in the reservoirs (C_0) and water effluent of the unactivated persulfate ……..……112

Figure 3.42: Average pH present in the effluent samples of all treatment and control bioreactors throughout the experimental trial ..……114

Figure 3.43: Average ORP (millivolts) present in the effluent samples of all treatment and control bioreactors throughout the experiment ..……..115

Figure A1: An illustration of the bench-scale set-up following bioreactor packing and attachment to GW reservoirs ……...166

Figure A2: This chart is a representation of the BIOLOG EcoPlate and the orientation of the 31 different carbon source wells in triplicate, including a blank well in triplicate ….167

Figure A3: An example of an inoculated Ecoplate used for carbon source utilization profiles ……167

Figure A4: An example of the qPCR completed for the bssA gene on one of the bioreactors (UP2) ……...168

Figure A5: An example of three excised bands that were sent for sequencing ……..169

Figure A6: Percent benzene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial ……170

Figure A7: Percent toluene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial ……170

Figure A8: Percent ethylbenzene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial ……171

Figure A9: Percent $_{p,m}$xylene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial ……171

Figure A10: Percent $_{o}$xylene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial ……172

Figure A11: Total BTEX concentration present in the reservoirs (C_0) and water effluent of the unactivated persulfate (plot A), alkaline-activated persulfate (plot B), alkaline-only (plot C), and control (plot D) bioreactors from September to mid-November ……...173

Figure A12: An example of the time-lapse before, during, and three-weeks post-exposure of AP2 bioreactor ……...……..174

Figure A13: A cross-section (bottom) and overhead (top) schematic of treatment train at Borden .……...…….175

Chapter 1. Introduction

1.1 General Background

For over a century, fossil fuels have been a prevalent and reliable source of energy. Fossil fuels like gasoline have been integral in sectors such as the transportation industry. However, the use of fossil fuels, and the subsequent dependence on them, have caused significant harm to the global environment. Over the past few decades, the extraction and storage of fossil fuels in depots and gas stations have led to the contamination of subsurface sediments and ground-water aquifers, resulting in contaminated sites (so-called "brownfields"). Not only are brownfields detrimental to ecosystems but they also limit urban development due to the restrictions placed on future uses of contaminated sites (Chen, 2013; Thornton, 2007). Internationally, the remediation sector has an estimated value of $50-60 billion US (Sutton *et al.*, 2010; Singh, 2009). With the increasing discovery of brownfield sites around the world, there is a need for the development of efficient and cost effective ways to remediate the various contaminants that plague our environment (Thornton, 2007). One promising mechanism is the use of *in situ* bioremediation (ISB) (Barker *et al*., 1987; Acton & Barker, 1992; Edwards *et al*., 1992). ISB implements indigenous microorganisms, with biodegradation potential, to clean a contaminated site. Over the past 2 decades, researchers have begun to study environmental microbiology and its predominant role in the processes involved in remediating petroleum hydrocarbons (PHCs) (Anderson and Lovley, 2000; Röling, *et al.*, 2004). With the development of different combinations of techniques to remediate sites, there is a continued need to study the effects of those techniques on indigenous microbial communities. Therefore, it is

important to better understand the structure and function of microbial communities, and the functional groups within that community, at sites undergoing remediation. Especially when other remediation (e.g. *in situ* chemical oxidation) techniques are used in unison with bioremediation/natural attenuation.

Land-based remediation is a developing area of research. There are many different ways to remediate contaminated sites, however, there is always a need to formulate remediation processes to be more cost effective and efficient. In order to do so, remediation processes have evolved to use a combination of methods to successfully clean contaminated sites (Petri *et al.*, 2011; Bombach *et al.*, 2010; Sutton *et al.*, 2010). More specifically, research developments have occurred which pertain to the use of oxidants, such as persulfate, permanganate or hydrogen peroxide, to initially degrade freshwater aquifer contaminants and the subsequent utilization of biodegrading bacteria as a long-term, polishing treatment (Sutton *et al.*, 2010). This sequential technology is known as an *in situ* chemical oxidation/*in situ* bioremediation (ISCO/ISB) treatment process. However, there are both positive and negative aspects associated with this process and those aspects will be discussed in further detail in a following section. A study done by Thomson *et al.* in 2004 demonstrated that the oxidant permanganate reduced activity and richness of the indigenous bacterial community at a remediation site. However, the microbial community was able to retain microorganisms capable of degrading naphthalene following the permanganate treatment. There have been other studies as well that have also examined the effects of oxidants such as Fenton's reagent, permanganate, ozone and persulfate on microbial communities (Kastner *et al.*, 2000; Klens *et al.*, 2001; Jung *et al.*, 2005; Bou-

Nasr *et al.*, 2006; Tsitonaki *et al.*, 2008; Richardson *et al.*, 2011; Sutton *et al.*, 2014; Cassidy *et al.*, 2015). However, these studies do not provide a detailed representation of the changes that occur within an anaerobic microbial community, specifically that of sulfate-reducing communities with PHC biodegradation capabilities, when exposed to oxidizing agents. In general, this combination of remediation techniques is still a relatively new area of research and there are knowledge gaps that need to be filled. Persulfate, in particular, is one of the more recently discovered oxidants used for *in situ* chemical oxidation (ISCO) and its effects on anaerobic bacteria are still relatively unknown (Sutton *et al*., 2010; Petri *et al*., 2011).

There is also a need to further research the microbiological processes as a part of the remediation approach for removing PHC contamination, in particular those that are coupled with an ISCO technology. In this study, the coupling of bacterial and chemical oxidative processes will produce an ISCO/ISB petroleum hydrocarbon treatment train. The term ‘treatment train’ is relatively new and a developing concept in environmental engineering (Shayan *et al*., 2015; Sutton *et al*., 2010). A treatment train refers to a set of processes that are linked together and flow in unison to reach the overall goal of completely remediating a contaminated area of concern. The treatment train approach can be implemented within a partially controlled setting which allows for the manipulation of remediation processes safely and with efficacy in the field. The controlled, and sometimes confined, system ensures that a contaminated site is not left to interact with the surrounding environment where the treatment process occurs. Closed systems can be made possible by including man-made barriers that section off portions of the aquifer (Shayan, 2015),

assuming that that the aquitard is somewhat impermeable (e.g. clay base). However, closed systems are not always possible due to physical barriers that may be present at a particular site.

As a relatively new approach, the treatment train design has limitations that are still being explored and assessed. For this particular study, a reflection of the treatment train method was implemented and examined through a bench-scale approach using bioreactors. The bioreactors will be described in further detail in the experimental design. Intrinsic bioremediation of PHC will be the main focus of the bioremediation treatment. However, a preliminary bench-scale model (data not shown in this thesis) using a bacterial community (containing a consortium of sulfate-reducing bacteria) from anaerobic waste water sludge (1% v/v inoculum) was examined initially to provide details (e.g. flow rate, developing anaerobic bioreactors, etc.) on how to proceed with the major column experiment described in this thesis (Shayan, 2015). The introduction of foreign microbial communities for contaminant biodegradation is known as a type of extrinsic bioremediation (EBR), which is not permitted/extremely restricted in Canada for field application (CEPA, 1999). The major column study in this project involved the use of actual core material containing a sulfate-reducing consortium from a previously contaminated field site, a simulation of an intrinsic bioremediation process.

1.2 Microbial Bioremediation:

Bioremediation can be defined as the use of biological organisms, such as plants (phytoremediation), fungi (mycoremediation), and/or bacteria, to degrade or absorb

contaminants in the environment. A recent focus of interest is the remediation of common PHCs (benzene, toluene, ethylbenzene, and $_{p,m,o}$xylenes (BTEX)) using microbial bioremediation. For this research, BTEX was the focus of this study as they are prevalent environmental contaminants and the components are some of the most mobile and soluble constituents in gasoline (Allen-King *et al*., 1994). It has long been known that microbial communities indigenous to freshwater aquifers can demonstrate biodegradation capabilities with respect to BTEX contamination (Barker *et al*., 1987; Barbaro *et al*., 1992).

Microbial bioremediation is the use of microorganisms to resolve, through biodegradation, prevalent environmental contaminants of groundwater and surface or sub-surface sediments (Barker *et al*., 1987; Margesin *et al*., 2000; Bamforth & Singleton, 2005). Many microorganisms utilize organics in the environment to sustain themselves; some even have the ability to consume organic pollutants that are introduced via environmental contamination (Juwarkar, 2010; Röling *et al*., 2004). It has been well-established that aerobic bacteria are capable of bioremediation but, more recently, there has been interest in extrapolating the biodegrading potential of anaerobes as there are typically anoxic regions in groundwater aquifers (Lovley, 1997; Bombach *et al*., 2010; Sun & Cupples, 2012). Biodegrading anaerobes are an important aspect to bioremediating subsurface sediments and aquifers because there are anoxic areas typically present to which contaminants can migrate. Anaerobic organisms that have pollutant-utilizing capabilities, such as denitrifying bacteria, sulfate-reducing bacteria and methanogens, are of particular interest in the study of bioremediation in groundwater aquifers. The use of these remediating organisms can occur in two ways. Typically, bioremediation can occur either

by 1) introducing pollution-consuming microbes (extrinsic bioremediation or EBR) or 2) enhancing the microbial community that is already present (intrinsic bioremediation or ISB). The second of the two provides a potentially more cost effective method and has, therefore, grown in interest globally (Sutton *et al*., 2010; Bombach *et al*., 2010).

Bioremediation can be a very effective and cost efficient way to remediate brownfield sites. However, the process of natural attenuation can be considered slow, taking years to fully remediate contaminated groundwater and sediment plumes. Longevity of the application is particularly affected if bioremediation of anaerobic aquifer environments is required, as it has been found to occur at a slower rate than aerobic biodegradation (Acton & Barker, 1992; Mathies *et al*., 2010; Weelink *et al*., 2010; Shah, 2014). To increase the efficacy of *in situ* bioremediation in anaerobic areas, biostimulation/ bioaugmentation is a common process where additional electron donors and/or nutrients are added to generate ideal redox conditions (Delvin and Barker, 1994; Mathies *et al*., 2010; Sutton *et al.*, 2010). To alleviate some aspects of this problem, chemical oxidants, such as persulfate and permanganate, are used initially to enhance the processes of remediation. However, oxidant treatment or ISCO does not necessarily reach all areas of contamination and, therefore, is typically used in unison with microbial bioremediation. Microbial bioremediation can be seen as a long-term, polishing treatment that completes the process of fully remediating a site. Given sufficient time, a PHC biodegrading microbial community can successfully remediate a site for future use (Shayan, 2015; Sutton *et al*., 2010; Edwards *et al*., 1992; Acton & Barker, 1992).

1.3 Microbial Communities in Freshwater Aquifers (Borden, ON, Canada)

The maintenance and protection of clean, potable groundwater is of utmost importance to humankind. When thinking about groundwater, one does not often speculate on how these aquifers operate. Freshwater aquifers are not only critical in that they supply us with useable water, but they also play a fundamental role in nutrient cycling. The main drivers of these processes are typically the microbial community within these aquifer zones. There are anaerobic and aerobic areas within freshwater aquifers, meaning that there is typically a vast and extremely diverse microbial composition present, typically working in unison to help drive biogeochemical processes (Lehman *et al*., 2001; Lehman *et al.*, 2001; Kleikemper *et al*., 2002; Flynn *et al*., 2012; Flynn *et al*., 2013).

With respect to the field site in this project (CFB Borden, ON, Canada) that was used for core material and groundwater, the physical parameters reflect a shallow, sandy aquifer that is mainly heterotrophic and aerobic in nature with oligotrophic components (Butler *et al*., 1997). In this type of environment, nutrients are relatively low compared to soils high in natural organic matter (NOM) (e.g. agricultural sediment). The microbial community composition consists primarily of aerobic, oligotrophic, psychrophilic organisms. Butler *et al.* (1997) found that the Borden aquifer contained a sparse and reduced viable population (dry weight: <10 to 10^4 CFU/g), where communities were localized, especially when considering the presence of anaerobes such as SRB. In general, total microbial numbers in the Borden aquifer tend to be on the low end in comparison to other pristine, shallow aquifers (Butler *et al*., 1997). The medium that showed the highest success rate at culturing the general, viable, microbial population, most notably anaerobes,

was R2A in the Butler *et al.* (1997) study. R2A is a general, non-selective, enrichment media used for the enumeration of cultivable organisms from environmental samples. Consequently, R2A was used in this study to analyze fluctuations in the general microbial community throughout the persulfate treatment process.

There can be a significant difference in microbial community structure between freshwater aquifers, depending on the aquifers' ability to sustain microbial life (bioavailability of nutrients). Therefore, the response/recovery of a biodegrading microbial community to different ISCO treatments can fluctuate between contaminated sites. However, one general thought for consideration is whether SRB have a higher potential for recovery since it has been shown SRB are able to handle environmental stresses (e.g. fluctuations in redox potential) more so than other microbes in the supporting community (Dolla *et al*., 2006). The return of SRB over other microbes, such as methanogens, is desirable as methane production is an unwanted by-product during bioremediation efforts. It is difficult to yield a solely SRB-dominated microbial community as SRB and methanogens co-exist and co-metabolize together in subsurface sediments (Stams *et al*., 2005; Dar *et al*., 2008). However, by changing sulfate concentrations and substrate levels (i.e. lactate, which is used for initial bioaugmentation of the bioreactors in this study), an SRB-dominant microbial community can develop with methanogens still present, but in lower numbers (Dar *et al*., 2005; Dar *et al*., 2008). Thus, the re-establishment of an SRB community following a persulfate treatment is an area of interest and the focus of this study.

It should also be noted that variability exists at a single site, with respect to attached and unattached (suspended) microbial communities (Lehman *et al*., 2001; Lehman *et al.*, 2001; Flynn *et al*., 2013). For example, Flynn *et al*. (2013) discovered that bacteria which respire insoluble substrates (i.e. iron oxides respired by *Geobacter* spp.) are in greater abundance in the attached community than the suspended community in pristine freshwater aquifers. Only the suspended microbial community (with exception of some particulate that leaves the bioreactors) was analyzed in this study. Further investigation into the changes that occur to attached microbial communities experiencing a persulfate treatment should take place to fully grasp the effects of an ISCO exposure on an anaerobic microbial community.

1.4 Sulfate-reducing Bacteria:

A class of microbes, known as sulfate-reducing bacteria (SRB), have the ability to remove PHCs from the environment (Acton & Barker, 1992; Reinhard *et al*., 1997; Pelz, 2001; Morash *et al*., 2004; Müller *et al*., 2004; Cassidy *et al*., 2015). SRB are present throughout the environment and are integral participants of the sulfur and carbon cycles (Fukui *et al*., 1999; Ghazy *et al.*, 2011; Castro *et al.*, 2011; Plugge *et al.*, 2011; Kondo *et al.*, 2012). SRB utilize sulfate as their electron acceptor (‘respire’ sulfate) while they degrade organics; they are strictly anaerobic and colonize subsurface sediments, making them a suitable organism for bioremediation of contaminated aquifer plumes (Acton & Barker, 1992; Chang *et al.*, 2001). Plumes, in this case, are the contaminated areas of the aquifer. The reduction of sulfate can transpire over an extended range of pressure, pH, temperature, and salinity environments (Ruwisch *et al.*, 1987; Atlas and Bartha, 1993).

SRB have even been found to form biofilms, aiding in nutrient sequestration and colony protection (Santegoeds *et al*., 1997; Wargin & Skucha, 2007). In the natural environment, sulfate-reducers have been found to utilize pyruvate, lactate and molecular hydrogen as sources of electron donors and produce H_2S as their main metabolic by-product (Postgate, 1979; Atlas and Bartha, 1993). H_2S can be indicative of metabolically active SRB in an aquifer. H_2S is toxic, even to SRB themselves, so a build-up of H_2S is not overly desirable in remediation processes, however, it can precipitate out as metal sulfides, becoming less toxic, unlike methane which can pose potential health risks (e.g. explosive above 50,000 ppm) (Reis *et al*., 1992; Ma *et al*., 2012). A general example of reduction of sulfate is as follows:

$$4H_2 + SO_4^{2-} \rightarrow H_2S + 2H_2O + 2OH^- \qquad \text{(Equation 1.1)}$$

Also, as mentioned, sulfate-reducers have been discovered to biodegrade environmental contaminants such as PHCs, and have thus been noted as a potential mechanism for facilitating bioremediation.

Sulfate-reducing bacteria that have been found and/or introduced at remediation sites include species of *Desulfovibrio*, *Desulfotomaculum*, *Desulfobulbus*, *Desulfobacter*, and *Desulfosporosinus* (Devereux *et al*., 1997; Robertson *et al*., 2001; Kleikemper *et al*., 2002; Sun & Cupples, 2012; Petri *et al*., 2011; Colin *et al*., 2013). Although we have some understanding of the key participants in bioremediation on an organismal/genus level, more research is needed on how to effectively use or manage a sulfate-reducing community as a remediation tool.

To further our understanding, the metabolic function of indigenous sulfate-reducing communities in bioremediation needs to be examined and profiled. The functionality of sulfate-reducing bacteria is extremely important to analyze. Functionality will give us an indication of the energetic needs of sulfate-reducing communities involved in bioremediation. The way to analyze function (i.e. metabolism) within a community is through culture-based approaches and the analysis of biodegradation potential. Culture-based approaches will allow for the ability to analyze the utilization of different carbon sources, and the effects of temperature, as well as other environmental stressors on the community (e.g. pH). In this study, an exposure to a high pH (pH 12) will be analyzed; a form of persulfate activation is the use of high alkaline conditions (≥ pH 10.5) (Sutton *et al.*, 2010). When ISCO is used as an initial form of remediation, oxidative stress, as well as changes in pH and redox potential, can alter subsurface soil conditions and potentially have a negative effect on bioremediating microbial communities (Sutton *et al.*, 2010; Cassidy *et al.*, 2015). Culture-based analysis is important as this approach will compliment genetic-based analysis and provide insight into the effects of ISCO on the viable portion of the anaerobic communities examined.

Knowledge of microbial community structure changes will also play an important role in the successful application of bioremediation (Li *et al*., 2007; Zucchi *et al.*, 2003). Molecular techniques, such as quantitative polymerase chain reaction (qPCR) and denaturing gradient gel electrophoresis (DGGE), will provide information on changes in relative abundance, as well as fluctuations in genetic richness and diversity with respect to the sulfate-reducing community (Dar *et al*., 2007; Miletto *et al*., 2007; Foti *et al*., 2007;

Talbot *et al*., 2008; Geets *et al*., 2006; Geets *et al*., 2005; Giloteaux *et al*., 2010; Zeleke *et al*., 2013). The practical meaning of richness and diversity in this case is the number of species/bands present in a samples (richness), as well as the evenness of band intensities in comparison to the number of bands in a single lane/sample (diversity). The basis of genetic analysis in this thesis was the study of fluctuations in the dissimilatory sulfite reductase gene (dsrAB; ~1.9 bp), which encodes for a conserved enzyme within the SRB phylogeny, during a persulfate-mediated ISCO treatment. The enzyme contains two subunits, dsrA and dsrB (alpha and beta subunits, respectively), and is a key component in the reduction of sulfate. The conserved gene segment(s) that encode for the enzyme, and its subunits, make it an appropriate target for molecular-analysis of various SRB communities (Wagner *et al*., 1998; Pérez-Jiménez *et al*., 2001; Neretin *et al*., 2003; Kondo *et al.*, 2004; Geets *et al*., 2006; Spence *et al*., 2008; Santillano *et al*., 2015; Müller *et al*., 2015).

With respect to DGGE analysis, changes in a microbial community 'fingerprint' profile/structure can be monitored by changes in a community's G+C composition (Muyzer *et al*., 1993). Monitoring differences in G+C content is a way to compare similarities in nucleotide composition of highly conserved bacterial 16s rDNA within a community or between communities and can infer phylogenetic similarities (Wayne *et al*., 1987). In DGGE analysis, an organism-based ladder, containing segments of DNA from known species of bacteria, is included, however, the identity of particular OTUs (operational taxonomic unit) or phylotypes cannot be inferred due to the potential for band co-migration on a gel. A phylotype refers to classes of organisms that are similar with respect to their evolutionary development. Since bands or OTUs have been known to co-

migrate through gels, OTU comparisons to an organismal-based ladder can be misleading. However, as mentioned, taxa with similar G+C content can infer phylogenetic similarities (Wayne, 1987). Band excision from gels and sending the band(s) for sequencing is one way to determine the classification/taxa of recovering organisms or dominant OTUs in a sample. For this study, SRB-based and universally-based DGGE was used to monitor shifts, or a lack thereof, in the recovering anaerobic microbial community following two types of persulfate treatment (unactivated and alkaline-activated). Shifts are also monitored with respect to changes in the community following inoculation into the columns at room temperature. Chandler *et al*. (1997) has shown that fluctuations in a microbial community fingerprint profile can occur when subsurface sediments are removed from their natural environment and brought into a lab setting.

The structure of the microbial community is expected to change throughout a remediation process, especially when including extenuating factors such as oxidants. Oxidants used for the remediation of petroleum contaminants can be very strong oxidizers, such as persulfate. Consequently, some or all of the bacterial community could be eliminated following an ISCO treatment. However, recent research suggests that a biodegrading community can actually be enhanced following a remediation process (Sutton *et al.*, 2010; Richardson *et al.*, 2011; Sutton *et al.*, 2014; Cassidy *et al.*, 2015). With all aspects considered, further investigation is needed with respect to the changes in the structure of a sulfate-reducing community involved in a remediation process that includes persulfate and its various activation methods. Some of the voids mentioned will be filled with the application of the results found throughout this study.

1.5 Persulfate-based *in situ* Chemical Oxidation (ISCO):

An ISCO treatment process typically involves subsurface injections of chemical oxidants, which are responsible for mass contaminant oxidation/degradation (Sutton *et al*., 2010; Siegrist *et al*., 2011). Any contaminants attached to a soil matrix, or in non-aqueous phase liquid form, can typically be treated by this aggressive method. However, treatment is more effective if the contaminant is not bound to dense natural organic matter (NOM) (Petri *et al.*, 2011). If the contaminant plume is mainly confined within sediment, the NOM usually needs to be broken down first by the oxidant to allow sorption of the contaminant. This can be more difficult if the NOM is dense and composed of only partially decomposed material. This is where the addition of enhanced bioremediation can come to the forefront. If a biodegrading community can withstand a persulfate application, then they can be used for 'polishing' a site by remediating any recalcitrant by-products remaining in areas where oxidant treatment is less effective (Sutton *et al*., 2010). Some of the reactions that are included in the activation of persulfate are as follows:

Electron transfer:

$$S_2O_8^{2-} + 2e^- \rightarrow 2SO_4^{2-} \qquad \text{(Equation 1.2)}$$

Acid generation:

$$S_2O_8^{2-} + H_2O \rightarrow 2HSO_4^- + \tfrac{1}{2}O_2 \qquad \text{(Equation 1.3)}$$

High pH:

$$2S_2O_8^{2-} + 2H_2O \xrightarrow{OH^-} SO_4^{\bullet -} + O_2^{\bullet -} + 3SO_4^{2-} + 4H^+ \qquad \text{(Equation 1.4)}$$
$$SO_4^{\bullet}{}_4^- + OH^- \rightarrow SO_4^{2-} + OH^{\bullet}$$

As mentioned, there are currently a variety of different oxidants used for ISCO, such as permanganate, Fenton's reagent, or persulfate. All methods are ongoing areas of remediation research in an effort to discover which chemical additions to a contaminated aquifer will provide the most efficient/cost-effective path to remediation (Sutton *et al.*, 2010). The versatility of ISCO as a remediation strategy makes it a developing research area. ISCO provides an extensive and rapid way to remove both dense and light recalcitrant, pure phase PHC compounds (Sutton *et al*., 2010; Thomson *et al*., 2004; Petri *et al.*, 2011). An important aspect to consider is the environmental effects of the radicals produced from the oxidant reacting with PHC. The radicals produced will also be considered when examining potentially effective chemicals. One particularly aggressive oxidant, persulfate ($Na_2S_2O_8$), produces sulfate when activated (Sutton *et al.*, 2010; Shayan, *et al.*, 2013). Sulfate ($\bullet SO_4^{2-}$) and hydroxyl ($\bullet OH^-$) radicals also develop following persulfate applications as well as other unknown agents, all of which are expected to be attribute to the oxidants aggressiveness as a contaminant treatment tool. An example of this is the degradation of benzene proposed by Sra *et al*. (2013):

$$C_6H_6 + 15H_2SO_8^{2-} + 12\ H_2O \rightarrow 6CO_2 + 30SO_4^{2-} + 30H^+ \qquad \text{(Equation 1.5)}$$

Following the reaction, it is proposed that the generated sulfate can then be utilized by PHC degrading SRB, potentially leading to a type of 'enhanced' bioremediation. Furthermore, if incomplete degradation of PHC compounds occurs, the partially degraded contaminant is potentially more bioavailable for biodegradation by indigenous microbes still present following the ISCO treatment. (Sutton *et al*., 2010; Shayan, 2015). However, it is mainly

the production of sulfate that has the potential to increase biological reaction rates of SRB via biostimulation (Sutton e*t al.*, 2010). Thus, the products of the chemical oxidation of petroleum contaminants could ultimately support or promote the bioremediation 'scrubbing' process that follows ISCO. The research on persulfate-mediated ISCO and its effects on microbial communities is relatively grey, with most studies focusing on the remediation of chlorinated hydrocarbons or bioremediation via aerobic bacteria (Tsitonaki *at al*., 2008; Cassidy *et al*., 2009). A study completed by Cassidy *et al*. (2015) looked at persulfates' effect on SRB, however, they were mainly looking at the recovery of naphthalene degraders, not BTEX degraders, which is the focus of this study.

The effectiveness of the secondary trait of the oxidant, the production of sulfate, is not as well understood (Sutton *et al.*, 2010). Thus, completing an analysis of a PHC-degrading SRB community throughout persulfate exposure reveals important information, such as where the SRB community increases in abundance along the remediation plume (Shayan, 2015) and/or if some types of organisms are enhanced over others. In this study, a SRB community analysis following a persulfate treatment will help elucidate whether the sulfate, produced during the activation of persulfate, aids in the development of a long-term bioremediation polishing step. The subsequent SRB-based bioremediation step following ISCO is described as 'long-term' because SRB are still slow biodegraders in general. Enhancement via sulfate may alleviate some of this delay in the polishing of a PHC contaminated site via SRB bioremediation (Cassidy *et al.*, 2015). The time of subsequent bioremediation will depend mainly on the mass removal potential of the ISCO treatment applied.

Since persulfate is a strong oxidant there is the potential for detrimental effects on the microbial community as well. Identifying community structure changes will help establish the severity of the negative effects on the community. It might be predicted that there will be a reduction in the overall abundances within the microbial community but portions of the community, namely SRB, should recover with biostimulation by electron acceptor (sulfate) generation, especially in a field setting where re-inoculation can occur from up-gradient portions of the aquifer (Sutton *et al.*, 2010).

With respect to the bioremediation processes involved with PHC contaminated ground aquifers, previous tests have been done to determine which types of bacteria are present and prospering in contaminated aquifers and surrounding sediments (Barker *et al.*, 1987; Dojka *et al.*, 1998; Staats *et al.*, 2011). As mentioned, the field site used in this research project, CFB Borden, has previously had its soil microbial community analyzed by Butler *et al.* during the 1990s (Butler *et al.*, 1997). However, indigenous SRBs were found to be rare/unculturable at C.F.B Borden in their study. Background sulfate concentrations are shown to be typically high at the Borden test site and it is suspected that there are sulfate-reducing conditions in anoxic areas of the groundwater aquifer (Acton & Barker, 1992). An earlier petroleum hydrocarbon bioremediation study done by Acton and Barker (1992) at Base Borden showed that toluene biodegradation occurred in aerobic and anaerobic areas of the Borden aquifer (Barker *et al.*, 1987; Acton & Barker, 1992). At the time, they were not able to distinctly determine whether sulfate-reduction was occurring and if it was SRB activity responsible for toluene biodegradation. The results seem to indicate that other methods of bioremediation and potentially abiotic sorption were

responsible for the remediation/natural attenuation of toluene in this case (Acton & Barker, 1992). However, it was noted that background sulfate concentrations (200-300 mg $L^{\sim 1}$) were considerably high. Since sulfate concentrations were so high during Acton and Barker's study, it is possible that the sulfate reduction, required for biodegrading the PHCs present in the aquifer, was too minute to be detected or the sulfate reduction was masked by the overall background sulfate concentration (Acton & Barker, 1992). In order to truly distinguish the presence and function of SRBs at Borden, further analysis needs to be completed. In this study, core material from the Borden aquifer was extracted in an attempt to develop bioreactors with sulfate-reducing bacteria that are indigenous to groundwater sediments. Once established, persulfate exposure tests will be applied.

1.6 Summary and Study Description

Recently, there has been an increased effort to promote the efficiency and cost effectiveness of remediating contaminated sites. In doing so, research effort has been invested in the examination of a combination of ISCO and *in situ* microbial bioremediation. The approach would be to use an oxidant for mass removal of the contaminant initially and then promote the growth of organisms capable of biodegradation/mineralization of the contaminant (to CO_2 and H_2O) during a final 'scrubbing' phase of the site (Beller *et al*., 1992; Sutton *et al*., 2010; Petri *et al*., 2011). As mentioned for this particular research project, microbiological aspects of a persulfate-mediated ISCO/ISB treatment train system are examined. The theory behind the treatment train application can be seen in the following illustration:

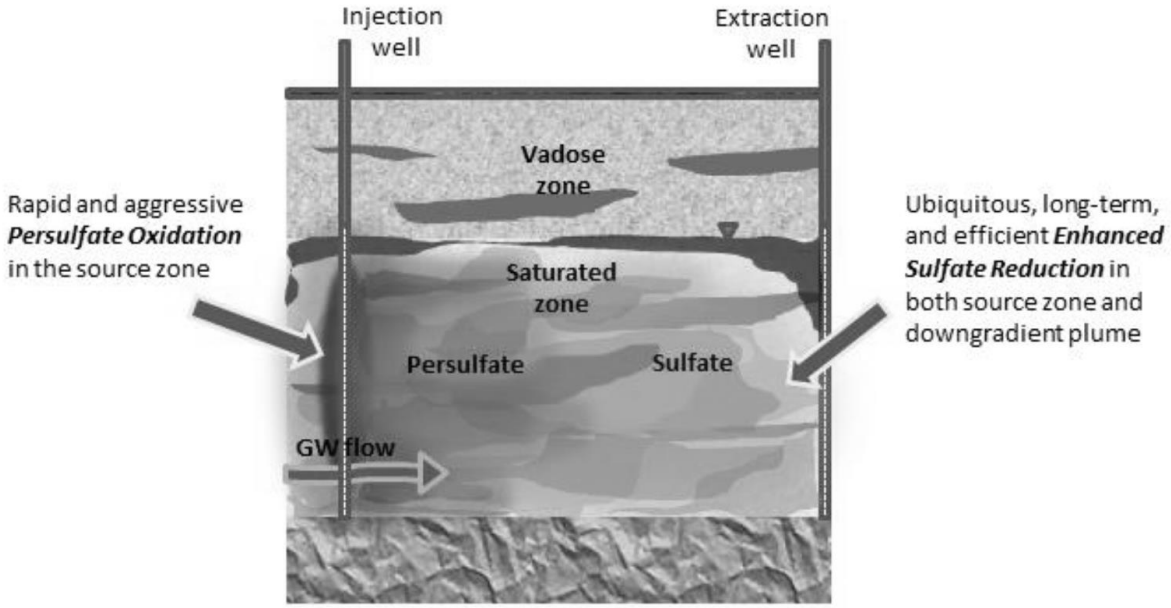

Figure 1.1: Schematic illustration of a persulfate/ISB treatment train reproduced from (Shayan, 2015) with permission from Dr. Shayan.

The particular oxidant proposed in this project is persulfate. As mentioned, persulfate is a strong oxidant. It can be implemented in both an 'active' and 'non-active' state in solution. Unactivated persulfate takes longer to react with contaminants in the environment. However, this form is useful as it can be delivered to contaminated areas that may be far from the injection point (Sutton *et al*., 2010). Unactivated persulfate becomes activated through interactions with substrates in the subsurface, such as NOM (Sutton *et al*., 2010; Petri *et al*., 2011). The forms of activation vary and include strategies such as metal activation, peroxide activation, heat activation and alkaline activation. All of these strategies vary in their effectiveness to treat a contaminated site, as well as their effects on subsequent bioremedmdiation. For example, with respect to alkaline-activated persulfate treatments, the high alkaline environments (11-13 pH) required can change/disrupt a

microbial community as well (not only the strong oxidant) (Padan *et al*., 2005; Baker *et al*., 1982).

It can be concluded that biphasic ISCO/bioremediation treatment is a feasible remediation method of PHCs. However, to further optimize 'treatment trains', further exploration into the effects of different oxidants, with their different forms of activation, on a residing microbial community, responsible for the final degradation processes of a site, is needed.

The study presented uses a combination of culture-based and molecular-based techniques to monitor changes in an anaerobic community from a freshwater aquifer. This was done by generating anoxic bioreactors that contain microbial communities from cores that were extracted from the Borden aquifer. Successful development and inoculation of indigenous microbes took place that had BTEX biodegration capabilities. BTEX exposure (3-4 ppm total) followed by a 24-hour, 20g/L injection of unactivated and alkaline-activated persulfate (pH 12) took place. The recovery phase was monitored for 2-months post-oxidation. As revealed by DGGE, the recovery of a novel, SRB-dominated, anaerobic microbial community took place within 4-weeks of treatment. The community that recovered had increased numbers of SRB, as shown by qPCR and the MPN method for sulfate-reducers, in comparison to the pre-oxidation community.

1.7 Research Needs:

It is known that sulfate-reducing bacteria have the capacity to be integral in remediating brownfield sites (Acton & Barker, 1992; Noh *et al.*, 2003; Sutton *et al.*, 2010;

Cassidy *et al.*, 2015; Shayan, 2015). Little is known about how the SRB community changes on a functional and structural level throughout bioremediation in either the short or long term (Pelz *et al.*, 2001). With that in mind, the proposed research will provide important information on how to implement PHC remediation trains that involve the use of microbial communities, in particular sulfate-reducing bacteria.

Further exploration into the microbial ecology of SRBs involved in PHC remediation trains is needed. The overall change in function and structure of sulfate-reducing communities, with respect to biogeochemical processes, following a persulfate-mediated ISCO treatment is relatively unknown. Discovering aspects about the changes in an indigenous bacterial community profile, with respect to diversity and richness, is essential as well in order to determine if a significant microbial rebound can occur (for essential processes such as nutrient cycling, as well as PHC biodegration). Knowledge of the fluctuations in the anaerobic microbial community will provide integral information in the development and maintenance of PHC treatment systems following ISCO treatment. It will provide insight into the manner in which indigenous microbial communities recover following remediation treatments and the communities' biodegradation potential after exposure to the strong oxidant persulfate. Results from this research will also help increase the overall efficacy and cost effectiveness of ISCO/SRB-related remediation projects world-wide. It is proposed that the generation of a treatment train through the coupling of ISCO via persulfate and intrinsic SRB bioremediation will produce enhanced, long-term sulfate-reducing biodegradation of PHC by SRBs in contaminated groundwater plumes. Initial exposure to persulfate will have detrimental effects on the microbial community

(Richardson *et al*. 2011; Sutton *et al*., 2014; Cassidy *et al*., 2015). However, as previous studies have noted, the biodegrading potential of the microbial community is anticipated to rebound as they become acclimatized and/or as conditions return to their representative indigenous profile (Cassidy *et al*., 2015; Shayan, 2015).

Overall, it is widely considered that an ISCO/ISB treatment train approach can be an effective method for remediating PHC contaminated sediment and groundwater aquifers. The hypothesis is that persulfate will have an initial negative impact on the general microbial community, including the SRB population, but the community will rebound after persulfate exposure and the environment will continue to support PHC biodegrading SRB activity for subsequent long-term bioremediation. Furthermore, the sulfate production from the persulfate-PHC reaction should support and even enhance the SRB community.

1.8 Research Objectives

The overarching goal of this research is to analyze and profile a sulfate-reducing community from a remediation test site in Borden, ON and determine if the community can recover following a 20g/L unactivated and alkaline-activated persulfate treatment. This goal will be achieved using a bench-scale remediation system designed in cooperation with environmental engineers and scientists from the University of Waterloo. The primary objectives of this research are to:

- **Objective 1**: Assess the presence/location of sulfate-reducing communities at a field-site in CFB Borden, ON

- **Objective 2**: Develop bioreactors that contain an active SRB community
    - assess PHC biodegradation capabilities
- **Objective 3**: Observe the effects of unactivated and alkaline-activated persulfate exposures on SRBs and their supporting microbial communities
    - monitor/assess any changes in SRB community **structure** and **function** as well as BTEX biodegradation capabilities post-treatment

Chapter 2. Experimental Approach and Methodologies

To monitor the effects of different ISCO treatments on indigenous anaerobic microbial communities, both a culture- and molecular-based approach was used. Also, the design, construction, and development of sets of bioreactors, in triplicate, took place to complete the types of analysis outlined in the experimental plan. Specific sampling procedures for the bench-scale and field-scale approach are also outlined.

2.1 Column Design and Development

A total of twelve bioreactors were used for this study. There were four sets of triplicates designated for three treatments and a control that address the primary objectives of the experimental design:

- 3x unactivated persulfate/ISB Bioreactors; referred to as UP1, UP2, and UP3
- 3x alkaline activated persulfate/ISB Bioreactors; referred to as AP1, AP2, and AP3
- 3x alkaline-only exposure/ISB Bioreactors; pseudo-control; referred to as AO1, AO2, and AO3
- 3x ISB-only (control) Bioreactors: referred to as Con1, Con2, and Con3

Each set of column experiments will have the same column dimensions with an injection port at the bottom and effluent port at the top in triplicate for each treatment. An illustration of the final set-up can be found in the Appendix section (Figure A1), however, a schematic of the initial design is provided as follows:

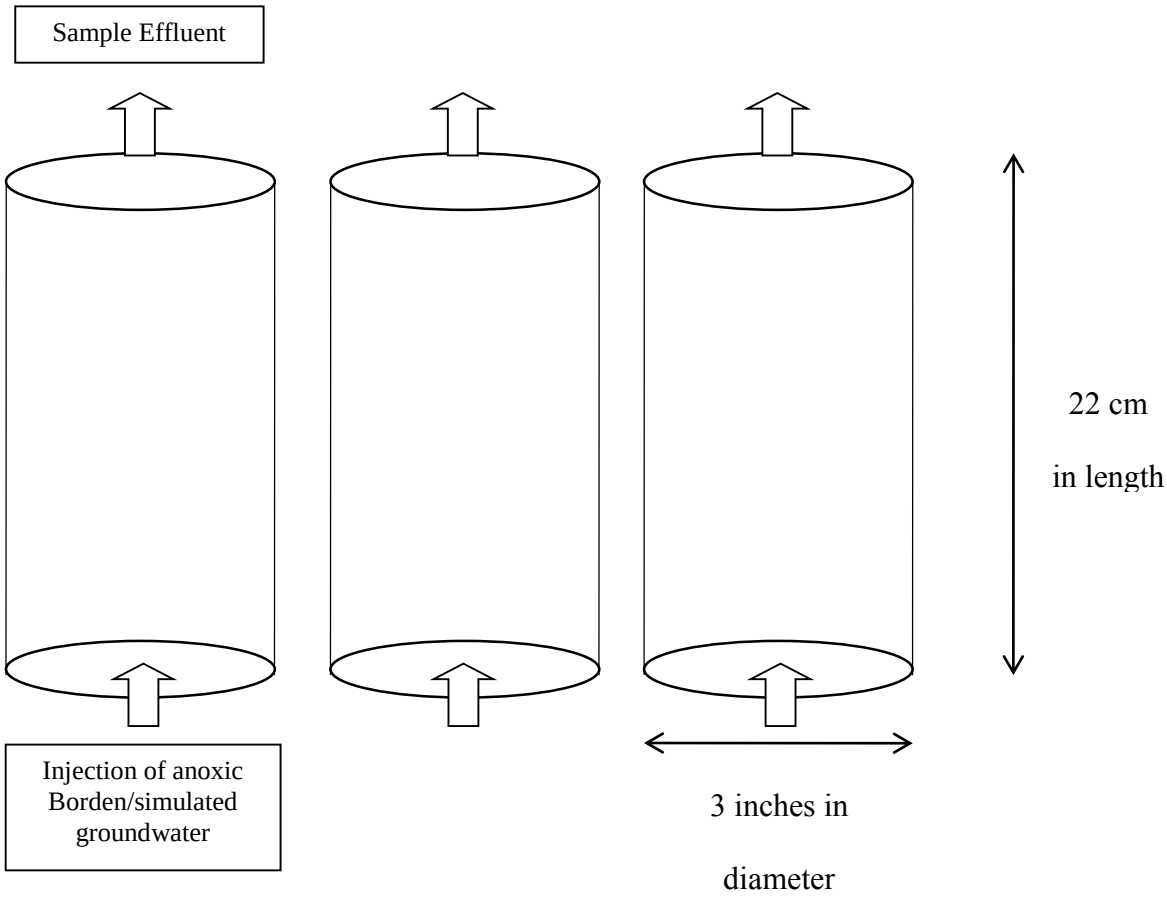

Figure 2.1: A general schematic of the bioreactor design for the different treatments. There were four sets of triplicates with this general design. The dimensions shown provide a 1L capacity with a pore volume of ~ 300mL.

The bioreactors were packed with core material from the Borden aquifer where SRB activity was detected in groundwater (GW) samples. Cores and groundwater were extracted from 7-11 feet deep (down to the aquitard) into the aquifer to provide anoxic material and a source of inoculation (of indigenous anaerobic bacteria, notably SRB) for the bioreactors. The core material was cut into small sections (~ 2 foot sections so they could be taken into an anaerobic chamber before opening to limit oxygen exposure),

marked with their respective extraction depth and capped off once extracted. Prior to extracting cores from the aquifer, groundwater samples were taken for analysis. Colony-confirmation PCR on the dsrAB gene for the dissimilatory sulfite reductase enzyme was completed (Figure 3.1 in Results section) to ensure that the core material taken contained sulfate-reducing microbial community. Core extraction took place with the assistance of Bob Ingleton and Paul Johnson from the University of Waterloo (Earth Sciences; Waterloo, ON, Canada) using a Geoprobe® extraction rig. Once extracted, the core material was taken back to the lab and mixed thoroughly in the anaerobic chamber to ensure a homogenous blend before being packed into the columns. Groundwater (including experimental components such as BTEX and persulfate) was injected using peristaltic pumps, at the lowest reliable flow rate (~0.2 mL/min), into each column using separate reservoirs for each triplicate set of columns. Once packed and running, samples were extracted from the effluent of the columns to ensure the successful inoculation/enumeration of a sulfate-reducing, anaerobic microbial community (Figure 3.2 in Results section).

Borden GW was initially used as the injection solution during bioreactor development for further inoculation/acclimation of the anaerobic microbial community. Due to restrictions of the season (winter), injection solutions were switched to 'simulated' GW. The simulated GW was prepared by adding background levels of sulfate and carbonate (~130 mg/L and ~90 mg/L, respectively). The water was then purged with CO_2 to lower the pH in order to dissolve the carbonate. This was followed by a nitrogen-purging step to raise the pH of the water and to ensure the DO of the water was low ($\leq$ 1 mg/L).

Once purged, the GW was transferred into 5L tedlar bags (Sigma-Aldrich®) which served as the reservoirs for the experiment.

During the treatment phase, 20g/L sodium persulfate (unactivated and alkaline-activated) solutions as well as a high alkaline solution (pH 12) were injected into the columns for a 24-hour period with an increased flow rate to ensure the passage of three pore volumes. The control replicates remained the same, with simulated GW containing 3-4 mg/L BTEX, during this stage. Three pore volumes were flushed through the reactors to ensure the activation of the oxidant and exposure of the oxidant to the microbial communities contained within the bioreactors. Following the 24-hour treatment phase, the solutions and injections were restored to pre-oxidation conditions. The bioreactors were then monitored for a 2-month 'recovery' period to observe if a microbial community could return/recover and if that community was novel or shared a similar structure to the pre-oxidation community. Monitoring was completed by analyzing the effluent of the bioreactors using a combination of chemical and microbiological techniques, which are described in the following sections of methodologies.

2.2 Culture-Based Methods

Culture-based analysis began immediately prior to the treatment phase of the experiment. One set of pre-oxidation samples and subsequently four sets of post-oxidation samples were analyzed throughout this study. A reduced amount of culture-based sampling took place in comparison to genetic-based sampling events due to space restrictions within the Bactron II anaerobic chamber (Sheldon Manufacturing, Inc). Incubation times for

anaerobes are typically long, as they are known to be slow growers, and therefore turnover times for conducting culture-based sample analysis can be delayed (Rockne *et al*., 2000).

