Biological Treatment of Naphthenic Acids and Other Organic Compounds in Oil Sands Process-Affected Waters

by

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Abstract

The Alberta oil sands contain one of the world's largest reserves of oil - over 169 billion barrels of bitumen are economically recoverable with current extraction technologies. Surface mining, whereby the ore is extricated from the earth and bitumen is obtained via a hot water extraction process, accounts for approximately half of current production of synthetic crude oil and generates about nine cubic meters of raw tailings per cubic meter of oil. Oil sands facilities are required to operate under a policy of zero water discharge, resulting in impoundments containing more than one billion cubic meters of tailings, a mixture of sand, fines and process-affected water. Effective treatment processes are required in the immanent future, especially for a class of compounds called naphthenic acids, identified as the primary source of acute toxicity of process-affected water. Aerobic biodegradation of oil sands naphthenic acids by indigenous microbial populations present in tailings ponds has been shown to be slow and incomplete, relative to biodegradation of petroleum-refined naphthenic acids available commercially. This research focused on treating oil sands process-affected waters by coupling oxidation, as a means of reducing the recalcitrance of naphthenic acids and other organic compounds, with biodegradation to remove organic matter, including the resulting daughter products of oxidation. In addition, microbial cultures, obtained from an oil sands tailings pond and enriched on organic matter that included naphthenic acids, were assessed for their capability to biodegrade naphthenic acid compounds. Ozone pretreatment significantly improved the biodegradability of dissolved

organic carbon in aged process-affected water samples, from 5 mg/L removal in untreated samples to 11-13 mg/L removal in ozone-treated samples. Ozone exposed indigenous microbial communities were able to degrade an amount of ozone treated organic matter equivalent to those communities not previously exposed, although community structure analysis with microbiological molecular methods indicated these communities were only 65% similar. Ozone pretreatment of model naphthenic acid compounds enabled a bacterial isolate from an oil sands tailings pond, *Acidovorax* sp., to remove 40% of the tricyclic naphthenic acid, adamantane-1-carboxylic acid, hypothesized to be cometabolized during biodegradation of more labile ozone by-products. A fungal isolate from an oil sands tailings pond, *Trichoderma harzianum*, was capable of degrading two tricyclic model naphthenic acid compounds. Ozonation coupled with biodegradation is a promising treatment technology for oil sands process-affected waters, as the need for treatment of organic matter more aggressive than natural attenuation has been established. Ozone pretreatment prior to placement of process-affected waters in reclamation environments, such as end-pit lakes or constructed wetlands, may result in adequate removal of organic matter and the associated toxicity by subsequent biodegradation, without the need for building costly ex-situ wastewater treatment facilities.

Preface

A portion of this research was conducted as part of a collaborative project funded by a National Sciences and Engineering Research Council Strategic Grant, led by Drs. Jonathon W. Martin, Phillip M. Fedorak, and Mohamed Gamal El-Din, from the Departments of Analytical and Environmental Toxicology, Biological Sciences, and Civil and Environmental Engineering, respectively, at the University of Alberta. Chapter 3 of this thesis has been published as Lisa D. Brown, Leonidas Pérez-Estrada, Nan Wang, Mohamed Gamal El-Din, Jonathan W. Martin, Phillip M. Fedorak, and Ania C. Ulrich. 2013. "Indigenous microbes survive in situ ozonation improving biodegradation of dissolved organic matter in aged oil sands process-affected waters." Chemosphere. 93: 2748-2755. I designed and conducted the experiments, with input from P.M. Fedorak. Water samples were ozone treated by N. Wang, under the direction of M. Gamal El-Din. Naphthenic acid sample analysis and data processing was completed by L. Pérez-Estrada. I completed the remainder of the data collection and all data analyses. The manuscript was completed by myself, with editing contributions from J.W. Martin, P.M. Fedorak, M. Gamal El-Din, and A.C. Ulrich.

The experimental designs for the research covered in Chapters 5 and 6 were conceptualized by Dr. Corinne Whitby from the University of Essex, Dr. Ania Ulrich, and myself. Dr. Whitby and I established the microcosms for Chapter 5 together at the University of Alberta, and a portion of Day 0 the naphthenic acid samples were processed by Dr. Whitby at the University of Calgary. The remainder of the samples were processed by myself at the University of Essex. Elena Dlusskaya established the enrichment culture and Dr. Simmon Hofstetter and Timothy Edwards obtained the two microbial isolates that were utilized in Chapter 6. A majority of the experimental work in Chapter 6 was completed by myself at the University of Essex; the Microtox analyses and molecular work were completed at the University of Alberta, again by myself.