2.2.1 SRB Enumeration Medium and the Most Probable Number Method for Sulfate-reducers:

A modified Baar's medium for sulfate-reducers (ATCC medium 1249) recipe was used to isolate and enumerate SRB from water and core soil samples (Tanner, 1989; Atlas, 2005; Butler *et al*., 1997). The medium contained lactate, a preferable carbon source for SRB growth, and was stored in an anaerobic environment (90% N_2, 5% CO_2, 5% H_2) (Acton & Barker, 1992). As a growth indicator, the medium turns a black colour if SRB are present. The colour generation is due to the reaction of ferrous salts with hydrogen sulfide, produced during sulfate reduction, generating a black precipitate known as iron sulfide. Bacterial growth in this medium was used as an indicator of viable SRB at the field site in Borden as well as in the bioreactors following inoculation and treatment.

Although Baar's medium is a general SRB enumeration media, it should be noted that not all SRB species may be cultivated/captured, as a large amount of environmental isolates are viable but non-culturable (VBNC) in a lab setting. It has been speculated that less than 1% of water and soil bacteria can be cultured *ex situ* (Barcina & Arana, 2009). However, as mentioned, culture-based methods are still highly complementary to genetic-based analysis as the combination provides extended insight into phenotypic and genotypic characterization/traits.

In order to monitor changes in the viable portion of SRB in the bioreactors during the treatment phase, the three-tube Most Probable Number Analysis for Sulfate-reducers was implemented using the Baar's medium (Britton & Greeson, 1987). Baar's medium was inoculated in triplicate and in dilution series ($10^0 \rightarrow 10^{-6}$), with samples from pre- and post-oxidation bioreactor effluent. Room temperature was used for incubation, which took place for 28 days. Tubes showing a development in black, iron sulfide deposits were considered positive and their respective dilutions were recorded. The dilutions displaying positive results for SRB growth were then compared to a MPN table which dictated an estimated number of bacterial cells per milliliter (MPN/mL) (Britton & Greeson, 1987). This method allowed for the effects of the persulfate treatments, on the viable/culturable SRB portions, to be illustrated.

2.2.2 Monitoring Fluctuations in the Anaerobic Supporting Microbial Community using a Spread Plating Technique: Colony-Forming Units per Milliliter (CFU/mL) on R_2A

In an attempt to capture the effect of the persulfate treatments and alkalinity exposure on the anaerobic viable microbial population, a spread plating and subsequent plate counting technique (CFU/mL) was implemented before and after the treatment phase. The theory behind CFU/mL is that a single bacterial cell can give rise to a colony and the colonies formed on a media plate can be counted and recorded. In essence, this technique reveals the culturable bacterial load of a sample.

The CFU/mL method requires a sample to be inoculated into a series of dilutions (i.e. $10^0 \rightarrow 10^6$) and the dilutions are then spread plated onto a chosen media type. In this study, R_2A, a general non-selective medium, was used as it has been documented to

perform the best when assessing anaerobic bacterial loads, most notably at Borden (Reasoner *et al*., 1985; Butler *et al.*, 1997; Bowman *et al.*, 2006; Rockne *et al.*, 2000). The calculation for CFU/mL is as follows:

CFU/mL= # of colonies X dilution factor X volume (mL) factor (Equation 2.1)

In this study, 0.2 mL of sample was added to each plate and plating was completed in duplicate. Plates were counted after 14 days of incubation in the anaerobic chamber at room temperature (21-23^{O}C) (Butler *et al*., 1997).

2.2.3 Community-Level Physiological Profiling Analysis using BIOLOG Ecoplates:

In an attempt to provide further details on the functional aspects of objective 3, BIOLOG MicroPlate^{M} technology was used to monitor changes in the anaerobic community metabolic capabilities (i.e. utilize different carbon sources). This system is culture-based and provides a way to analyze spatial and temporal fluctuations in microbial communities. Ecoplates were inoculated with samples, which provide a Community-Level Physiological Profile (CLPP) (Christian, 2007). Ecoplates are comprised of 31 different carbon sources and a blank, all of which are present in triplicate (see Appendix Figure A2). In order to measure metabolic activity, a tetrazolium dye is used which develops a purple colour in correlation with the rate of cellular respiration, and inherently, substrate utilization. CLPP analysis was developed by Mills and Garland in 1991 and has established itself as an effective method to determine carbon source utilization profiles (CSUP). This method is typically used with aerobic microbial communities due to the difficulties in inoculating and reading plates inoculated with anaerobic bacterial communities. However,

recent studies suggest that it could potentially be better applied to anaerobic conditions with specific precautions taken, such as incubating plates in impermeable, transparent microplate bags (Christian, 2007; Borglin *et al*., 2009). Although the precautions taken in these studies were implemented, an unforeseen difficulty was eventually discovered which hindered the further use of this technique in this study. Unfortunately, abiotic reduction (false positives) of tetrazolium dye can occur (within minutes of inoculation) in the presence of a select few compounds including H_2S, which was in high concentrations in the bioreactor effluent throughout the experiment (refer to Appendix Figure A3) (Bhupathiraju *et al*., 1999). Although this technique would have yielded a great source of complementary data, the method had to be abandoned. Further investigation into using CLPP with anaerobic microbial communities is a necessity in order for this technique to be incorporated into studies such as the one presented in this thesis.

2.3 Molecular-based Methods

Polymerase chain reaction (PCR) confirmation, real-time or quantitative polymerase chain reaction (qPCR), and denaturing gradient gel electrophoresis (DGGE) was used to help satisfy the community structure aspects of objectives 2 and 3. In order to complete these methods, DNA was extracted from samples and isolates using the MO BIO Powersoil DNA Isolation Kit. Once extracted from the samples, the DNA is stored in a freezer at -20°C for future use.

DNA extraction from control strains was completed for use in DGGE ladders and for the creation of the standard curve for *dsrB*-based quantitative polymerase chain

reaction. The organisms were first grown in 5-10 mL of their respective growth mediums to late-exponential growth phase and then the broth tubes underwent a 13,000 xg centrifugation for 10 minutes with a 5702 Centrifuge (Eppendorf®). Following centrifugation, the supernatant was expelled. The remaining pellet was then resuspended in Powerbead tube buffer, used for the DNA extraction protocol, and the solution was decanted into the Powerbead tubes for completion of the DNA extraction protocol. Once the DNA was extracted, its concentration was taken spectrophotometrically using a Biodrop Duo (Montreal Biotech Inc., Montreal, QC, Canada). The purity of the DNA was collected (A_{260}/A_{280}) as well as the quantity at 260 nm.

Molecular techniques are a necessity in the study of environmental bacterial samples due to the inability to cultivate a significant amount of environmental bacteria *in vitro.* Exact conditions for growth can be difficult to establish and, therefore, molecular techniques are required to establish a true community profile. It should be mentioned that it is difficult to determine what is metabolically active with molecular techniques and that is why it is important to study both phenotypic community aspects (culture-based analysis) and genotypic profiles (molecular-based analysis) (Bochner, 2003).

2.3.1 Colony Polymerase Chain Reaction Confirmation

Polymerase chain reaction confirmation analysis was used initially to determine the presence of SRB species within the samples obtained from Borden and from the column tests in lab. For all polymerase chain reactions, the Bio-Rad iCycler IQ was used. Two universal SRB primer sets (DSR1F and DSR4R/DSRpF2060 and DSR4R) were also used.

Both primer sets select for a highly conserved, ubiquitous enzyme within SRB phylogeny, dissimilatory sulfite reductase (DSR). The gene is known as *dsrAB*. DSR1F and DSR4R primer set selects for the entire ~1.9 kb gene whereas the DSRpF2060 and DSR4R primer set selects a more specific part of the gene that encodes the dsrB sub-unit of the enzyme. The running parameters and materials for this procedure are outlined in Giloteaux *et al.* (2010) for DSR1F and DSR4R and Geets *et al.* (2006) for DSRpF2060 and DSR4R. The Geets *et al.* (2006) protocol is described below under qPCR. The Giloteaux protocol uses 1x PCR buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 1.5 mM $MgCl_2$, 0.2 µM each primer, 2.5 U of Go-Taq Flexi DNA polymerase (Promega), and 5 µL DNA template. The thermal cycling was as follows: initial denaturation step of 94^{O}C for 5 min, followed by 35 cycles of denaturation at 94^{O}C for 45 secs, annealing at 55^{O}C for 45 secs, elongation at 72^{O}C for 1:30 min followed by a final elongation step at 72^{O}C for 10 mins. In addition to these protocols, it has been noted in a study completed by Wagner *et al*. (1998) that bovine serum albumin (BSA) may also be an important additive to the PCR master-mix to prevent unwanted enzyme binding and/or to increase enzyme stability throughout each cycle of the protocol. A colony PCR was also performed to see if methanogens were present in the samples using a protocol developed by Steinberg *et al.* (2008). However, no band appeared on the agarose gel and without a pure strain (due to limited growth restrictions; requires a different atmospheric conditions (100% CO_2) than what was present in the anaerobic chamber), a successful PCR run could not be confirmed.

Following each protocol, PCR product was run on a 1.8% agarose gel, with a 100 bp ladder, at 100 mV for ~45 mins. Following the run time, the gels were stained in ethidium bromide and imaged to determine that the correct amplicon was acquired.

2.3.2 Real-time Quantitative Polymerase Chain Reaction

The use of qPCR provided a highly sensitive approach for the amplification of genes of interest (e.g. *dsrB* gene fragments). As such, this technique can help determine the presence and abundance of SRBs in environmental samples (Santegoeds *et al.*, 1998; Kondo *et al.*, 2004; Beller *et al.*, 2008). Since qPCR allows for the detection of a bacterial group (e.g. species) based on a specific gene, this technique will also aid in satisfying objective 1. A limitation to qPCR is that it can portray an overestimation of viable gene copies in a sample as the technique amplifies all target sequences, even if the sequence does not come from viable cells (Taskin *et al*., 2011).

More specifically, as the process goes through amplification cycles, concentrations of the amplicon rise, which proportionally increases the amount of fluorescence produced. During the exponential growth phase, fluorescence is detected and analyzed by the qPCR apparatus to distinguish the amount of gene expression that was present within the sample. To determine relative gene expression in the original sample, the amplicons are referenced to a standard dilution curve of 10^{0}-10^{-7} to reveal the gene copy numbers in each reaction well. Results are depicted as a 'cycle number versus the fluorescence generated' graph (refer to Figure 2.1). The plot also has a fluorescence threshold above the background levels during the amplification cycle`s linear phase. The starting concentrations of the

template DNA in each sample is known as the cycle threshold (C_t). C_t is reached when the detected fluorescence exceeds the determined threshold value. A melt curve was also run initially to ensure no non-specific binding was occurring with the primers selected.

The reaction that occurs is similar to that of the standard PCR previously described. The exception is that fluorescent probes/dyes are integrated into the technique so that only the template DNA that contains the gene of interest sequence is detected. For this particular study, the SsoFast EvaGreen Supermix protocol from Biorad® was implemented. This protocol used a dye (very similar to SYBR Green) that binds to the dsDNA that is amplified during qPCR. Once bound to the dsDNA, the dye fluoresces and produces a signal corresponding to the quantity of target sequence present in each sample; which can be read and quantified via the iQCycler optical system software version 3.1. The protocol used a final reaction volume of 20 µL; 0.4 µM of each primer, 10 µL of EvaGreen Supermix, 1 µL template and the remaining volume was Milli-Q (Millipore) water. The thermal cycling protocol was modified from Geets *et al.* (2006) with the addition of an enzyme activation step (98^{O}C for 2 mins) and the removal of the final elongation step. The previously mentioned SRB primers for PCR confirmation, DSRpF2060 and DSR4R, were used with this protocol as this primer set showed promising/consistent results with the qPCR standard curve that was developed as seen in Figure 2.2:

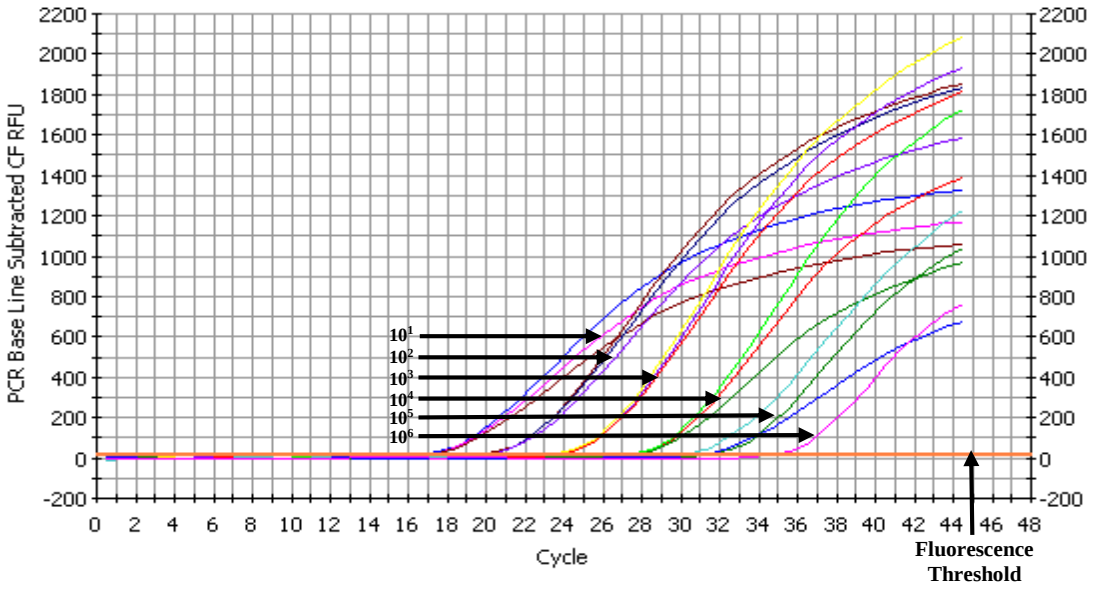

Figure 2.2: qPCR results illustrating the relationship between gene copy numbers/reaction (or DNA amount) and cycle threshold (Ct). *D. meridiei* genomic DNA was serially diluted over 6 orders of magnitude and subjected to SsoFast EvaGreen real-time PCR to generate a standard curve for sample comparison.

A melt curve was run between 65-95^{O}C to ensure non-specific binding of the primers was not occurring. Once the standard curve was run and the quantity of DNA was known with respect to *D. meridiei* genomic DNA that was extracted, the following equation was used to calculate gene copies/reaction:

$$\text{Gene copies/reaction:} \quad \frac{6.02 \times 10^{23} \times [\text{DNA}]\ \text{g/µL} \times 1\ \text{µL}}{(\text{size genome in bp})(660\ \text{g/mol})(\#\ \text{of copies in genome})}$$

Where: (Ritalahti *et al*. 2006; Equation 2.2)

- 6.02×10^{23} is Avogadro's number
- [DNA] is the concentration of DNA isolated from pure strain
- 1 µL is the template added to each real-time PCR reaction
- 660 g/mol is the single base pair weight (in mol) of double-stranded DNA

Following the creation of the standard curve, samples were run against it to determine gene copies in the unknown samples. A qPCR protocol developed by Beller *et al*. (2008) was also performed on a gene sequence, bssA, which is specific to SRB. bssA encodes for the enzyme benzyl-succinate synthase (BSS), which is involved in the anaerobic degradation of toluene and xylene. Unfortunately, when the protocol was run with the samples from the bioreactors there were multiple peaks in the melt curve analysis. This indicated that the PCR reaction had primer dimer formation and/or unspecific binding of the primers to non-target DNA sequences. The need for a higher purity primer set or changes to the protocol, as thermal cycling can have slight variations between instruments used, may help alleviate this problem. An example of the melt curve analysis and amplification cycle of bioreactor 2 is provided in the Appendix section (Figure A4).

Table 2.1: The target genes and primers explored in this study for colony PCR and qPCR

Target Gene	Primers	5'-3' sequence	Amplicon size (bp)	Reference
dsrAB (PCR)	DSR1F DSR4R	AC[C/G]CACTGGAAGCACG GTGTAGCAGTTACCGCA	~1.9 kb	Wagner *et al*., 1998
dsrB (qPCR)	DSRp2060F DSR4R	CAACATCGTYCAYACCCAGGG GTGTAGCAGTTACCGCA	~350 bp	Geets *et al*.,2006
bssA (qPCR)	SRBF SRBR	GTSCCCATGATGCGCAGC CGACATTGAACTGCACGTGRTCG	~97 bp	Beller *et al*., 2008

2.3.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a molecular fingerprinting technique that enables an individual to determine the integrity of or changes in a microbial community's structure by analyzing gene fragments (Muyzer *et al.*, 1993; Santegoeds *et al.*, 1998; Devereux *et al.*, 1997). This method can reveal an abundance of information with respect to objective 3 by identifying fluctuations in the microbial community structure within the bioreactors; genetic diversity and richness of the general microbial community and sulfate-reducing bacteria being among the most important aspects that was better elucidated.

Both an SRB-based and universal-based DGGE analysis was completed using DGGE. Extracted DNA from bioreactor effluent samples was amplified using the SRB primers previously described (DSRpF2060 and DSR4R). It was also of interest to complete an analysis using the universal primers (e.g. 357F-GC and 518-R) to see the effect of

persulfate on the entire community throughout the remediation process. A constructed DGGE ladder was a requirement for this procedure in order to have a reference for the different fragments of the PCR products and for normalization of a gel. The ladder created consisted of the following eleven organisms: *Flavobacterium* spp. (ATCC® 51823), *Aeromonas hydrophilia* (ATCC® 49140), *Bacillus cereus* (Ward's Science Plus), *Alcaligenes faecalis* (ATCC® 33950), *Bacillus megaterium* (ATCC® 10778), *Pseudomonas aeruginosa* (Ward's Science Plus), *Streptomyces griseus* (ATCC® 10137), *Nitrosomonas europaea* (ATCC® 25978), *Pseudomonas chlororaphis* (ATCC® 13985), *Desulfotomaculum nigrificans* (ATCC® 19998), and *Clostridium perfringens* (NCTC® 8237). The SRB-based DGGE had the addition of two extra SRB species in the ladder: *Desulfovibrio vulgaris* (ATCC® 29579) and *Desulfosporoinus meridiei* (ATCC® BAA-275).

Prior to running the gels, sample DNA was amplified using universal and SRB degenerate primers for universal DGGE and SRB-based DGGE, respectively. Universal DGGE used 50 µL reactions whereas SRB DGGE used 100 µL reactions. Universal DGGE required primer 357f (5'-CCTACGGGAGGCAGCAG-3), which has a GC-clamp attached to the 5' end, and 518r (5'-ATTACCGCGGCTGCTGG- 3') (Sigma Aldrich; Oakville, ON, Canada) whereas SRB-based DGGE uses the *dsrB* primers described in Table 2.1, but with the addition of a GC-clamp as well. The universal primer set anneals to the V3 region of 16S rDNA in bacterial genomes (Ogino *et al*., 2001). The PCR reaction contained 1 x Go-Taq™ Flexi buffer, 1.5 µM MgCl, 0.5 µM of forward and reverse primers, 200 µM dNTPs, 1.5 U Go-Taq™ flexi and 21.3 µL of Milli-Q (Millipore) water. 5 µL of template

(sample DNA) was used in each reaction. A touchdown PCR was used to meet the conditions suggested by Muyzer *et al*. (1993). For the PCR protocol there was an initial denaturation step of 94^{O}C for 5 mins, then 20 cycles of 94^{O}C, 65^{O}C, and 72^{O}C for 1 min. This was followed by a decreasing annealing temperature (every 2 cycles) starting at 65^{O}C and declining by 1^{O}C to 56^{O}C by the 20th cycle. The touchdown protocol was completed by 10 cycles of 94^{O}C, 55^{O}C, and 72^{O}C for 1 min each. A final extension step was included for 7 min at 72^{O}C. PCR products were then run on a 1.8% agarose gel and stained with ethidium bromide to ensure the proper gene segment was amplified. Once confirmed, the final PCR product(s) was then used for DGGE analysis.

With respect to the SRB-based DGGE protocol, a procedure developed by Geets *et al*. (2006) was followed with slight modifications to the annealing temperature. PCR reactions contained a total volume of 100 µL. Each PCR mixture contained 1 µL template, 20 µL reaction buffer, 100 µM of dNTPs, 2.5 U of Go-Taq Flexi DNA polymerase and 1 µM of each primer. The cycling protocol was as follows: an initial denaturation step of 94^{O}C for 4 min, followed by 40 cycles of denaturation at 94^{O}C for 1 min, annealing at 53.8^{O}C for 1 min, elongation at 72^{O}C for 1 min followed by a final elongation step at 72^{O}C for 10 mins. PCR products were then run on a 1.8% agarose gel and stained with ethidium bromide to confirm proper amplicon size.

DGGE provided a molecular fingerprint approach that revealed denaturing profiles of same-sized DNA fragments (PCR products). These profiles are based on the separation of those fragments on a polyacrylamide gel due to differences in nucleic acid (more

specifically G+C content) composition. The DNA is drawn through the gel via linear electrophoresis. The separation occurs as the PCR product moves through a gel that has an increasing concentration of chemical denaturant, urea/formamide. Once the DNA fragment has reached its threshold denaturant concentration on the gel, the DNA unwinds and halts its movement through the gel. Once running time is complete, the gel is stained with SYBR gold stain and imaged using a transilluminator. Imaging reveals banding pattern profiles of the microbial community. Each band is representative of a particular microbial population/OTU within an environmental sample. OTUs are described in more detail in the introduction section. The banding profiles can be compared, typically within the same gel for consistency, to determine any dynamic changes within the microbial community over time and space. As a result, this technique can be very useful in determining changes in a microbial community throughout a remediation process that uses a strong oxidant such as persulfate.

Once the PCR products and ladder were loaded onto the denaturing gel, the protocol was run using a CBS apparatus system in accordance with the procedure outlined in Green *et al.* (2009). The denaturing gels can contain different gradients depending on the size of fragment being run. Universal and SRB DGGE both used 8% acrylamide gels, however, universal DGGE required a 40-65% denaturing gradient which was run for 17 hours whereas SRB-based DGGE call for a 40-70% denaturing gradient and a 14 hour run time. Once the gels were stained for 1-2 hours in SYBR Gold and imaged, the banding pattern profiles were analyzed using a GelCompar II software system.

A band excision technique was also completed in an attempt to reveal the phylogeny of dominant operational taxonomic units (OTU) present in samples pre- and post-oxidation. OTUs are typically groups of genera/species or closely related groups of bacteria that are represented as bands on a DGGE gel (Wayne *et al*., 1987; Bowman *et al*., 2006; Flynn *et al.*, 2013). Bands were excised from gels using a UV 'Mighty Bright' Table (Hoefer Scientific Instruments, Inc.). Following excision, the excised bands were purified using a gel extraction clean-up kit (Omega bio-tech) and re-amplified to ensure a good product yield. The PCR products were then sent to the University of Guelph for sequencing. Unfortunately, the results came back containing mixed/contaminated sequence and, therefore, could not be compared to gene banks to determine homologies (refer to Appendix Figure A5). The next step would be to re-run DGGE gels following the re-amplification of excised bands, excise the band again and repeat multiple times to ensure pure product isolation before sending for sequencing (Priha, 2013).

2.4 Chemical Analyses

Sulfate, methane, hydrogen sulfide, ORP, pH and BTEX were the main physicochemical properties examined throughout the central phase of the experiment (treatment phase). For sulfate analysis a 2.0 mL sample was removed and added to a plastic 5mL Dionex IC autosampler vial. The sample was then positioned on a Dionex AS-40 autosampler. 25 µL of sample was then injected onto a Dionex ICS-2000 Ion Chromatograph which was equipped with an Ion-Eluent Generator and conductivity detector. A Dionex IonPac AS18 column (4x250mm) was used. The mobile phase used was 30mM KOH (flow rate: 1.0 mL/min). The chromatograph was acquired using Dionex

Chromeleon 6.5 software (Institute for Groundwater Research, University of Waterloo, ON, Canada). The detection limit was 0.5 (mg/L). Lactate concentrations were also acquired via this protocol.

With respect to the methane analysis protocol, provided by Institute for Groundwater Research, University of Waterloo, ON, Canada, the following steps were taken: a headspace was created in the aqueous samples with a ratio of 2.5 mL headspace to 2.5 mL aqueous sample. Samples were then positioned on a shaker for 15 mins, allowing equilibration between gas and water phases. For the analysis, a 250 µL sample was injected onto a Hewlett Packard 5790 gas chromatograph (GC) equipped with a flame ionization detector (FID) using an GS-Q plot capillary column. The GC had an initial temperature of 60^{O}C, which was held for 3 mins; the temperature was then increased at a rate of 15^{O}C/min reaching a final temperature of 120^{O}C and then was maintained at 125^{O}C for 10 mins. The detector was set for 280^{O}C and the injector temperature was 200^{O}C. The carrier gas was ultra pure N_2 (purity: 5.0) with a flow rate of 20 mL/min. The detection limit for methane was 0.9 µg/L.

H_2S analysis was completed using the Hach methylene blue method (method 8131) for dissolved H_2S. A spectrophotometer was used for the analysis. The detection limit for this method were 5-800 µg/L. If the sample concentrations were higher than 800 µg/L, dilutions to samples were done. With respect to pH and ORP, a pH/conditioning probe was used for the measurements in the effluent samples (Orion^{TM}, Thermo Scientific).

For the analysis of BTEX, samples were extracted using methylene Chloride (DCM) from water at a 2mL to 20mL DCM:Water ratio. Samples were then placed on a rotary shaker for 15 mins to allow equilibration between the organic phase and water phase. For analysis a 1µL sample is then injected onto a Hewlett Packard 7890 series II gas chromatograph (GC) equipped with a FID detector the uses a J&W DB-5MS column. (30mm x 0.30mm). The GC had an initial temperature of 50^{O}C, with a temperature program of 15^{O}C/min reaching a final temperature of 150^{O}C. This was then held at 150^{O}C for 7 mins. The detector is set for 300^{O}C and the injector temperature is 220^{O}C. The carrier gas was ultra pure helium with a flow rate of 7 mL/min.

The culture-based, molecular-based and chemical analysis techniques used for the bench-scale analysis helped provide a more complete examination of the SRB community and the manner in which the community was altered in response to the oxidant exposure.

Chapter 3: Results

3.1 The Creation and Development of Anaerobic Bioreactors

In order to test the effects of unactivated and alkaline-activated persulfate on indigenous anaerobic bacteria from subsurface sediments, a bench-scale setup using 1L bioreactors was developed to simulate what could occur in the field. To produce comparable results between the lab- and field-scale settings, core material was extracted from a freshwater aquifer as a source of indigenous organisms for inoculation of the bioreactors. The following section includes results from the pre-contaminant injection (development) phase of the bioreactors. The 'history' section presented is less intensively analyzed than the treatment portion of the experiment that follows this section. The treatment phase represents the overarching objective of this thesis: the impact of unactivated/alkaline-activated persulfate treatments on an indigenous sulfate-reducing bacterial community.

3.1.1Colony-Polymerase Chain Reaction for SRB confirmation at Borden and in the Bioreactors

The following gel illustrates a colony PCR that was completed to reveal where SRB may be prevalent within areas of the Borden aquifer. A 1.9 kbp segment of DNA that encodes for the dissimilatory sulfite reductase gene was detected within various areas of the aquifer as depicted in Figure 3.1 below. Areas showing the densest bands were chosen as locations for core extraction. Density of bands does not entirely dictate abundance of viable SRB, as dead cells are included, but can provide suggestive evidence for the

potential of their presence. The primers used do have a partial affinity for binding to the alpha- and beta-subunits of the enzyme and that is the reasoning for the appearance of smaller gene fragments in some samples.

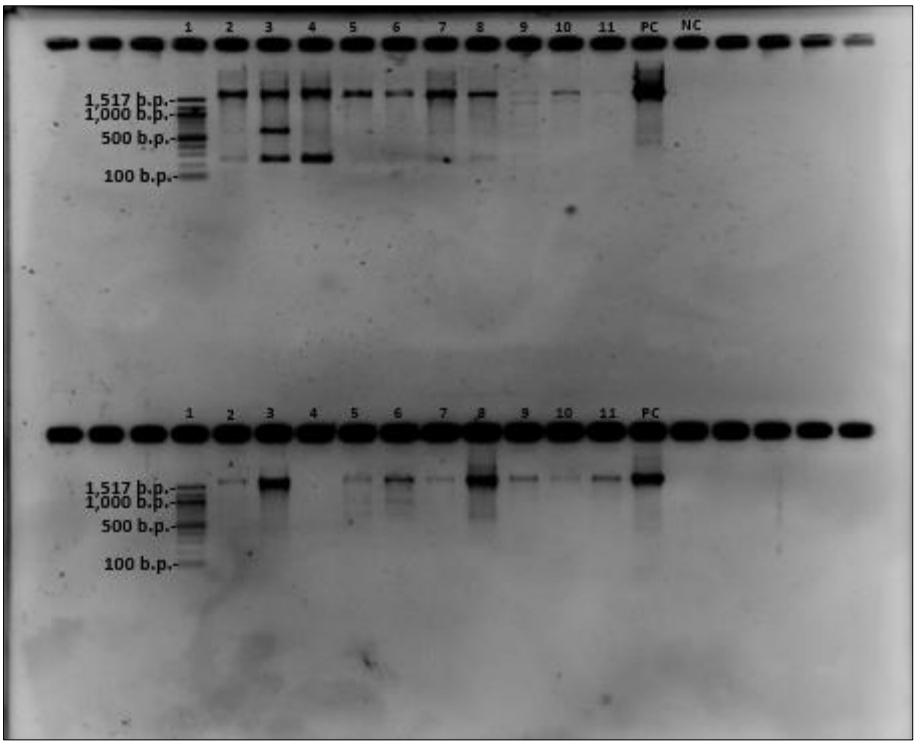

Figure 3.1: Colony-PCR confirmation for ~1.9kb dsrAB gene from the aquifer at Borden. In both rows of lanes, lane 1 contains a 100bp ladder, lanes 2-11 are samples taken from various wells. PC (positive control) is a pure strain of SRB (*D. nigrificans*).

Following core collection, bioreactors were packed and attached to reservoirs that pumped groundwater previously collected from the Borden site into the columns. The following colony-PCR in Figure 3.2 illustrates the successful inoculation of indigenous SRB into bioreactors in the bench-scale setting. The top gel illustrates the presence of SRB in the bioreactors immediately following set-up. The bottom gel displays the continued

presence of SRB one month following bioreactor set-up. The increased density of the ~1.9 kbp band seen in the bottom row potentially indicates an increase in the dsrAB gene one month following bioreactor inoculation.

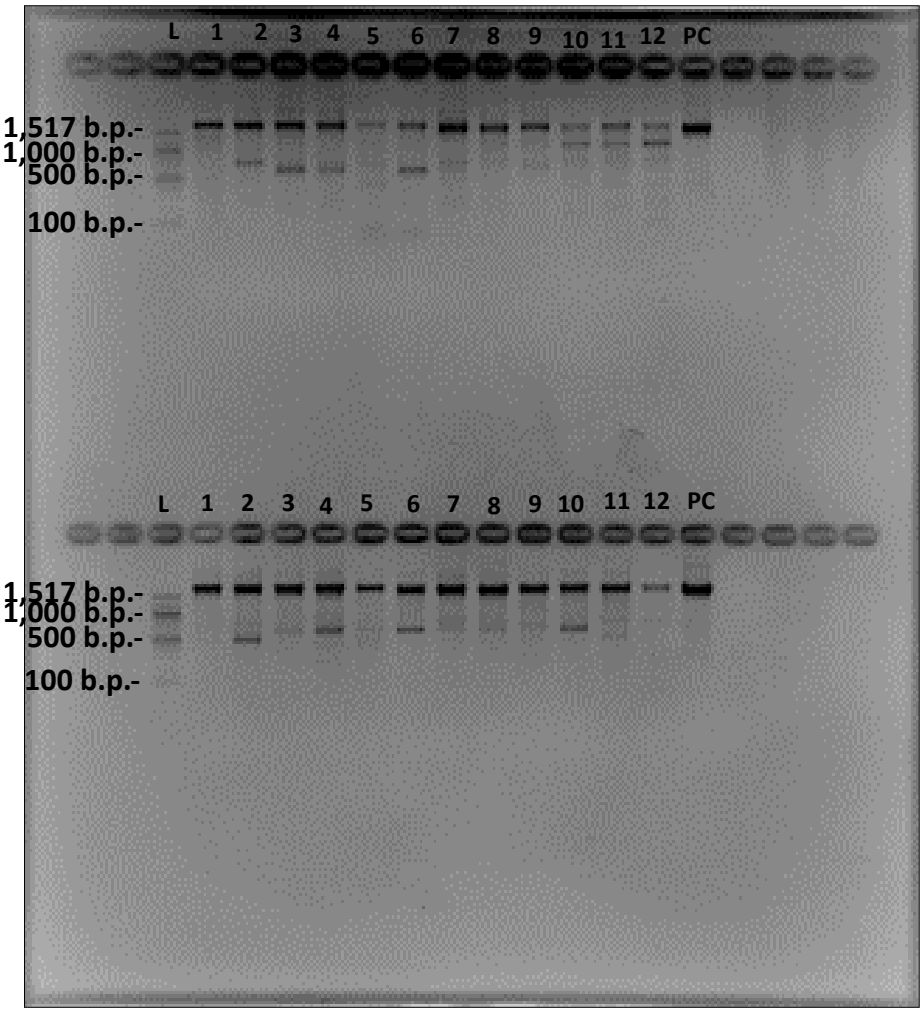

Figure 3.2: Colony-PCR confirmation for the ~1.9kb dsrAB gene in the bioreactors (lanes 1-12) once running. Lane 'L' is a 100bp ladder. PC (positive control) is a pure strain of SRB (*D. nigrificans*).

3.1.2 Structural Fingerprints of Microbial Communities Prior to Treatment Phase

The following section displays the historical progression of the entire microbial community via universal-DGGE prior to the treatment phase of the experiment. The raw images are provided to give a visual depiction of the pattern of community fluctuation from November 2014 (initial set-up) to July 2015 (second injection of BTEX).

Figures 3.3-3.6 display the progression of community structure for all bioreactor triplicate sets (UP1-3, AP1-3, AO1-3, & Con1-3). Two ladders (L; not considered 'lanes') were included in the left and middle lanes for comparison and normalization of the gels. Nine samples were included on each gel to ensure that a depiction of the community structure for each month (from November to July) was obtained in order to monitor any changes. The bioreactors began to exhibit some consistency between samples, with respect to banding profiles, toward the last three sampling events. An initial contaminant injection was completed between February and March (lanes 5 and 6, respectively) which caused a shift in the community to a structure that contained dominant OTUs with a lower G+C content. Once contaminant injection was ceased in March, due to unsaturation development, the community began to return to a more moderately rich G+C dominated structure.

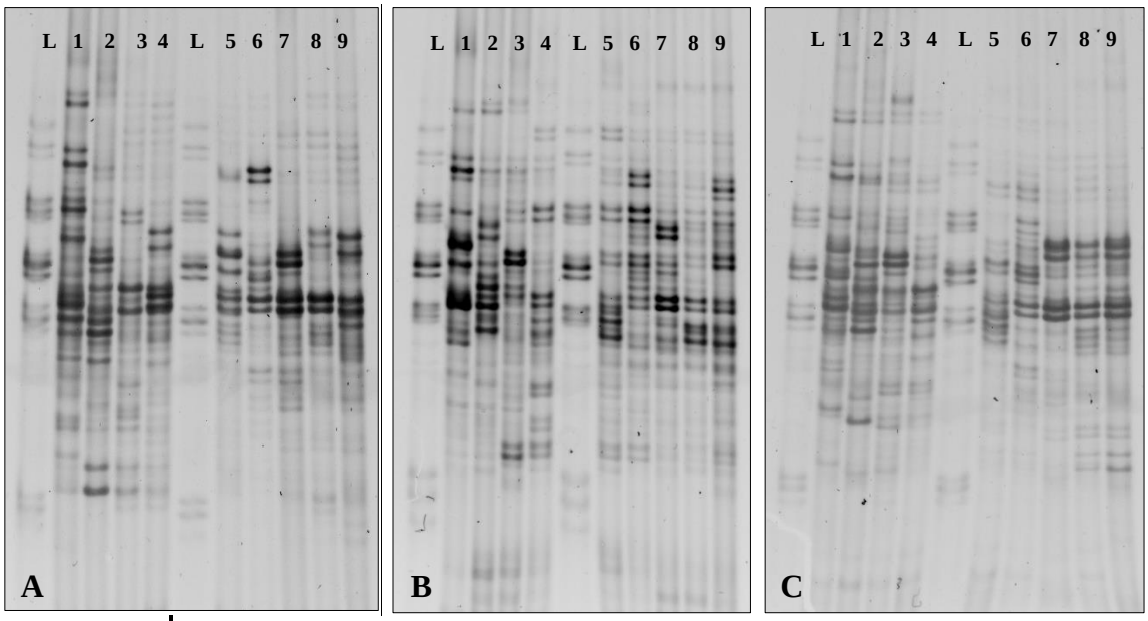

Figure 3.3: Unactivated-Persulfate Bioreactors (UP1-3). Universally-based DGGE analysis for the development of UP1 (A), UP2 (B), and UP3 (C) bioreactors prior to contaminant injection in August. Lanes 1-9 represent samples taken each month from October (2014) through June (2015). Lanes `L` represent the organismal ladder.

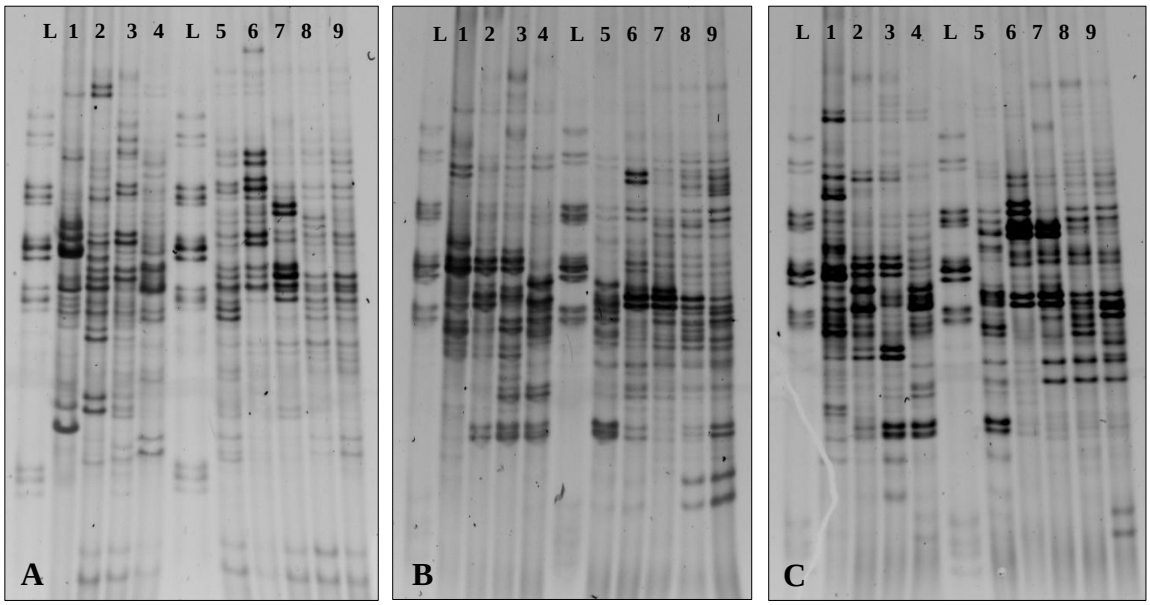

Figure 3.4: Alkaline-activated Persulfate Bioreactors (AP1-3). Universally-based DGGE analysis for the development of AP1 (A), AP2 (B), and AP3 (C) bioreactors prior to contaminant injection in August. Lanes 1-9 represent samples taken each month from October (2014) through June (2015). Lanes `L` represent the organismal ladder.

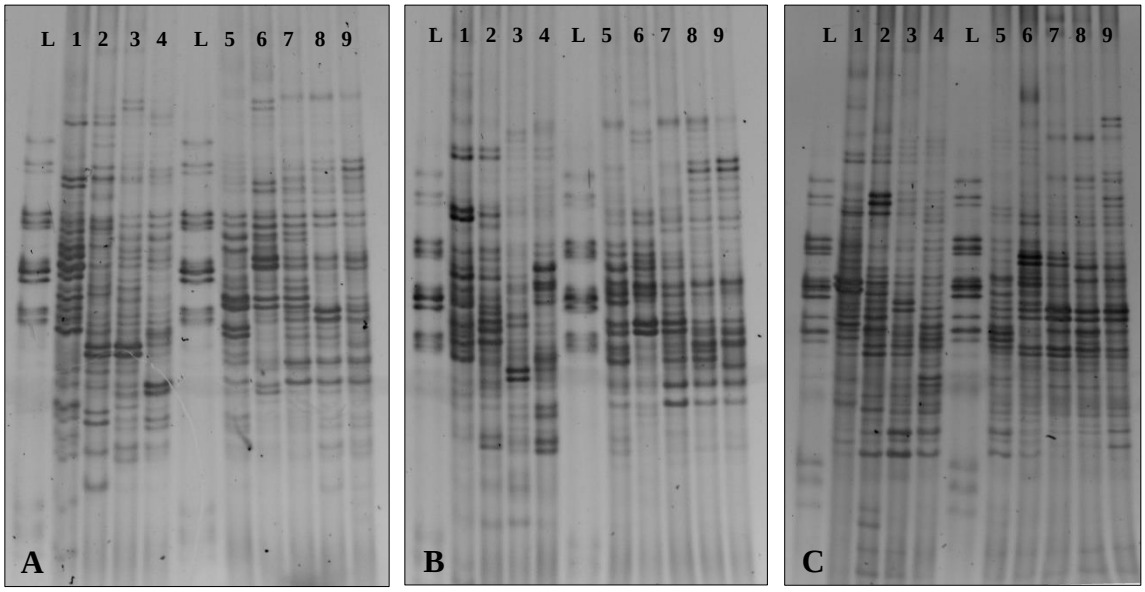

Figure 3.5: Alkaline-Only Bioreactors (AO1-3). Universally-based DGGE analysis for the development of AO1 (A), AO2 (B), and AO3 (C) bioreactors prior to contaminant injection in August. Lanes 1-9 represent samples taken each month from October (2014) through June (2015). Lanes `L` represent the organismal ladder.

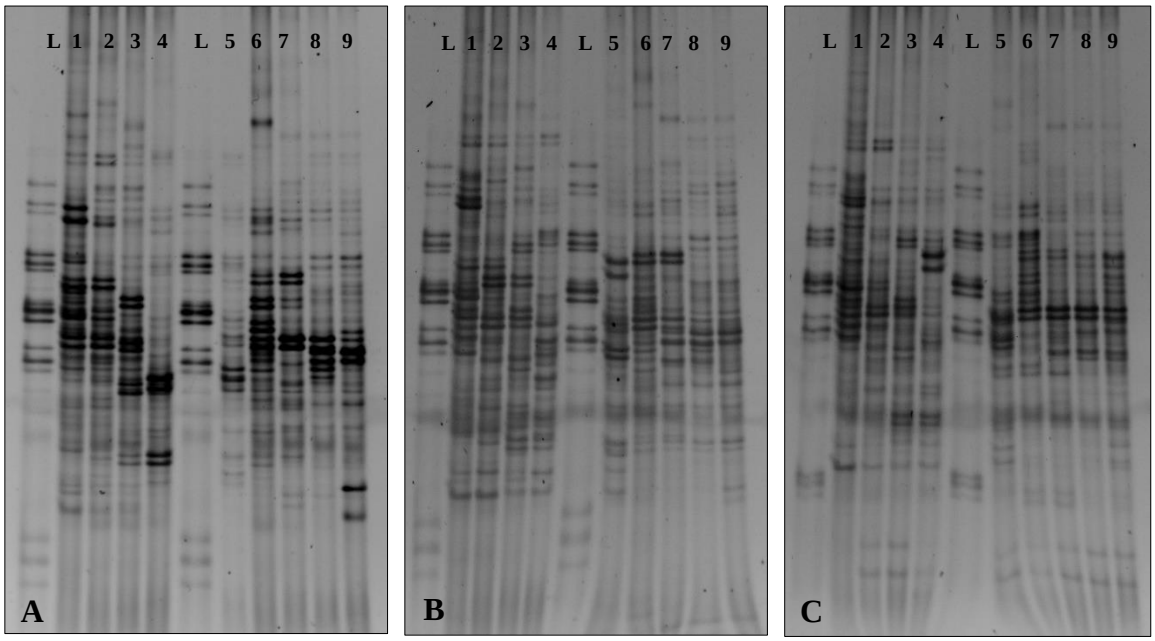

Figure 3.6: Control Bioreactors (SRB-only). Universally-based DGGE analysis for the development of Con1 (A), Con2 (B), and Con3 (C) bioreactors prior to contaminant injection in August. Lanes 1-9 represent samples taken each month from October (2014) through June (2015). Lanes `L` represent the organismal ladder.

3.1.3 Structural Fingerprints of the Sulfate-reducing Consortia in the Bioreactors Prior to Treatment Phase

The following section displays the historical progression of the sulfate-reducing bacterial portion of the community via *dsrB*-based DGGE prior to the treatment phase of the experiment. The raw images in Figures 3.7 and 3.8 are provided to give a visual depiction of the community fluctuation from November 2014 (initial set-up) to July 2015 (injection of BTEX).

Unlike the universal-DGGE gels previously displayed, each SRB-based gel represents two bioreactors. Therefore, each month is not represented. Samples from November, December, February, March, May, and July are represented for each bioreactor. It is difficult to discern any particular trends, most notably between lanes 4-5 and 11-12 of each gel respectively, which contain samples prior to and after the initial contaminant injection. Some OTUs are consistently present within the SRB community throughout the development of each bioreactor.

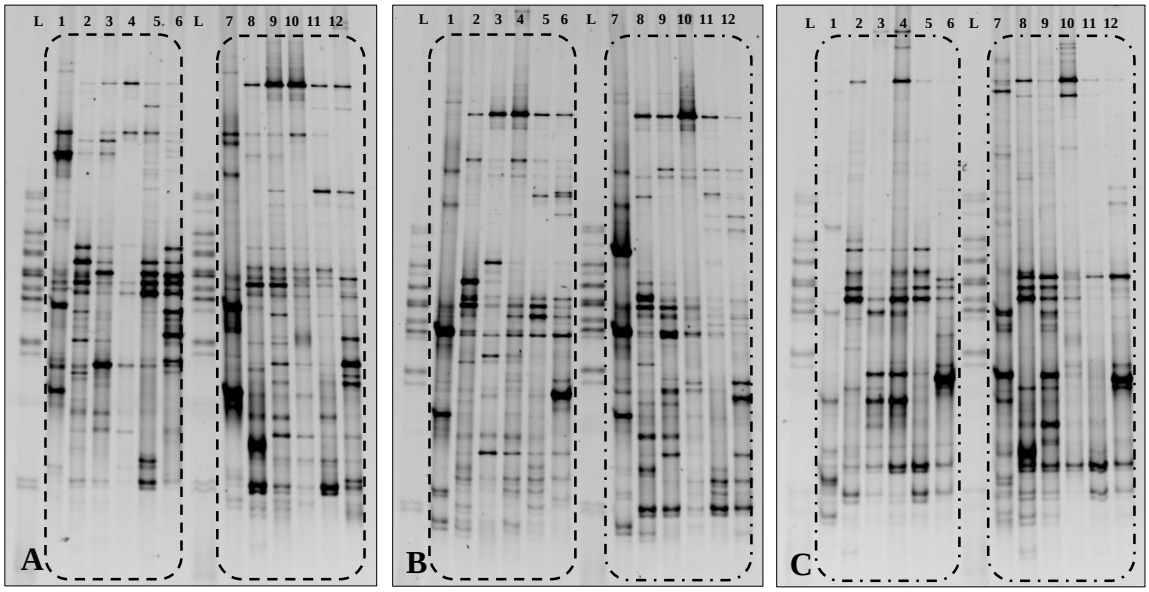

Figure 3.7: SRB-based DGGE analysis for the development of UP1-2 (A), UP3/AP1 (B), and AP2-3 (C) prior to contaminant injection in August. Lanes 1-6 ('UP' reactors) and 7-12 ('AP' reactors) represent samples from November, December, February, March, May, and July for the respective bioreactor sets.

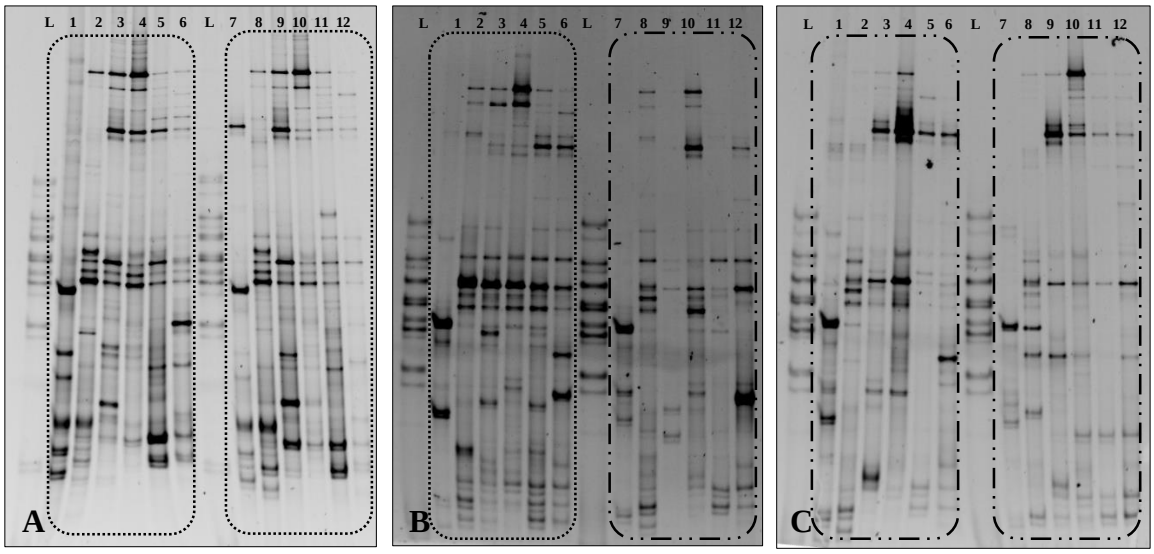

Figure 3.8: SRB-based DGGE analysis for the development of AO1-2 (A), AO1/Con1 (B), and Con2-3 (C) prior to contaminant injection in August. Lanes 1-6 ('AO' reactors) and 7-12 ('Con' reactors) represent samples from November, December, February, March, May, and July for the respective bioreactor sets.

3.1.4 Supporting Parameters

In order to promote SRB growth and development, lactate amendment was included. Sulfate and lactate were plotted to show the relationship between lactate consumption and sulfate reduction. For the majority of the experiment, sulfate concentration in the reservoirs was ~ 130 mg/L. Conversely, lactate concentration (~ 80 mg/L) in the reservoirs were kept below sulfate concentrations to ensure SRB development over potentially competing microbes. Lactate amendment was reduced by 20 mg/L per week over a one-month span, starting July 6th, until the complete exclusion of lactate by mid-August, approximately one-month prior to the treatment phase. During lactate amendment, lactate was being completely consumed while sulfate was being reduced by up to 74%. As lactate was excluded, sulfate reduction began to decrease. By mid-August, ~10 mg/L of sulfate was consumed in the bioreactors on average without the addition of lactate.

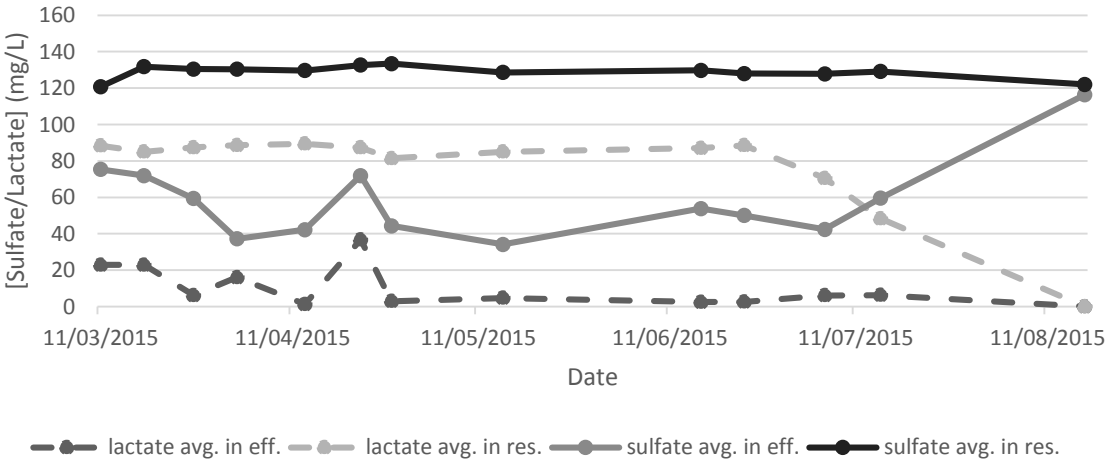

Figure 3.9: The average [sulfate] and [lactate] present in the reservoirs (res.) and bioreactor effluents (eff.) during the SRB bioaugmentation portion of the experiment.

Figure 3.10 displays the BTEX concentrations present in the bioreactor sets throughout July and August. The contaminant exposure phase began on June 30th, 85 days prior to the 'treatment' phase. BTEX degradation gradually increased until August, when up to 70% of the BTEX was being consumed. A plateau was then reached followed by a slight decrease in BTEX reduction. Once the plateau was reached, it became apparent that the experiment could move towards the treatment phase.

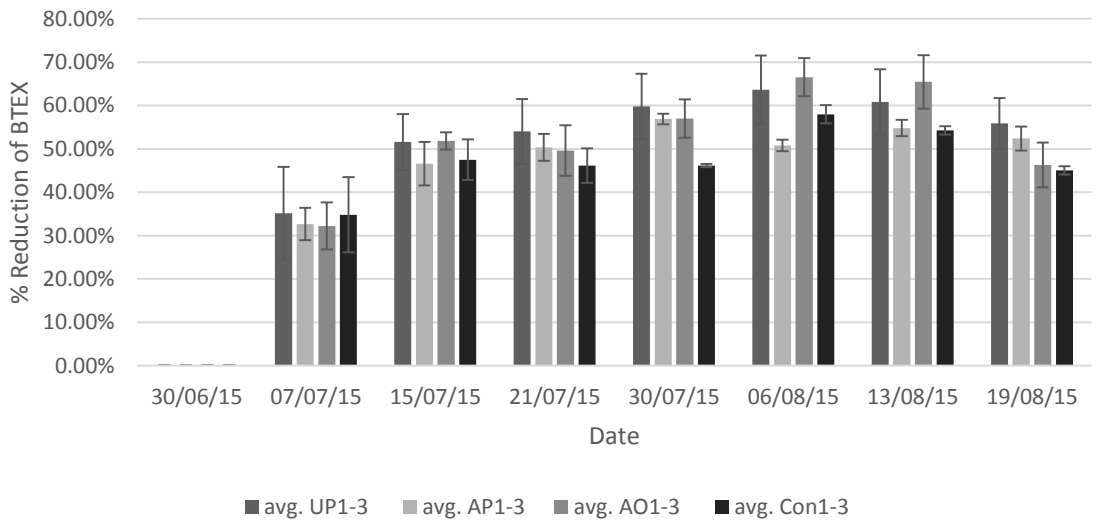

Figure 3.10: Average percent reduction in BTEX throughout July and August in the unactivated persulfate (UP1-3), alkaline-activated persulfate (AP1-3), alkaline-only (AO1-3) and control (Con1-3) bioreactors.

Finally, the last indicator used to determine when to begin the persulfate treatment was a return in the columns' weights following an 'unsaturation' event during the first

contaminant injection at the end of Febuary. The illustration below depicts the bioreactor weights from Febuary to August (2015). Weights were monitored every week following the unsaturation event. The peak (not noted on the graph), around the end of March, seen in columns AO1-3 is due to a leak in the reservoir that allowed for partially oxygenated air into the columns, presumably causing an initial increase in biomass of microaerophilic bacteria. The reservoir was replaced and the columns returned to a more consistent weight trend within a few days. Overall, by mid-June the weights returned to the approximate weights of the columns prior to the initial contaminant injection at the beginning of March that caused the weights to decline (see Figure 3.11). Foil was added to the columns at the beginning of May which caused a slight increase in weight as well. AO1 was heavier than the rest of the bioreactors because it had a metal clamp attached to it to ensure a tight seal.

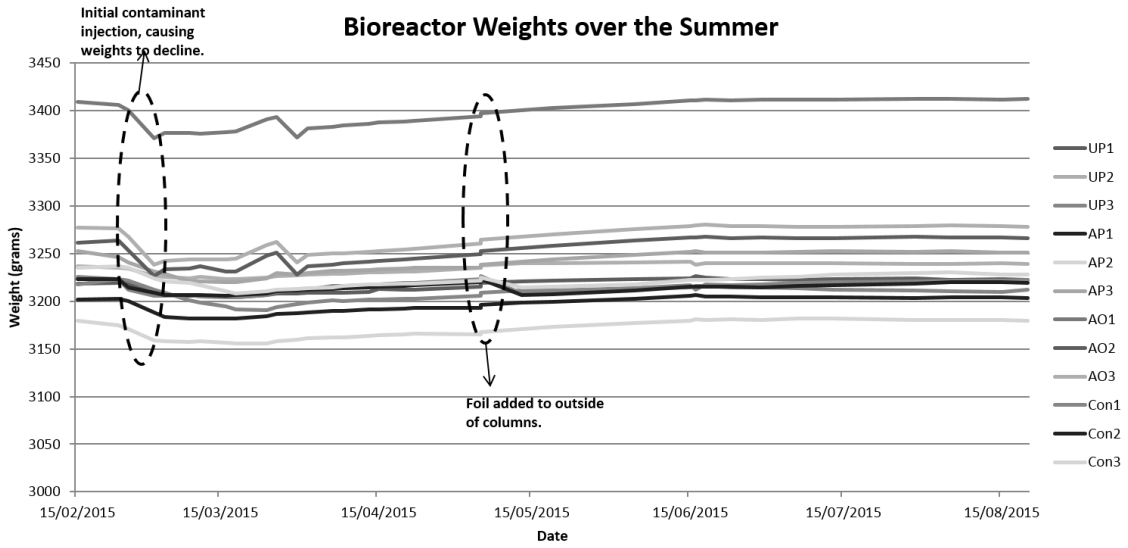

Figure 3.11: The bioreactor weights, in grams, throughout the spring and summer months (Febuary (baseline) to August).

3.2 Treatment Phase

The following section presents all of the results, including chemical and microbial results completed during the treatment phase of the experiment. The main range of analysis took place from mid-September to the end of November, 2015. The 'treatment episode' for unactivated persulfate, alkaline-activated persulfate, and alkalinity occurred for a 24-hour period between September 23rd-24th, 2015. The treatment episode/exposure period is indicated by a thick, dashed line on the figures and tables. The control replicates had an increased flow rate during the treatment exposure phase and, thus, their respective plots include an indicator as well.

3.2.1 Culture-Based Results:

3.2.1.1 Colony-forming-units per millilitre (CFU/mL) observed in all bioreactors

The plate count method was performed to determine the total abundance of anaerobic bacteria in all bioreactors during the exposure trial. A total of five sampling events took place within a two a month period (September 18th, 2015 → November 22, 2015). The September 18th samples were taken prior to the persulfate/alkaline treatments that took place September 23rd-24th. The remaining four sampling episodes took place following the treatment episode.

Unactivated Persulfate Bioreactors (UP1-UP3)

Similar plate count trends were observed throughout all replicates in the unactivated persulfate treatment. The total anaerobic bacterial loads prior to the 24-hour persulfate treatment were determined to be in the range of 10^3-10^4 CFU/mL, with UP3 having the

highest counts (20 CFU/mL) and UP1 having the lowest (10 CFU/mL). Immediately following the treatment exposure counts decreased to approximately 10^1, with UP2 being the highest and UP3 the lowest (refer to Figure 3.12). The subsequent sampling event on October 21st saw an increase in CFU/mL to a magnitude ranging from 10^5 to 10^6 CFU/mL. A similar number of culturable cells were maintained throughout the remaining sampling events, which reflects a stable microbial load.

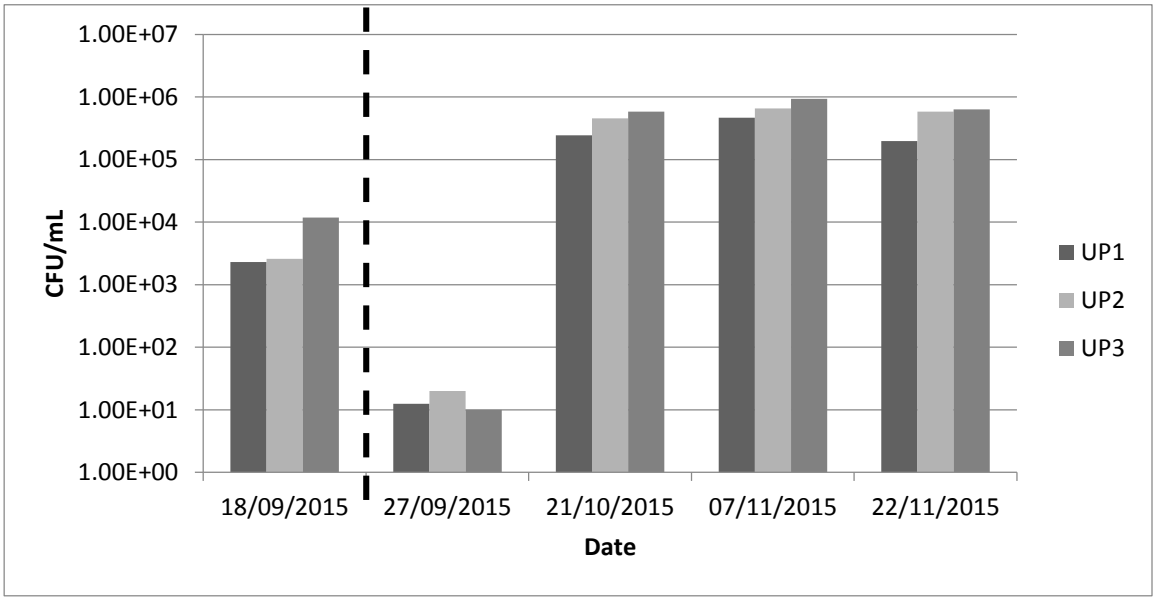

Figure 3.12: Colony-forming-units per mL (CFU/mL) observed in the unactivated persulfate column replicates. The dashed line represents the treatment episode. UP1, UP2, and UP3 are replicates.

Alkaline-activated Persulfate Bioreactors (AP1-AP3)

Similar CFU/mL trends were observed throughout all replicates in the alkaline-activated persulfate treatment columns (Figure 3.13). The total anaerobic bacterial loads prior to the 24-hour persulfate treatment were determined to be in the range of 10^3-10^4,

with AP1 having the highest counts ($1.27x10^4$ CFU/mL) and AP2 having the lowest ($3.53x10^3$ CFU/mL). Immediately following the treatment, counts were lower, ranging from $10^{2.5}$ to as low as 10^1, with AP2 being the highest and AP3 the lowest. The subsequent sampling event on October 21st saw an increase in CFU/mL to a magnitude ranging from 10^5 to 10^6. As in the unactivated persulfate columns, a similar range was maintained throughout the remaining sampling events.

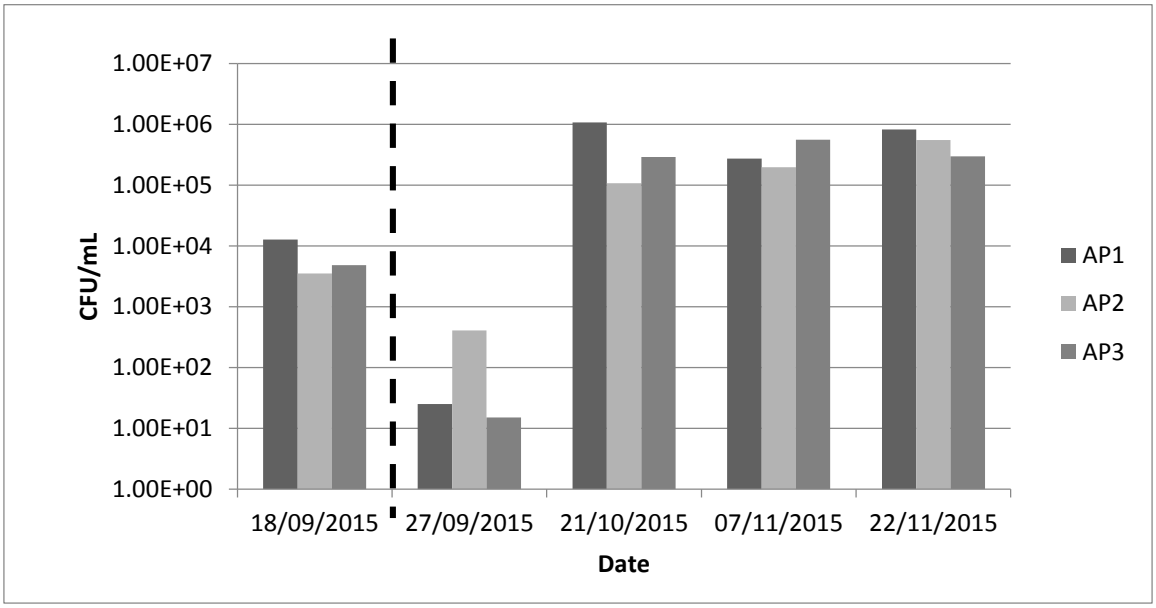

Figure 3.13: Colony-forming-units per mL (CFU/mL) observed in the alkaline-activated persulfate column replicates. The dashed line represents the treatment episode. AP1, AP2, and AP3 are replicates.

Alkaline-only Bioreactors (AO1-AO3)

The total bacterial loads prior to the 24-hour alkaline treatment (pH 12) were determined to be in the range of 10^3-10^4 CFU/mL, with AO1 containing the highest counts (8.15×10^3 CFU/mL) and AO2 having the lowest (2.3×10^3 CFU/mL) (refer to Figure

3.14). Immediately following the alkaline treatment, AO1 experienced the largest decrease (~ 2 log reduction), followed by AO3 then AO2 (~ 1 log reduction). The subsequent sampling showed an increase in CFU/mL in all replicates to a magnitude just below 10^5 CFU/mL. A similar range was maintained throughout the remaining sampling events in all replicates, with a slight increase in CFU/mL seen in AO1 at approximately a month and a half (Nov. 7th) after the 24-hour pH 12 solution exposure.

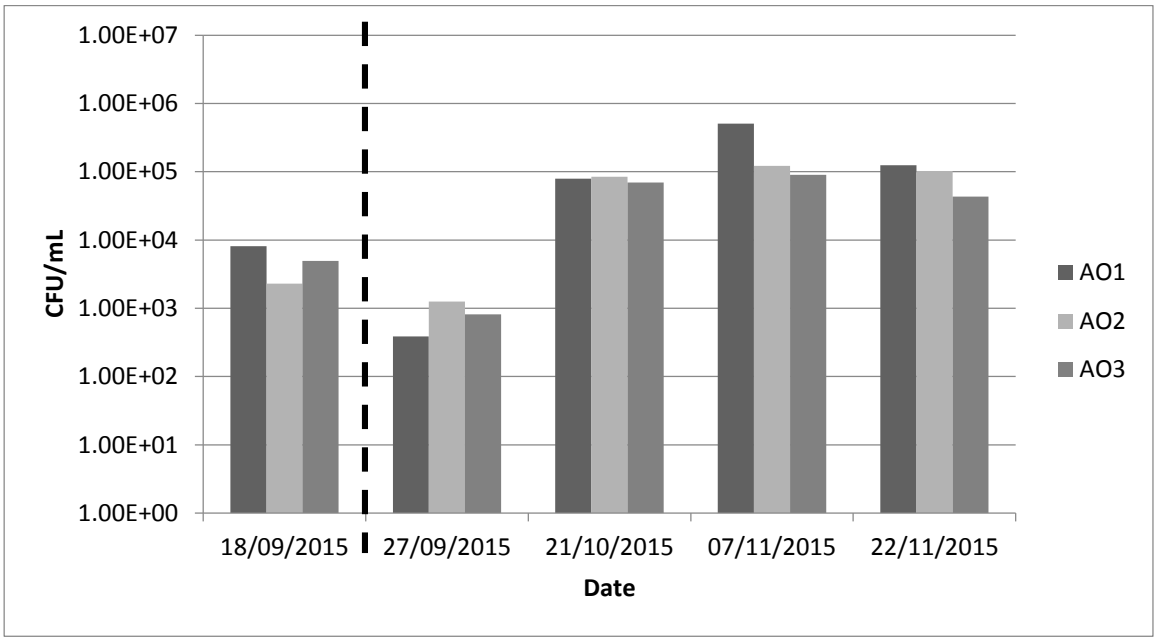

Figure 3.14: Colony-forming-units per mL (CFU/mL) observed in the alkaline-only bioreactor replicates. The dashed line represents the treatment episode. AO1, AO2, and AO3 are replicates for the pH 12 exposure.

Control Bioreactors (Con1-Con3)

The CFU/mL observed in the control bioreactors (Con1-Con3) did not demonstrate any reductions in microbial load. By this method, the general trend displayed a slow increase in CFU/mL between sampling events in all replicates. Con2 and Con3 showed a

plateau between October 21st and November 7th (~ 10^5 CFU/mL) whereas Con1 continued to increase to 3.83 x 10^5 CFU/mL in microbial load throughout the final sampling events.

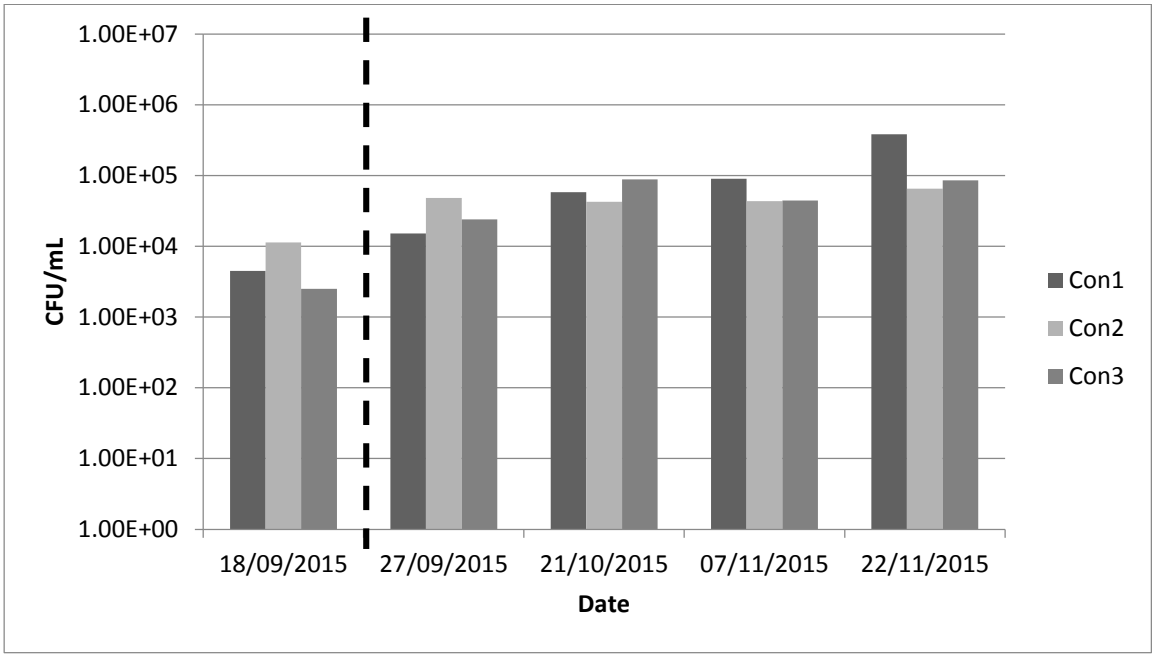

Figure 3.15: Colony-forming-units per mL (CFU/mL) observed in the control bioreactor replicates. The dashed line represents the treatment episode. Con1, Con2, and Con3 are replicates.

Treatment comparison:

Figure 3.16 for CFU/mL represents the log average of each set of triplicates for each treatment (UP1-3, AP1-3, & AO1-3) and control (Con1-3) plotted against each other. UP1-3 showed almost a 3 log reduction (~10^4 → ~10^1 CFU/mL) in the second sampling event and then experienced substantial increase (almost to 10^6 CFU/mL) in the next sampling episode. Between 10^5-10^6 CFU/mL was maintained throughout the final three sampling events. AP1-3 also experienced a reduction (~10^4 → ~10^2 CFU/mL) in the second sampling event and then experienced substantial increase (almost to 10^6 CFU/mL) in the

next sampling episode. Between 10^5-10^6 CFU/mL was maintained throughout the final three sampling events. AO1-3 also experienced a reduction (~10^4 → ~10^2 CFU/mL) in the second sampling event and then experienced substantial increase (almost to 10^6 CFU/mL) in the next sampling episode. Between 10^5-10^6 CFU/mL was maintained throughout the final three sampling events. With respect to the control replicates, a slight increase was seen in CFU/mL between each sampling event, beginning at just below 10^4 CFU/mL and finishing just above a CFU/mL of 10^5 CFU/mL.

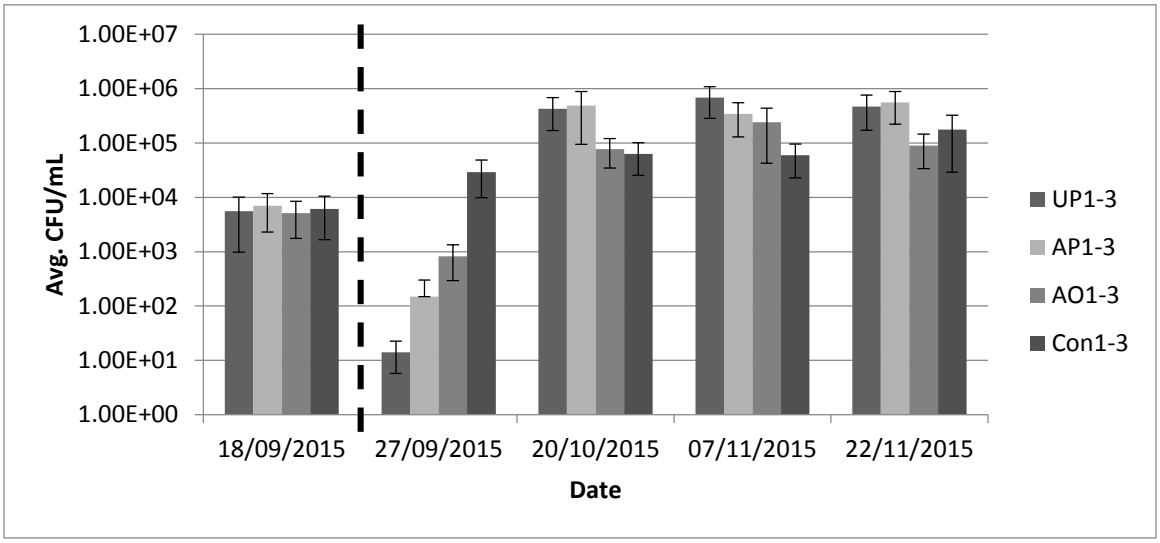

Figure 3.16: Average colony-forming-units per mL (CFU/mL) observed for each treatment, pre-injection (18/09/2015) and post-injection (Sept. 27th → Nov. 22nd) samples. UP1-3, AP1-3, AO1-3, and Con1-3 represent all replicates for respective treatments. The dashed line represents the treatment episode.

3.2.1.2 Analysis of the Most-Probable-Number method for Sulfate-reducing Bacteria

As previously mentioned, the MPN method for SRB provides insight into the amount of SRB cells present per millilitre of bulk phase liquid (effluent). Table 3.1 below shows the MPN analysis for all treatment replicate samples taken before and after the treatment episode. All replicates began with below 100 cells per mL. The general trend

noticed in UP1-3 was that there was an initial decrease in viable SRB in the bioreactors. However, the following sampling events saw an increase to levels 1-2 logs higher than pre-treatment counts, with UP1 experiencing the largest increase. Treatment columns AP1-3 showed a similar trend, with an initial decrease in counts throughout all replicates and then a subsequent increase in bacterial numbers in the following sample, with AP1 displaying the largest increase. AP2 did not show as much of a decrease in numbers immediately following treatment. The alkaline only reactors (AO1-3) also saw an initial decrease in the number of culturable SRB. However, they too experienced a rebound in numbers following the first post-treatment sample, with AO1 experiencing the largest increase. The control bioreactors displayed fluctuations throughout the monitoring process. Con2 and Con3 displayed an increase in SRB approximately one-month post-treatment, with Con3 returning to pre-treatment counts in the following sampling event. Con2 continued to show a slight increase, to 430 cells per mL, in SRB counts until the two-month sampling period, when a slight decline, to 93 cell per mL, was observed.

Table 3.1: Most-Probable-Number (MPN) analysis observed for each treatment, pre-injection (18/09/2015) and post-injection (Sept. 27th → Nov. 22nd) samples.

MPN #	18/09/2015	95% confidence intervals	27/09/2015	95% confidence intervals 2	20/10/2015	95% confidence intervals 3	07/11/2015	95% confidence intervals 4	22/11/2015	95% confidence intervals 5
UP1	46	7.1	0	0	4300	700	15,000	3,000	93,000	15,000
		240		0		21,000		44,000		380,000
UP2	46	7.1	0	0	430	70	930	150	23,000	4,000
		240		0		2,100		3,800		120,000
UP3	28	10	0	0	4300	700	430	70	93,000	15,000
		150		0		21,000		2,100		380,000
AP1	64	15	0	0	4300	700	15,000	3,000	43,000	7,000
		380		0		21,000		44,000		210,000
AP2	24	3.6	10	1.5	2300	400	4,300	700	430,000	70,000
		130		38		12,000		13,000		2,100,000
AP3	43	7	1	0.1	930	150	4,300	700	43,000	7,000
		210		2.3		3,800		13,000		210,000
AO1	64	15	1	0.3	120000	30,000	2,300	400	21,000	3,500
		380		3.6		380,000		12,000		47,000
AO2	64	15	2	0.3	930	150	750	140	23,000	4,000
		380		3.7		3,800		2,300		120,000
AO3	75	14	1	0.1	2300	400	2,300	400	4,300	700
		230		3.6		12,000		12,000		21,000
Con1	46	7.1	46	7.1	43	7	21	4	9,300	15,000
		240		240		210		47		38,000
Con2	46	7.1	46	7.1	150	30	430	70	93	15
		240		240		440		2,100		380
Con3	15	3	15	3	230	40	43	7	43,000	7,000
		44		44		1,200		210		210,000

Legend: UP1, UP2, UP3= unactivated persulfate bioreactors; AP1, AP2, AP3= alkaline-activated persulfate bioreactors; AO1, AO2, AO3= Alkaline-only treatment bioreactors; Con1, Con2, Con3= Control bioreactors. The numbers shown are representative of SRB cells per millilitre.

3.2.2 Molecular-Analysis

3.2.2.1 Quantitative Detection of Changes in Sulfate-reducing Bacterial Loads using Quantitative Real-time PCR (qPCR)

Through the use of real-time qPCR, a conserved gene within sulfate-reducing bacteria was successfully enumerated to detect changes in the SRB population following the treatment episode. The degenerate primers used allowed for the detection of a part of the dissimilatory sulfite reductase gene, *dsrB*. See Table 2.1 for the primer set used for this portion of the experiment. qPCR was completed on two pre-treatment samples (Sept. 11 & Sept 21) and 5 post-treatment samples taken from Sept 28th-Nov 18th. The standard deviation from the mean is indicated by the error bars present on each graph. All graphs for qPCR are displayed on a log scale.

Unactivated Persulfate Bioreactors (UP1-UP3)

qPCR analysis of the 'UP' replicates displayed initial gene copies per mL in the range of 10^3-10^5 copy numbers/mL, with a log difference between UP1 (highest) and UP3 (lowest) (Refer to Figure 3.17). Immediately following treatment, the bioreactors experienced approximately a 2 log decrease, with copy numbers ranging between 10^1-10^3. UP1 continued to display the highest number and UP3 the lowest, with UP2 ranging in the middle between the two. Two weeks following the first post-treatment sample, all bioreactors displayed an increase in gene copies/mL with comparable values. UP3 exhibited the largest increase, showing approximately a 3 log increase. UP1 displayed the second largest enumeration with almost a 2 log increase. UP2 had the slowest re-establishment, with less than a log increase from September 28th to October 9th. The

subsequent sampling events showed a slight increase (less than a log) in UP1 and UP3, with UP3 showing an observable slight decrease until the last sampling event. UP2 experienced almost a 3 log increase between the October 9th and October 20th, reaching gene copy quantities over 10^5, and then slightly tapering off towards the last sampling event.

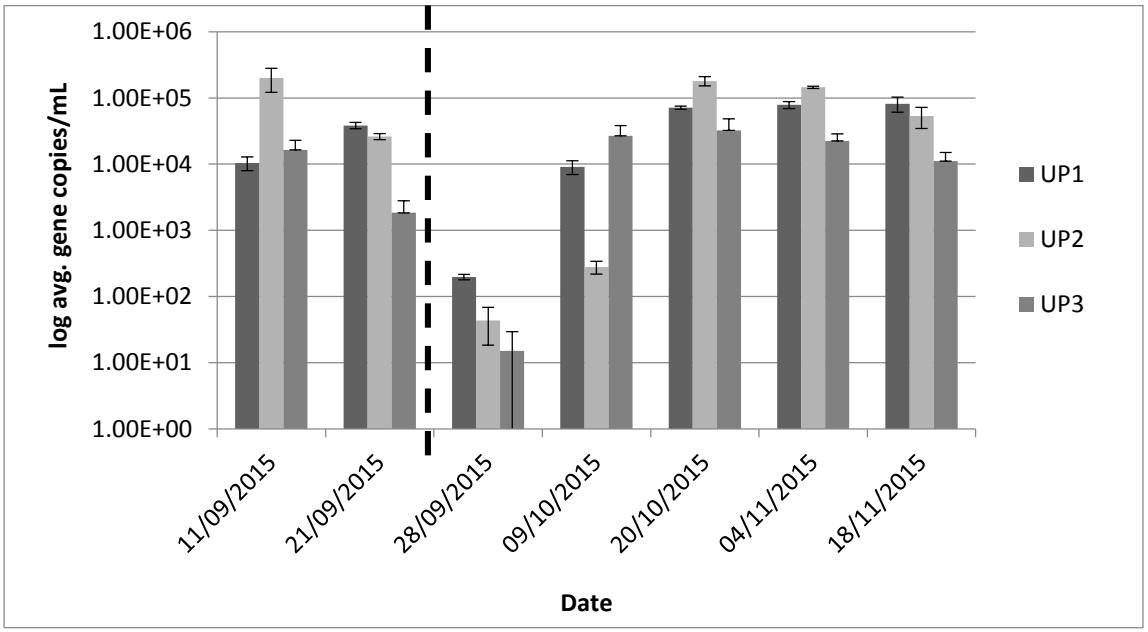

Figure 3.17: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the unactivated persulfate bioreactors throughout the experiment. The dashed line represents the treatment episode.

Alkaline-activated Persulfate Bioreactors (AP1-AP3)

qPCR analysis of the 'AP' replicates displayed initial gene copies per mL in the range between 10^3-10^5 gene copies/mL, with slightly less than a one log difference between AP2 (highest) and AP3 (lowest). Immediately following treatment, the bioreactor replicates experienced somewhat different trends. AP1 experienced the largest reduction, decreasing by nearly a magnitude of 2 logs. AP3 experienced approximately a 1 log

reduction. On the contrary, AP2 did not display any reduction immediately following the treatment. However, two weeks following the first post-treatment sample, AP2 did display a slight decrease in gene copies. With respect to the other replicates, AP3 exhibited a log increase whereas AP1 only showed a slight increase between September 28th and October 9th. One month (Oct. 20th) after the treatment episode, all replicates displayed an increase in gene copy number above pre-treatment levels. AP1 displayed the largest enumeration, increasing by more than 3 logs to between 10^5-10^6. AP2 and AP3 also increased by approximately one log, reaching levels just above 10^5. The following sampling events (Nov. 4th & Nov. 18th) showed slight fluctuations (~1 log or less) in gene copies in all columns, with AP3 experiencing a decreasing trend towards the last sampling event.

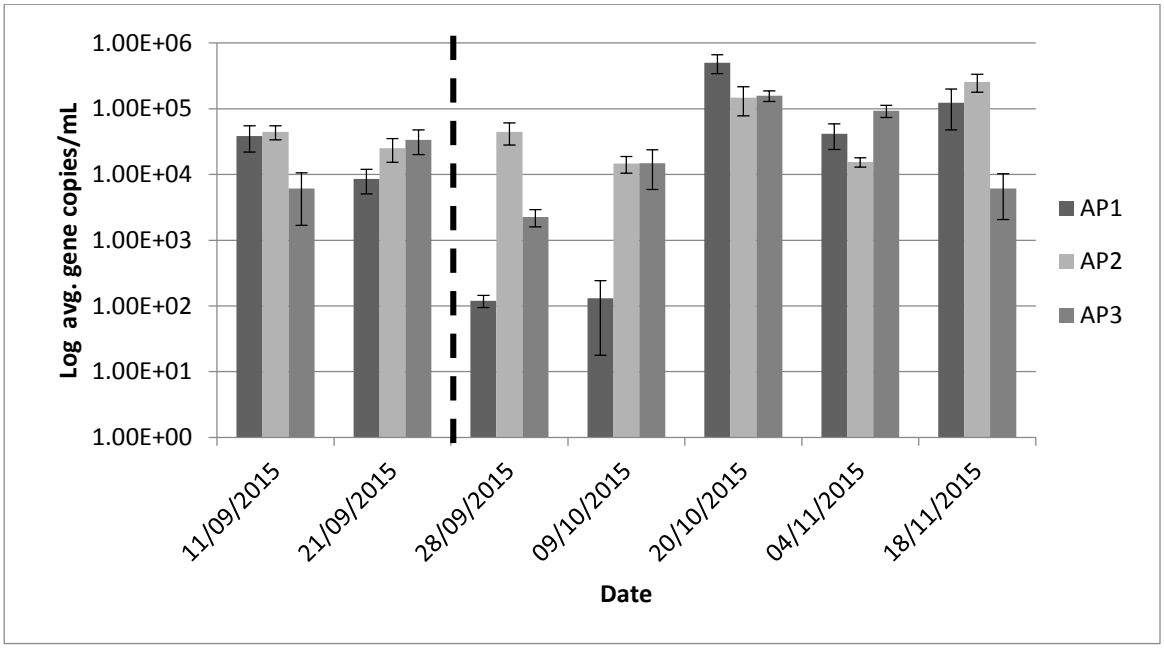

Figure 3.18: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the alkaline-activated persulfate bioreactors throughout the experiment. The dashed line represents the treatment episode.

Alkaline-only Bioreactors (AO1-AO3)

The qPCR results for the alkaline-only bioreactors displayed an interesting overall trend. Two weeks prior to the treatment episode, *dsrB* were at their lowest (between 10^2-10^4 gene copies/mL) in all columns, indicating slight fluctuations in gene copies within the community without treatment. 2 days prior to the treatment, all bioreactors had increased in gene copies numbers to just above 10^4. Immediately following the alkaline treatment, gene copy numbers rose in all bioreactors by approximately 2 logs. Two weeks following the first pre-treatment sampling event, gene copies numbers were reduced to just below 10^4 in AO1 and AO3, whereas AO2 was just above 10^4. One month after the alkalinity exposure, copy numbers rose again, by approximately 1 log in all replicates in comparison to the sampling event prior on Oct. 9th. Following the increase on Oct. 20th, all replicates began to see a general decrease in levels just above (AO2) and below (AO1 & AO3) 10^4 gene copies/mL.