Dedication

I dedicate this body of work to my horses,
whom give me strength and courage,
whom teach me patience and gratitude,
whom feed my soul, whom make my heart sing.

Dusty, who has been my only constant;
Milo, for instilling my faith in hope and miracles;
Promise and Chiron, for reminding me how to dream;
and Pedro, for showing me how blessed I am.

Rest in Peace, Pedro 1999 - 2012

Horses lend us the wings we lack. ~Pam Brown

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List of Abbreviations

OSPW = oil sands process-affected water

MFT = mature fine tailings

NA = naphthenic acid

DOC = dissolved organic carbon

PCR = polymerase chain reaction

DGGE = denaturing gradient gel electrophoresis

AEO = acid-extractable organics

EC = effective concentration

1 Biological Treatment of Naphthenic Acids and Other Organic Compounds in Oil Sands Process-Affected Waters

1.1 Introduction

One of the world's largest oil reserves, containing over 169 billion remaining barrels (27 x 10^9 m^3) of recoverable bitumen, is located in northern Alberta, Canada (ERCB, 2012). Bitumen is extracted from the surface mined Athabasca oil sands deposit using a caustic hot water extraction process, resulting in significant volumes of tailings. Fine tailings, consisting of oil sands process-affected water (OSPW), clay and residual sand particles, are currently stored in tailings ponds, in part due to a "zero discharge policy" maintained by the regulatory framework (Allen, 2008a). Use of tailings ponds as process-affected water retention basins enables water recycling for use in the extraction process, as well as allows for the settling of solids and, as originally hoped, partial mineralization of residual organics (Allen, 2008b; Scott et al., 2008b). Due to water recycling efforts, actively utilized tailings ponds continue to exhibit increasing concentrations of dissolved salts, minerals, trace metals, residual bitumen and organics, including naphthenic acids (NAs) (Allen, 2008a). The development of appropriate treatment technologies for OSPW is one of the most formidable challenges facing the oil sands industry (Allen, 2008a). This research focused on biologically treating organic compounds found in OSPW, including NAs, which have been linked to acute toxic responses of aquatic life to OSPW (Clemente and Fedorak, 2005; MacKinnon and Boerger, 1986).

1.1.1 Naphthenic Acids

The term "naphthenic acids" is used to describe the polar organic carboxylic acids that occur naturally in petroleum (Brient et al., 1995). Those occurring in the Athabasca oil sands likely resulted from the biodegradation of mature petroleum (Clemente and Fedorak, 2005). Although NAs are found in many crude oil deposits (Tissot and Welte, 1984), development in the Athabasca oil sands presents unique challenges due to the concentration of NAs in tailings ponds, intensified with water recycling efforts. MacKinnon and Boerger (1986) identified NAs as the primary source of acute toxicity when aquatic life is

exposed to oil sands process affected water, driving the increase in research focused on NAs in OSPW (Grewer et al., 2010).

A number of difficulties have plagued NA research. To date, a cost-effective, accurate analytical technique does not exist, nor does an appropriate calibration standard. Commercially available NA mixtures differ from those found in OSPW (Headley and McMartin, 2004; Scott et al., 2005), thus requiring the extraction of NAs from OSPW for relevant biodegradation, analytical, and toxicity studies. Grewer et al. (2010) unequivocally demonstrated, however, that more than half of the organic compounds in the acid-extractable fraction of OSPW are not "classical" NA compounds, yet would be measured with most analytical techniques, calling into question conclusions drawn from studies on the acid-extractable fraction of OSPW. Despite these challenges, resistance of OSPW NAs to biodegradation by indigenous tailings microorganisms has been established (Han et al., 2009; Quagraine et al., 2005b; Scott et al., 2005).

A robust, high resolution analytical technique enabled the identification of numerous organic compounds found in OSPW, including individual NAs (Rowland et al., 2011a, 2011c, 2011f). Subsequent synthesis of individual compounds has facilitated, and will continue to facilitate, investigation of biodegradation metabolic pathways (Johnson et al., 2013, 2012, 2011) and structure-reactivity relationships, to help elucidate mechanisms of toxicity (Jones et al., 2011; Scarlett et al., 2012).