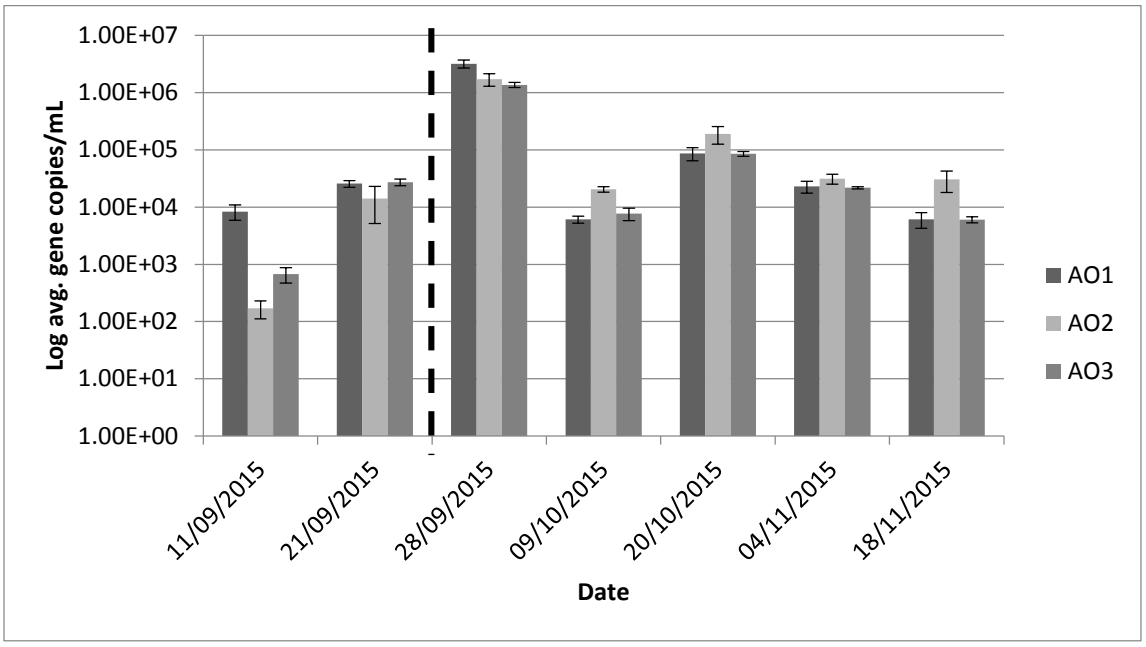

Figure 3.19: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the alkaline-only bioreactors throughout the experiment. The dashed line represents the treatment episode.

Control Bioreactors (Con1-Con3)

For the majority of the experiment, the control replicates maintained a relatively constant state with respect to the amount of *dsrB* gene copies. The bioreactors fluctuated between 10^3-10^5 throughout the sampling events, with the exception of the Sept. 28th and Nov. 11th sampling events, where Con1 and Con2 displayed gene copy numbers slightly above 10^5. Con2 displayed the lowest (Sept. 11) and highest gene copy (Sept. 28th) numbers among the control replicates recorded throughout the experiment.

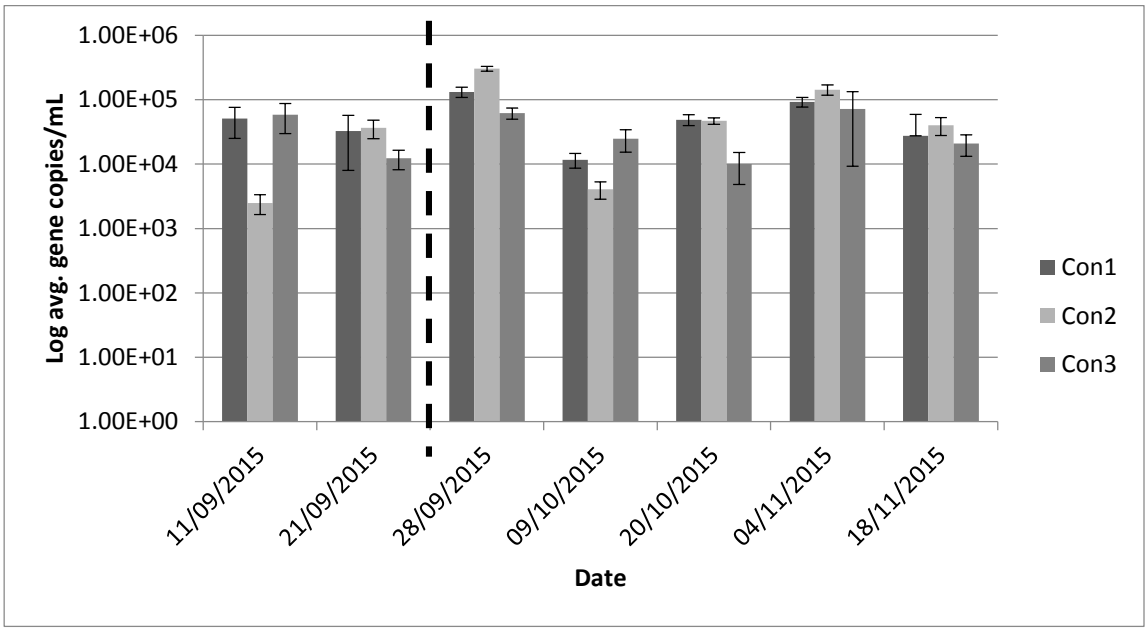

Figure 3.20: Relative abundance (target gene copies/mL) fluctuations of *dsrB* in the control bioreactors throughout the experiment. The dashed line represents the treatment episode.

Treatment comparison:

In order to establish a comparison between treatments, the average *dsrB* copies per mL between replicates in each treatment were calculated to provide insight into differences among treatments (Figure 3.21). The pre-treatment samples display similar gene copy numbers just above 10^4 gene copies/mL. Following the treatment phase, UP1-3 experienced the largest reduction, dropping from above 10^4 to below 10^2 in copy numbers. AP1-3 displayed a slight reduction but still had an average copy number above 10^4 immediately following treatment. However, AO1-3 and Con1-3 experienced an increase in average copy number to above 10^6 and 10^5, respectively. Two weeks following the treatment episode, all bioreactors (for treatment(s) and control replicates) returned to levels

just below that which was present in the pre-treatment samples. One month following the injection phase, all of the `treatment` bioreactors (UP1-3, AP1-3, AO1-3) increased almost one order of magnitude from 10^4 to 10^5 gene copies/mL, whereas the control reactors increased only slightly. The last two sampling events, leading up to two months' post-treatment, displayed mild observable fluctuations between 10^4 and 10^5 gene copies/mL for all treatment and control bioreactors.

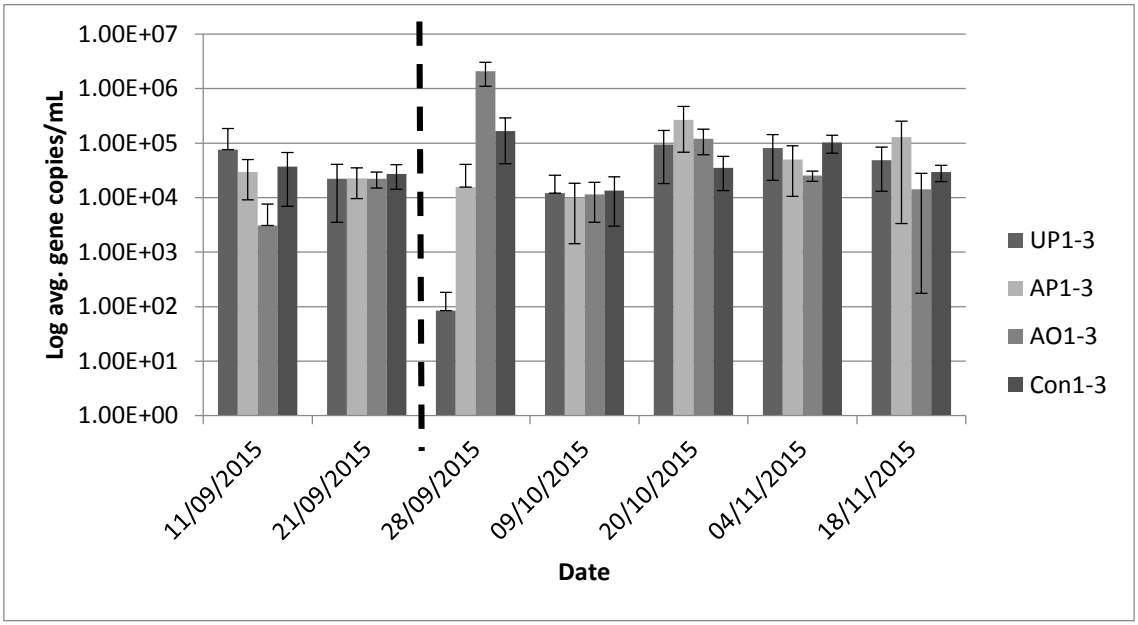

Figure 3.21: Average Relative abundance (target gene copies/mL) fluctuations observed for each treatment, pre-injection (Sept. 11 & Sept. 21) and post-injection (Sept. 27th → Nov. 22nd) samples. UP1-3, AP1-3, AO1-3, and Con1-3 represent all replicates for respective treatments. Standard deviations represent significant changes from the average gene copies/mL. The dashed line represents the treatment episode.

3.2.2.2 Structural Fingerprinting of SRB Communities within Bioreactors

The following section includes the results obtained from the SRB-based DGGE analysis. For each treatment, three types of analyses were completed. This includes: 1.) species diversity, which uses the Shannon index (H') to measure a community's evenness and richness proportionally; 2.) community species richness, which is the amount of bands present in a single lane, 3.) averaged banding pattern profiles, which provides further insight into changes in SRB community structure with respect to dominant OTUs in band movement groups. This type of analysis was completed on two pre-treatment samples (Sept. 11 & Sept 21) and five post-treatment samples taken from Sept 28th-Nov 18th (indicated by black 'boxes').

Unactivated Persulfate Bioreactors (UP1-UP3)

Species diversity in the unactivated persulfate treatment replicates displayed a steep decline following the 24-hour exposure (Figure 3.22). This decline was met with a gradual increase in diversity in the subsequent sampling events, returning to levels similar to the pre-treatment status. Species richness exhibited a similar trend with respect to the initial depletion in the number of bands present in a sample following the exposure period and the respective rebound, reflected in increased band number, seen as the bioreactors recovered. UP2 and UP3 showed recovery beyond the species richness present in the pre-treatment samples whereas UP1 only saw a moderate return in species richness.

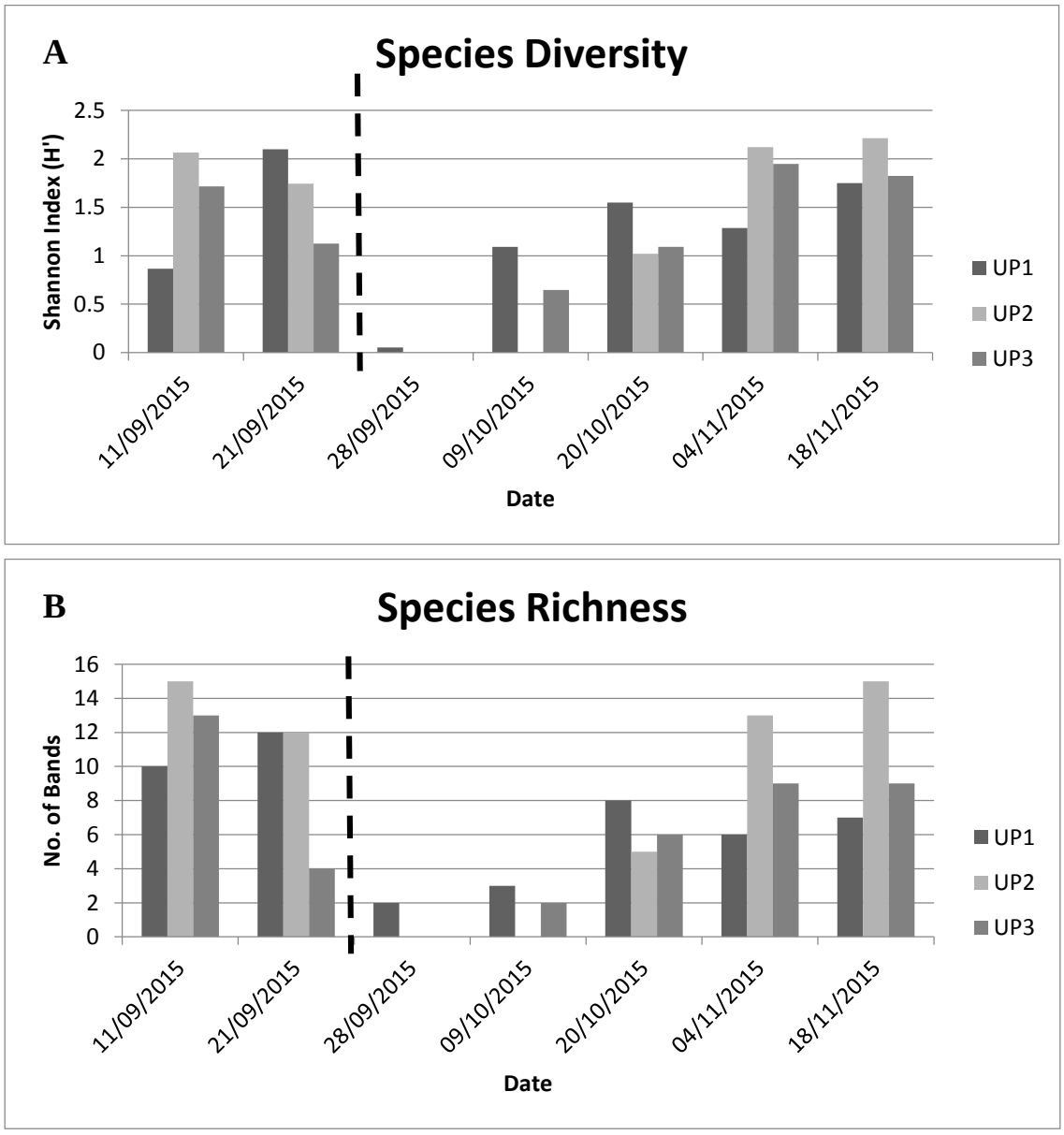

Figure 3.22: SRB DGGE-oriented analysis of the SRB consortia(s) present in the unactivated persulfate bioreactor replicates. Graph A represents the species diversity measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

For the unactivated persulfate treatment, abundance changes in operational taxonomic units (OTUs) across all three replicates can be seen in Figure 3.23. Prior to the treatment phase for replicate UP1, OTUs were distributed throughout the 8th to 17th

movement group. A dominant OTU, moderately rich in G+C, was observed at the 8th movement group, comprising 70% and 25% of the total OTU relative band intensity in the September 11th and 21st pre-treatment samples. That dominant band movement group was maintained in the first two sampling events that took place post-treatment. However, in the October 9th sample a second OTU, rich in G+C content, began to appear between the 16th and 18th band movement group. The following three sampling events displayed a community that began to be dominated by G+C rich OTUs, spanning from the 11th to 18th movement group, with a dominant peak seen at the 16th movement group, comprising 50%-60% of the relative band intensities.

A similar shift to OTUs with higher G+C content was observed following treatment in replicates UP2 and UP3 as well. UP2 was more proportionally distributed with respect to relative band intensity pre-treatment but a dominant OTU was still present (13th movement group) in the bioreactors. UP3 displayed a dominant OTU(s) between the 13th and 14th movement group. Both UP2 and UP3 had no OTUs appear in the first post-treatment sample and nothing appeared for the 2nd post-treatment sample for UP2. However, there was a general shift again to a more G+C rich SRB community in both bioreactors in the following post-treatment samples. These post-treatment samples (i.e. Oct. 20th → Nov. 18th) became more proportionally distributed as more OTUs began to appear.

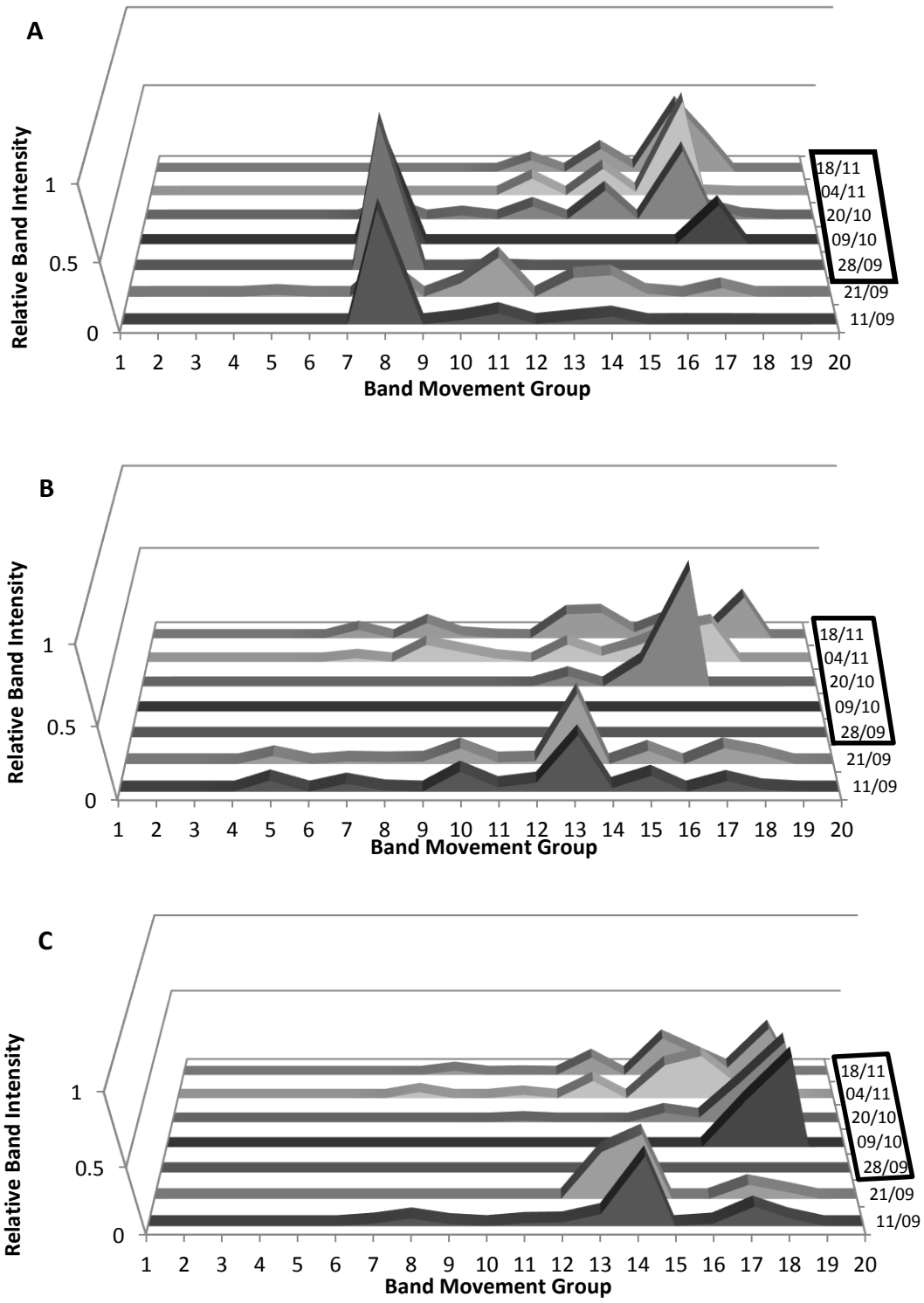

Figure 3.23: SRB DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the unactivated persulfate bioreactor replicates: A) UP1; B) UP2; C) UP3.

Alkaline-activated Persulfate Bioreactors (AP1-AP3)

Prior to the treatment episode in the alkaline-activated persulfate replicates, SRB species diversity and richness was highest in AP2 and lowest in AP1 (Figure 3.24). Following treatment on September 28th, AP1 displayed a steep decline in diversity and richness, whereas AP2 and AP3 only experienced a slight reduction. However, the sampling event on October 9th showed a continued reduction in both diversity and richness in AP2 and AP3, with AP2 showing almost no richness and diversity. At this point, AP1 began to rise in diversity and richness. All replicates began or continued to gradually increase in diversity throughout the last three sampling events. AP1 and AP3 experienced an increase above that of the pre-treatment samples where as AP2 never fully returned to its pre-treatment richness and diversity.

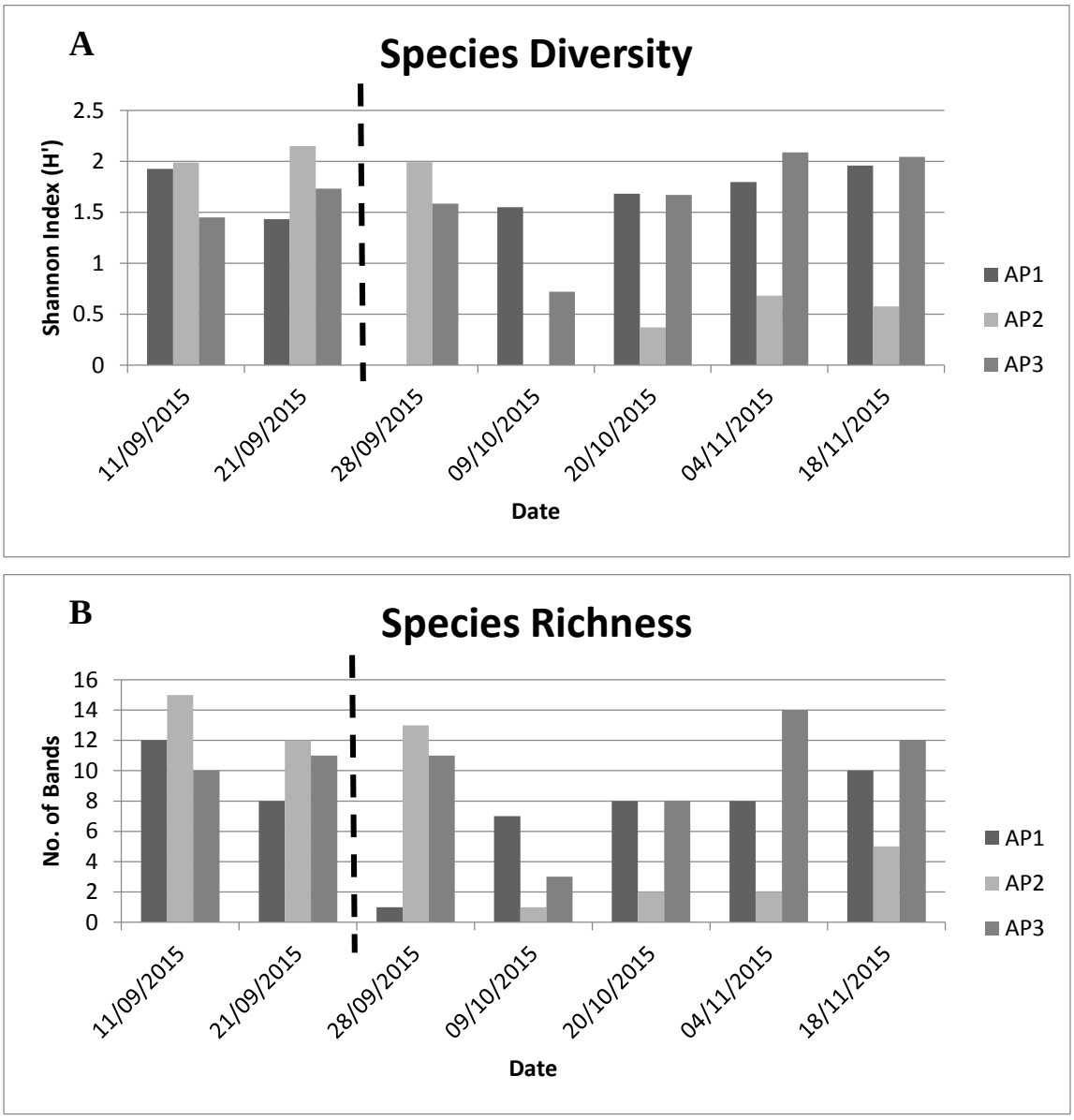

Figure 3.24: SRB DGGE-based analysis of the SRB consortia(s) present in the alkaline-activated persulfate bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

For the alkaline-activated persulfate treatment, abundance changes in OTUs across all three replicates are identified in Figure 3.25. Prior to the treatment phase for replicate

AP1, OTUs were expansively and proportionally distributed throughout the 8th to 17th band movement groups. Dominant OTUs, moderately rich in G+C, were observed at the 8th and 14th movement groups, comprising 35% and 40% (respectively) of the total OTU relative band intensity in the 21st pre-treatment samples. That dominant band movement group was maintained in the first sampling event that took place post-treatment. However, in the October 9th sample there was shift in the banding profile to a more G+C rich community structure between the 13th and 18th band movement groups and a second dominant OTU developed. The following three sampling events continued to show a community that was dominated by G+C rich OTUs, gradually increasing in the amount of band movement groups that include OTUs, indicating an increase in diversity.

A similar shift in OTUs with higher G+C content was observed, following the treatment episode, in replicate AP2. Pre-treatment, AP2 was more proportionally distributed than the other replicates (AP1 and AP3) with respect to relative band intensity, with bands appearing from the 5th to the 18th movement groups. The band movement span was decreased significantly in the post-treatment samples. The span was reduced to include the band movement groups 11 to 18 with the dominant OTU(s) profiles becoming more rich in G+C content. A dominant OTU was observed at the 17th band movement group with an additional OTU displaying partial dominance in the November 4th sample. These post-treatment samples (i.e. Oct. 20th → Nov. 18th) became slightly more proportionally distributed as more OTUs began to appear.

In replicate AP3, a shift in the community banding profile and inherent structure was not as noticeable. Band presence fluctuated throughout the 5th to 17th band movement groups. In most samples, pre-treatment and post-treatment, a dominant OTU was observed at the 14th band movement group. A slight shift was seen in the post-treatment samples to include G+C rich OTUs that are more proportionally equivalent to other OTUs in a respective sample. Samples taken on the 4th and 18th of November displayed a more proportional distribution with respect to relative band intensities.

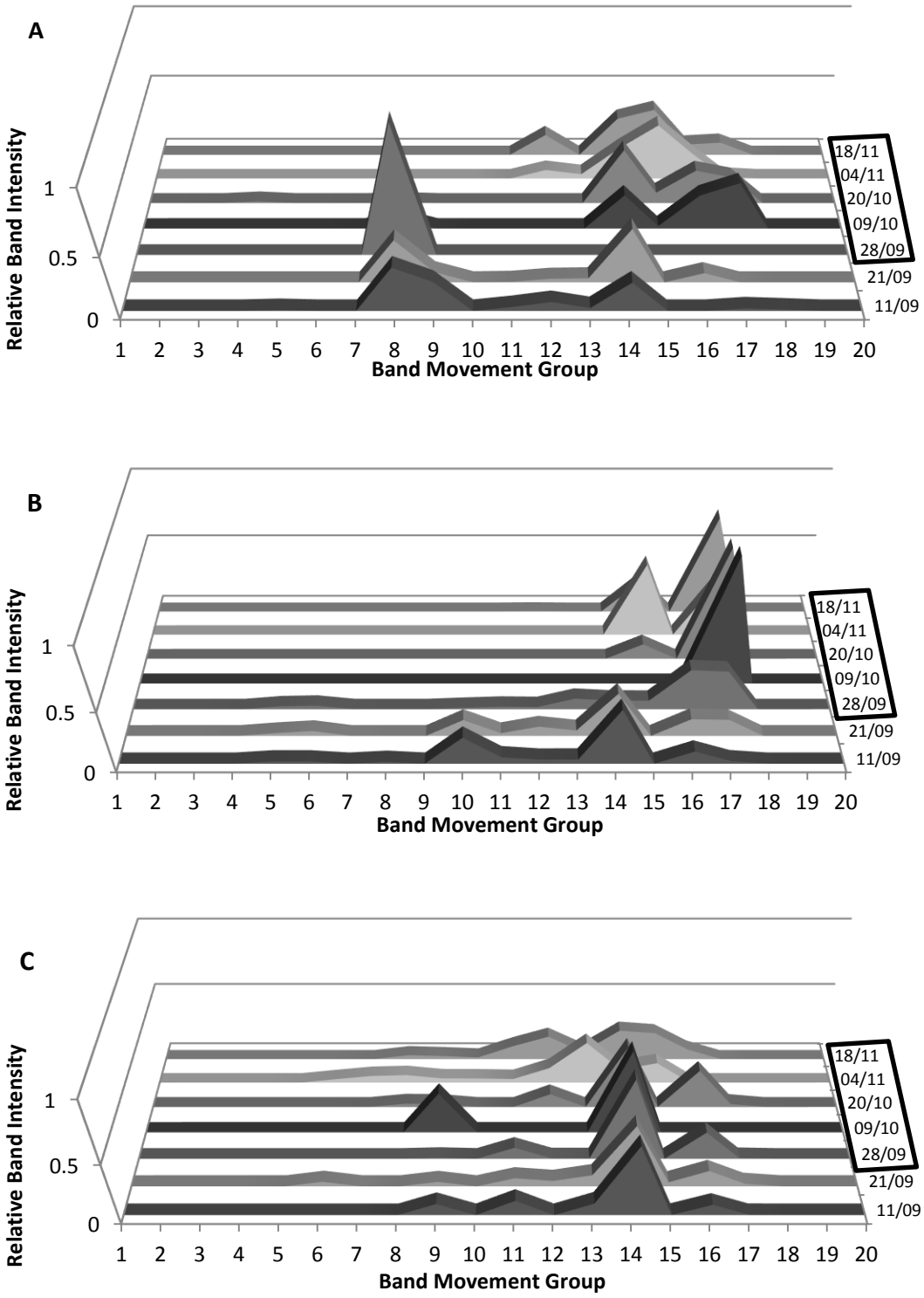

Figure 3.25: SRB DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-activated persulfate bioreactors: A) AP1; B) AP2; C) AP3.

Alkaline-only Bioreactors (AO1-AO3)

Prior to the treatment episode in the alkaline-only replicates, SRB species diversity and richness was not similar in the two sampling events prior to the injection episode (refer to Figure 3.26). This is believed to be due to an oxygen leak in one of the sample heads prior to the sampling event on September 11th. The sampling event prior (August 25th) displayed increased levels in species richness and diversity which was more comparable with the September 21st pre-treatment sample. Following treatment on September 28th, AO2 displayed a slight decline in diversity and richness, whereas AO1 remained relatively constant and AP3 experienced a slight increase. However, the sampling event on October 9th showed a reduction in both diversity and richness in all replicates, with AO1 showing the least amount of observable change. All replicates began to gradually increase in diversity and richness throughout the last three sampling events, with AO1 exhibiting a slight decline on November 4th. None of the replicates fully returned to their respective pre-treatment richness and diversity. Although AO1, the bioreactor least affected initially, almost returned to a similar SRB species richness and diversity.

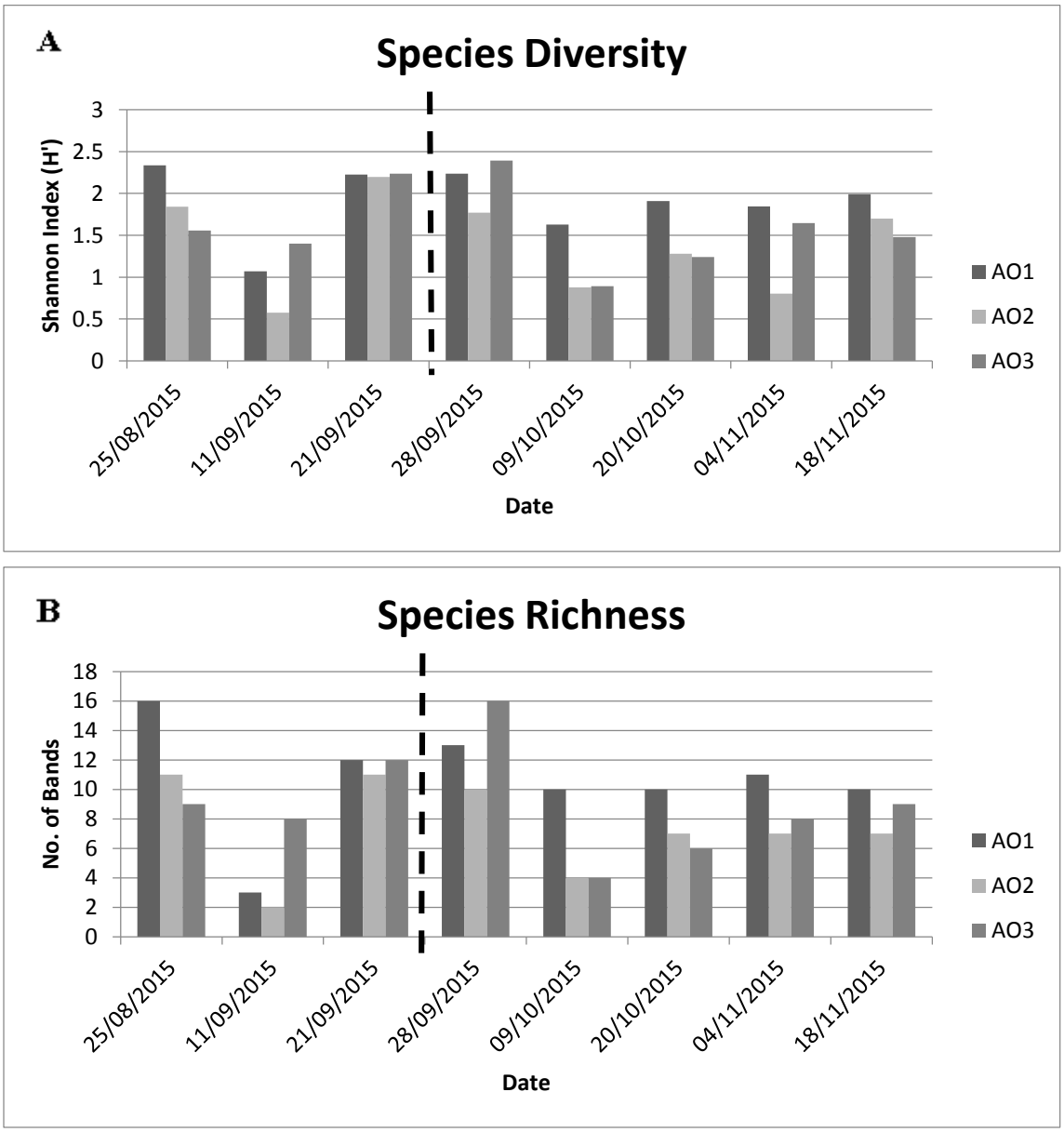

Figure 3.26: SRB DGGE-based analysis of the SRB consortia(s) present in the alkaline-only bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

The general trend observed in all of the 'AO' replicates as seen in Figure 3.27 was that the communities shifted to a more G+C rich community following the alkaline exposure. However, this shift in dominant band movement groups was not displayed until approximately one month after the treatment episode. Prior to the October 18th sampling event, the community profile in all replicates were typically distributed proportionally in the middle and later band movement groups (~7th to 18th) with the dominant OTUs appearing closer to the middle. However, on October 18th and thereafter, the SRB community structure was reduced and the dominant OTUs that were present fell in later band movement groups (~14th to 18th). The last sample set, taken on the 18th of November, began to display a more proportional distribution with respect to relative band intensities and the banding profile was slightly more expansive.

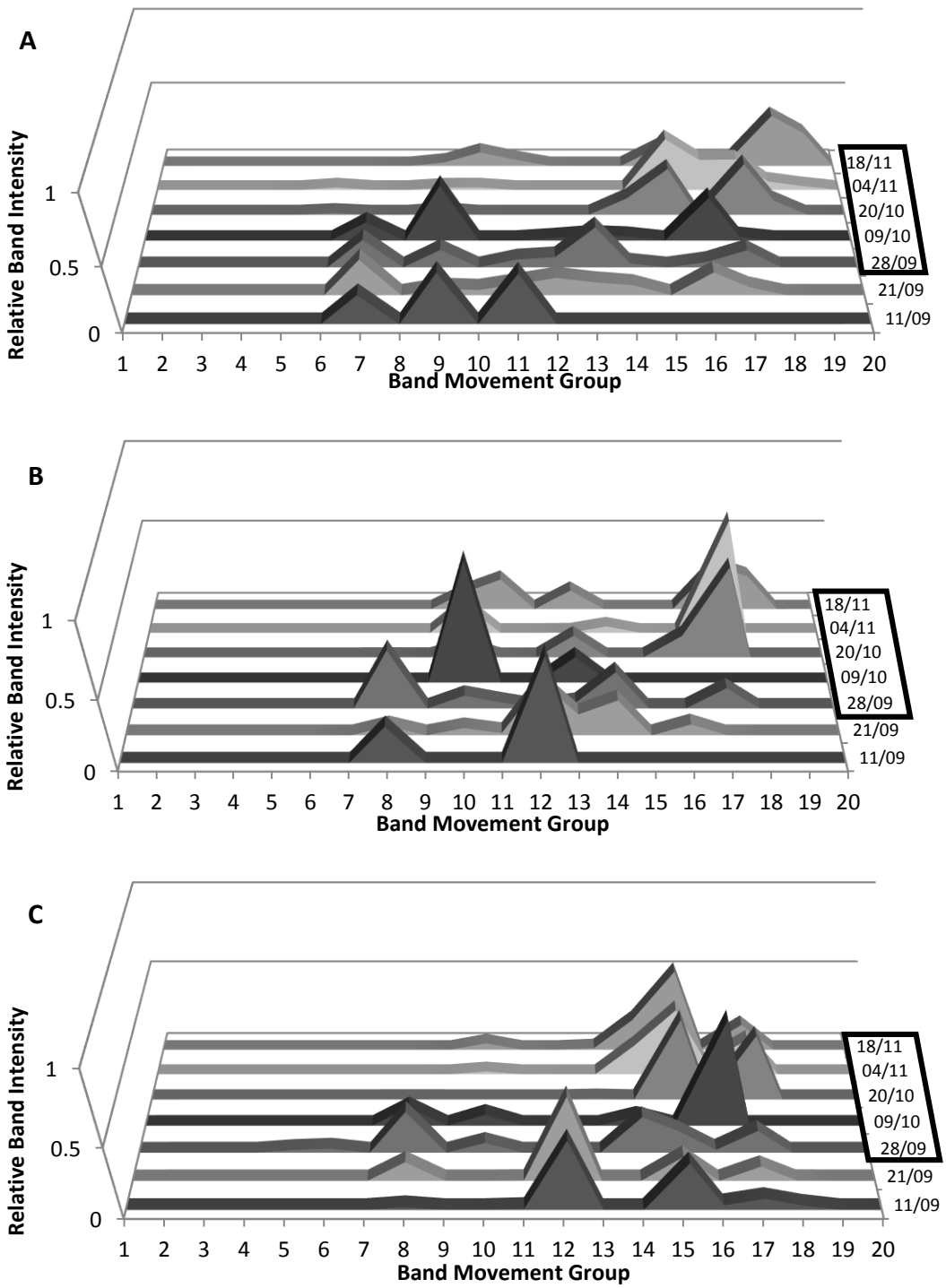

Figure 3.27: SRB DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-only persulfate bioreactor replicates: A) AO1; B) AO2; C) AO3.

Control Bioreactors (Con1-Con3)

The control bioreactor replicates all appeared to stay relatively constant throughout the experiment with respect to species diversity and richness (refer to Figure 3.28). There was an increase in species richness and diversity seen for all replicates on September 28th.

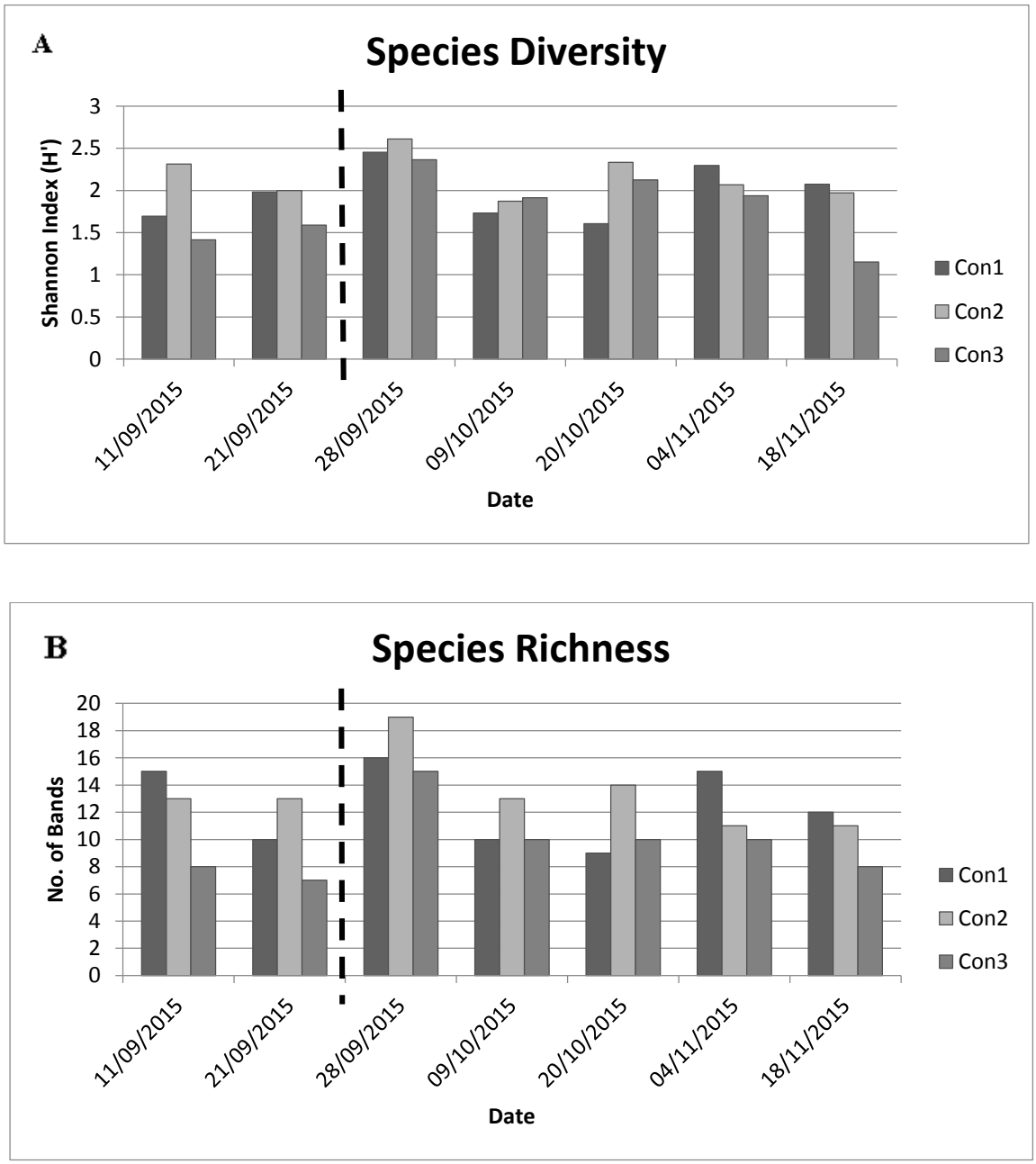

Figure 3.28: SRB DGGE-based analysis of the SRB consortia(s) present in the control bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

With respect to averaged banding profiles seen in Figure 3.29, Con1 exhibited a fairly constant profile throughout the experiment. The profile was mainly restrained within

the 7th to 18th band movement groups. In the September 11th sample, the main OTU resided at the 8th movement group. The rest of the samples displayed the highest peak at the 17th band movement group and that was maintained throughout the rest of the sampling events.

Replicate 'Con2' experienced some fluctuation with respect to the dominant OTU that was present in each sample. Proportionally, however, the samples were similar in their expansiveness across the band movement groups, staying within the 7th to 17th band movement groups typically.

Replicate 'Con3' was not as consistent with respect to averaged banding patterns. Samples take on September 11th and 21st showed 2 dominant OTUs present between the 10th and 15th band movement groups. The samples following displayed more consistency with respect to proportional band intensities across a larger span of band movement groups. The November 18th samples displayed a dominate OTU at the 10th movement group, which was only present with a reduced band intensity in previous samples. The same OTU appeared to be present in the other replicates at the same sampling time point, but it was not as dominant in comparison to other supporting OTUs.