1.1.2 Other Organic Pollutants

Although NAs are a major component of the OSPW toxic fraction (Frank et al., 2008; Garcia-Garcia et al., 2011b; Jones et al., 2011; Scarlett et al., 2012), a majority of the organic compounds in OSPW do not fit the strict formula for NAs (Grewer et al., 2010; Headley et al., 2011, 2009) and NAs are not the only organic compounds in OSPW which could contribute to chronic toxicity of OSPW (Garcia-Garcia et al., 2011a; Garcia-Garcia et al., 2011b). Identifying all organic compounds present in OSPW, and determining their potential contribution to toxicity, continues to be an area of research in the oil sands industry. Utilization

of both synthesized representative organic compounds, especially NAs, and the entire OSPW matrix in the investigation of appropriate treatment technologies is essential while analytical challenges prevail.

1.1.3 Treatment of OSPW

Remediation options for OSPW are an imminent requirement as industry-wide expansion increases demand on local water resources and the public and government mount pressure to decommission tailings ponds. Decades-long retention times have allowed acclimated microbial communities to develop in tailings ponds and utilize NAs and other organic compounds as substrates, but a fraction of oil sands NAs has proven to be recalcitrant (Quagraine et al., 2005b; Scott et al., 2005) such that natural attenuation is not a feasible treatment option for OSPW.

Ozone treatment has been proposed as pretreatment to break down NAs in OSPW to render these compounds amenable to biodegradation (Scott et al., 2008b; Martin et al., 2010; Wang et al., 2013). Advanced oxidation processes are often used to oxidize complex organic compounds in wastewaters, enabling or enhancing their biodegradability (Metcalf and Eddy, 2003).

Bioremediation of organic compounds in OSPW remains an attractive treatment option due to low cost and minimal waste generation (Allen, 2008b). Exploration of candidate microorganisms capable of significantly biodegrading NAs and other organics (Johnson et al., 2011) may enable bioaugmentation of tailings ponds or reclamation environments as well as provide appropriate microbial seeds for *ex situ* biological treatment technologies (Quagraine et al., 2005b).

1.2 Objectives

The aim of this body of work was to elucidate potential biological treatment options for OSPW, particularly NAs and other organics. The specific objectives are highlighted, followed by the novelty of this work:

1. *Explore ozone pretreatment coupled with aerobic biodegradation as a treatment option for NAs and other organic compounds in aged OSPW.* For the first time, aged OSPW is treated with ozone and the capacity for microbial communities to degrade organic carbon following ozone exposure is assessed.
2. *Investigate the dynamic responses of indigenous microbial communities from aged OSPW upon exposure to ozone, followed by aerobic incubation.* For the first time, microbial communities in aged OSPW are characterized and community dynamics after ozone exposure are assessed.
3. *Determine if ozone pre-treatment will improve biodegradation of model naphthenic acid compounds.* Model NA compounds are utilized for the first time to test ozone coupled with biodegradation as treatment regime for OSPW.
4. *Examine the capacity for enriched microbial cultures from OSPW to degrade model naphthenic acid compounds.* For the first time, a fungus isolated from OSPW is utilized for NA degradation.

Components of this research include the use of microbiological molecular methods to explore community dynamics of indigenous microbes from OSPW, in response to ozone exposure (Chapters 3 and 4) and aerobic incubation (Chapters 4 and 6). Also model NA compounds were utilized to enable thorough investigation of the capabilities of indigenous microbes to degrade NA compounds of particular structure (Chapters 5 and 6).

1.3 Organization of Thesis

This thesis follows a paper format. Chapter 1 provides a brief introduction to the topic of research. Chapter 2 reviews all relevant literature and theory, including a summary of the oil sands industry, analytical techniques for NAs, NA biodegradation, treatment of OSPW, and microbial communities in tailings ponds. Chapters 3 through 6 address the objectives, as highlighted above, individually. Chapter 7 provides a summary of the contributions of this work and recommendations for future investigation.