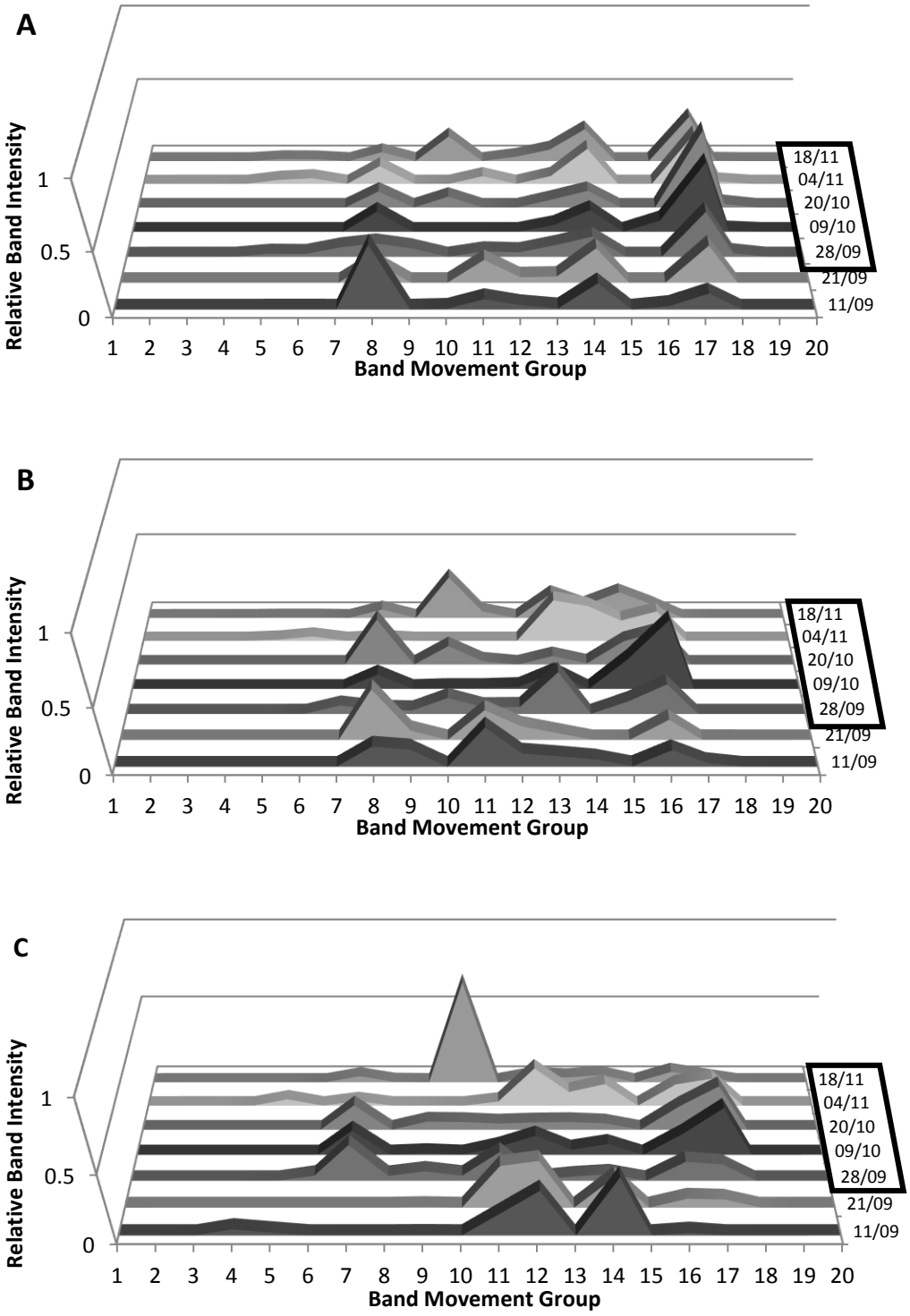

Figure 3.29: SRB DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the control bioreactor replicates: A) Con1; B) Con2; C) Con3.

3.2.2.3 Structural Fingerprinting of Supporting Microbial Communities within Bioreactors

The following section displays the results obtained from the 'universal-based' DGGE analysis. For each treatment, the same types of analyses were conducted. This includes: 1.) species diversity, which uses the Shannon index (H') to measure a samples' evenness and richness proportionally; 2.) species richness, which is the amount of bands present in a single lane, 3.) averaged banding pattern profiles, which provides further insight into changes in SRB community structure. Overall, these help reflect changes in a microbial community's structure and helps distinguish if a similar community returns following the treatment phase or whether a novel community, different from pre-treatment, arises. This type of analysis was completed on two pre-treatment samples (Sept. 11 & Sept 21) and 5 post-treatment samples taken from Sept 28th-Nov 18th.

Unactivated Persulfate Bioreactors (UP1-UP3)

Overall, species diversity in the unactivated persulfate treatment replicates displayed a steep decline following the treatment episode (Refer to Figure 3.30). The decline on September 28th was then met with a gradual increase in diversity in the subsequent sampling events (October 9th to November 18th), returning to levels similar to the pre-treatment samples. Throughout the post-oxidation phase, UP1's SRB population experienced the least amount of reduction with respect to species diversity whereas UP3's SRB consortium experienced the largest decline. Species richness exhibited a similar trend with respect to the initial depletion in the number of bands present in a sample following the exposure period and the respective rebound seen as the bioreactors recovered. After a

month of recovery (October 20th), UP1 and UP3 had recovered in species richness whereas UP2 microbial diversity returned after a month and a half.

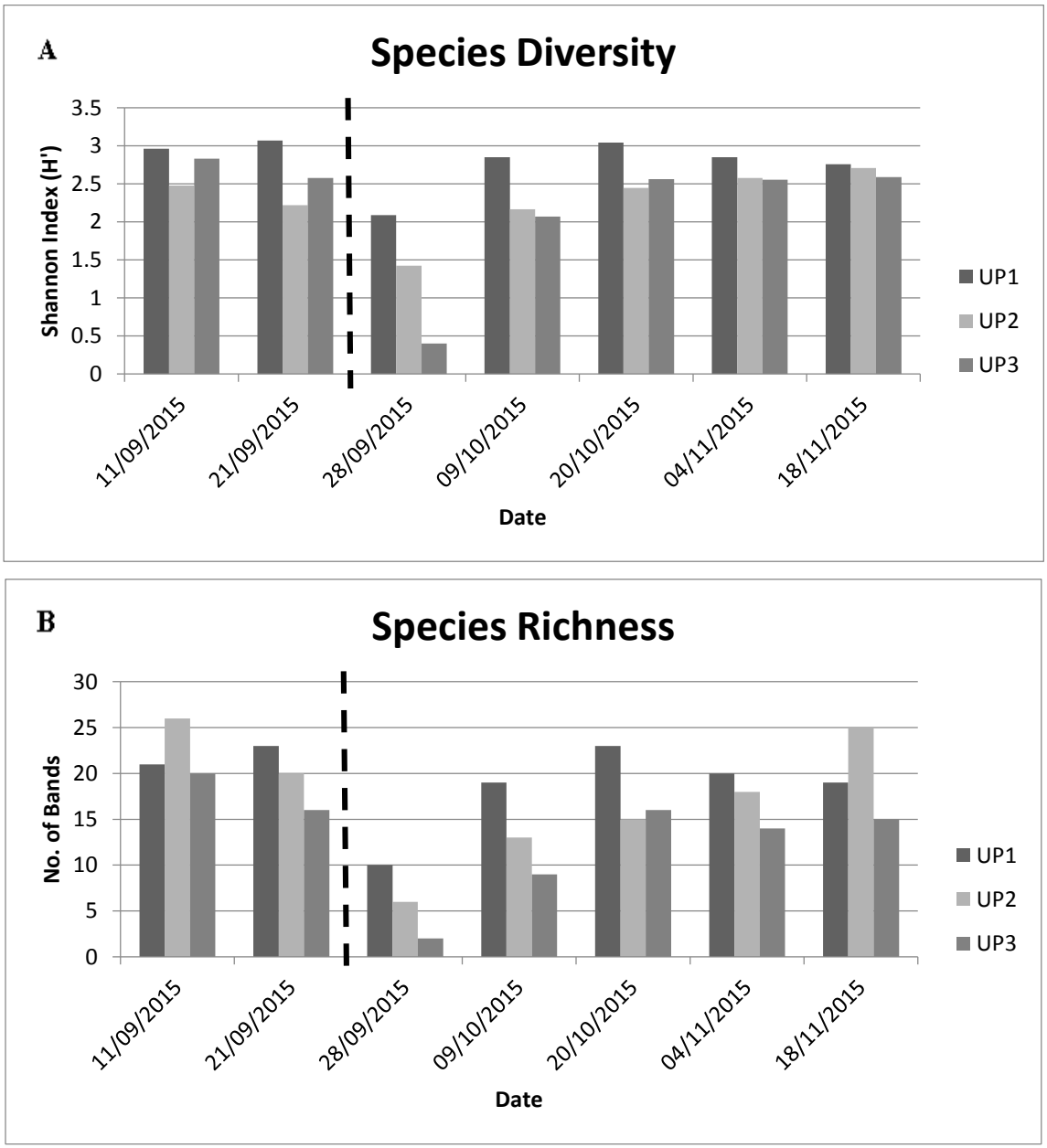

Figure 3.30: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the unactivated persulfate bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

With respect to the averaged banding patterns in the unactivated persulfate column replicates as seen in Figure 3.31, the microbial community appeared to stay within moderately G+C rich region. UP1 displayed a wider range of movement groups, which contained OTUs more proportionally spread apart, prior to treatment and after treatment. UP2 and UP3 seemed to have their relative band intensity more centralized. The majority of OTUs were located between band movement groups 10 to 15 throughout the experiment. Prior to the persulfate injection, UP3s' relative band intensity was more proportionally divided between movement groups. Immediately following treatment, it became even more centralized around 1-2 OTUs. However, as the weeks progressed, OTUs with increased prevalence/increased relative band intensity began to appear and develop. This is indicated by the smoother/flatter peaks (Figure 3.31C).

A general trend seen throughout all three replicates when looking at the raw data was that as the columns proceeded through the post-treatment phase, OTUs in the community became more centralized with individual OTUs comprising more of the relative band intensity per sample. Dominant OTUs started to appear more consistently in moderate-high G+C rich regions/band movement groups.

Overall, a shift in dominant OTUs was not particularly noticeable between the replicates. Some details, as previously mentioned, about the community can be gathered but it was difficult due to a significant fluctuation in the averaged banding patterns from week to week in each replicate. The fluctuations illustrated little consistency week to week.

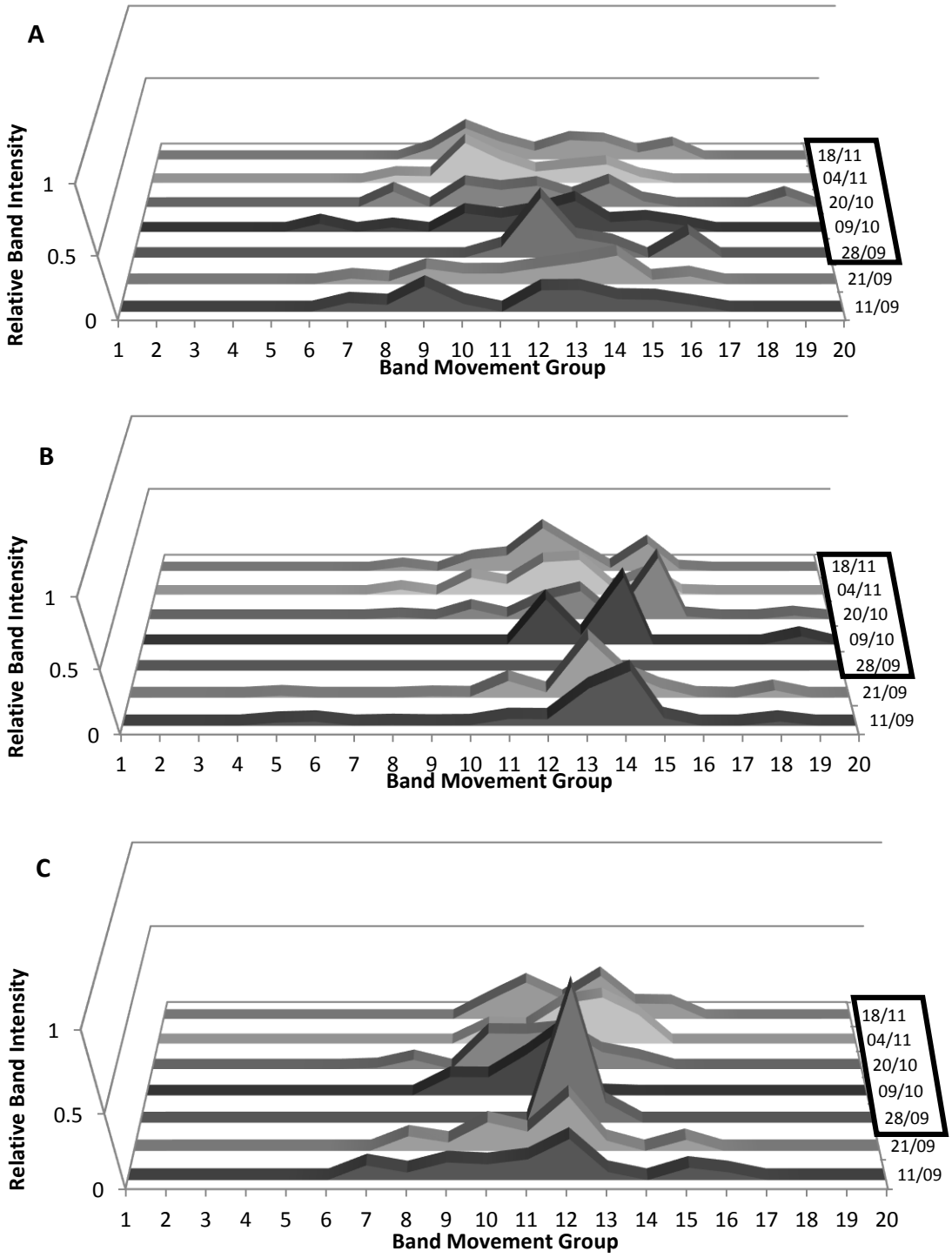

Figure 3.31: Universal DGGE-based averaged banding patterns from Sept. 11th to Nov. 18th for the unactivated persulfate bioreactors: A) UP1; B) UP2; C) UP3.

Alkaline-activated Persulfate Bioreactors (AP1-AP3)

Prior to the treatment episode in the alkaline-activated persulfate replicates, SRB species diversity and richness was highest in AP2 and lowest in AP1 (refer to Figure 3.32). Following treatment, AP1 displayed a steep decline in diversity and richness, whereas AP2 and AP3 experienced a reduction but not quite to the same observable extent. After time (October 9th) reflected a continued reduction in both diversity and richness in AP2 and AP3 while AP1 maintained the same richness but increased slightly in diversity. In the subsequent sampling event, all of the replicates increased in diversity almost to the levels seen in the pre-treatment samples. The level was maintained in the following two sampling events. With respect to species richness, the four-weeks post-oxidation sampling event revealed an increase in all replicates to levels just below that which was present in the pre-treatment samples. AP1 was the only replicate to reach the species richness that was present before the persulfate injection episode.

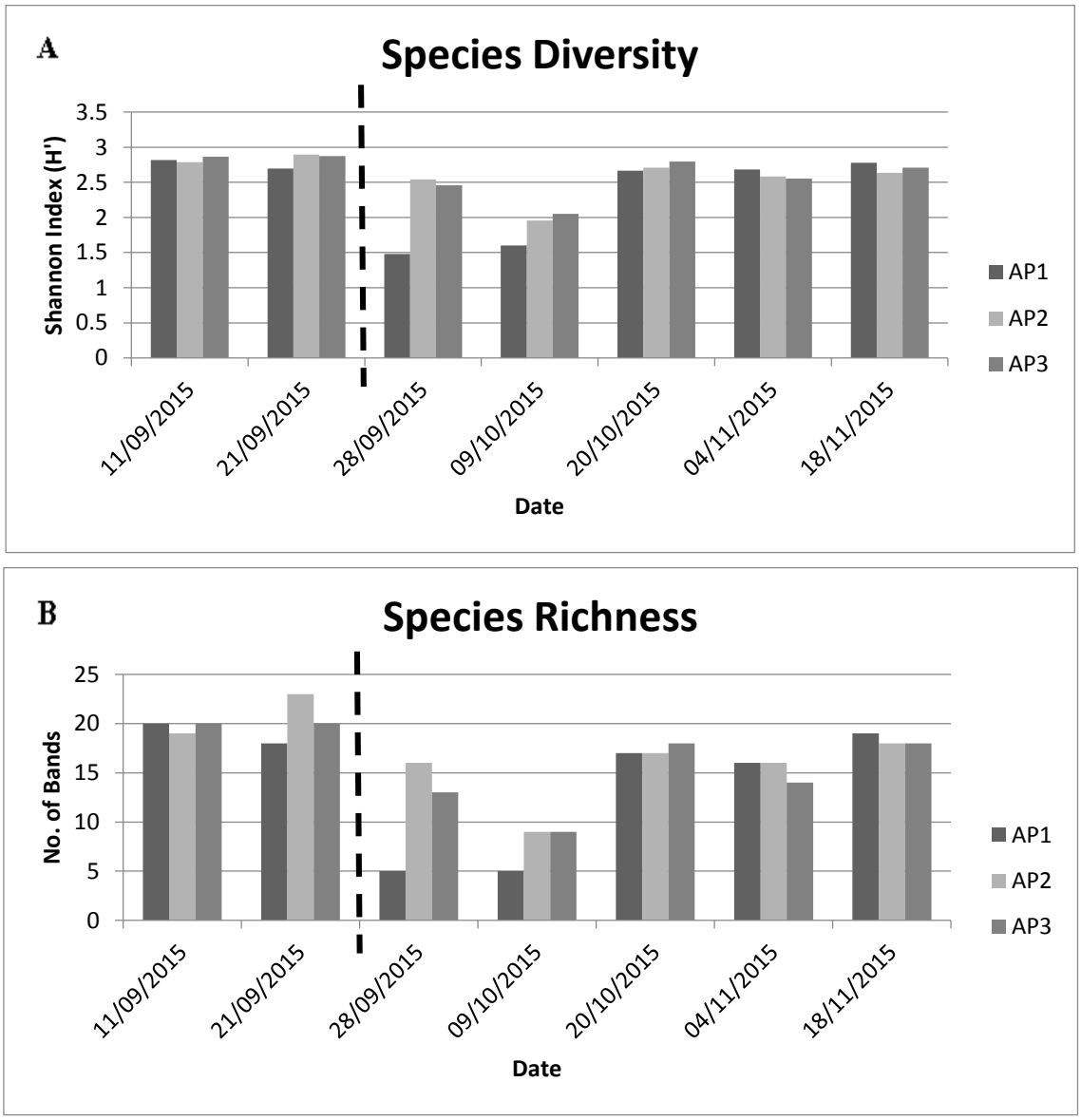

Figure 3.32: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the alkaline-activated persulfate bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

Following the alkaline activated persulfate injection, the replicates maintained dominant OTUS within similar band movement groups as shown in Figure 3.33. For replicate AP1 (Figure 3.33A), the relative band intensity was proportionally spread out,

spanning from the 7th to 18th band movement groups. Prior to treatment, OTUs within the 11-13th group made up 35-50% of the relative band intensities. For the first two sampling events following treatment, the same span comprised 80-100% of the relative band intensities. As the columns developed, the band movement groups containing OTUs began to broaden, with band movement groups 10 and 15 containing up to 33% of the relative band intensities. A peak also began to appear in movement group 7, comprising 16% of the relative band intensity in the final sampling event, 2 months after the treatment episode.

With respect to replicate AP2 (Figure 3.33B), the OTUs were relatively constrained within a few moderately G+C rich movement groups (10-13), containing 80-95% of the relative band intensities prior to the treatment episode. For the first two sample events following treatment, the dominant OTUs (in band movement groups 10 and 13) became increasingly prevalent, with no outlying OTUs in 'exterior' band movement groups. However, as the columns developed, more movement groups contained OTUs (increase in diversity), with movement group 8 comprising 26% of the relative band intensity in the last sample on November 18th.

With respect to AP3 (Figure 3.33C) prior to the injection episode, the relative band intensity was proportionally spread out, spanning from 5th to 18th band movement groups. OTUs were more dominant between the 11th and 15th movement groups. Immediately following treatment, on September 28th, 100% of the OTUs present fell in between band movement groups 10-15. The community was further restricted in the following sampling events, with 90% of the relative band intensities falling between the 11th and 14th band movement groups. As seen in the other two replicates, the last three sampling events saw

the community expand. OTUs in band movement groups 8 and 16 began appearing, comprising up to 12% of the relative band intensity in the last sampling event.

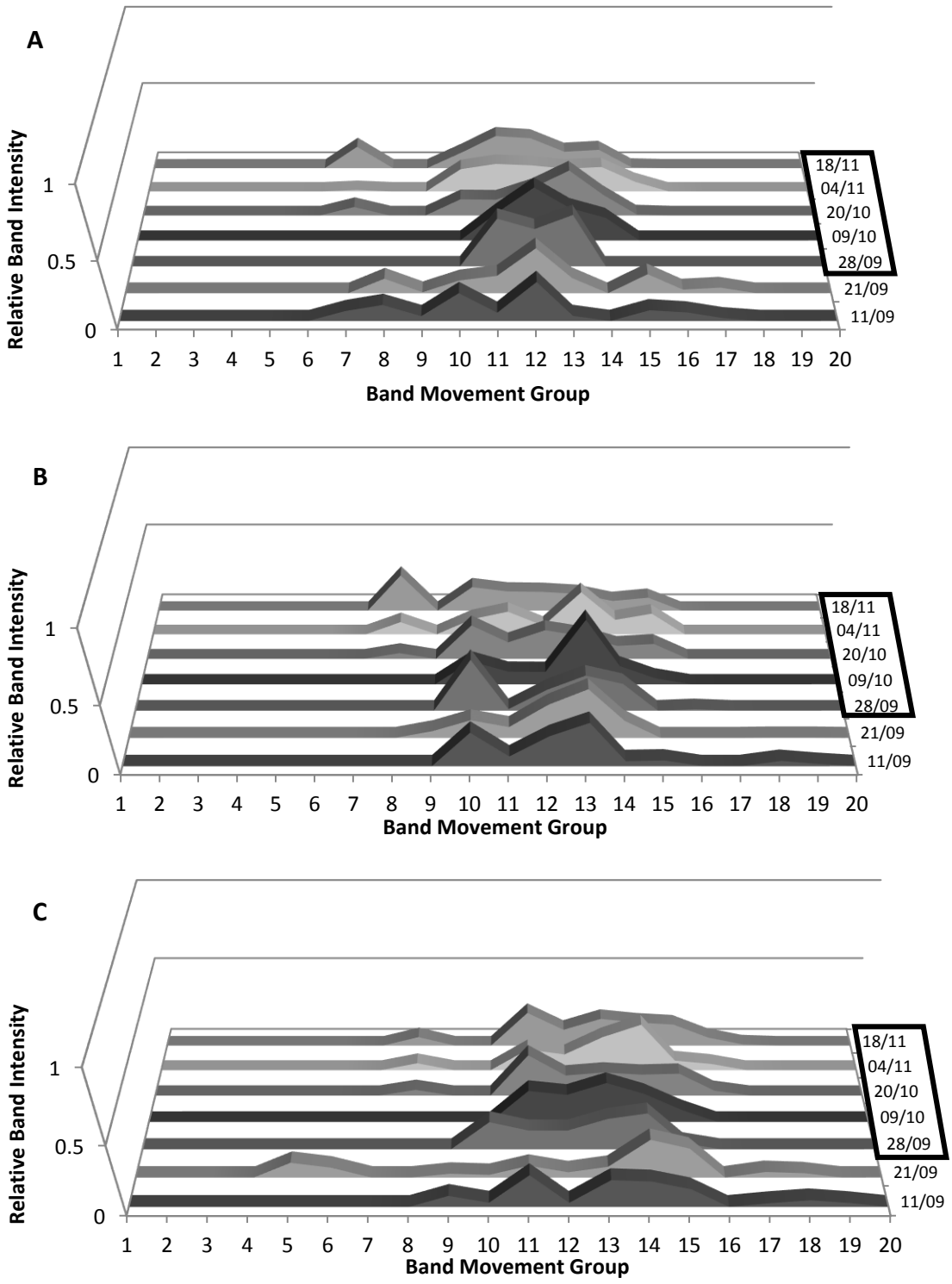

Figure 3.33: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-activated persulfate bioreactors: A) AP1; B) AP2; C) AP3.

Alkaline-only Bioreactors (AO1-AO3)

In the alkaline-only replicates, species diversity and richness was not similar in the two sampling events prior to the injection episode (refer to Figure 3.34). This is believed to be due to an oxygen leak as mentioned previously. The August 25th and September 21st pre-treatment samples showed consistency with respect to species diversity and richness whereas the September 11th sample was a bit of an outlier, displaying decreased levels of richness and diversity in comparison to the other two pre-treatment samples. Following treatment on September 28th, AO1 and AO2 displayed a slight decline in diversity and richness, whereas AP3 demonstrated a slight increase. The subsequent sampling events on October 9th and 20th showed an increase in diversity and richness in replicates AO1 and AO2, followed by a slight decline and then increase in the last two sampling events. AO3 maintained relatively constant overall species diversity and richness throughout the post-treatment sampling events.

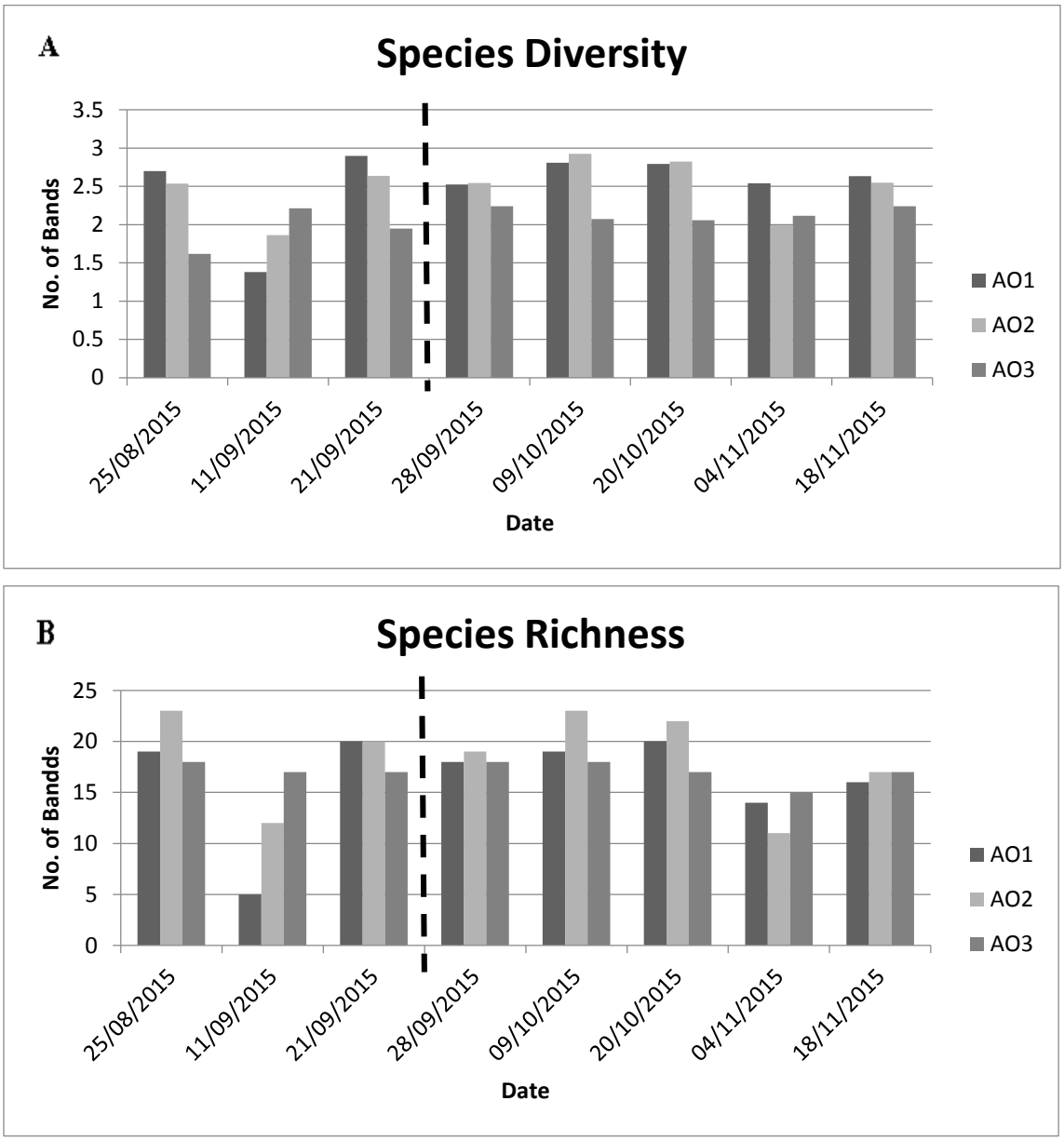

Figure 3.34: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the alkaline-only bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

In comparison to the other treatment tests, the alkaline-only replicates did not see a noticeable die-off and then resurgence with respect to genetic analysis (refer to Figure

3.35). Rather, the replicates for this test seemed to undergo a shift in the averaged banding profile. Essentially, there was a slight shift towards dominant OTUs with a lower in G+C content (lower band movement group), although the OTUs were still in a moderate G+C range, as the post-treatment recovery occurred. Replicates AO1 and AO3 exhibited this trend most clearly, as seen in Figure 3.35 (A & C). Pre-treatment, all replicates had the majority (50-80%) of their dominant OTUs fall in between band movement groups 11 through 14, for AO1 and AO3, and 12 through 14 for AO2.

Following alkalinity exposure, band movement groups 8-12 began to contain the majority of dominant OTUs. This can be said for all replicates, whereby individual movement groups, in that span, slightly fluctuated in relative intensity. In replicates AO1 and AO3, the post-treatment samples seem to contain OTUs slightly more proportionally distributed amongst the moderately G+C rich band movement groups, and therefore OTUs. AO2 displayed a similar trend in the October 9th and 20th samples but then individual OTUs become more dominant amongst the community. A trend was seen in the last sampling event for AO3 as well. With 3 OTUs in band movement groups 11, 12 and 13 comprising 88% of the relative band intensity in the November 18th sample.

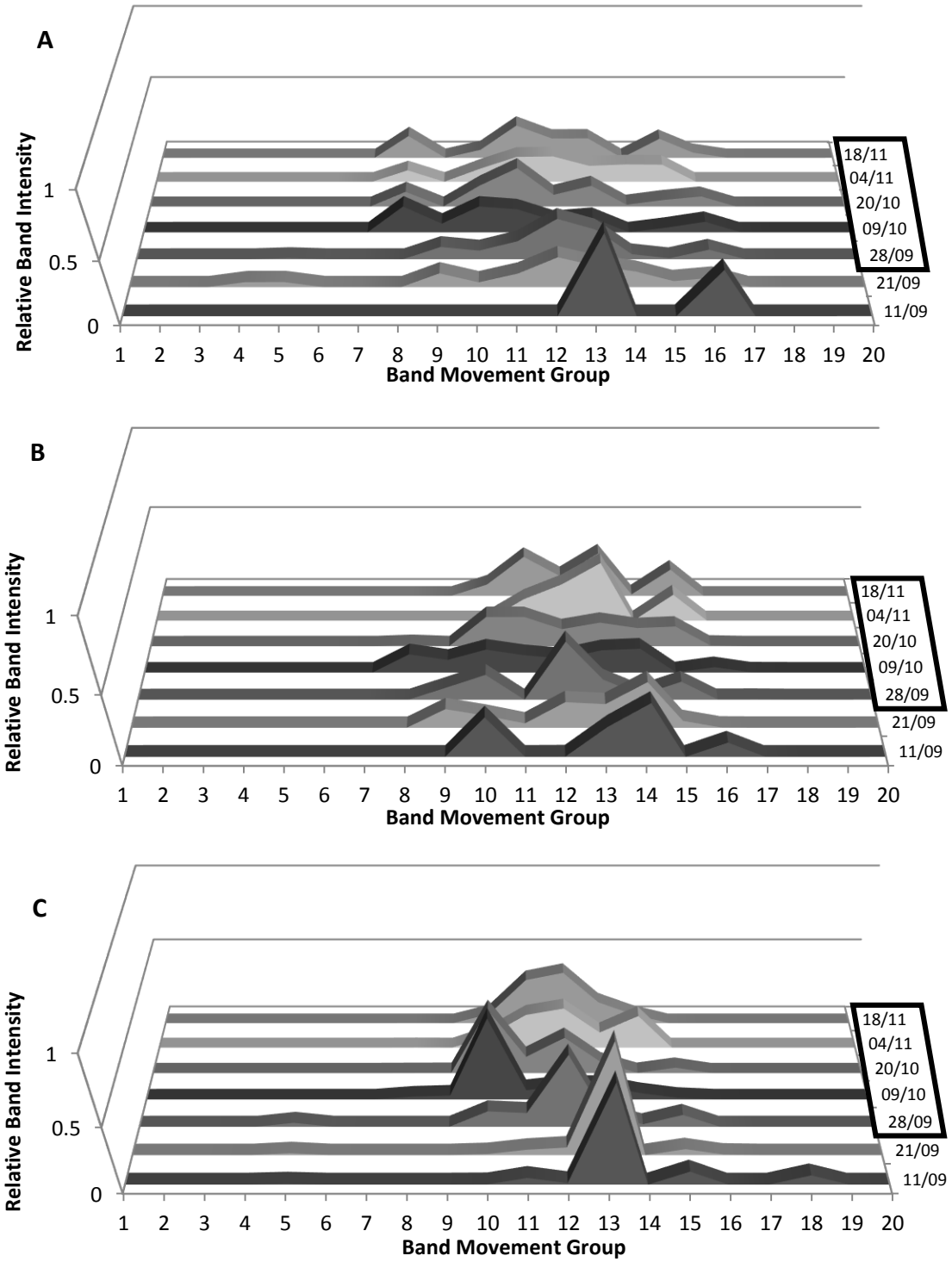

Figure 3.35: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the alkaline-only persulfate bioreactor replicates: A) AO1; B) AO2; C) AO3.

Control Bioreactors (Con1-Con3)

With respect to species diversity and richness, the control bioreactor replicates all appeared to stay relatively constant prior and immediately following the treatment phase of the other bioreactor experiments (refer to Figure 3.36). There was an increase in species richness and diversity seen for all replicates on September 28th. Following September 28th the samples begin to show a decrease in diversity and richness. This trend continued until the last sampling date, when the community experienced a slight increase, returning to levels seen in the September 21st samples.

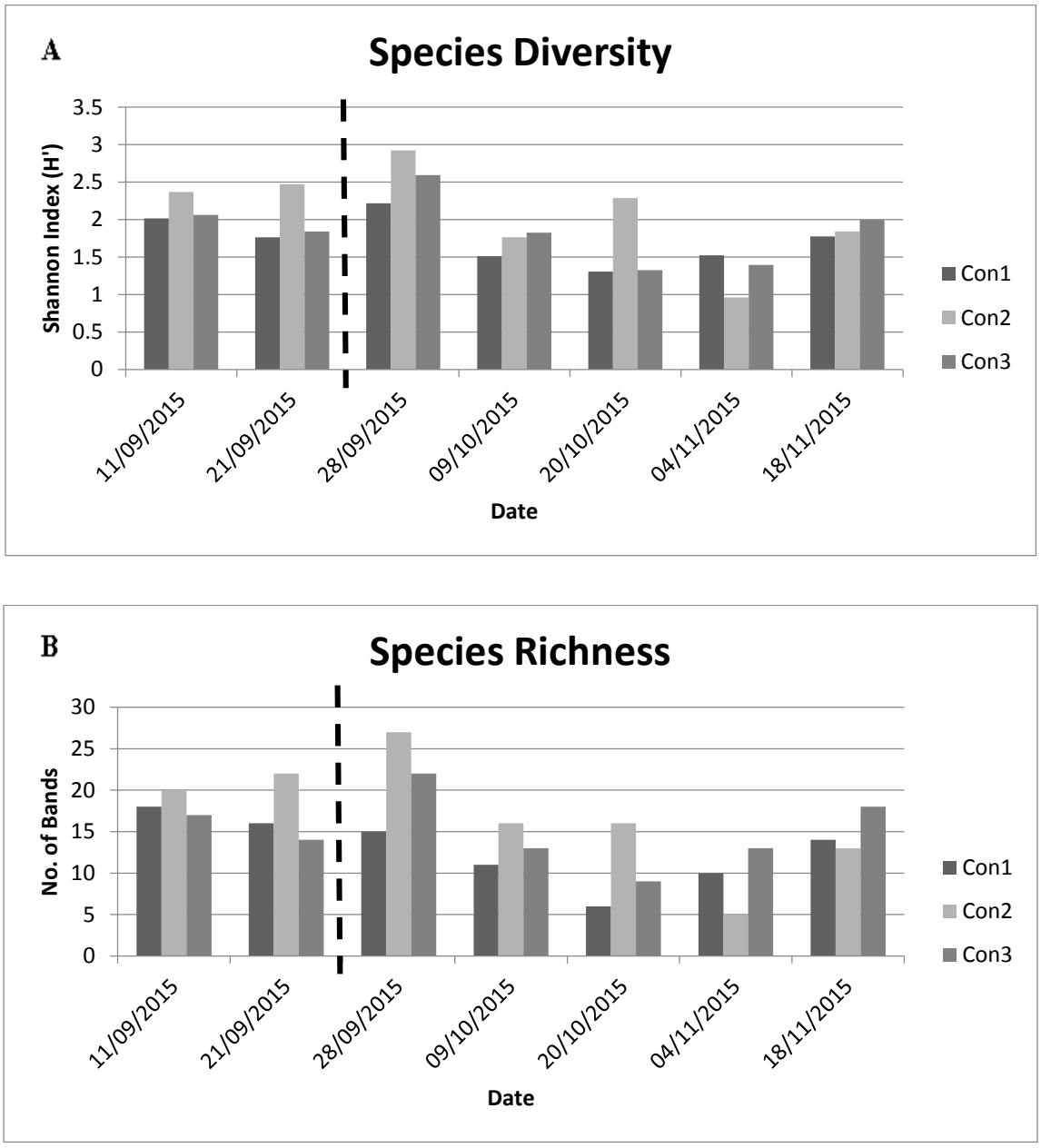

Figure 3.36: Universal DGGE-based community analysis of the supporting microbial consortia(s) present in the control bioreactor replicates. Graph A represents species diversity which is measured by the Shannon index (H'). Graph B illustrates species richness in the replicates, measured in number of bands. The dashed line represents the treatment episode.

The control bioreactor series maintained a relatively consistent averaged banding pattern overall. The dominant OTUs seen in all replicates were moderately rich in G+C, with most of the relative band intensity falling between the 10th and 14th band movement groups.

In Con1 replicate, the presence of a dominant OTU at band movement group 13 was present in all of the samples (refer to Figure 3.37). The OTU comprised 35-90% of the relative band intensity in every sample throughout the experiment, typically falling in the higher end of that range. A second OTU in band movement group 11 shared prevalence within samples from October 20th and November 11th samples, with relative band intensities of 52% and 65%, respectively. A similar trend was seen in Con2 and Con3 replicates. However, Con2 and Con3 showed wider peaks indicating OTUs in close proximity with similar G+C content. Overall, the averaged banding patterns in the control bioreactors showed more consistency between samples than in the treatment replicates.

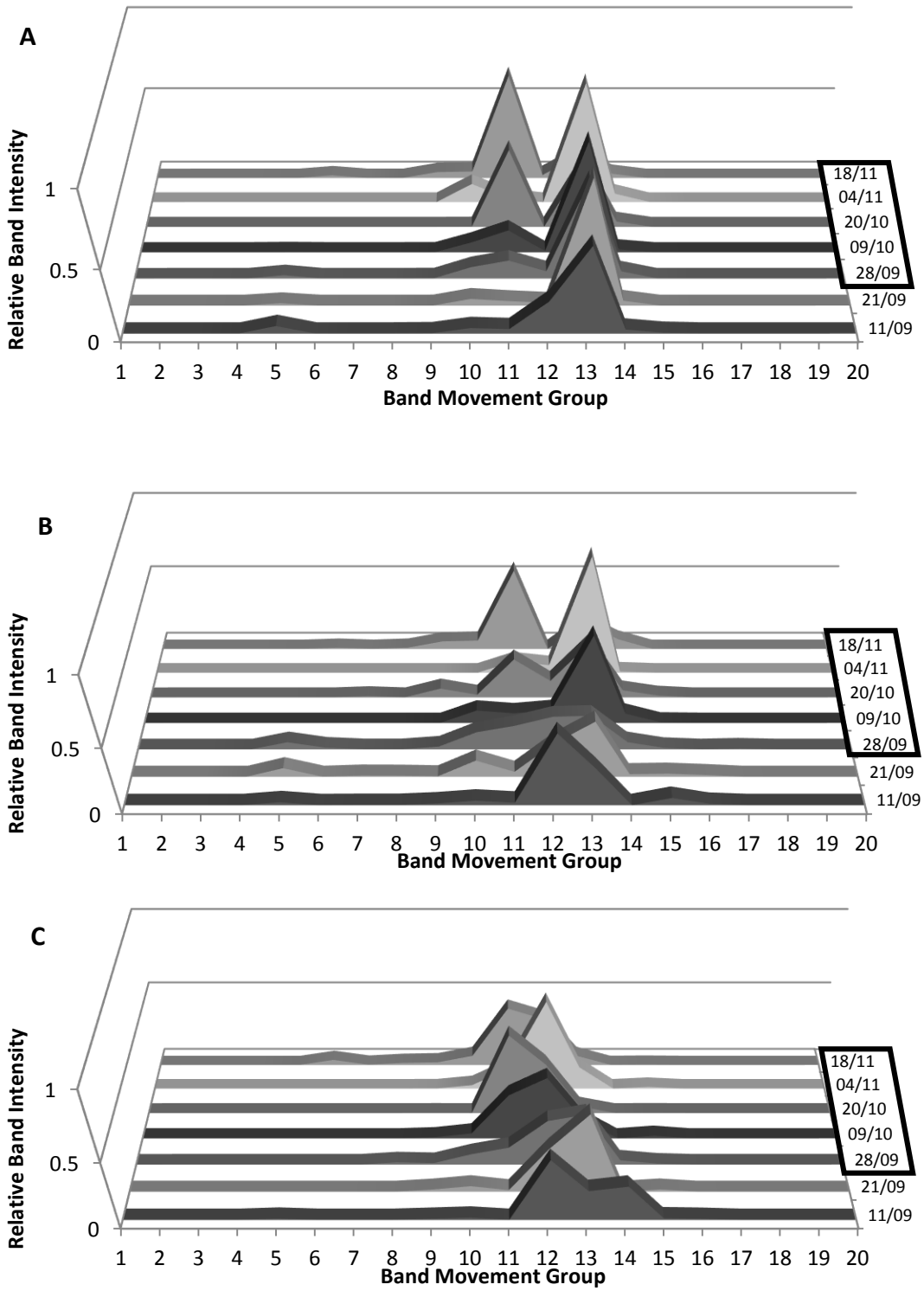

Figure 3.37: Universal DGGE averaged banding patterns from Sept. 11th to Nov. 18th for the control bioreactor replicates: A) Con1; B) Con2; C) Con3.

3.2.3 Chemical Analyses

Throughout the experimental phase, a collection of physicochemical parameters were monitored. This included total BTEX, dissolved hydrogen sulfide, dissolved methane, sulfate, pH, and oxidation-reduction potential (ORP). By measuring these parameters, it was possible to better assess the physicochemical condition of the bioreactors which may impact the microbial consortia following the treatments. Biodegradation potential was reflected by analyzing the environment inside the columns, through effluent sampling, to assess whether conditions were favourable for anaerobic communities, namely that of a sulfate-reducing consortia.

3.2.3.1 Total Petroleum Hydrocarbon concentration throughout Experimental Trial

A balanced mixture of benzene, toluene, ethylbenzene and xylenes (BTEX) was used as the contaminant source during the experimental trial. The mixture of petroleum hydrocarbons (PHC) compounds was monitored throughout the experiment. Figure 3.38 depicts how the PHC concentrations changed before and after the experiment in each of the bioreactor sets, through analysis of the effluent samples. The reservoirs were also monitored for comparison throughout the experiment. There was a loss of total BTEX through the reservoir walls of approximately 100-400 ppb between fillings (one-week); typically falling on the lower end of that range. This was taken into effect when calculating percent reduction. Prior to treatment, all bioreactors were experiencing almost complete mineralization of toluene (See Appendix Figure A7) but this trend ceased in all of the

treatment sets (except controls) following the persulfate/alkaline injection phase. The control replicates maintained relatively constant BTEX reduction in the effluent throughout the experiment.

Prior to treatment, UP1-3 bioreactors displayed over a 50% consumption in total BTEX from the reservoir to the effluent end of the reactors. With respect to the UP1-3 bioreactors, the general trend observed following persulfate treatment was that [BTEX] in the effluent was higher than what was being observed in pre-treatment samples (20% less BTEX reduction). Biodegradation potential continued to decline to 25% total BTEX reduction three weeks' post-oxidation. The next three sampling events displayed a fluctuation in BTEX degradation between 20-30%, never reaching pre-treatment biodegradation potential by the final sampling date (8-weeks post-oxidation).