2 The Oil Sands Industry, Naphthenic Acids, Oil Sands Process-Affected Water Treatment: A Review

2.1 Background

2.1.1 Alberta Oil Sands

Alberta is home to one of the largest oil deposits in the world, with 169 billion barrels (at the end of 2011) of crude bitumen, economically recoverable by current technologies, available in three major deposits, shown in Figure 2-1, located in northern Alberta (ERCB, 2012). Only 2.4%, by area, of the oil sands deposits is sufficiently shallow to allow for surface mining, which occurs in the Athabasca oil sands deposit, north of the community of Fort McMurray and near the Athabasca River; 20% of this deposit will be mined (Government of Alberta, 2008). Mining has occurred in the Athabasca deposit since the late 1960's (ERCB, 2012). Currently, five companies operate large-scale mining operations: Suncor, Syncrude, Shell Albian Sands, Imperial Oil, and CNRL (Alberta Energy, 2013). To date, over 760 square kilometres of land has been disturbed by oil sands mining operations (Alberta Environment and Sustainable Resource Development, 2013).

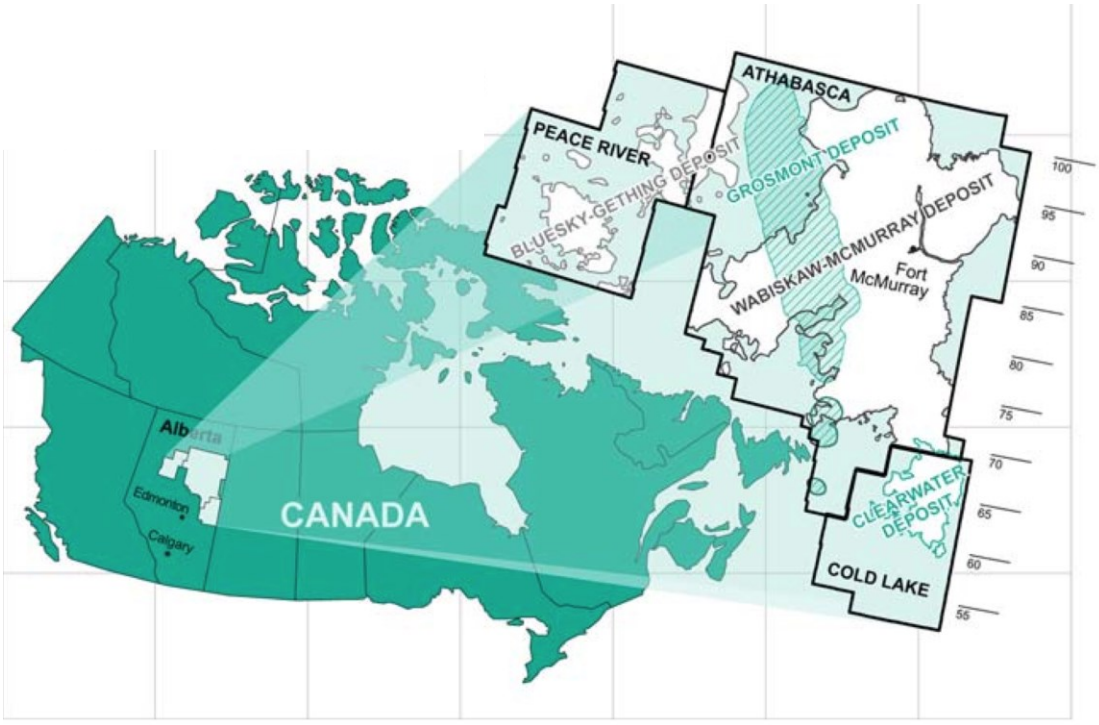


Figure 2-1: Athabasca, Peace River, and Cold Lake oil sands deposits (ERCB, 2012)

2.1.1.1 Oil Sands Processing

Oil recovered from oil sands deposits is considered unconventional due to the intense recovery methods required (open pit mining and *in situ* intensive heating) and the upgrading needed to generate synthetic crude oil. The oil sands ore consists of sand impregnated with bitumen, a highly viscous hydrocarbonaceous material. The sand grains are wet with a film of water and then surrounded with bitumen (Speight, 2007).