AP1-3 displayed a similar trend to that of UP1-3, where BTEX reduction decreased by approximately 25% (to less than 30% reduction) immediately following the treatment phase. The reduction potential of the 'AP' bioreactors continued to decline to approximately 20% reduction on October 15th. BTEX reduction did increase up to 45% on 6-week post oxidation but declined again by the 8-week mark to approximately 30% reduction.

AO1-3 bioreactors also experienced an obvious reduction in BTEX degradation in the effluent samples. Prior to the alkalinity exposure, BTEX reduction was approximately 45%. Ten-days following treatment BTEX degradation dropped to 27%. On October 15th, three-weeks post-injection, AO1-3 replicates were consuming approximately 38% total

BTEX. However, the subsequent sampling event displayed a return to more limited BTEX degradation values (less than 20%). BTEX reduction slightly increase in the final two sampling events but was still experiencing less than a 30% reduction therefore never returning to pre-treatment biodegradation levels, on average.

Overall, it can be deduced that BTEX biodegradation potential was reduced throughout all of the treatment sets in comparison to pre-treatment samples, with the exception of the control set. Although, the communities did still display BTEX biodegradation capabilities after treatment (in all cases), just at reduced rates.

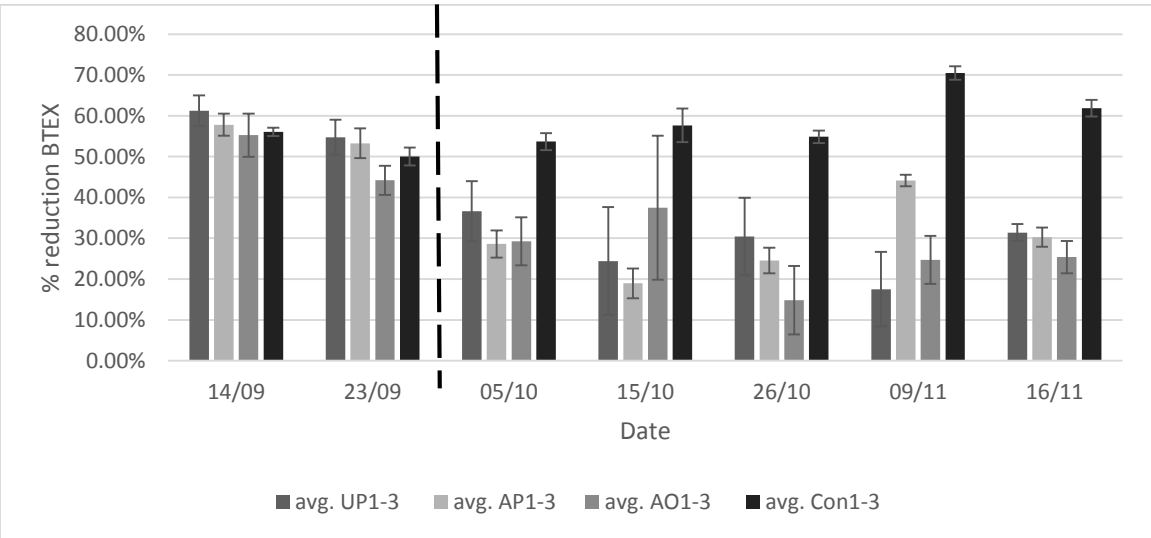

Figure 3.38: Average percent reduction in BTEX September to November in the unactivated persulfate (UP1-3), alkaline-activated persulfate (AP1-3), alkaline-only (AO1-3) and control (Con1-3) bioreactors. The dashed line represents the treatment phase.

3.2.3.2 Dissolved Hydrogen Sulfide (H_2S)

Dissolved H_2S was monitored throughout the experiment to provide a quantitative assessment of changes in sulfate reduction (refer to Figure 3.39). H_2S increase or decrease reflects a reduction in sulfate (i.e. SRB activity). Prior to treatment, the H_2S in all treatment replicates was approximately 2.5 ppm. The two sampling events following the treatment phase noted a reduction in the amount of H_2S detected in all treated column sets. AO1-3 bioreactors experienced the least reduction, dropping to 700 ppb in dissolved H_2S. UP1-3 and AP1-3 bioreactor sets experienced a more notable drop in H_2S down to 2 and 23 ppb, respectively. However, within just over one-month post-treatment, all columns experienced an increase in H_2S above concentrations observed in pre-treatment samples. By the last sampling event, dissolved H_2S was almost one order of magnitude higher than that present in pre-injection samples.

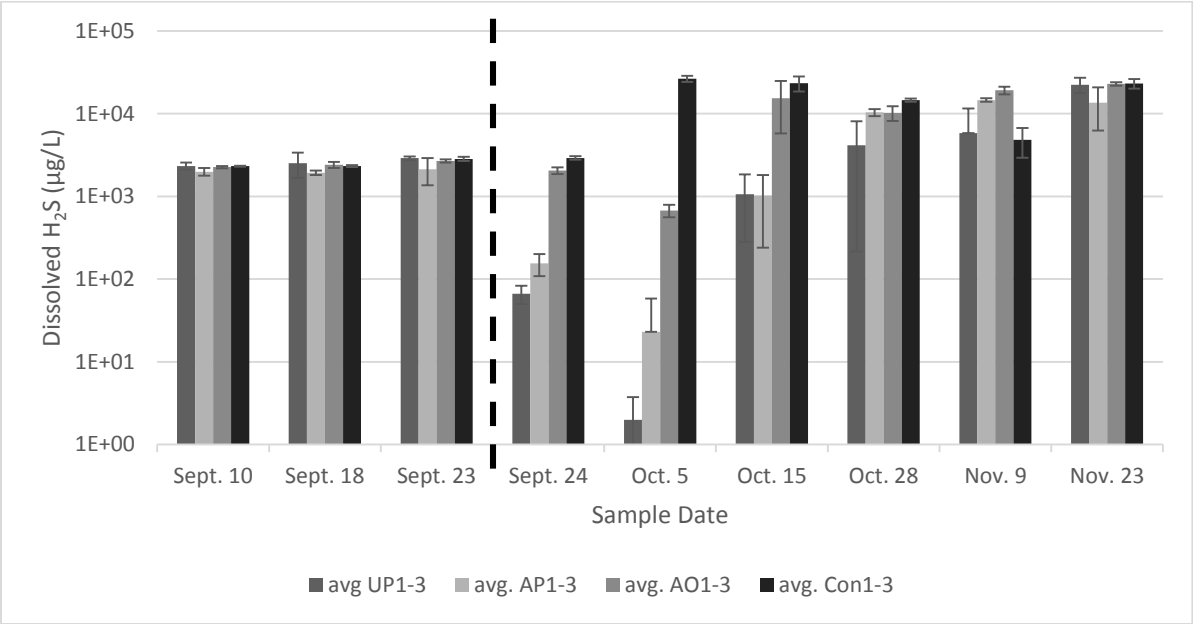

Figure 3.39: Average dissolved hydrogen sulfide (H_2S) present in the effluent samples of all treatment and control bioreactors throughout the experiment. The dashed line represents the treatment phase.

3.2.3.3 Dissolved Methane (CH_4)

Prior to the treatment phase, all of the bioreactor effluents displayed methane concentrations in the 5-7 ppm range (refer to Figure 3.40). Immediately following the treatment phase UP1-3, AP1-3 and AO1-3 bioreactor sets demonstrated a reduction in methane levels. On average, AP1-3 and AO1-3 bioreactor sets dropped in methane levels to less than 1 ppm whereas AP1-3's bioreactors' methane concentration declined only slightly to 1.9 ppm. By October 5th, the methane concentration in the treatment column sets was less than 100 ppb. This concentration continued in these sets of columns until the November 9th sampling date, when methane levels slightly increased to the 200 ppb range.

200 ppb was observed in the last sampling event on November 23rd as well. The control bioreactor replicates remained relatively constant throughout, with slight fluctuations.

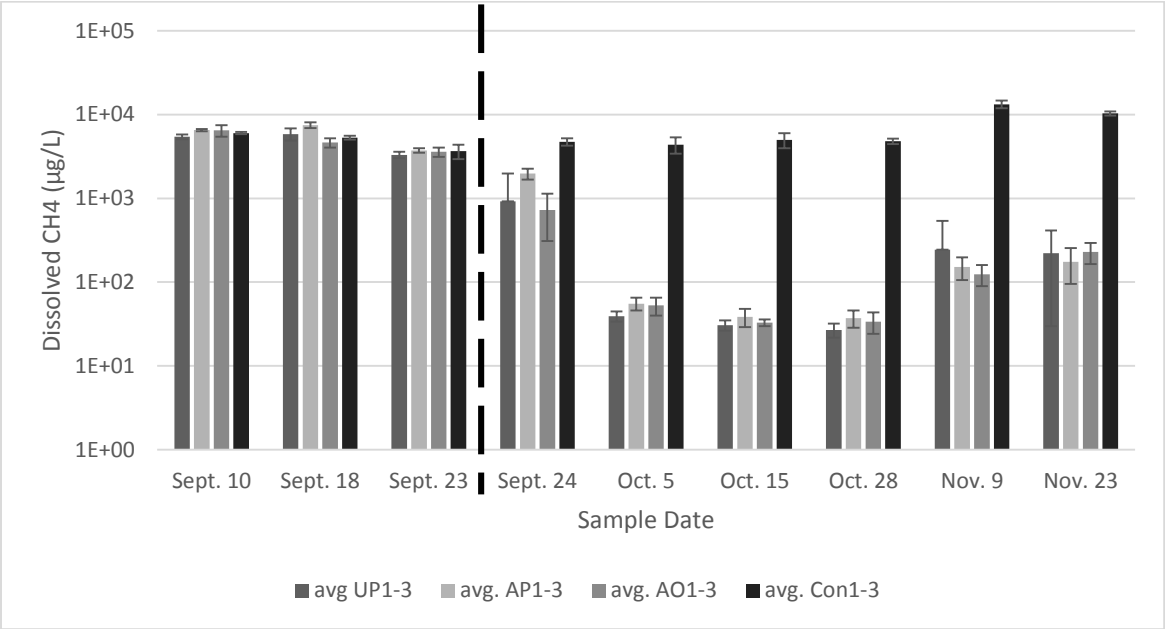

Figure 3.40: Average dissolved methane (CH_4) present in the effluent samples of all treatment and control bioreactors throughout the experiment. The dashed line represents the treatment phase.

3.2.3.4 Monitoring Sulfate

The sulfate concentration in the effluent of each bioreactor was monitored throughout the experiment as a complementary method to monitor SRB activity. Figure 3.41 represents the average sulfate concentration in each set of treatments. The Y axis in plots A and B are shown in a log scale due to a large increase in sulfate concentration (approximately 6000 ppm) in the sample set following the treatment phase. The sulfate concentrations in the reservoirs ranged between 110-130 mg/L. Prior to treatment, sulfate reduction remained relatively low when compared to the post-treatment samples across all

replicates. Immediately following treatment, sulfate reduction increased to 6-7 g/L in the treatment columns sets. The subsequent sampling event displayed a return to similar effluent [sulfate] as the pre-treatment samples. The sample set taken approximately three weeks after treatment, on October 14th, began to show an increase in sulfate consumption by the microbial consortia(s) across all treatment sets as reflected by a drop in concentration. UP1-3 decreased by almost 50% from 125 mg/L on October 6th to 60 mg/L on the 14th. The following samples in UP1-3 replicates continued to display a decreased [sulfate] throughout the rest of the experiment. A similar trend was noticed in AP1-3 and AO1-3 treatments. By the final sample, sulfate concentration had decreased by more than 50% in comparison to the pre-treatment samples. The control columns also experienced a progressive increase in sulfate reduction. Sulfate levels began to slightly decrease (by 10 mg/L) from September 18th to September 23rd. Two-weeks post-oxidation, from September 24th to October 6th, a large decrease (73 mg/L decrease from 113 mg/L to 40 mg/L) was observed. Decreased levels continued until the November 9th sampling episode, where sulfate levels increased from 34 mg/L to 103 mg/L. Concentrations decreased across all control replicates by the last sampling date, falling to its lowest point throughout the experiment, 2mg/L.

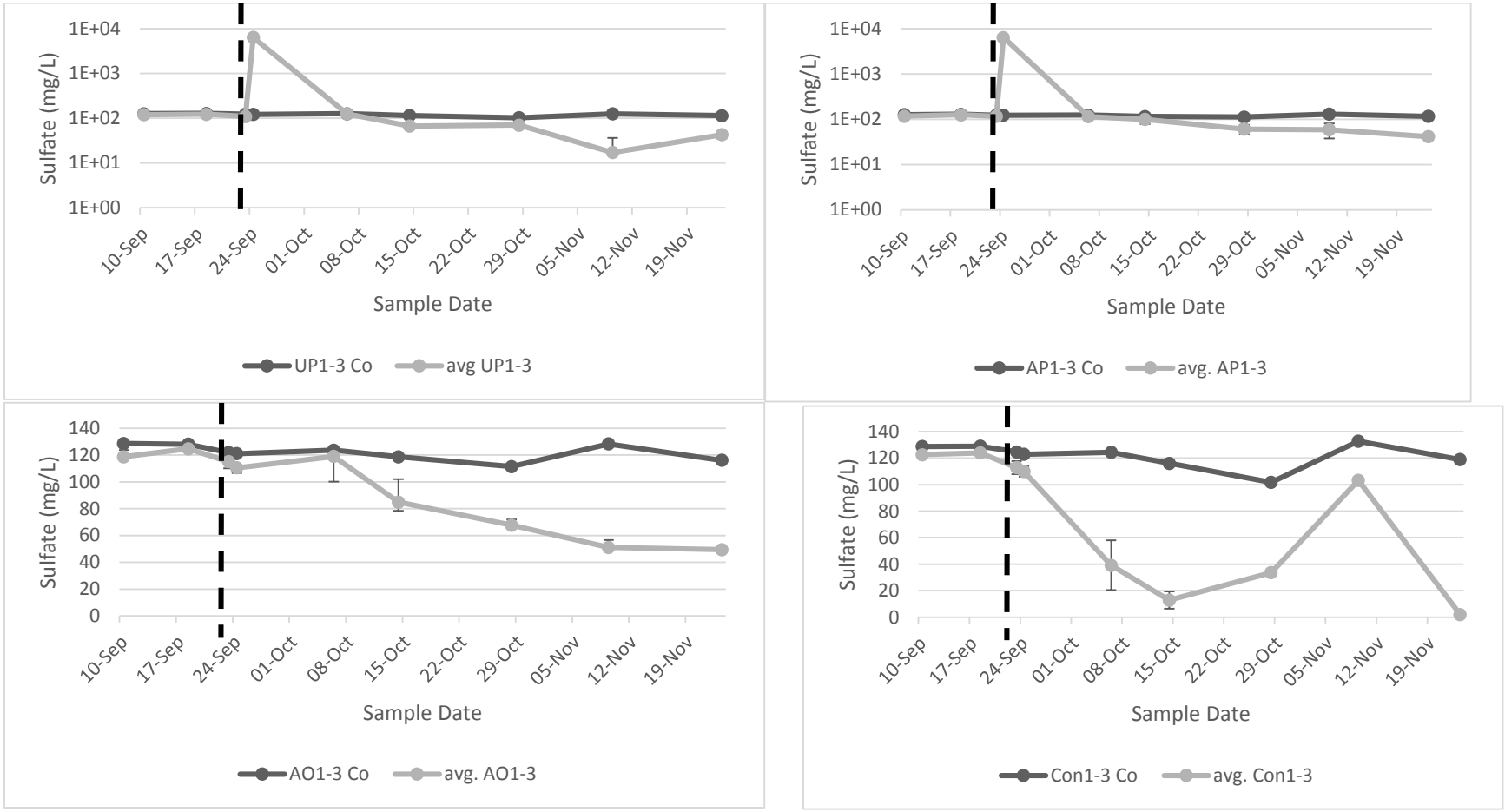

Figure 3.41: Sulfate concentration in the reservoirs (C_0) and water effluent of the unactivated persulfate (plot A), alkaline-activated persulfate (plot B), alkaline-only (plot C), and control (plot D) bioreactors from September to mid-November. The dashed lines indicate the 24-hour treatment phase.

3.2.3.5 Monitoring pH

The pH conditions of the columns were monitored both before and after the treatment process. Throughout the experiment, the simulated groundwater in the reservoirs was a pH of 7.4; with the exception of the alkaline solutions (pH 12) during the 24-hour treatment phase. In Figure 3.42, the pH in the effluent from September 10th to November 23rd is illustrated. Prior to treatment, the column effluent was above the reservoir pH. The pH of the effluent pre-treatment was between 7.7-7.8 in all bioreactors. Following the treatment, on September 24th, the pH in the effluent of the AP1-3 bioreactors had dropped to approximately 6.38. In the alkaline-activated persulfate bioreactors the pH dropped from 12, in the reservoir, to 6.32 in the effluent. The alkaline-only reactors continued to show the same pH as that was present in the reservoir (pH 12). The control columns maintained a pH similar to the pre-treatment samples. Eleven days following the treatment phase, on October 5th, the pH in the effluent of the AP1-3 and AO1-3 was 9 and 8.24, respectively, in comparison to 7.4 in the reservoir(s). Also on October 5th, on average UP1-3 showed a pH above 8. This was mainly due to the pH measured in UP2, which was 9, as the other replicate showed a pH of approximately 7.75. The remaining sampling events saw a return to a pH ranging between 7.7-7.8 in all bioreactors. The control replicates maintained a relatively constant pH throughout the experiment, with the exception of the November 9th sampling date when the pH was just above 8 in the effluent of all replicates. This was due to an increase in reservoir pH, to 7.7, owing to microbial contamination from the bioreactors. With a new reservoir bag, the pH in the effluent of the control columns returned to approximately 7.5.

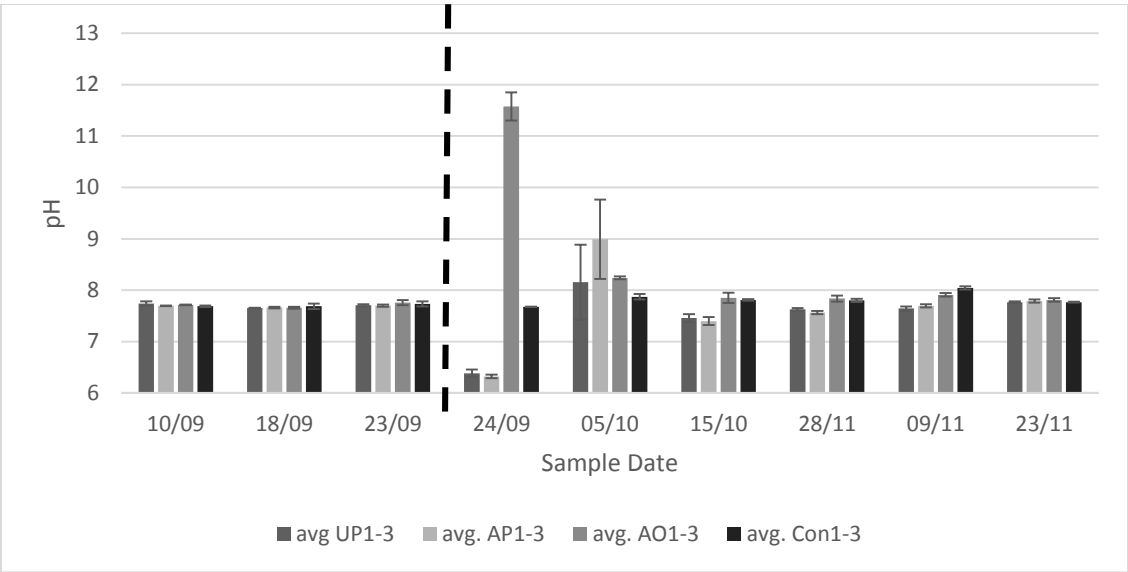

Figure 3.42: Average pH present in the effluent samples of all treatment and control bioreactors throughout the experimental trial.

3.2.3.6 Monitoring ORP

ORP was measured throughout the experiment to ensure that reducing conditions were prevailing prior to the treatments and to monitor how ORP changes after the injection phase. For the two persulfate systems, the ORP increased to approximately +700 mV after the treatment episode. On October 5th, 11 days after treatment, the redox conditions had dropped to 300-400 mV. ORP continued to drop in the two persulfate systems and by approximately one-month post-treatment, the conditions were more reducing than pre-treatment. This trend (prevalent reducing conditions) continued in UP1-3 and AP1-3 for the rest of the monitoring phase. With respect to AO1-3, conditions remained reducing. An

increase in ORP was noted in the first two sampling events post-oxidation but by October 15th, conditions had returned to pre-treatment levels. The control replicates maintained reducing conditions as well, fluctuating slightly around the -100 mV range throughout the experiment (Figure 3.43).

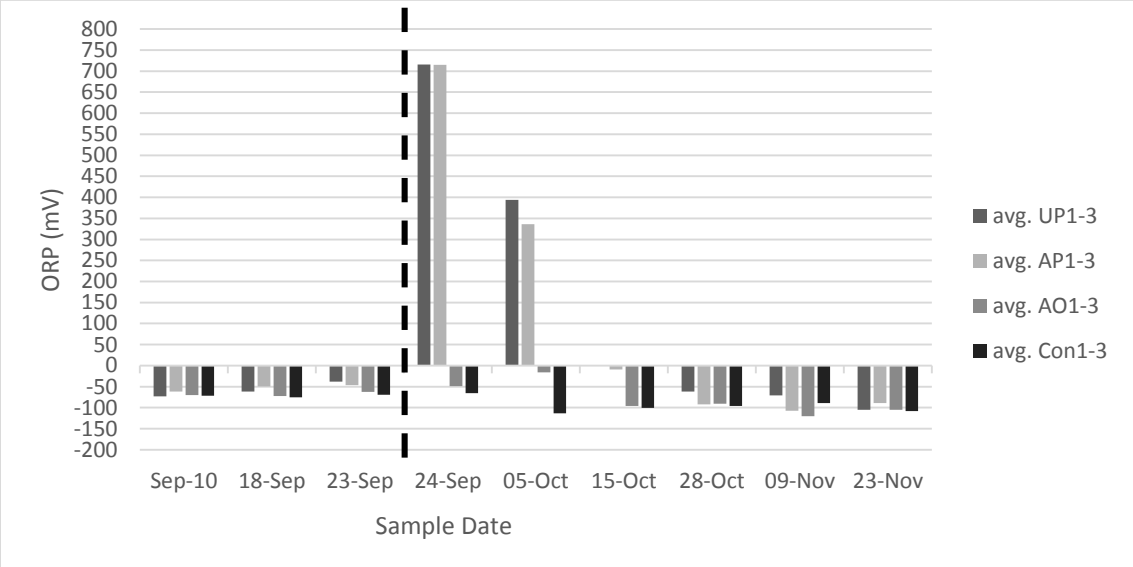

Figure 3.43: Average ORP (millivolts) present in the effluent samples of all treatment and control bioreactors throughout the experiment.

The results section encompassed a wide range of analysis, mainly revolving around microbial community analysis using molecular and culture-based techniques. Microbial analysis was supported with chemical analysis based on physicochemical parameters monitored from the bioreactors. By combining these techniques, a clear depiction of the factors influencing and changes in an anaerobic microbial community, exposed to different types of persulfate treatments, was illustrated.

Chapter 4. Discussion

There is increasing awareness of contaminants that can pose a risk to the environment as well as to human health. Groundwater contamination is of particular interest for a variety of reasons, one of which is its direct impacts on water supply. In particular, petroleum hydrocarbons (PHCs), including persistent and toxic aromatic PHCs (such as the contaminants studied in this research (benzene, toluene, ethylbenzene, and xylenes (BTEX))), have been found to be a main source of groundwater contamination throughout the world. The subsurface and groundwater remediation sector, on the international stage, was predicted to be valued between $50-60 billion dollars in 2009 (Singh, 2009). From a human, environmental health and economical standpoint, it is important to conduct studies such as the one presented in this thesis to ensure that viable economically, feasible methods for remediation are available for use. This particular study helps provide further insight into a technique that is beginning to see application in the field (ISCO/ISB treatment), and will aid in more accurate predictions on the outcomes of a contaminated site undergoing ISCO/ISB treatment. Oxidants such as persulfate (synthetically or naturally activated) are strong, aggressive detergents used to remove the bulk mass of contaminants. As detergents, the oxidants have initial negative impacts on indigenous microbial communities that may be present in liquid and solid phases of groundwater aquifers. In recent studies it is suggested that microbial communities may be able to partially rebound with respect to diversity and richness, although the remediation capabilities of the recovered community may be reduced (Richardson *et al.*, 2011; Sutton *et al.*, 2014; Cassidy *et al.*, 2015). However, microbial recovery needs to be further

investigated with respect to different types of ISCO treatments and the effects of those treatments on different microbial communities (i.e. anaerobic communities) that are present in heterogeneous environments like groundwater aquifers. The results of this study will be discussed, for each treatment approach, in order to more thoroughly assess the impact of persulfate-mediated ISCO treatments on the indigenous and degradative anaerobic microbial communities present in freshwater aquifers.

4.1 The Development of Anoxic Bioreactors Containing Indigenous Microbial Communities from a Freshwater Aquifer

In order to properly depict the effects of persulfate on an indigenous microbial community on a bench-scale level, bioreactors were created that contained subsurface sediment from a freshwater aquifer in Borden, Ontario. The microbial communities within the core sediment were allowed to acclimate to the bioreactor/lab setting until the end of February (90 days), when the first contaminant injection took place. The successful inoculation of a sulfate-reducing community can be attributed to a few factors. The core material was immediately brought into an anaerobic chamber for packing into the columns so that by-products, oxygen etc. would not accumulate in the cores, potentially causing a loss of viable anaerobic microorganisms. Also, once the bioreactors were packed, groundwater from the Borden aquifer was used to saturate the columns, as well as provide a further source of inoculation for indigenous microbes. Ensuring that the DO and ORP of the water was relatively low (below 1 mg/L and $\leq$ 0 mV, respectively) also attributed to the successful development of anaerobic bioreactors (Müller *et al*., 2009; Shayan, 2015).

It can be concluded following an examination of the raw images of both the universal (Figures 3.3-3.6) and SRB-based (Figures 3.7 & 3.8) DGGE gels that the microbial community structure alters following the transfer from the environment to a lab-scale setting, even though the community was contained within the same sediment. This type of alteration has been reported by others previously (Chandler *et al*., 1997). Changes from the environment to the lab are a common and unavoidable factor when moving from *in situ* to *ex situ* environments, even when significant steps are taken to limit changes (such as the ones mentioned in this study). Structural changes are illustrated between the first 4 sample sets (lanes 1-4) on the universal-based DGGE gels, as the communities appear to contain dominant OTUS with a lower G+C content (lower band movement groups) initially and then fluctuate to a more moderately rich G+C OTU-dominated community. From this an observable shift in the community dynamics from the field-scale to the bench scale was evident. A build-up of by-products and the bioavailability of nutrients can be partially attributed to the changes observed in community structure (Chandler *et al*., 1997; Müller *et al*., 2009). It is difficult, if not impossible, to perfectly mimic environmental geochemical conditions, especially for anaerobic bacterial communities, even with initially injecting groundwater from the site. In this study, the main contributor to structural changes is most likely temperature as the bioreactors were maintained at room temperature (~21^{O}C). The aquifer at Borden fluctuates around 10^{O}C (Butler *et al*. 1997). In turn, the microbial community experiences a fluctuation of more than 10^{O}C from the natural environment to the lab-setting which can drastically affect the metabolic activity of different microbes, causing a portion of the population to die or become dormant while others become

metabolically active (Müller *et al*., 2009). The fluctuations in population structure present some unknowns with respect to the application of this bench-scale experiment to a pilot-scale study. However, the microbes present in the bioreactors were still indigenous organisms to a freshwater aquifer and likely reflect a more realistic study with respect to aquifer conditions than an outside source inoculum.

During the development phase of the bioreactors, lactate-amendment was used to ensure the growth and conservation of SRB prior to the ISCO treatment. Lactate is a source of electron donor that only SRB can utilize and it was used in order to limit methanogens from prospering over SRB (Acton & Barker, 1992; Stams *et al*., 2005; Dar *et al*., 2008). Methanogens are not desirable as they produce methane, and if trapped, could generate potentially dangerous pockets of the combustible compound within the subsurface sediment (Ma *et al*., 2012). Typically, SRB and methanogens are found together co-metabolizing substrates (e.g. acetate) in the environment (Barker and Acton, 1992; Stams *et al*., 2005) and this was the same in the bioreactors for this study, as methanogenesis was observed in all bioreactors, as well as sulfate reduction (Figure 3.9). As mentioned, methanogens can utilize acetate, which is an intermediate by-product in the breakdown of lactate, and thus it seems inevitable that some methanogenesis would occur. However, if sulfate is not in limited supply, SRB can typically co-exist or outcompete methanogens for electron donors (Dar *et al*. 2008; Stams *et al*. 2005; Talbot *et al*. 2008; Barker & Acton, 1992). In this study, sulfate was always kept above 1mM to ensure the sulfate-reducers could dominate over methanogens (Lovely & Klug, 1983; Barker & Acton, 1992).

An initial BTEX injection took place at the beginning of March (2015) to begin initial steps towards the oxidant injection phase. A relatively large concentration of BTEX (~80 mg/L; in comparison to the ~3-4 mg/L used in the second contaminant injection) was injected into the bioreactors in unison with arresting lactate addition. The introduction of 80 mg/L BTEX caused a significant 'unsaturation' event immediately following PHC exposure. Unsaturation of the bioreactors can create unpredictable side-effects and thus became a concern (Petri *et al*., 2011). Initially, the unsaturation was speculated to be due to an increase in methane production in the columns, which was between 4-6 ppm following the contaminant injection. Since methanogenesis is also not desirable, contaminant injection was stopped. However, it is difficult to place the responsibility of the unsaturation event on a methanogenesis dominated system as methane concentrations in the bioreactors prior to the contaminant injection were not analyzed. Furthermore, sulfate concentrations were kept above 1 mM, limiting methanogens from outcompeting SRB. Once contaminant exposure was ceased, lactate was re-introduced into the columns to biostimulate SRB. In addition, methane concentrations did not decrease significantly, which also questions whether the unsaturation was due strictly to methane production. In the future, other avenues of responsibility for the unsaturation event need to be explored. Speculation about the formation of CO_2, from microbial mineralization of BTEX, causing the unsaturation event could be a future avenue of exploration (Beller *et al*., 1992; Affek *et al*., 1998). The unsaturation event was also exemplified by a measurable reduction in column weights (≥20 g reduction; Figure 3.11), indicating a potential reduction in biomass as well as loss of water. Although the unsaturation was always visibly present, a return of

bioreactor weights was observed and a second contaminant injection took place in July, which was followed by the 20 g/L persulfate treatment phase in September.

The second attempt at BTEX exposure began at the start of July. BTEX biodegradation throughout July gradually increased (Figure 3.10) up to 70% BTEX reduction 5-weeks post-contaminant exposure. The residence time (the time it takes for one pore volume to pass through the bioreactors) of BTEX in the columns was approximately 1.5 days. Overall, the biodegration potential of the bioreactors was as high as 5.71 µg/L per hour. The plateau in percent reduction was met by a slight decline on the August 18th sampling date. The slight decline in BTEX biodegration corresponded with the cessation of lactate amendment. However, even without lactate, the anaerobic microbial community was able to biodegrade over half the contaminants injected within the 1.5-day residence period (refer to Figure 3.38).

4.2 Treatment Phase

The treatment phase of the experiment included a 24-hour, three pore volume (PV), exposure to 20 g/L unactivated persulfate, alkaline-activated (pH 12; 20 g/L) persulfate, and alkaline (pH 12; pseudo-control for AP1-3) containing solutions. Following the 24-hour treatment phase, the bioreactors were monitored for the recovery of an ‘indigenous’ anaerobic microbial community (that is predominately sulfate-reducing), as well as a return in biodegradation potential. A control bioreactor set was also included which contained anaerobic microbial communities and a BTEX plume (solely ISB) throughout the treatment phase of the experiment.

4.2.1 Unactivated Persulfate Treatment

When used as an oxidant for remediation, persulfate can be implemented with or without the use of an activating substance or condition. When persulfate is used without the introduction of an activation catalyst, it can react with natural organic matter (NOM) and other reactive constituents within the soil matrix, becoming activated and splitting into sulfate radicals (Petri *et al*., 2011) . Once activated, persulfate can have deleterious effects on indigenous microorganisms that may otherwise be helpful in subsequent bioremedation/'polishing' of a contaminated site. The following section discusses the effect of unactivated persulfate on anaerobic microbial consortia(s) that are indigenous to a ground water aquifer.

The two culture-based methods used displayed similar trends throughout the experiment. An observable reduction (over two logs) in culturable organisms and SRB, specifically, occurs following an unactivated persulfate treatment. However, a rebound in microbial numbers above pre-treatment levels takes place within one month of the recovery phase. Specifically, in the UP1-UP3 bioreactors a 2-3 log reduction in CFU/mL was observed 4 days (approximately 2 PV) following the unactivated persulfate exposure, whereby nearly complete loss of viable anaerobes occurred, with an average of 14 CFU/mL remaining immediately following treatment. Even without the use of an activator, it is apparent that a 24-hour persulfate exposure has a highly deleterious effect on viable bacteria that may be residing within anoxic portions of a groundwater aquifer. This can be mainly attributed to fluctuations in geochemical conditions such as redox conditions (from reducing to highly oxidizing conditions (≥ +700 mV; Figure 3.43) and pH (~7.71 → ~6.37;

Figure 3.42) generated from the persulfate transition into its constituents (Sutton *et al*., 2010; Petri *et al*., 2011). Twenty-eight days following the persulfate treatment, a noticeable increase in the general bacterial population was detected as counts rose over 4 log, resulting in CFU/mL between 10^5-10^6. The CFU/mL remained between 10^5-10^6 for the remainder of the two-month recovery period. Others have noted 'Enhancement' of the community beyond counts found in pre-injection samples may be attributed to a return to more 'natural' geochemical conditions, as well as the mobilization of NOM from persulfate reacting with the soil matrix, generating increased bioavailability of supporting nutrients for microbial growth (Sirguey *et al.* 2008; Westersund *et al.* 2006). The Most Probable Number method applied to assess relative SRB abundance changes displayed a similar overall trend to that of the CFU/mL analysis. SRB cell counts per mL ranged between ≤ 100 cells per mL prior to treatment (refer to Table 3.1). Immediately following treatment, no evidence of viable SRB cells were recorded. Again, the changes in geochemical conditions due to the aggressiveness of persulfate was most likely the cause of a complete eradication of SRB in the effluent samples. After twenty-eight days post-oxidation phase displayed a return of a viable SRB community, with counts exceeding 1-2 log from pre-treatment samples. Again, this may be attributed to a multitude of factors, including increased availability of nutrients such as NOM or sulfate, since persulfate releases sulfate as it breaks down into sulfate ($\bullet SO_4^{2-}$) and hydroxyl ($\bullet OH^-$) radicals (Petri *et al*., 2011). It can also make sulfate bioavailable due to the breakdown of substances in the soil. Sulfate concentrations increased 2 logs above pre-treatment levels due mainly to its liberation from persulfate (Figure 4.32), indicating a flux of bioavailable sulfate in UP bioreactors.

Although the increase in sulfate occurred immediately following the treatment, the SRB community was no longer present to utilize it. However, with the continued input of sulfate into the bioreactors, as well as any sulfate that had become more bioavailable (from the degradation of the soil matrix) and a return to pre-oxidation geochemical conditions (e.g. ORP: ~ -100 mV (Figure 3.43); pH ~7.7 (Figure 3.42)), an SRB community was able to rebound in numbers exceeding pre-treatment MPN counts. Less competition between microbial groups (e.g. with methanogens) may also play a role in the subsequent proliferation of SRB. Dissolved methane (Figure 3.40) experienced an observable decline (~ 2 log reduction from ~ 6000 ppb to ≤ 100 ppb) following persulfate treatment, and it did not recover to pre-treatment levels for the duration of the trial. This can be an indicator of lower methanogen levels, which would decrease competition with SRB for available nutrients (Stams *et al*., 2005; Dar *et al*., 2008).

The *dsrB* gene is a good molecular indicator for metabolically active SRB as it encodes for an integral and highly conserved enzyme in the sulfate reduction pathway (Geets *et al*., 2006; Kondo *et al*., 2012; Priha *et al*., 2013; Zeleke *et al*., 2013). Quantitative polymerase chain reaction analysis for *dsrB*, combined with the culture-based MPN for SRB, helped provide a more complete picture of the presence of viable SRB. qPCR on the *dsrB* gene revealed relatively similar results to the MPN results with respect to the initial decrease immediately following treatment. Evidently, there was a subsequent increase in the weeks following the first post-oxidation sampling event (Figure 3.17). Immediately following the persulfate injection, *dsrB* gene copies per mL showed a 2 log reduction in all replicates, dropping from 2.21E+04 to 8.51E+01 gene copies per mL on average.

Following the initial post-oxidation sampling event, *dsrB* levels began to rise, and by the 4-week point post-treatment, *dsrB* levels had risen above pre-treatment levels (to 9.48E+04 genes/mL). *dsrB* gene copies remained above pre-treatment levels for the remainder of the experiment, lowering slightly towards the last sampling date, 2-months post-treatment. The slight reduction in the latter sampling events coincides with a slight increase in methane levels (Figures 3.17 and 3.40, respectively), indicating that some competition for resources may be developing as methane may be indicative of an increase in methanogens. Previous research formed the basis for this experiment, found that methanogenesis begins to eventually increase following a persulfate treatment on SRB (Shayan, 2015). Shayan (2015) also observed increases in *dsrB* following a persulfate-mediated ISCO treatment, as was observed in this experiment, indicating a return of an SRB consortium as well. Recently, a similar study completed by Cassidy *et al.* (2015) also revealed a rebound in dissimilatory sulfite reductase (alpha sub-unit) after a persulfate exposure indicating the recovery of an SRB population.

In order to monitor changes in the supporting community, as well as the SRB community more specifically, denaturing gradient gel electrophoresis was implemented. Two types of DGGE were employed. The first was a technique used to obtain/monitor changes in the entire microbial community's genetic 'fingerprint' in each bioreactor. The second provides a similar method to monitor changes in the SRB sub-portion of the community. As previously mentioned, DGGE provides a fingerprint of the community by distributing and grouping G+C similar bands of DNA. By doing so, richness, diversity and shifts in the microbial community were monitored throughout the pre- and post-oxidation

phases of the experimental trial. Changes/recovery in richness (number of species) and diversity (species evenness and richness) helps illustrate the return of a robust microbial community following the treatments. Monitoring shifts in the community structure (average banding patterns) reveals if a similar microbial community returns following treatment or if a novel community, different to the pre-treatment community, recovers.

With respect to the SRB community profile, a large reduction in species richness and diversity occurred in the unactivated persulfate treated columns. Pre-treatment, diversity in UP1-3 ranged between 1 and 2 on the Shannon index for diversity indicating the presence of a robust community within the bioreactors. Following treatment, diversity was reduced to almost zero as die-off ensued meaning the presence of fewer unique microorganisms. Species richness exhibited a similar trend with respect to a notable reduction immediately following oxidant exposure, displaying only 2 bands in UP1 and 0 bands in the other two bioreactors. A decrease in richness indicates that the unactivated persulfate treatment had deleterious effects on the number of species present in the bioreactor microbial communities. UP2 and UP3 bioreactors did however experience a recovery with respect to SRB diversity and richness indicating an increase in the number of SRB species, as well as the ability of indigenous SRB to withstand/recover from the persulfate exposure. UP2 and UP3 SRB communities reached levels slightly higher than the last sample taken pre-treatment which aids in confirming enhancement of the SRB community following treatment. This is most likely due to a decrease in competing microbes, bioaugmentation from sulfate, as well as potential increases in intermediate by-products from the breakdown of PHC and the soil matrix which can be more readily used

by SRB (Shayan, 2015, Cassidy *et al*., 2015). UP1 also experienced an increase following treatment but never reached pre-treatment levels with respect to diversity and richness. This may be attributed to slower development of the SRB consortia in UP1 due to lack of bioavailable nutrients in comparison to the other two replicates although this cannot be confirmed in this study (Sutton *et al*., 2014). The average banding patterns of the replicates revealed a shift in the SRB community from a moderately-high G+C content to very high G+C rich community, with respect to the *dsrB* gene, indicating the development of a novel SRB consortium in comparison to the pre-oxidation community. No precedence exists for monitoring SRB communities exposed to oxidants via *dsrB*-based DGGE so it is difficult to determine why such a change in community structure occurred. Sutton *et al.* (2014) did discover a similar trend via universal DGGE with respect to variation in composition of the general community post-oxidation. New by-products are released through the breakdown/mobilization of constituents in the soil matrix via oxidation which can provide competitive advantages for other bacterial groups, which may not have been previously metabolically active.

Universal DGGE, used to fingerprint the surrounding/supporting community in the bioreactors, displayed a rapid rebound in comparison to the SRB-based DGGE, with respect to species diversity and richness. Within two weeks of post-oxidation, UP1-UP3 began to show richness and diversity levels closer to pre-treatment levels than observed with the SRB-DGGE method. This is most likely due to the gradual decrease in redox conditions to more reducing environments (Figure 3.43). Dominance of different organisms or groups of organisms occurs at specific redox conditions which could attribute

to the delay in a return in SRB richness in diversity in comparison to the surrounding microbes still present after the oxidant exposure (Sutton *et al*., 2014). Shifts in the average banding patterns were not as noticeable as the variation seen between pre- and post-treatment SRB community fingerprints. Community fluctuations (with respect to G+C content) were still observed (Figure 3.31) but the shifts were less noticeable most likely to the smaller denaturing gradient used for universal DGGE. Communities tended to be within a smaller range of band movement groups in universal DGGE than SRB DGGE and that may be attributed to the differences in denaturing gradients. Overall, a different community developed, as predicted from the study completed by Sutton *et al*. (2014), but the community remained moderately rich in G+C content. This could possibly indicate some similarity between pre- and post-oxidation microbial communities as G+C content similar taxa are typically phylogenetically similar (Wayne, *et al.* 1987).

The remaining chemical parameters monitored that were not previously discussed for the unactivated persulfate bioreactors are dissolved hydrogen sulfide (H_2S), ORP and BTEX concentrations (% reduction). With respect to H_2S concentrations, a substantial decrease was observed following the treatment phase indicating a reduction in metabolically active SRB due to the deleterious effects of the oxidant (e.g. increase in redox conditions). Pre-oxidation, H_2S was around 3000 µg/L in the 'UP' replicates. Also, metal sulfide deposits were apparent throughout the reactors, indicated by the darkening of the sediment inside (refer to Appendix Figure A12 (pre-exposure)). Post-oxidation, the H_2S dropped to an average of 67 ppb (September 24th) and then to 2 ppb by October 5th (11 days' post-oxidation). The observed decline is indicative of the cessation of sulfate-

reduction and thus active SRBs two-weeks following a persulfate injection. However, by October 15th (3-weeks post-oxidation) dissolved H_2S began to rebound (~1000 ppb) and by October 28th (34 days' post-treatment) levels escalated beyond pre-treatment levels (~4160 ppb). An increase in H_2S continued until the last sample date, where over a log (≥ 200 mg/L) increase was measured in comparison to pre-treatment levels, further indicating the enhancement of an SRB consortium within 8-weeks following an unactivated persulfate treatment. ORP levels were indicative of a return to reducing conditions as ORP in the effluent was recorded to be ~100mV. Sulfate reduction does not occur until after -150 mV and is optimal at -200mV and these levels were never observed (Acton & Barker, 1992; Cassidy *et al*., 2015). However, it should be noted that measuring ORP in the effluent of the columns may not be completely indicative of redox conditions inside the bioreactors. Including a probe inside the bioreactors may improve representation as all other parameters measured indicate the presence of a high rate of sulfate reduction. Although -150mV was never observed, it was promising that there was a return to reducing conditions. A return that surpassed pre-treatment conditions of approximately -50 mV. A study completed by Geets *et al*. (2005) suggest that monitored redox conditions around or below -100mV is sufficient for sulfate-reduction to occur and that level was reached in all bioreactors, thus indicating the development of an SRB-dominated community.

The BTEX concentrations in the effluent changed significantly following the oxidant injection phase. Prior to treatment exposure, total BTEX concentration being degraded in the bioreactors was between 45-50% of that being injected into the columns (on average 2660 µg/L). Following the injection, the biodegradation potential of the

microbial community was severely hindered, as evidenced by only 20-30% of the contaminant mass being reduced. The reduced biodegradation rate continued throughout the rest of the experiment (refer to Figure 3.38). As a result, the microbial community that recovered within a 2-month time span did not have as strong a BTEX bioremediation capability as the pre-treatment community. Richardson *et al.* (2011) and Sutton *et al*. (2014) discovered similar findings with respect to reduced biodegradation capabilities following persulfate treatments, even with aerobic communities. Sutton *et al.* (2014) revealed that out of the ISCO treatments they tested (Fenton's reagent, permanganate, persulfate, and ozone), persulfate and permanganate were the least suitable for a 'polishing' step via bioremediation. Furthermore, they recorded no biological activity in these columns during the monitoring phase post-oxidation (8 weeks). However, they did suggest that longer recovery times (90 days +) may reveal a better return in the recovery communities' ability to remediate any recalcitrant products.