The primary oil sands processing stages are mining, extraction, and upgrading. In the extraction process, the bitumen is separated from the deposit material, leaving behind sand and process water contaminated with clay minerals, residual bitumen, dissolved salts, and non-bitumen organics (Allen, 2008a). Because the sand in the Athabasca deposit is water-wetted and bitumen becomes less dense than water at temperatures greater than 80°C, hot water extraction is successful at separating bitumen from the sand (Speight, 2007). A modified version of the Clark Hot Water Process, developed by Dr. Karl Clark of the Alberta Research Council in the 1920's, is still employed today (Allen, 2008a). Water recycling efforts have greatly reduced the need for oil sands mining companies to draw from surface water bodies in the region; in 2012, 2.2 barrels of fresh water was used for every barrel of synthetic crude oil produced (Alberta Environment and Sustainable Resource Development, 2013).

Mined oil sand is mixed with hot water, and occasionally caustics to improve bitumen separation, and then hydro-transported to the extraction plant as a slurry, which conditions the material in preparation for separation (Allen, 2008a; Gosselin et al., 2010). In the primary extraction vessel, aeration promotes bitumen flotation and it is skimmed off the surface as froth, while the remaining water is treated further to recover bitumen and the settled sand removed (Allen, 2008a). Solvent addition during froth treatment enables greater removal of water and solids (Allen, 2008a).

The extraction stage of processing results in up to 10% of total bitumen from the deposit being unrecovered (Alberta Chamber of Resources, 2004; Allen,

2008a), resulting in a significant amount of hydrocarbons being present in the tailings impoundments.

2.1.1.2 Managing Waste – Tailings Impoundments

Process waters and tailings, mostly generated during the extraction process, must be contained on-site and release to the surrounding environment prevented, due to a "zero discharge policy." Over 180 square kilometres of the land disturbed by oil sands operations is occupied by tailings impoundments (Alberta Environment and Sustainable Resource Development, 2013), and total volumes of stored tailings exceed 1 billion m^3 (Martin et al., 2008).

For every cubic metre of synthetic crude oil produced, approximately 6 m^3 of sand and 1.5 m^3 of fine tailings are generated (Alberta Chamber of Resources, 2004). The fine tailings are stored in a settling basin called a tailings pond, eventually forming mature fine tailings (MFT), and the sand is utilized as a construction material for the impoundment containment structures, called dykes. The primary purpose of the tailings pond is to provide sufficient retention time for sedimentation to occur, enabling both water recycling and, eventually, reclamation.

2.1.1.3 Closure and Reclamation

Oil sands development occurs on public land leased to companies who purchase the rights to explore for, extract and develop the resources (Government of Alberta, 2008). Both the *Public Lands Act* and *Environmental Protection and Enhancement Act* require that disturbed land be returned to a productive state (Government of Alberta, 2008). All disturbed areas, including tailings ponds, will need to be biologically self-sustaining. Therefore, the water contained in tailings ponds will need to be treated for eventual release to the environment, ponds filled for land reclamation, or end-pit lakes created that are able to sustain aquatic life.

The Alberta Energy Resources Conservation Board, now the Alberta Energy Regulator, introduced Directive 074 in 2009, in an attempt to reduce the

growing volumes of tailings in the oil sands industry (ERCB, 2013). Requirements include increasing solids content in MFT to enable development of trafficable landscapes and reducing volumes of OSPW (ERCB, 2013). In the 2011-2012 reporting period, none of the operators met the fines capture requirement (ERCB, 2013).

2.2 Naphthenic Acids

2.2.1 Properties of NAs

Naphthenic acids (NAs) are classically defined as a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where *n* indicates the carbon number and *Z* specifies a homologous series, or the degree of cyclization (Brient et al., 1995). The *Z* variable is an even negative integer between 0 and -12, which indicates the loss of covalently bonded hydrogen due to the presence of ring structures (Marsh, 2006). The saturated ring structures predominantly contain five or six carbon atoms, and each multiple of -2 indicates the presence of another ring. Thus, NAs contain three components: one or more five- or six- carbon cycloalkane rings, an aliphatic side chain of various lengths, and a carboxylic group (Herman et al., 1994). The carboxyl functional group is usually bonded to the side chain versus directly to the ring structure (Headley and McMartin, 2004). Values of *n* range from 5 to 33, resulting in molecular weights between approximately 100 to 500 g/mol (Clemente et al., 2003). Some typical NA structures are shown in Figure 2-2.

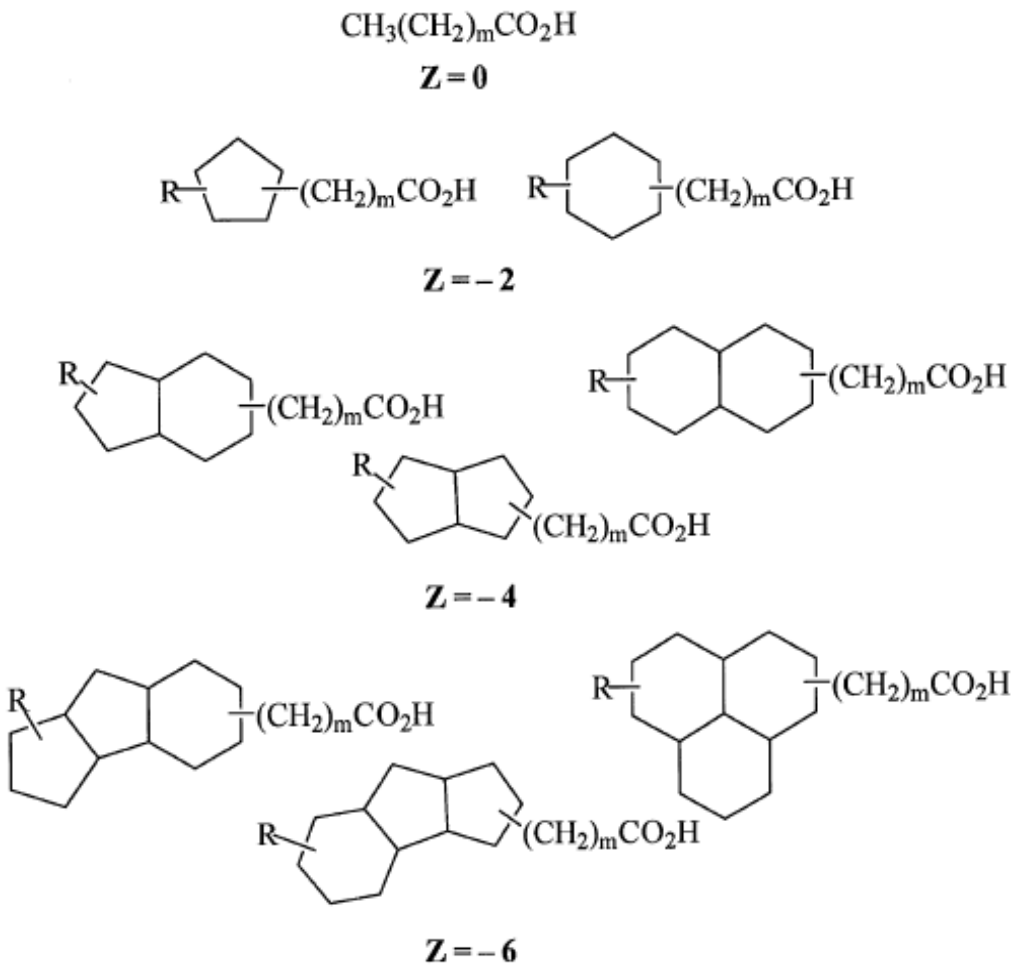


Figure 2-2: Typical naphthenic acid structures, where *R* is an alkyl chain and *m* is number of CH_2 units (Holowenko et al., 2001)

Fatty acids also satisfy the general chemical formula for NAs, but are differentiated based on the biomolecules' absence of highly branched alkyl groups (Scott, 2007). In addition to NAs, aromatic, olefinic, hydroxyl, and dibasic acids also contribute to the overall acid content of crude oil deposits (Marsh, 2006). Before Grewer et al. (2010) and Headley et al. (2011) discovered numerous organic acids that are not classically defined NAs, a majority of the research to date has focused on those compounds that fit the strict definition of NAs above.

Completely soluble in organic solvents and oil, NAs are considered water insoluble, with a solubility under 50 mg/L (Brient et al., 1995). Naphthenic acids are weak acids, with dissociation constants on the order of 10^{-5} to 10^{-6} (Brient et al., 1995). Like most weak acids, NAs forms metal salts, such as sodium naphthenate. Based on the above pKa range, Young et al. (2008) calculated that over 99% of NAs exist in their protonated form, soluble naphthenates, at the alkaline pH of OSPW and river water. Thus, "NAs", or sodium naphthenates, remain in the aqueous phase in tailings (Clemente and Fedorak, 2005).