4.2.2 Alkaline-Activated Persulfate

There are a multitude of ways to activate persulfate upon injection into subsurface sediments and aquifers, such as iron activation, chelated-iron activation, hydrogen peroxide activation, heat activation, and alkaline-activation (Petri *et al*., 2011). As persulfate is one of the relatively newer oxidants being tested for its efficacy in remediation, the different sources of activation are still being explored for effectiveness at sites with vary geochemical characteristics (Petri *et al*., 2011). The type of activation explored in this study is alkaline-activated persulfate. In this case, not only does the persulfate become activated synthetically and potentially have a more immediate effect on

the microbial community, but the presence of pH 12 in the injection solution would presumably also have a deleterious effect (Padan *et al*., 2005). For this reason, a triplicate set of alkaline only (pH 12, no persulfate) bioreactors was used as a 'pseudo-control' to see if the effect was additive or not.

With respect to the culture-based analysis included in this experiment, a similar trend was noticed as seen in the unactivated persulfate treated bioreactors. Figure 3.2 illustrates that the supporting community experienced a noticeable reduction following the treatment phase. On average, between replicates, CFU/mL in AP1-3 showed over a 1.5 log reduction from 7.01E+03 to 1.49 E+02 CFU/mL. Although very similar, the alkaline-activated persulfate columns displayed a smaller/delayed overall reduction in viable cells than the unactivated persulfate replicates. This delay/lessened effect can be attributed to a reducing radical that is produced during an alkaline-activated persulfate application (Petri *et al*., 2011). Although, it should be noted that UP2 was somewhat of an outlier as the reduction was less than one log (3.53E+03 to 4.80E+02 CFU/mL). This may be attributed to preferential flow paths that may have developed in UP2, limiting persulfate exposure to the periphery of the reactor that may have 'pockets' or dead end pores that harbour microbes (Sutton *et al.* 2010; Shayan, 2015). In comparison to the alkaline-only treatment (discussed later), the reduction was more prominent, presumably due to the presence of persulfate. However, it is difficult to determine if the effect is additive (in comparison to persulfate-only and alkaline-only) as both the unactivated and activated treatments displayed CFU/mL reductions to almost 0. One aspect that can be deduced is that persulfate is the main detriment to the indigenous supporting community, with respect to initial

impact. The recovery phase was also similar to that of the unactivated persulfate bioreactors (Figures 3.12 and 3.13). The 2nd sampling date for culture-based results, 28 days post-oxidation, revealed a substantial recovery by the microbial community within the AP bioreactors. The recovery was as large as a 2 log increase (AP1; ~ 10^4 to 10^6). A robust community was maintained throughout the remainder of the 8-week sampling period, indicating the possibility of re-development of an anaerobic microbial community after an oxidant shock by alkaline-activated persulfate. Using qPCR with a universal primer set, Richardson *et al.* (2011) also observed a full recovery of a microbial community within a 30-day time period, post-oxidation.

The MPN method for sulfate reducers revealed a recovery of viable SRB beyond pre-treatment levels (≤ 100 cells per mL) within a 30-day period; to an average of 2510 cells per mL, similar to that of the unactivated persulfate treatment (~ 3010 SRB per mL). The remaining 4-weeks of recovery displayed a continuous increase in viable SRB, with AP2 becoming an outlier on the final sampling date, with an estimated count of 4.3 x 10^5 SRB per mL. UP2s large quantity of SRB could be attributed to the fact that it was the most successful at withstanding the persulfate treatment, displaying 10 cells per mL immediately following treatment (refer to Table 3.1).

The qPCR (Figure 3.18) results on *dsrB* gene indicated a similar trend to the MPN analysis with respect to UP2 withstanding the persulfate treatment, although much more exaggerated. qPCR also revealed a 2 log reduction in AP1, a 1 log reduction in AP3, and a slight increase in *dsrB* in AP2 immediately following the treatment phase, which suggests

that this form of persulfate treatment is detrimental as well. However, it is difficult to depict the true effect of alkaline-activated persulfate from these results solely. If one was to consider AP2 as an outlier, then it would be apparent that this form of ISCO has a substantial negative effect initially. A negative effect can be confirmed by combining the culture-based result with the genetic-based results. Both CFU/mL and MPN for SRB reveal a substantial reduction in the viable microbial community after an alkaline-activated persulfate treatment. It is difficult to determine why the genetic analysis does not display such a trend as vividly as the culture-based results. This is especially portrayed in the alkaline-only columns (discussed later) as the qPCR results (Figure 3.9) revealed a 2 log increase in *dsrB* following pH 12 exposure even though the culture-based results revealed a reduction in the community. qPCR on DNA can provide potentially skewed results as the technique amplifies all targeted gene segments in a sample, not just those from the viable portion of the microbial community (Taskin *et al*., 2011). Therefore, supporting methods are essential, such as the culture-based methods (e.g. MPN for SRB) used in this study. AO1-3 trends will be discussed more in the following section. With respect to the recovery phase of AP1-3, there was a minor lag in the recovery of *dsrB* gene copies when compared to the unactivated persulfate replicates. On the October 9th sampling date, fifteen days post-oxidation, only AP3 displayed a significant increase (2.25E+03 to 1.48E+04 gene copies/mL) in *dsrB* whereas AP1 remained at ~ 10^2 gene copies/mL and AP2 slightly declined. The lag is most likely due to the pH fluctuations experienced in the columns (refer to Figure 3.42). pH was measured as low as 6.28 immediately following the treatment and as high as 9.25 11 days following treatment. The pH did not return to pre-treatment

levels (7.5-7.7) 3-weeks following treatment. ORP fluctuations displayed a similar trend to that of the unactivated persulfate treatment and, therefore, may not contribute as significantly to the lag in *dsrB* presence/recovery. *dsrB* gene copies did recover beyond pre-treatment numbers before one-month post-oxidation, aligning with the observations by others of community recovery within a 4-week span (Cassidy *et al.* 2015; Sutton *et al.* 2014, Richardson *et al.* 2011).

Overall, the SRB community (DGGE) results displayed a similar lag time with respect to initial reductions and then increases in species richness and diversity (refer to Figure 3.24); again with regard to meaning number bands/species (richness) and the combination of species richness and evenness (variation in band intensities) in a single lane (diversity). However, replicate AP1 was an exception to this trend as it experienced almost complete reduction of *dsrB* gene fragments immediately following the treatment phase. This is consistent with the MPN and qPCR for SRB. Although it should be mentioned that AP1 had the lowest community diversity, richness etc. prior to treatment, which can promote/exaggerate the oxidants deleterious effects (Richardson *et al.*, 2011) on an indigenous microbial community. On the other hand, AP2 and AP3 essentially maintained pre-oxidation SRB richness and diversity immediately following treatment. It was not until approximately 2-weeks post-oxidation that the detrimental effects of the alkalinity and the oxidant were observed in AP2 and AP3. Again, the pH fluctuation may have played a role in the delayed negative effects on the community, although this is just speculation. By the October 20th sampling date, 28 days post-oxidation, all replicates were recovering but only AP1 had reached pre-treatment levels at that point. Approximately 6-weeks following

treatment, AP1 and AP3 displayed enhancement of the SRB community with respect to diversity and continued to show that into the 8-week post-treatment period. AP2 never portrayed full recovery throughout the 8-week post-oxidation monitoring period. This was an unexpected result as AP2 displayed a similar or even higher *dsrB* gene copy number throughout most of the experiment in comparison to the other replicates. One possible explanation is that AP2 may contain only a limited variety of strains of SRB that are dominating the bioreactor post-oxidation and thus reduced SRB diversity/richness was observed. With all aspects considered, an SRB consortia did return, although differences in community structure were noted via average banding patterns as the general SRB population became higher in G+C content and more concentrated in fewer band movement groups (Figure 3.14). Band congregation may indicate the recovery of phylogenetically similar organisms (Wayne *et al*. 1987). One might expect that those able to withstand an oxidation treatment may be similar in resistance abilities and potentially genetic orientation.

Immediate impacts of the alkaline-activated persulfate treatment were more evident when examined through universally-based DGGE; in other words, considering the broader community. However, a slight 'lag' in microbial reduction of diversity and richness, in comparison to unactivated persulfate treatment, was still observed. As observed in SRB DGGE, AP1 experienced the largest reduction in community diversity/richness immediately following treatment, whereas AP2 and AP3 exhibited a more noticeable response at the two-week post-oxidation point. Although AP1's microbial community remained reduced at the two-week post-oxidation point as well, indicating a prolonged

delay in community recovery. By the 28-day post-treatment mark, all bioreactors experienced a recovery in the supporting microbial community as evidenced by increases in species diversity and richness (refer to Figure 3.32). However, the recovery was not enhanced beyond pre-treatment levels and it can be argued that on average, complete recovery was never reached within the 8-week recovery period. Incomplete recovery after an alkaline-persulfate treatment is consistent with the results found by Cassidy *et al*. (2015). Of the different forms of persulfate treatment they tested, NaOH/persulfate treatment led to the least degree of recovery, as defined by increases in the number of species (richness). Nevertheless, a supporting microbial community did recover during the post-oxidation monitoring period, as evidenced by a return in contaminant biodegradation. The average banding patterns did not reveal an observable change/shift in the community structure but the communities did become slightly more centralized around moderately rich GC band movement groups. However, as the community recovered a return in diversity was noticed, including OTUs with lower GC content. An increase in the diversity of the structural profiles suggests the development of a more robust community, capable of long-term biodegradation of recalcitrant products (Richardson *et al*., 2011; Sutton *et al*., 2014).

The chemical parameters monitored supported the trends observed in microbial-based analysis. A return to pre-treatment pH (7.5-7.7) (Figure 3.42) around one-month post-treatment is consistent with the return of a microbial community in the AP bioreactors. Also, an initial increase in sulfate, similar to that in the AP bioreactors, and then subsequent decrease was observed following treatment, with the community reducing up to 65% of the sulfate in the respective reservoir (116 mg/L background SO_4^{2-}). The increase in H_2S

following treatment also indicates an increase in sulfate reduction and, therefore, metabolically active SRB (Acton & Barker, 1992). An ORP below -100 mV on average, (Figure 3.43) also indicates a return to reducing conditions within approximately one month's time, providing conditions for anaerobic bacteria to thrive. Additionally, dissolved methane was also significantly reduced, as seen in UP bioreactors as well, following treatment and was maintained at a lower concentration throughout the recovery phase. Methane did begin to increase but never came close to the concentrations that were present in the pre-treatment samples (Figure 3.40). This may have helped with the development a SRB- dominated community as there is less competition for available resources with methanogens, who typically live syntrophically in the anaerobic areas of subsurface sediments during biodegradation processes (Muyzer and Stams, 2008; Plugge *et al*. 2011). The increase in methane near the end of the recovery phase can be reflected in the continual drop in ORP, as methanogenesis takes place at ever lower redox conditions (~-250 mV) (Fetzer and Conrad, 1993; Plugge *et al*. 2011). From the chemical parameters measured in unison with the microbial analysis, it becomes increasingly apparent that a robust SRB community can develop following a 20 g/L alkaline-activated (pH 12) persulfate treatment with an 8-week timeframe under bench-scale conditions.

Since it has been established that a SRB community can recover following a 20 g/L alkaline-activated persulfate treatment, another important aspect was to determine if that community can biodegrade any remaining contaminants. Prior to the treatment, AP microbial communities were degrading approximately 50% of the BTEX in a 2-day time period (length of time to pass 1 pore volume through bioreactors). Following treatment,

the biodegradation rates were reduced by over one-half, with the recovering communities biodegrading 17-26% of the total contaminant mass throughout the 8-week recovery phase. The reduction in BTEX degradation is noticeable but it is promising that the community is still able to reduce/mineralize the recalcitrant to a measurable degree. Assuming that in the natural environment the contaminant plume concentration would not be continuous, unlike this experiment where contaminant concentration remains constant in the reservoir, it is reasonable to propose the recovering indigenous community would be able to eventually 'polish' a contaminated site (Richardson *et al*., 2011; Sutton *et al*., 2014; Cassidy *et al*., 2015).

4.2.3 Alkaline-Only Bioreactors and SRB-Only Bioreactors

The following section will be described in less detail as these replicate sets have been touched on in previous sections. The two types of 'control' column sets were used to distinguish and reflect on the effects of the two different persulfate treatments examined in this study, alkaline-activated and unactivated persulfate.

In order to test whether there was an additive, synergistic or antagonistic detrimental effect(s) of alkaline-activated persulfate on an indigenous, bioremediating SRB community, an alkaline-only treatment (AO1-3) was explored. The results depicted that a change in pH (~7.5 to 12) for a 24-hour time period does have detrimental effects on an indigenous, anaerobic subsurface microbial community. The CFU/mL and MPN for SRB indicated an initial reduction (less than one log on average) in the microbial community, but overall the decline in CFU/mL was not nearly to the extent observed in the persulfate

treated columns (Figures 3.12-3.15; Table 3.1). qPCR for *dsrB* revealed quite the opposite, with *dsrB* genes increasing by almost 2 log (~ 10^4 to 10^6 gene copies per mL) immediately following treatment. However, by the two-week post-treatment mark, the *dsrB* gene copies returned to pre-oxidation levels. A slight increase in *dsrB* was noted around the one-month mark, which may be partially attributed to SRB utilizing nutrient availability due to the lack of competition from other groups such as methanogens, as methane levels were substantially decreased in the AO1-3 post-oxidation as well. This corresponded with an increase in H_2S concentrations following pH 12 exposure. This suggests SRB were able to withstand the shock of a change in pH over methanogens. Other researchers have noted that SRB can typically manage physiological stresses, such as a change in pH or redox potential (as in the persulfate treated column sets), better than their community members (Dolla *et al*. 2006; Cassidy *et al*. 2015). The physical robustness of SRB are likely responsible for the observed increases in sulfate reduction and development of SRB dominated communities throughout the 'treated' bioreactor sets, including AO1-3.

SRB diversity and richness (Figure 3.15) did decline following the alkalinity exposure, suggesting that some SRB strains can withstand physiological stresses more than others. A shift in the SRB community structure further indicated the robustness of some SRB strains over others as the community shifted to a more G+C rich structure, indicating the recovery of a novel SRB consortium. The broader community profile (universal-DGGE; Figure 3.23) did not show reductions (or shifts) in community diversity and richness as much as observed in the SRB-DGGE for AO bioreactors or as seen in the alkaline-persulfate bioreactors. The lack of overall microbial destruction following an

alkalinity exposure is suggestive that persulfate, as a strong oxidant, is the stronger stressor in the initial deterioration of a subsurface, anaerobic microbial community following an alkaline-mediated ISCO treatment. However, it should be noted that the biodegradation of the alkaline-only bioreactors exhibited a similar decrease in biodegradation potential to that of the persulfate treatments. Therefore, it is further elucidated that some microbial groups have the capacity to withstand a physicochemical shock when exposed to environmental extremes better than others. Unfortunately, in the case of the 'treated' column sets (UP1-3, AP1-3, and AO1-3), it seems that the some of the organisms that were functional in the BTEX biodegradation were not as robust as the community that recovered or they may take longer to recover (i.e. $\geq$ 8-weeks). In that case, a longer monitoring period post-oxidation may be required.

In comparison to the treatment columns sets, the control replicates (Con1-3) maintained a relatively constant state. There were no notable fluctuations in the community structure, with respect to reductions or community shifts following the 24-hour treatment phase on September 23rd-24th, observed in the unamended control bioreactors.

However, it should be noted that there were still some minor fluctuations observed in the SRB community structural profile throughout the experiment. The SRB consortia remained relatively diverse throughout the experiment, with OTUs located across more band movement groups (i.e. Con1 had OTUs spread across movement groups 6 through 18 throughout the experiment) than seen in any of the treatment column sets. The diversity and fluctuations observed in Figures 3.17 and 3.18 may be attributed to constant cycling

of metabolic by-products and intermediates, as some SRB can only utilize a small range of nutrients whereas others, such as PHC-degrading *Desulfosporosinus meridiei*, can degrade intermediate by-products as well (Beller *et* al. 1992; Morasch *et al*. 2003; Plugge *et al.* 2011). Nevertheless, band movement groups that contained OTUs remained relatively constant which was important as a reference point for monitoring the treatment responses.

When examining the universal-DGGE results, species diversity and richness fluctuated and declined to some extent. This may be attributed to the build-up of toxic metabolic by-products, such as H_2S, which can be toxic in high concentrations (250-550 mg/L for SRB and even lower for non-SRB) to all bacteria, even SRB themselves (Reis *et al*. 1992). Although some fluctuations were present in Con1-3 throughout the experiment, the structural fingerprint of the community remained constant (Figure 3.26). A community comprised mainly of moderately G+C rich OTUs (and therefore phylogenetically similar OTUs (Wayne *et al*., 1987)) was maintained which provides a good comparison for the treatment bioreactor sets, which all experienced structural changes following the 24-treatment phase between September 23rd-24th.

The chemical parameters for Con1-3 yielded reasonably consistent results throughout the experiment as well, with the exception of H_2S which experienced a one log increase (~10^3 to ~10^4) between September 24th and October 5th. Methane, pH and BTEX degradation remained relatively constant throughout the experiment. With respect to H_2S, it is difficult to determine what caused such an increase in the control columns. An increase in MPN for SRB was noted in the control columns, but not to the extent that would correlate

with the increase in H_2S. The *dsrB* gene copies also remained relatively constant (~between 10^4-10^5 throughout). It can be speculated that the available metals (e.g. iron), which H_2S binds with to form metal sulfides, became saturated or unavailable, allowing some of the produced H_2S to leave the columns as dissolved H_2S in the effluent. However, when examining the sulfate concentration in the effluent, it is apparent that sulfate reduction did increase noticeably following September 24th. Therefore, increased sulfate reduction may be attributed to SRB gaining a competitive advantage over other competing microbes in the columns. Furthermore, ORP decreased even more in the control columns following September 24th (Figure 3.43). This may be indicative of a change in dominance towards a mainly SRB-based community. However, the reason for increased sulfate reduction may go beyond that. For example, on October 5th bacterial contamination due to back-feed of microbes from the columns into the reservoir was noticed. This continued to be a problem throughout the remainder of the experiment even though pre-injection lines were sterilized and reservoir bags were changed. Continual back-feed of microbes was most likely due to the deterioration of the glass wool/screens put in place to hold the soil matrix in the columns. In turn, the microbes in the reservoirs began to drop the ORP before the injection solution even reached the columns, providing an even further reduced medium for SRB to thrive, along with the competitive advantages they already experienced (i.e. increased electron acceptors (sulfate), physical robustness). Also, it may be possible that the SRB producing the H_2S are not culturable or detectable (qPCR) with the growth conditions and primers used, although this seems unlikely due to highly conserved nature of the target gene (Geets *et al.*, 2006).

Although the control columns experienced some unexpected fluctuations, they worked well for the purpose they served. They maintained BTEX biodegradation levels throughout experiment which help indicate comparatively that unactivated and alkaline-activated persulfate treatments do have a negative impact on a PHC-degrading anaerobic community and, therefore, lowers bioremediation potential of indigenous microbial communities at a contaminated site. Even though a more robust SRB-specific consortia recovered during the 8-week monitoring period, the community's biodegradation potential did not. However, biodegradation by an SRB community is known to be slow to begin with and the remediation potential following these treatments is still promising (Cassidy *et al*., 2015). Other studies have observed reduced biodegradation following ISCO treatments, most notably during persulfate treatments (Shayan, 2015) and more specifically, alkaline-activated persulfate treatments (Sutton *et al.* 2014; Cassidy *et al*. 2015). They also stated that ISCO/ISB remediation techniques using persulfate and SRBs can work. An extended study beyond an 8-week recovery period, such as the 32-week recovery period monitored by Cassidy *et al*. (2015), may have strengthened the results presented as an increased capacity for biodegradation of recalcitrant contaminants following the ISCO treatments may have developed with an extended time-frame.

Chapter 5: Conclusions and Future Perspectives

5.1 Summary

The research presented in this thesis provided significant insight into the changes that can occur following an *in situ* chemical oxidation/*in situ* bioremediation treatment using persulfate, unactivated and alkaline-activated, and a sulfate-reducing bacterial consortium. The primary goal of this study was to determine whether or not an anaerobic microbial community could recover following two types of ISCO treatment: 1) Unactivated persulfate; 2) Alkaline-activated persulfate. Conventional diagnostic methods, mainly microbiology-based, were implemented in order to assess the effects of these persulfate treatments, to mineralize the bulk of the contaminant plume, on the subsequent recovery/use of an indigenous SRB consortia to biodegrade any remaining recalcitrant, as a final 'polishing' step for a PHC-contaminated site. The results depicted in this thesis suggest that indigenous anaerobic communities from aquifer sediments will be temporarily reduced following a 24-hour, 20 g/L persulfate exposure. However, recovery of a novel SRB-dominated community can occur within four-weeks of treatment. The two types of ISCO/ISB techniques explored can be effective, and relatively cost efficient, remediation methods in comparison to conventional approaches, which typically involves more costly removal of contaminated sediments for treatment *ex situ*.

The general conclusions discovered with respect to the primary objectives made are as follows:

Objective 1: Assess the presence/location of sulfate-reducing communities at a field-site in CFB Borden, ON

- It was discovered that there are anoxic portions in the Borden aquifer that support a viable SRB consortia. Determined from the groundwater samples taken and the cores extracted from the aquifer, anaerobic microbial communities, containing SRB, are typically found between 7-11 feet below the ground surface, just above the aquitard.

Objective 2: Develop bioreactors that contain an active SRB community

- The anaerobic consortia extracted from the freshwater aquifer at CFB Borden is culturable in a lab-setting. More specifically, the successful enumeration of indigenous, sulfate-reducing consortia in bioreactors was completed. Furthermore, the anaerobic microbial community present in the bioreactors had BTEX biodegradation capabilities prior to the treatment phase, degrading up to 5.7 µg/L per hour (total PHC).

Objective 3: Observe the effects of the persulfate exposures on SRBs and their supporting microbial communities

- As revealed by SRB- and universally-based DGGE, the microbial community that returns following a persulfate exposure differs in its structure dynamics. Changes

were expected as bacterial groups can have different capabilities to withstand environmental stresses. However, when discerning the aspects of field-scale application, differences in structure may only be temporary as the influx of indigenous organisms and the ensuing return to 'natural' geochemical conditions will more than likely preference the microbial community structure to return to that present prior to site contamination.

- Following the initial post-oxidation decline, a novel microbial community (different in structure from the pre-treatment community), most notably SRB, recovered beyond that which was present pre-treatment. The increase observed can potentially be viewed as an enhancement of the community, even though the recovered community exhibited a decrease in BTEX biodegration capability throughout the 8-week post-oxidation observation period. This decrease in biodegradation capability was similar between the unactivated and alkaline-activated persulfate treatments (reaction rates decreased from ~ 5.7 µg/L per hour to 2.5-2.7 µg/L per hour total PHC, on average). Longer recovery may reveal a full return in biodegradation potential of the microbial community (Cassidy *et al*., 2015).

Further contributions of the findings contained within this thesis study are as follows:

- This study illustrated the importance of continuing the implementation of culture-based analysis to complement more modern genetic-based techniques. An example of this was the use of Most Probable Number (MPN) method for sulfate-reducers and qPCR technique for the *dsrB* gene segment. An increase in *dsrB* gene was observed following the alkaline-based treatments, which would suggest an immediate increase of SRB following alkaline-activated persulfate treatment. However, as MPN for sulfate-reducers revealed, viable/metabolically active SRB were severely hindered following treatment and numbers did not begin to increase until at least a week post-exposure. CFU/mL also confirmed the reduction in microbial load.
- Confirmation of anaerobic microbial communities containing sulfate-reducing bacteria in shallow, sandy aquifers (most notably at CFB Borden). The SRB consortia contained within these sediments can be enhanced via reduction of competing microbes and inclusion of bioaugmentation (e.g. sulfate/lactate amendment), which occurs during a persulfate treatment (with the exception of lactate addition). It was also discovered that eliminating a source of bioaugmentation, in this case lactate, can initially reduce the activity of a target organism or group of organisms, such as SRB, prior to an ISCO treatment.
- The amount of contaminant present at a site may dictate the diversity of the biodegrading portion of the microbial community. Following the initial contaminant injections in March, an unsaturation event occurred in the columns due

to an increase in gas production potentially attributed to methanogenesis. This was most likely a result of too high a level of BTEX introduced into the bioreactors in parallel with the elimination of biostimulation of SRB. The abundance of organics available, with a subsequent halt in bioaugmentation of SRB via lactate, likely contributed to a shift in community dynamics.

- The generation of sulfate during a persulfate-based ISCO treatment may stimulate the recovery of a SRB-dominated community. However, with the way the bioreactor(s) setup was structured (small (1L) capacity with a relatively fast turnover rate (~ 1.5 days to pass 1 pore volume), it is difficult to determine the true effect of sulfate production during a persulfate treatment. The sulfate was flushed out of the columns within 2-days' post-treatment. However, sulfate was maintained above 100 mg/L following treatment, providing significant amounts of electron acceptors for SRB recovery and enhancement.
- Sulfate-reducing bacteria have the ability to withstand an unactivated and alkaline-activated persulfate treatment more so than many of the supporting anaerobic microbial population, notably methanogens. The recovery of a SRB dominated community, as well as the observed reduction in methanogens, supports the idea that a persulfate treatment can be implemented in combination with a SRB bioremediation 'polishing' step.
- Although an SRB-dominated community returned following the treatments examined in this thesis, it appears that over time methanogenic activity begins to return as well, as illustrated by the increase in methane levels during the last two

sampling events. An increase in methanogenesis is most likely due to a combination of aspects, most notably the continued decrease in redox conditions following a persulfate treatment and the availability/production of by-products that accumulate, increasing the bioavailability of nutrients that can support methanogenic activities. Methanogens and sulfate-reducing bacteria are typically found together in anaerobic portions of subsurface sediments and, thus, the re-establishment of methanogens is highly likely. In particular in field-scale applications, indigenous organisms can be re-inoculated into treatment zones, following the implementation of ISCO, from up-gradient portions of an aquifer.

As an aggressive oxidant, persulfate has deleterious effects on the microbial communities that are found in groundwater aquifers. Overall, the results presented in this study indicate the theory of using an ISCO/ISB treatment train can be very useful when looking at *in situ* remediation technology. Richness and diversity of a microbial community can rebound after being treated within a 4-week period. However, an anaerobic bacterial community can experience negative effects with respect to biodegradation capabilities initially. A decrease in biodegradation potential was seen at the lab-scale level. However, with a longer recovery time, re-inoculation of indigenous microbes from up-stream gradients, and the fact that SRB exhibit relatively low biodegradation rates, the treatment process examined would be suitable to support long-term bioremediation of PHC-saturated zones at a site by native SRB and their supporting microbial community members.

5.2 Future Directions and Recommendations

1. In order to generate a more thorough evaluation of a 'treatment train' that utilizes persulfate and *in situ* bioremediation via SBR, the application needs to be further assessed at the field scale. Field scale applications can present challenges not presented in lab. Dr. Shayan's experiment attempted to conquer some of these aspects by taking the proposed method to a 'closed' system set-up in the field. Overall, the study illustrated the successful application of the treatment train technique, however, difficulties may arise as not all brownfield sites can be contained within such a controlled/closed system.
2. With respect to the lab-scale setting, a more precise and clear depiction of the physicochemical parameters would be provided with constant monitoring via flow through probes at the source zone of contamination rather than just monitoring the dissolved portion of the plume. Cassidy *et al*. (2015) were able to capture a more precise and representable interpretation of ORP values by including stationary probes within their bioreactors which provide real-time monitoring and analysis at the point of contaminant mineralization.
3. Considering different methods of activation of persulfate is also an area of ISCO/ISB treatment that needs to be further examined. There are numerous activation methods available for persulfate treatment (e.g. iron-activators, heat application, hydrogen peroxide) and their effects on an indigenous microbial community are not well-documented. It is recommended that these types of applications be examined first at the bench-scale level and then taken to a pilot-

scale level in the field. Also, the application and effects of alkaline-activation at the pilot-scale level is an area that needs to be tested and better understood.

4. Further monitoring of geochemical parameters and by-products (e.g. dissolved inorganic carbon, sulfate, sulfide, methane) generated may provide a more in-depth view of the effects of the treatment train 'footprint'. The ISCO/ISB remedy may be further described and the impact of such a treatment on the natural geochemical conditions following treatment can be established. It is important to know whether conditions can return to that pre-contamination, following a source contamination and subsequent remediation, which involves manipulation/changing of subsurface conditions for an extended period of time.
5. Isotope analysis would provide a detailed view of the mass removal processes completed by both microbial biodegradation and chemical oxidation. Compound-Specific Isotope Analysis (CSIA) would be an excellent method for this type of analysis. CSIA would provide a more complete quantitative measurement of the temporal changes in rates of chemical oxidation/SRB processes through a persulfate treatment.
6. With respect to the microbial-based analysis completed in this study, there are extenuations of methods used that will provide a more descriptive analysis of the effects of the 24-hour persulfate treatment on the indigenous, anaerobic microbial community examined herein. With regard to universal- and SRB-based DGGE, the next step would be to continue the band excision protocol for sequencing. DGGE band sequencing is a common technique implemented to reveal homologies

between organisms in a microbial community. As mentioned, this technique was attempted but the bands revealed were a mixture of sequences. Re-amplification of PCR products, re-running gels, and excising bands multiple times is an addition to the protocol that will aid in the successful acquisition of single sequences (Priha *et al*. 2013).

7. With regard to qPCR analysis, further investigation and optimization using a wider range of primer sets (as well as the primer sets in this study) is recommended. Since this study focused on an anaerobic bacterial consortium, it is difficult to obtain and maintain pure cultures that are used for qPCR standards, as anaerobes can require very specific environments for growth. Without a pure strain, it is difficult to determine primer specificity as unknown samples contain multiple sequences with a high degree of homology, but differ slightly in nucleotide content, therefore, producing differences in melting peaks during qPCR. These differences make it difficult to determine if the proper segment of DNA is being amplified during qPCR that uses a double-stranded DNA binding dye, like that used in this study. Taqman-based qPCR, which includes the addition of probes as well as primers, helps increase specificity of amplification and may provide a more precise method of quantification when a pure strain is not available as a standard.
8. In addition to the suggestions for further microbial analysis, an investigation into which bacteria are specifically present/attached to the soil matrix would provide a more comprehensive view of the microbial populations within the bioreactors. Attached and suspended microbial communities can display variability in

subsurface sediments/aquifers and thus, optimally, both should be explored (Lehman *et al*., 2001; Lehman, Colwell & Bala, 2001). In this thesis, only the microbial community suspended in the liquid portion of the media was examined.

9. Finally, model parameters (e.g. water chemistry, soil composition, flow and transport kinetics etc.) for this experiment were based on site specific conditions. In turn, further exploration into uncertain/different model parameters, and the inherent performance of these ISCO/ISB remedies, may yield a more complete illustration of effectiveness of persulfate applications at contaminated sites with different physical and chemical boundaries/properties.

Chapter 6. Integrative Nature of this Research

Integrative biology is an essential part of a somewhat divided scientific community. It allows for the sub-disciplines of biology, and other research areas of science, to be analyzed on a broad spectrum and as a unified collection. With reference to research presented in this thesis, a variety of expertise is valued as there are many variables. More specifically, working on a remediation process for petroleum-contaminated ground aquifers presents a lot of different biological, geochemical and environmental engineering barriers. The research presented revolves around the bioremediation aspects of this process, using sulfate-reducing bacteria as the catalyst for long-term revival of a brownfield/contaminated site. However, the integration of research areas such as molecular biology, microbiology (microbial ecology), hydrogeology (e.g. porosity of the sediment present), geochemistry, as well as physics are necessary to understand the scope of applying an ISCO/ISB 'treatment train' at various brownfield sites.

REFERENCES

Acton, D. W., & Barker, J. F. (1992). In situ biodegradation potential of aromatic hydrocarbons in anaerobic groundwaters. *Journal of Contaminant Hydrology*, *9*(4), 325–352.

Affek, H. P., Ronen, D., & Yakir, D. (1998). Production of CO 2 in the capillary fringe of a deep phreatic aquifer. *Water Resources Research*, *34*(5), 989–996.

Allen-King, R. M., Barker, J. F., Gillham, R. W., & Jensen, B. K. (1994). Substrate- and nutrient-limited toluene biotransformation in sandy soil. *Environmental Toxicology and Chemistry*, *13*(5), 693–705.

Anderson, R. T., & Lovley, D.R. (2000). Anaerobic Bioremediation of Benzene under Sulfate-Reducing Conditions in a Petroleum-Contaminated Aquifer. *Environmental Science & Technology*, *34*(11), 2261 – 2266.

Baker, M. D., Inniss, W. E., Mayfield, C. I., & Wong, P. T. S. (1982). Effect of pH on the growth and activity of heterotrophic sediment microorganisms. *Chemosphere*, *11*(10), 973–983.

Bamforth, S. M. & Singleton, I. (2005). Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology & Biotechnology*, *80*(7), 723 – 736.

Barbaro, J., Barker, J., Lemon, L., & Mayfield, C. . (1992). Biotransformation of BTEX under anaerobic, denitrifying conditions: Field and laboratory observations. *Journal of Contaminant Hydrology*, *11*(3-4), 245–272.

Barcina, I., & Arana, I. (2009). The viable but nonculturable phenotype: a crossroads in the life-cycle of non-differentiating bacteria? *Reviews in Environmental Science and Bio/Technology*, *8*(3), 245–255.

Barker, J. P., Patrick, G. C., & Major, D. (1987). Natural Attenuation of Aromatic Hydrocarbons in a Shallow Sand Aquifer. *Ground Water Monitoring & Remediation*, *7*(1), 64–71.

Beller, H. R., Kane, S. R., Legler, T. C., McKelvie, J. R., Sherwood Lollar, B., Pearson, F., Mackay, D. M. (2008). Comparative Assessments of Benzene, Toluene, and Xylene Natural Attenuation by Quantitative Polymerase Chain Reaction Analysis of a Catabolic Gene, Signature Metabolites, and Compound-Specific Isotope Analysis. *Environmental Science & Technology*, *42*(16), 6065–6072.

Beller, H. R., Reinhard, M., & Grbić-Galić, D. (1992). Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. *Applied and Environmental Microbiology*, *58*(9), 3192–5.

Bhupathiraju, V. K., Hernandez, M., Landfear, D., & Alvarez-Cohen, L. (1999). Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria. *Journal of Microbiological Methods*, *37*(3), 231–243.

BIOLOG, Inc. BIOLOG EcoPlate™ Microbial Community Analysis [Internet]. Hayward (CA) [cited 2014 May 10]. Available from http://www.BIOLOG™.com

Bombach, P., Richnow, H. H., Kästner, M., & Fischer, A. (2010). Current approaches for the assessment of in situ biodegradation. *Applied Microbiology and Biotechnology*, *86*(3), 839–52. doi:10.1007/s00253-010-2461-2

Borglin, S., Joyner, D., Jacobsen, J., Mukhopadhyay, A., & Hazen, T. C. (2009). Overcoming the anaerobic hurdle in phenotypic microarrays: generation and visualization of growth curve data for Desulfovibrio vulgaris Hildenborough. *Journal of microbiological methods*, *76*(2), 159–68.

Bowman, K. S., Moe, W. M., Rash, B. A., Bae, H.-S., & Rainey, F. A. (2006). Bacterial diversity of an acidic Louisiana groundwater contaminated by dense nonaqueous-phase liquid containing chloroethanes and other solvents. *FEMS Microbiology Ecology*, *58*(1), 120–33.

Britton, L. J., & Greeson, P. E. (1987). Chapter A4: Methods for Collection and analysis of Aquatic Biological and Microbiological Samples. In *Techniques of Water-Resources Investigations of the United States Geological Survey* (pp. 73–77).

Butler, B. J., Barbaro, S. E., Crocker, F. H., & Mayfield, C. I. (1997). Characterization of microbial populations of the borden aquifer. *Geomicrobiology Journal*, *14*(4), 253–268.

Canadian Environmental Protection Act (1999). *New Substances Notification Regulations (Organisms).* Accessed online: May 18, 2014 from: https://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=1B4C0B62-1

Cassidy, D., Northup, A., & Hampton, D. (2009). The effect of three chemical oxidants on subsequent biodegradation of 2,4-dinitrotoluene (DNT) in batch slurry reactors. *Journal of Chemical Technology & Biotechnology*, *84*(6), 820–826.

Cassidy, D. P., Srivastava, V. J., Dombrowski, F. J., & Lingle, J. W. (2015). Combining in situ chemical oxidation, stabilization, and anaerobic bioremediation in a single

application to reduce contaminant mass and leachability in soil. *Journal of Hazardous Materials*, *297*, 347–55.

Castro, H. F., Williams, N. H., & Ogram, A. (2000). Phylogeny of sulfate-reducing bacteria1. *Fems Microbiology Ecology*, *31*(1), 1–9.

Chandler, D. P., Brockman, F. J., & Fredrickson, J. K. (1997). Use of 16S rDNA clone libraries to study changes in a microbial community resulting from ex situ perturbation of a subsurface sediment. *FEMS Microbiology Reviews*, *20*(3-4), 217–230.

Chang, Y. J., Peacock, A. D., Long, P. E., Stephen, J. R., McKinley, J. P., Macnaughton, S. J., … White, D. C. (2001). Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. *Applied and Environmental Microbiology*, *67*(7), 3149–60.

Chen, I. -chu. (2013). Using risk maps to link land value damage and risk as basis of flexible risk management for brownfield redevelopment. *Chemosphere*, *90*(7), 2101 – 2108.

Christian, B. W. (2007). Key Issues Concerning Biolog Use for Aerobic and Anaerobic Freshwater Bacterial Community-Level Physiological Profiling. *International Review of Hydrobiology*, *91*(3), 257 – 268.

Colin, Y., Goñi-Urriza, M., Caumette, P., & Guyoneaud, R. (2013). Combination of high throughput cultivation and dsrA sequencing for assessment of sulfate-reducing bacteria diversity in sediments. *FEMS Microbiology Ecology*, *83*(1), 26–37.

Dar, S. A., Kleerebezem, R., Stams, A. J. M., Kuenen, J. G., & Muyzer, G. (2008). Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. *Applied Microbiology and Biotechnology*, *78*(6), 1045–55.

Dar, S. A., Kuenen, J. G., Muyzer, G., & Al, D. A. R. E. T. (2005). Nested PCR-Denaturing Gradient Gel Electrophoresis Approach To Determine the Diversity of Sulfate-Reducing Bacteria in Complex Microbial Communities, *71*(5), 2325–2330.

Dar, S. A., Yao, L., van Dongen, U., Kuenen, J. G., & Muyzer, G. (2007). Analysis of diversity and activity of sulfate-reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and *dsrB* genes as molecular markers. *Applied and Environmental Microbiology*, *73*(2), 594–604.

Devereux, R., Willis, S. G., & Hines, M. E. (1997). Genome Sizes of Desulfovibrio desulfuricans , Desulfovibrio vulgaris , and Desulfobulbus propionicus Estimated by

Pulsed-Field Gel Electrophoresis of Linearized Chromosomal DNA. *Current Microbiology*, *34*(6), 337–339.

Devlin, J. F., & Barker, J. F. (1994). A Semipassive Nutrient Injection Scheme for Enhanced In Situ Bioremediation. *Ground Water*, *32*(3), 374–380.

Dojka, M. A., Hugenholtz, P., Haack, S. K., & Pace, N. R. (1998). Microbial Diversity in a Hydrocarbon- and Chlorinated-Solvent-Contaminated Aquifer Undergoing Intrinsic Bioremediation. *Appl. Envir. Microbiol.*, *64*(10), 3869–3877.

Edwards, E. A., Wills, L. E., Reinhard, M., & Grbić-Galić, D. (1992). Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Applied and Environmental Microbiology*, *58*(3), 794–800

Fetzer, S., & Conrad, R. (1993). Effect of redox potential on methanogenesis by Methanosarcina barkeri. *Archives of Microbiology*, *160*(2), 108–113.

Fichtel, K., Mathes, F., Könneke, M., Cypionka, H., & Engelen, B. (2012). Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. *Frontiers in Microbiology*, *3*, 65.

Flynn, T. M., Sanford, R. a., Santo Domingo, J. W., Ashbolt, N. J., Levine, A. D., & Bethke, C. M. (2012). The active bacterial community in a pristine confined aquifer. *Water Resources Research*, *48*(9), n/a–n/a.

Flynn, T. M., Sanford, R. A., Ryu, H., Bethke, C. M., Levine, A. D., Ashbolt, N. J., & Santo Domingo, J. W. (2013). Functional microbial diversity explains groundwater chemistry in a pristine aquifer. *BMC Microbiology*, *13*(1), 146.

Foti, M., Sorokin, D. Y., Lomans, B., Mussman, M., Zacharova, E. E., Pimenov, N. V, … Muyzer, G. (2007). Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Applied and Environmental Microbiology*, *73*(7), 2093–100.

Fukui, M., Teske, a, Assmus, B., Muyzer, G., & Widdel, F. (1999). Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (genus desulfonema). *Archives of Microbiology*, *172*(4), 193–203.

Geets, J., Borremans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D., & Vanbroekhoven, K. (2006). *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. *Journal of Microbiological Methods*, *66*(2), 194–205.

Geets, J., Borremans, B., Vangronsveld, J., Diels, L., & Lelie, D. van der. (2005). Molecular Monitoring of SRB Community Structure and Dynamics in Batch Experiments to Examine the Applicability of in situ Precipitation of Heavy Metals for Groundwater Remediation (15 pp). *Journal of Soils and Sediments*, *5*(3), 149–163.

Ghazy, E. A., Mahmoud, M. G., Asker, M. S., Mahmoud, M. N., Elsoud, M. M. A., & Sami, M. E. A. (2011). Cultivation and Detection of Sulfate Reducing Bacteria (SRB) in Sea Water, *7*(2), 604–608.

Giloteaux, L., Goñi-Urriza, M., & Duran, R. (2010). Nested PCR and new primers for analysis of sulfate-reducing bacteria in low-cell-biomass environments. *Applied and Environmental Microbiology*, *76*(9), 2856–65.

Green, S.J., Leigh, M.B., Neufeld, J.D. 2009. Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. In: Timmins, K.N. (ed). *Microbiology of Hydrocarbon, Oil, Lipids and Derived Compounds*. Springer-Verlag. Heidelberg, Germany. Pp. 4137-4158.

Juwarkar, A. (2010). A comprehensive overview of elements in bioremediation. *Reviews in Environmental Science and Bio/Technology*, *9*(3), 215 – 288.

Kleikemper, J., Schroth, M. H., Sigler, W. V, Schmucki, M., Bernasconi, S. M., & Zeyer, J. (2002). Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. *Applied and Environmental Microbiology*, *68*(4), 1516–23.

Kondo, R., Mori, Y., & Sakami, T. (2012). Comparison of Sulphate-reducing Bacterial Communities in Japanese Fish Farm Sediments with Different Levels of Organic Enrichment. *Microbes and Environments*, *27*(2), 193–199.

Kondo, R., Nedwell, D. B., Purdy, K. J., & Silva, S. Q. (2004). Detection and Enumeration of Sulphate-Reducing Bacteria in Estuarine Sediments by Competitive PCR. *Geomicrobiology Journal*, *21*(3), 145–157.

Lehman, R. M., Colwell, F. S., & Bala, G. A. (2001). Attached and unattached microbial communities in a simulated basalt aquifer under fracture- and porous-flow conditions. *Applied and Environmental Microbiology*, *67*(6), 2799–809.

Lehman, R. M., Roberto, F. F., Earley, D., Bruhn, D. F., Brink, S. E., O'Connell, S. P., Colwell, F. S. (2001). Attached and unattached bacterial communities in a 120-meter corehole in an acidic, crystalline rock aquifer. *Applied and Environmental Microbiology*, *67*(5), 2095–106.

Li, H., Zhang, Y., Kravchenko, I., Xu, H., & Zhang, C. (2007). Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds: a laboratory experiment. *Journal of Environmental Sciences (China)*, *19*(8), 1003–13.

Lovley, D. R. (1997). Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. *Journal of Industrial Microbiology & Biotechnology*, *18*(2-3), 75 – 81.

Lovley, D. R., & Klug, M. J. (1983). Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. *Applied and Environmental Microbiology*, *45*(1), 187–92.