Naphthenic acids exhibit properties of surfactants due to the hydrophilic carboxyl functional group and the hydrophobic alicyclic end, and specific densities are less than 1 (Brient et al., 1995), resulting in their concentration at the water surface in tailings ponds. The presence of naphthenates at the elevated pH of the alkaline bitumen extraction process improves bitumen recovery, due to this surfactant property (Masliyah et al., 2004). Headley and McMartin (2004) highlight that the reported toxicity of NAs is often associated with their surfactant characteristics; narcosis, whereby a hydrophobic compound enters the lipid bilayer of the cell membrane causing disruption and cell death, has been suggested as the likely mode of action in an acute toxic response (Frank et al., 2010, 2009; Tollefsen et al., 2012).

Naphthenic acids are natural components of nearly all crude oils (Brient et al., 1995) and occur because either the deposit has not undergone sufficient catagenesis, the conversion of kerogens to hydrocarbons, or the oil has been biodegraded by bacteria (Tissot and Welte, 1984). The benzene-soluble fraction of heavy bituminous hydrocarbons extracted from the Athabasca oil sands deposit is dominated by complex cyclic and branched aliphatic compounds, with only a low concentration of acyclic and straight chain alkane compounds (Quagraine et al., 2005a). In the Athabasca oil sands deposit, NAs likely are the result of biodegradation of mature petroleum (Tissot and Welte, 1984) and constitute approximately 2%, by weight, of the total bitumen (Headley and McMartin, 2004). Naphthenic acids found in oil sands deposits, and ultimately tailings ponds, are much more complex than commercial mixtures of NAs that

have been refined from other crude oils (Headley and McMartin, 2004), which must be considered when commercial NAs are utilized as surrogates in experimentation (Bataineh et al., 2006; Garcia-Garcia et al., 2012, 2011b; Marsh, 2006; Tollefsen et al., 2012).

Naphthenic acids are a concern to the oil and gas sector not only because of their environmental significance, but also because they have been found to be a cause of corrosion (Slavcheva et al., 1999). Deposits with a high acid content must be processed in facilities constructed of stainless steel.

To reduce reliance on the Athabasca River for fresh water supply to processing operations, oil sands companies have strived to increase water recycling rates, with recycled water accounting for up to 80 to 85% of the water used in extraction (Allen, 2008a). Although beneficial for a number of reasons, including reducing stress on the Athabasca River flow and ultimately controlling the amount of process affected water requiring disposal, water recycling concentrates constituents in process affected waters, including NAs. Due to the corrosive nature of NAs, without a treatment system to remove these compounds, oil sands operators will always require fresh water to use in susceptible equipment, such as cooling towers and boilers (Scott, 2007).

Because the Athabasca oil sands deposit is ubiquitous in the area, background levels of NAs are expected in surface waters and groundwater that interact with the oil sands deposit. Headley and McMartion (2004) reviewed literature to date and provided typical NA concentrations in the Fort McMurray area: river water contained less than 1 mg/L, tailings ponds averaged 110 mg/L, near-surface aquifers less than 4 mg/L and there was greater than 55 mg/L in basal aquifers. Merlin et al. (2007) sampled the Athabasca River upstream, downstream, and within the region of oil sands development; NAs were only detected next to mining and processing operations, with a method detection limit of 10 μg/L. While a number of attempts have been made to distinguish anthropogenic (ie. originating from oil sands processing and OSPW storage) from “natural” NAs that occur in surface and ground waters due to the ubiquity of the oil sands deposit in the region (Frank et al., 2014; Grewer et al., 2010;

Headley et al., 2013b, 2011; Ross et al., 2012), analytical challenges continue to render development of appropriate surface and ground water guidelines difficult (Ross et al., 2012).

2.2.2 Toxicity of NAs

Research began on oil sands NAs in the 1980s (Miskimmin et al., 2010) with MacKinnon and Boeger (1986) observing simultaneous reduction in NAs (actually carboxylic acids) and toxicity with OSPW treatment. Strong evidence now exists to suggest NAs cause a toxic response in many organisms. Naphthenic acids have been found to be toxic to microorganisms, aquatic algae, aquatic organisms such as fish, invertebrates, and vegetation, birds, and mammals (reviewed in Headley and McMartin, 2004; Kindzierski et al., 2012; Miskimmin et al., 2010).