Ma, J., Rixey, W. G., DeVaull, G. E., Stafford, B. P., & Alvarez, P. J. J. (2012). Methane bioattenuation and implications for explosion risk reduction along the groundwater to soil surface pathway above a plume of dissolved ethanol. *Environmental Science & Technology*, *46*(11), 6013–9.

Margesin, R., Zimmerbauer, A., & Schinner, F. (2000). Monitoring of bioremediation by soil biological activities. *Chemosphere*, *40*(4), 339–346.

Mathies, C., Xiong, W., & Bradshaw, K., Carlson, T., Tang, K. (2010). Enhanced Anaerobic Biodegradation through Sulfate Reduction Induced by the Addition of Nitrate and Phosphate. Case Study. (Stantec Consulting Ltd., Saskatoon, Saskatchewan, Canada; Federated Co-operative Ltd., Saskatoon, Saskatchewan, Canada).

Miletto, M., Bodelier, P. L. E., & Laanbroek, H. J. (2007). Improved PCR-DGGE for high resolution diversity screening of complex sulfate-reducing prokaryotic communities in soils and sediments. *Journal of Microbiological Methods*, *70*(1), 103–11.

Miletto, M., Loy, A., Antheunisse, A. M., Loeb, R., Bodelier, P. L. E., & Laanbroek, H. J. (2008). Biogeography of sulfate-reducing prokaryotes in river floodplains. *FEMS Microbiology Ecology*, *64*(3), 395–406.

Morasch, B., Schink, B., Tebbe, C. C., & Meckenstock, R. U. (2004). Degradation of o-xylene and m-xylene by a novel sulfate-reducer belonging to the genus Desulfotomaculum. *Archives of Microbiology*, *181*(6), 407–17.

Müller, A. L., Kjeldsen, K. U., Rattei, T., Pester, M., & Loy, A. (2015). Phylogenetic and environmental diversity of DsrAB-type dissimilatory (bi)sulfite reductases. *The ISME Journal*, *9*(5), 1152–65.

Müller, S., Vogt, C., Laube, M., Harms, H., & Kleinsteuber, S. (2009). Community dynamics within a bacterial consortium during growth on toluene under sulfate-reducing conditions. *FEMS Microbiology Ecology*, *70*(3), 586–96.

Muyzer, G., de Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, *59*(3), 695–700.

Muyzer, G., & Stams, A. J. M. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews. Microbiology*, *6*(6), 441–54. doi:10.1038/nrmicro1892

Neretin, L. N., Schippers, A., Pernthaler, A., Hamann, K., Amann, R., & Jørgensen, B. B. (2003). Quantification of dissimilatory (bi)sulphite reductase gene expression in Desulfobacterium autotrophicum using real-time RT-PCR. *Environmental Microbiology*, *5*(8), 660–671.

Ogino, A., Koshikawa, H., Nakahara, T., & Uchiyama, H. (2001). Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *Journal of Applied Microbiology*, *91*(4), 625–635.

Padan, E., Bibi, E., Ito, M., & Krulwich, T. A. (2005). Alkaline pH homeostasis in bacteria: New insights. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1717*(2), 67–88.

Pelz, O. (2001). Tracing toluene-assimilating sulfate-reducing bacteria using 13C-incorporation in fatty acids and whole-cell hybridization. *FEMS Microbiology Ecology*, *38*(2-3), 123–131.

Pérez-Jiménez, J. R., Young, L. Y., & Kerkhof, L. J. (2001). Molecular characterization of sulfate-reducing bacteria in anaerobic hydrocarbon-degrading consortia and pure cultures using the dissimilatory sulfite reductase (dsrAB) genes. *FEMS Microbiology Ecology*, *35*(2), 145–150.

Petri, G. B., Watts, J. R., Teel, a. M., Huling, S. G., & and Brown, A. (Ed.) (2011). *In Situ Chemical Oxidation for Groundwater remediation*.

Plugge, C. M., Zhang, W., Scholten, J. C. M., & Stams, A. J. M. (2011). Metabolic flexibility of sulfate-reducing bacteria. *Frontiers in Microbiology*, *2*, 81.

Postgate, J. R. 1984. The sulphate reducing bacteria, 2nd ed. Cambridge: Cambridge University Press.

Priha, O., Nyyssönen, M., Bomberg, M., Laitila, A., Simell, J., Kapanen, A., & Juvonen, R. (2013). Application of denaturing high-performance liquid chromatography for monitoring sulfate-reducing bacteria in oil fields. *Applied and Environmental Microbiology*, *79*(17), 5186–96.

Reasoner, D. J.; Geldreich, E. E. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49:1-7.

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

Reis, M. A., Almeida, J. S., Lemos, P. C., & Carrondo, M. J. (1992). Effect of hydrogen sulfide on growth of sulfate reducing bacteria. *Biotechnology and Bioengineering*, *40*(5), 593–600.

Richardson, S. D., Lebron, B. L., Miller, C. T., & Aitken, M. D. (2011). Recovery of phenanthrene-degrading bacteria after simulated in situ persulfate oxidation in contaminated soil. *Environmental Science & Technology*, *45*(2), 719–25.

Robertson, W. J., Bowman, J. P., Franzmann, P. D., & Mee, B. J. (2001). Desulfosporosinus meridiei sp. nov., a spore-forming sulfate-reducing bacterium isolated from gasolene-contaminated groundwater. *International Journal of Systematic and Evolutionary Microbiology*, *51*(Pt 1), 133–40.

Rockne, K. J., Chee-Sanford, J. C., Sanford, R. A., Hedlund, B. P., Staley, J. T., & Strand, S. E. (2000). Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Applied and Environmental Microbiology*, *66*(4), 1595–601.

Röling, W. F. M., Milner, M. G., Jones, D. M., Fratepietro, F., Swannell, R. P. J., Daniel, F., & Head, I. M. (2004). Bacterial community dynamics and hydrocarbon degradation during a field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil. *Applied and Environmental Microbiology*, *70*(5), 2603–13.

Ruwisch, R. C., Kleinitz, W., & Widdel, F. (1987). Sulfate Reducing Bacteria and Their Activities in Oil Production. *Journal of Petroleum Technology*, (January), 97–106.

Santegoeds, C. M., Ferdelman, T. G., Muyzer, G., & de Beer, D. (1998). Structural and Functional Dynamics of Sulfate-Reducing Populations in Bacterial Biofilms. *Appl. Envir. Microbiol.*, *64*(10), 3731–3739.

Santillano, D., Boetius, A., & Ramette, A. (2010). Improved dsrA-based terminal restriction fragment length polymorphism analysis of sulfate-reducing bacteria. *Applied and Environmental Microbiology*, *76*(15), 5308–11.

Shah, M. (2014). An Application of Sequencing Batch Reactors in Microbial Degradation of Benzene, Toluene & Xylene under Anoxic and Micro Aerobic Condition. *Journal of Applied & Environmental Microbiology*, *2*(5), 231–236.

Shayan, M. (2015). Persulfate Oxidation Coupled with Microbial Sulfate Reduction as a Combined Remedy. Doctoral dissertation, Department of Civil and Environmental Engineering, University of Waterloo, ON, Canada. Retrieved from: https://uwspace.uwaterloo.ca/bitstream/handle/10012/9106/Shayan_Moghadam_Mahsa.pdf?sequence=3

Siegrist, R. L., Crimi, M., & Simpkin, T. J. (Eds.). (2011). *In Situ Chemical Oxidation for Groundwater Remediation* (Vol. 3). New York, NY: Springer New York.

Sirguey C, Silva P, Schwartz C, Simonnot MO (2008) Impact of chemical oxidation on soil quality. Chemosphere 72:282–289

Spence, C., Whitehead, T. R., & Cotta, M. A. (2008). Development and comparison of SYBR Green quantitative real-time PCR assays for detection and enumeration of sulfate-reducing bacteria in stored swine manure. *Journal of Applied Microbiology*, *105*(6), 2143–52.

Sra, K. S., Thomson, N. R., & Barker, J. F. (2013). Persulfate Treatment of Dissolved Gasoline Compounds. *Journal of Hazardous, Toxic, and Radioactive Waste*, *17*(1), 9–15.

Staats, M., Braster, M., & Röling, W. F. M. (2011). Molecular diversity and distribution of aromatic hydrocarbon-degrading anaerobes across a landfill leachate plume. *Environmental Microbiology*, *13*(5), 1216–27.

Stams, A. J. M., Plugge, C. M., Bok, F. A. M. De, Houten, B. H. G. W. Van, Lens, P., Dijkman, H., … J, W. (2005). Metabolic interactions in methanogenic and sulfate-reducing bioreactors, 13–20.

Steinberg, L. M., & Regan, J. M. (2008). Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. *Applied and Environmental Microbiology*, *74*(21), 6663–71.

Sun, W., & Cupples, A. M. (2012). Diversity of five anaerobic toluene-degrading microbial communities investigated using stable isotope probing. *Applied and Environmental Microbiology*, *78*(4), 972–80.

Sutton, N. B., Atashgahi, S., van der Wal, J., Wijn, G., Grotenhuis, T., Smidt, H., & Rijnaarts, H. H. M. (2015). Microbial dynamics during and after in situ chemical oxidation of chlorinated solvents. *Ground Water*, *53*(2), 261–70.

Sutton, N. B., Grotenhuis, J. T. C., Langenhoff, A. A. M., & Rijnaarts, H. H. M. (2010). Efforts to improve coupled in situ chemical oxidation with bioremediation: a review of optimization strategies. *Journal of Soils and Sediments*, *11*(1), 129–140.

Sutton, N. B., Langenhoff, A. A. M., Lasso, D. H., van der Zaan, B., van Gaans, P., Maphosa, F., … Rijnaarts, H. H. M. (2014). Recovery of microbial diversity and activity during bioremediation following chemical oxidation of diesel contaminated soils. *Applied Microbiology and Biotechnology*, *98*(6).

Talbot, G., Topp, E., Palin, M. F., & Massé, D. I. (2008). Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Research*, *42*(3), 513–37.

Tanner, R. S. (1989). Monitoring sulfate-reducing bacteria: comparison of enumeration media. *Journal of Microbiological Methods*, *10*(2), 83–90.

Taskin, B., Gozen, A. G., & Duran, M. (2011). Selective Quantification of Viable Escherichia coli Bacteria in Biosolids by Quantitative PCR with Propidium Monoazide Modification. *Applied and Environmental Microbiology*, *77*(13), 4329–4335.

Thomson, N. R., Lamarche, C, Butler, B. J., Magar, V. S., & Kelley, M. E. (2004). ISCO of a creosote source zone: impacts on the microbial community. In *Seventh International In Situ and On-Site Bioremediation Symposium, Orlando, Florida, USA, 2-5 June 2003. Part D. Bioremediation of Creosote, Chlorophenols, and Pesticides.* Battelle Press.

Thornton, G., Franz, M., Edwards, D., Pahlen, G., & Nathanail, P. (2007). The challenge of sustainability: incentives for brownfield regeneration in Europe. *Environmental Science and Policy*, *10*(2), 116 – 134.

Tsitonaki A, Smets BF, Bjerg PL. Effects of heat-activated persulfate oxidation on soil microorganisms. Water Res. 2008; 42:1013–1022. [PubMed: 17942135]

Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A., & Stahl, D. A. (1998). Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *Journal of Bacteriology*, *180*(11), 2975–82.

Wargin, A., & Skucha, M. (2007). Sulphate-Reducing Bacteria , Their Properties and Methods of Elimination from Groundwater, *16*(4), 639–644.

Weelink, S. A. B., Eekert, M. H. A., & Stams, A. J. M. (2010). Degradation of BTEX by anaerobic bacteria: physiology and application. *Reviews in Environmental Science and Bio/Technology*, *9*(4), 359–385.

Zeleke, J., Lu, S.-L., Wang, J.-G., Huang, J.-X., Li, B., Ogram, A. V, & Quan, Z.-X. (2013). Methyl coenzyme M reductase A (mcrA) gene-based investigation of methanogens in the mudflat sediments of Yangtze River estuary, China. *Microbial Ecology*, *66*(2), 257–67.

Zucchi, M., Angiolini, L., Borin, S., Brusetti, L., Dietrich, N., Gigliotti, C., Daffonchio, D. (2003). Response of bacterial community during bioremediation of an oil-polluted soil. *Journal of Applied Microbiology*, *94*(2), 248–57.

Appendix-A

Figure A1: An illustration of the bench-scale set-up following bioreactor packing and attachment to GW reservoirs.

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure A2: This chart is a representation of the BIOLOG EcoPlate and the orientation of the 31 different carbon source wells in triplicate, including a blank well in triplicate. (www.biolog.com)

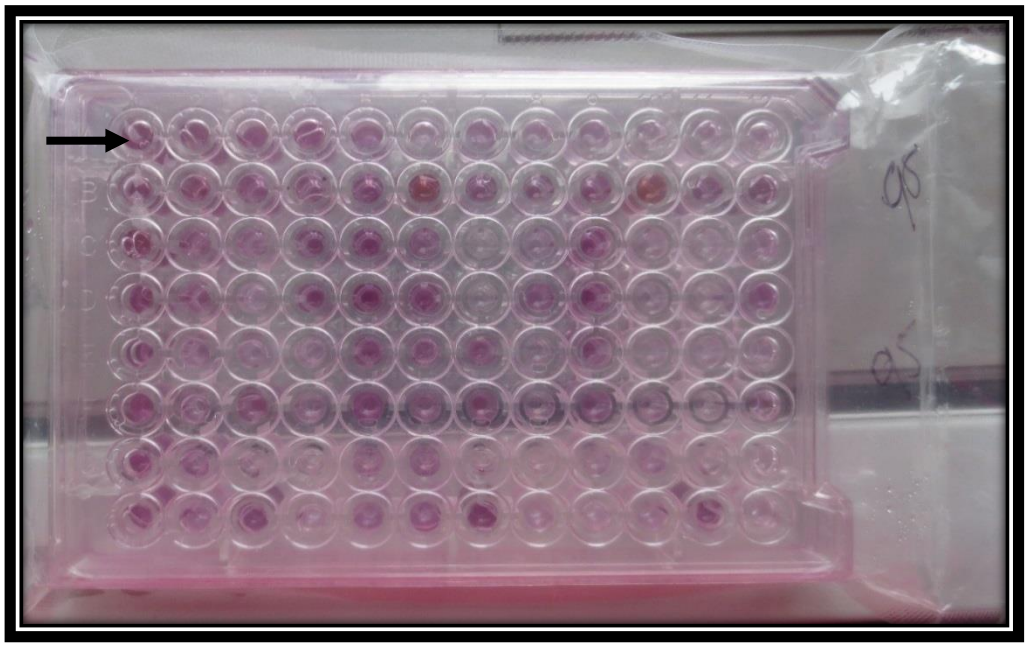

Figure A3: An example of an inoculated Ecoplate used for carbon source utilization profiles. This illustration shows the abiotic reduction, by sulfide in the samples, of the tetrazolium dye 30 mins following inoculation, indicating false positives for carbon source utilization, most notably in the control well where no dye reduction should occur (indicated by arrow).

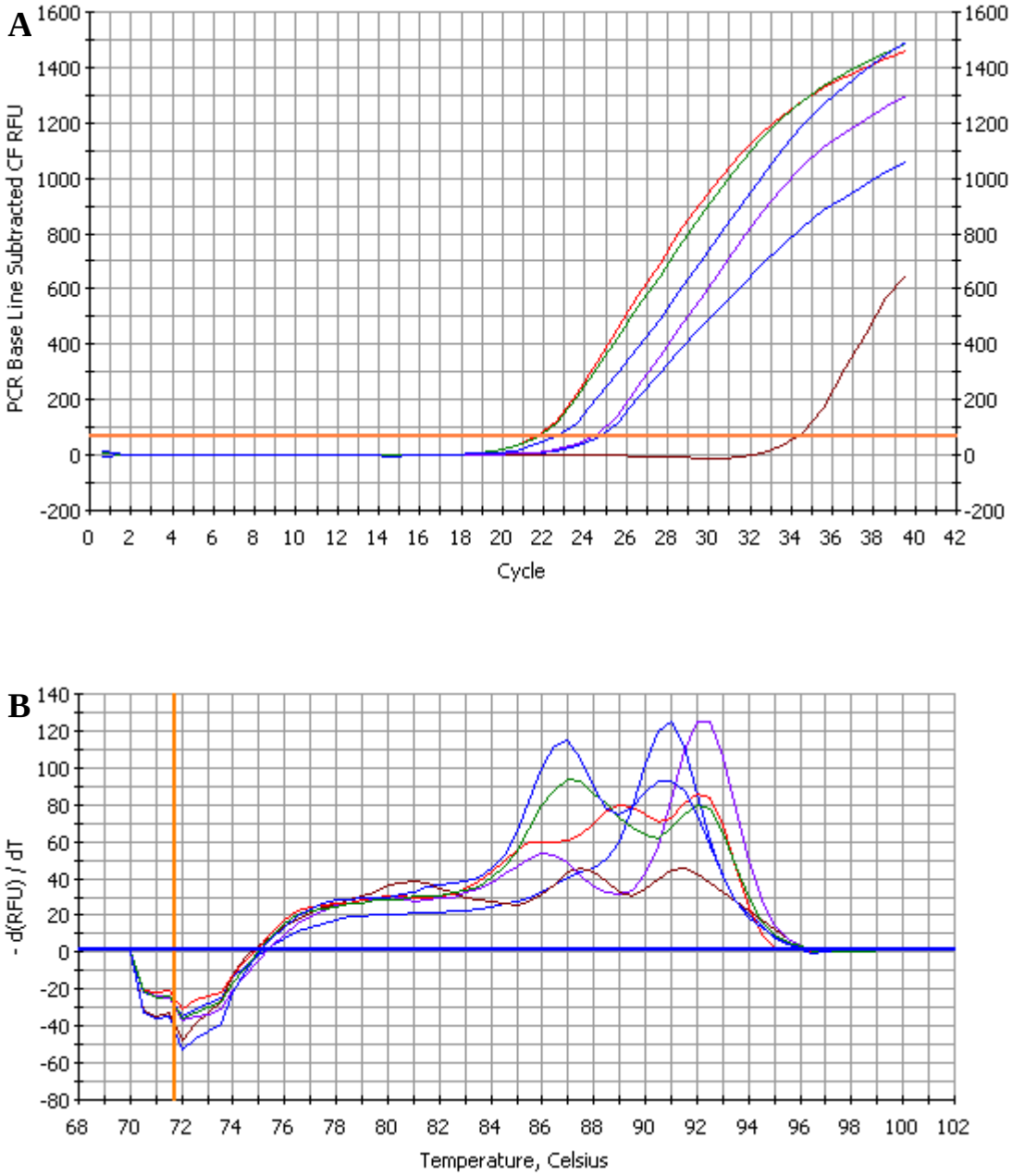

Figure A4: An example of the qPCR completed for the bssA gene on one of the bioreactors (UP2). Part A represents the amplification graph with respective cycle thresholds for samples taken on Sept. 21st, Sept. 28th, Oct. 9th, Oct. 20th, Nov. 4th and Nov. 18th. Part B represents the melt curve analysis showing multiple peaks (non-specific amplification or primer dimers) which indicates potentially flawed results.

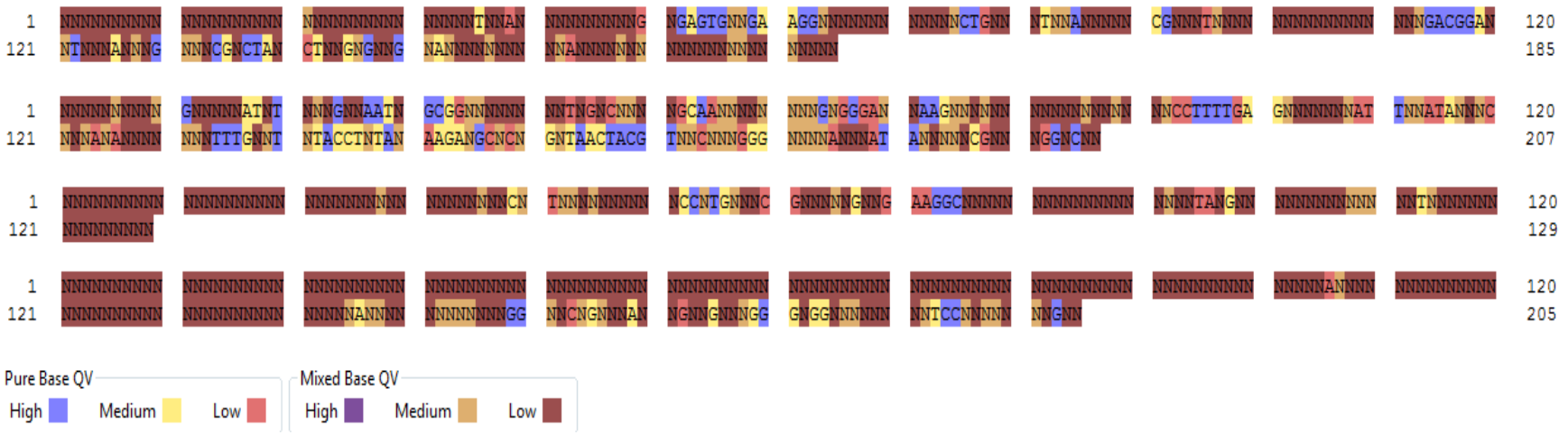

Figure A5: An example of three excised bands that were sent for sequencing. Although some base pairs were revealed, the purity of the product sent was not high enough. Therefore, band sequences came back with mixed base pairs (N).

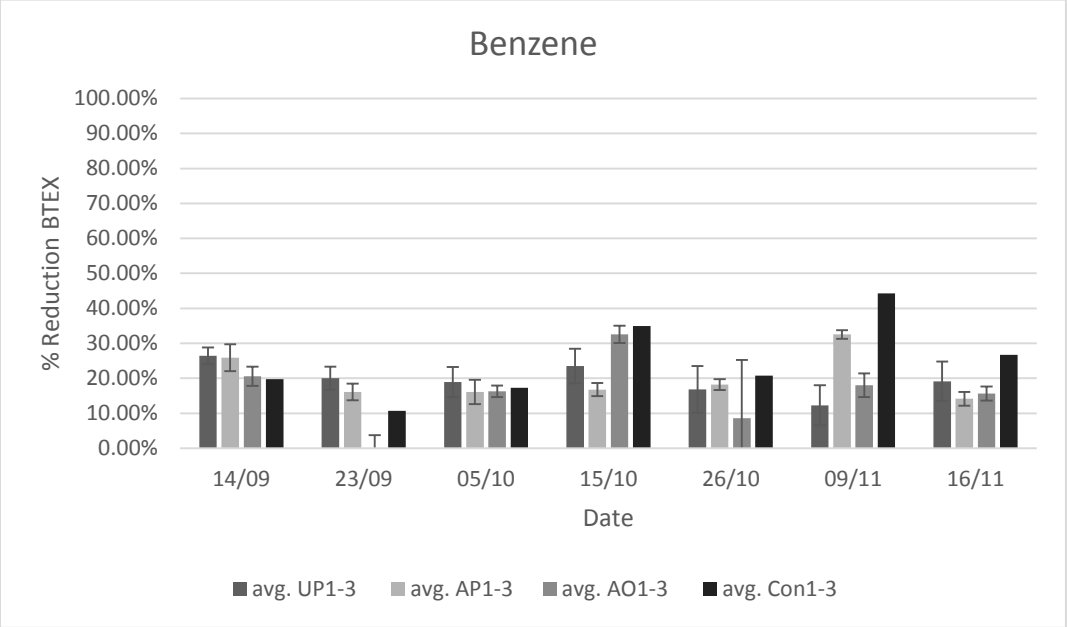

Figure A6: Percent benzene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial.

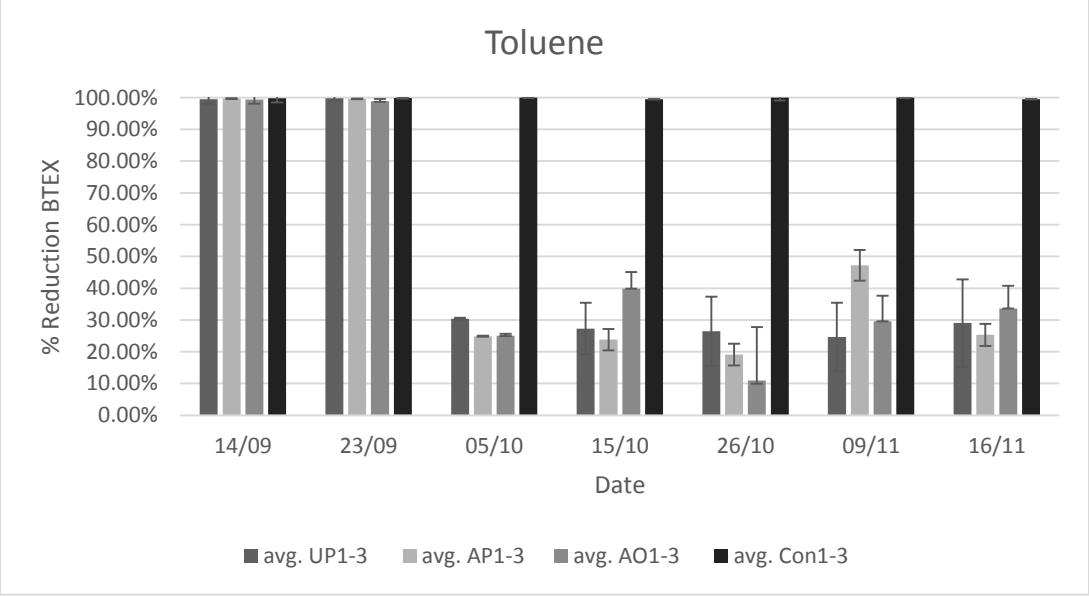

Figure A7: Percent toluene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial.

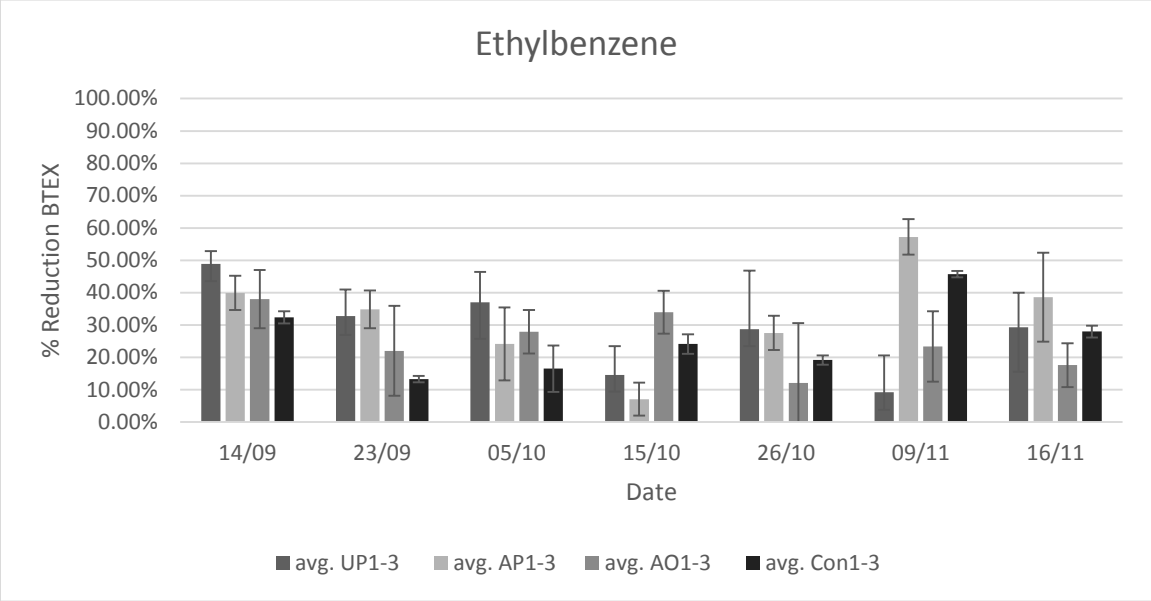

Figure A8: Percent ethylbenzene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial.

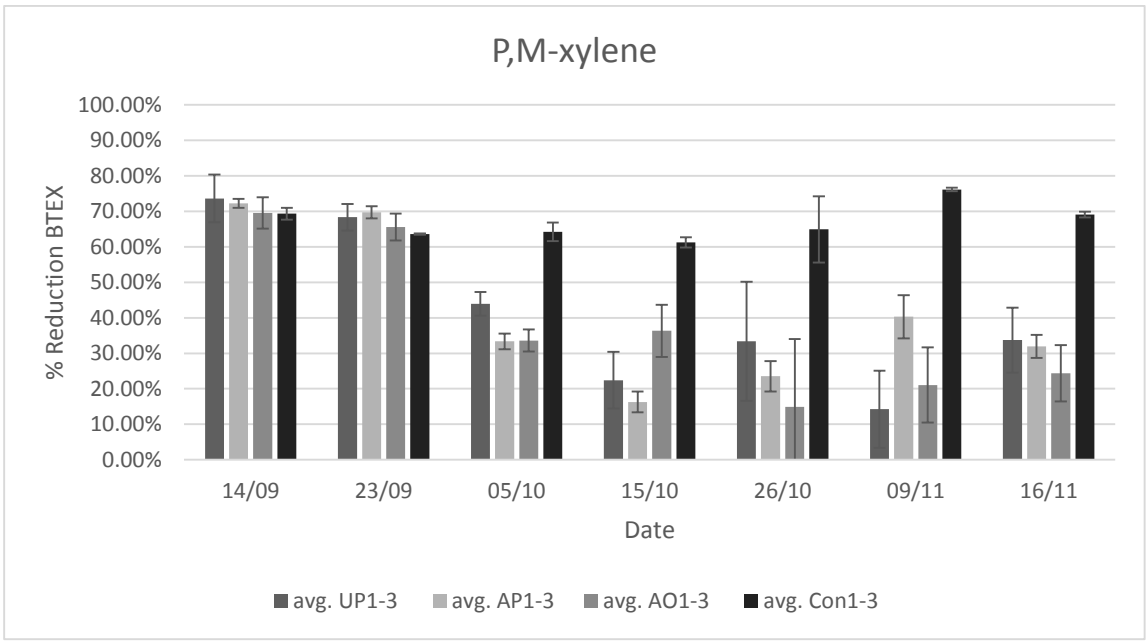

Figure A9: Percent $_{p,m}$xylene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial.

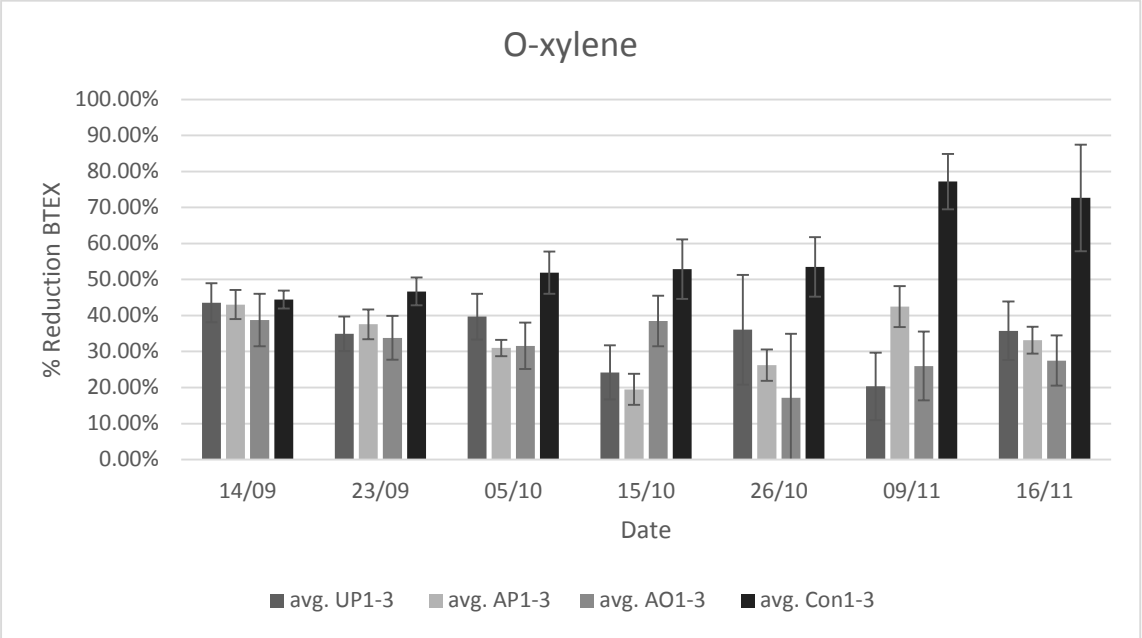

Figure A10: Percent $_{o}$xylene reduction in the bioreactor treatment and control sets throughout the treatment phase of the experimental trial.

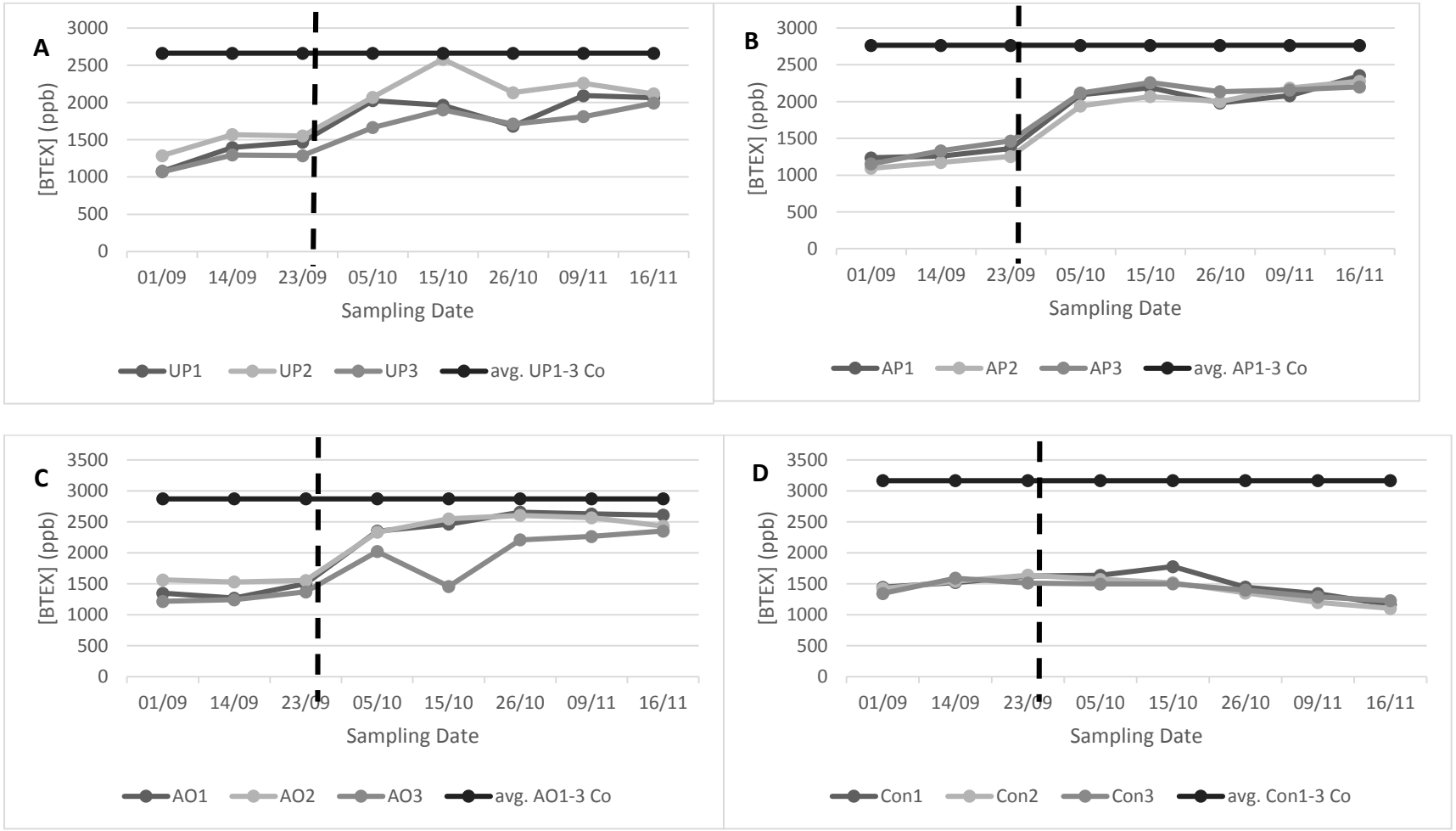

Figure A11: Total BTEX concentration present in the reservoirs (C_0) and water effluent of the unactivated persulfate (plot A), alkaline-activated persulfate (plot B), alkaline-only (plot C), and control (plot D) bioreactors from September to mid-November. The dashed line represents the treatment phase.

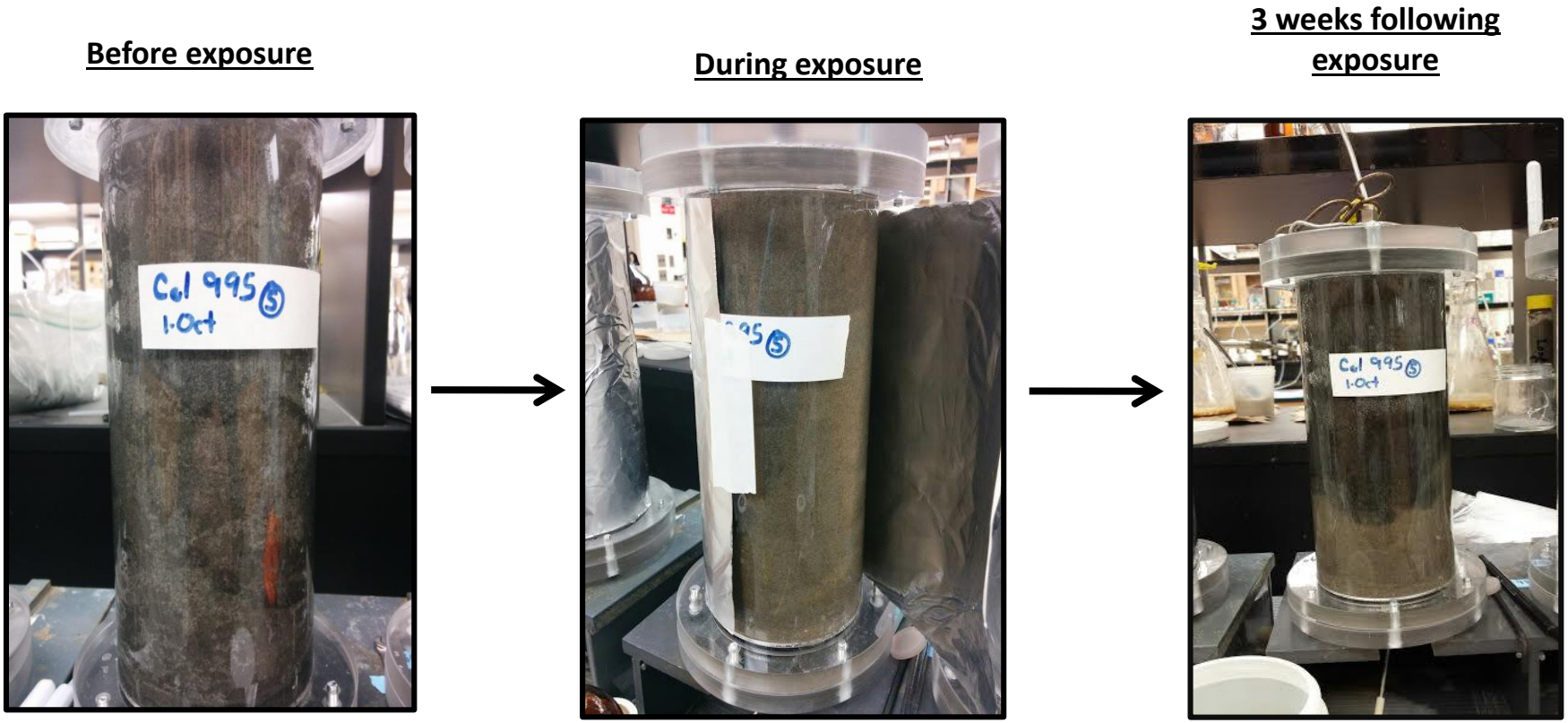

Figure A12: An example of the time-lapse before, during, and three-weeks post-exposure of AP2 bioreactor. This illustration shows the presence of metal sulfide deposits (darkening of the columns) prior to treatment, the subsequent stripping of the deposits during the 24-hour exposure as well as the return of the metal sulfide deposits following treatment, indicating a return in SRB activity.

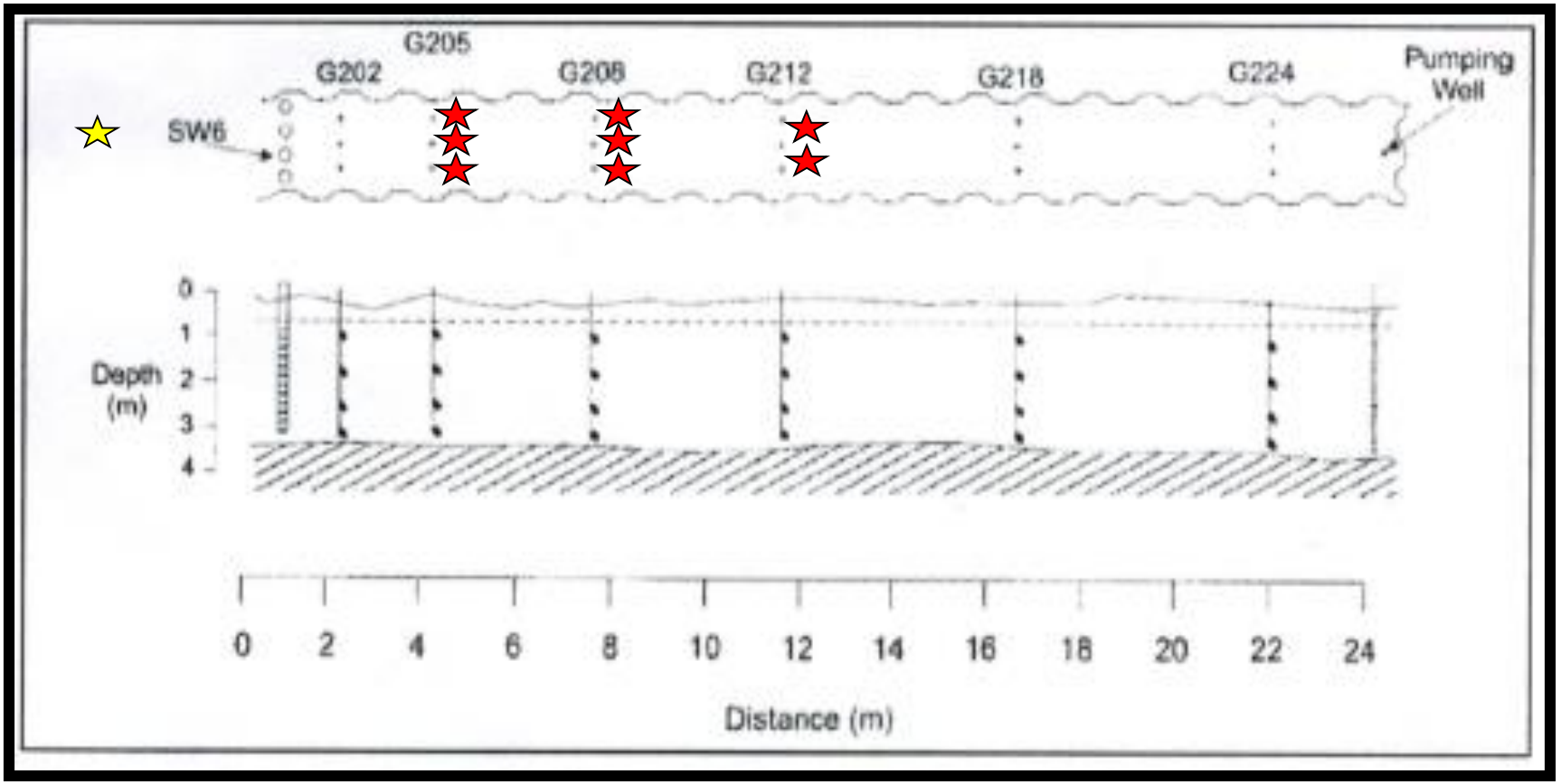

Figure A13: A cross-section (bottom) and overhead (top) schematic of treatment train at Borden. This represents a sheet-piled walled gated showing the multilevel monitoring fences, extraction well and source wells (SW6). The stars within the treatment train portion indicate where cores were taken for reactor packing and the star outside indicates where groundwater was extracted.