Determining the total NA concentration is insufficient in describing toxic effects because molecular structure and composition of the NA mixture need to be understood and identified (Clemente and Fedorak, 2005; Kindzierski et al., 2012). Higher proportions of lower molecular weight compounds in a NA mixture correlate with greater toxic response in various organisms (Frank et al., 2008; Holowenko et al., 2002; Lo et al., 2006; Rogers et al., 2002). Frank et al. (2009) hypothesized that this observation is due to presence of multiple carboxylic acid groups in higher molecular weight NA-like compounds, which reduce hydrophobicity and thus toxicity, as increasing molecular weight in structurally similar model NAs increased toxic response. Thus, structural differences between low and high molecular weight NAs, not just increasing carbon number, are likely to be contributing to differences in measured toxicity. It is still not known which NA compounds in OSPW are most toxic, or what structural characteristics of these compounds cause toxic responses (Kindzierski et al., 2012; Rowland et al., 2011c).

The identification and synthesis of individual, structurally representative acid compounds found in OSPW (Rowland et al., 2011a, 2011c, 2011f) has enabled study of structure-toxicity relationships (Jones et al., 2011; Scarlett et al.,

2012) in the absence of the ability to fractionate individual OSPW NA compounds. Of the ten classes of acid compounds tested, polycyclic monoaromatic acids were predicted, with modeling analysis, to present the greatest threat to various endpoints, including fish and mammals (Scarlett et al., 2012). For NAs, toxicity decreased with decreasing carbon number, decreasing cyclicity (Frank et al., 2010) and increasing branching (Jones et al., 2011; Scarlett et al., 2012). Little difference in toxicity to both the Microtox bioassay and hepatocytes from rainbow trout was observed with different NA isomers (Tollefsen et al., 2012).

Recent research, however, now indicate that NAs are not the only cause of OSPW toxicity (Garcia-Garcia et al., 2012, 2011b). That said, Miskimmin et al. (2010) highlight that exposure to NAs through direct contact with OSPW is more probable because of their high solubility at alkaline pH of OSPW. Given this, it is appropriate for toxicity research on OSPW to continue in two directions; one focused on identifying the most toxic components of OSPW to enable targeted treatment regimes, and the second to utilize whole OSPW due to the potential for synergistic effects between compounds.

2.3 Literature Review

Following is a review of published research to date related to the research objectives. First, a discussion of analytical techniques for NAs will be conducted, as the method greatly impacts what compounds are being measured. An extensive review of the biodegradation of NAs is presented, focussing on those occurring in the Athabasca oil sands region. In addition, treatment options for, and characterization of indigenous microbial communities in, oil sands process-affected water will be discussed.

2.3.1 Analytical Techniques for NA Measurement

Since MacKinnon and Boerger (1986) identified NAs as the cause of tailings water toxicity, much research has been conducted to develop accurate

and precise analytical methods to quantify and characterize NA compounds. Numerous methods of chemical analyses have been utilized to estimate total NA concentrations in environmental samples and to delineate individual NAs. Significant progress in all areas of research surrounding NAs has been hampered by the abundance of published analytical methods and the lack of a uniformly accepted, adequately quantitative analytical methodology (Headley and McMartin 2004). Recently, the pursuit of a robust analytical technique with very high resolution power has been driven by the need to distinguish NAs in surface waters that originate from natural sources, due to water bodies scouring the oil sands formation, from anthropogenic NAs, released from tailings ponds (Headley et al., 2013b; Ross et al., 2012). In addition, deciphering an NA signature to determine bitumen ore source, age of OSPW, and extraction procedure, for example, would be a powerful forensics tool.

Quantification of NAs in crude oil and distillates is typically achieved by neutralization via titrating with potassium hydroxide (Brient et al., 1995). The current oil sands industry standard for quantifying NAs in environmental samples was developed by Syncrude Canada (Clemente and Fedorak, 2005) and involves dichloromethane extraction of an acidified water sample, concentration of extract, followed by absorbance measurement of carboxylic acids by Fourier transform infrared (FTIR) spectroscopy (Scott et al., 2008a). This method detects both the single and double bonds between carbon and oxygen in the carboxylic acid functional group, and so will detect compounds that do not strictly adhere to the chemical formula for NAs (Miskimmin et al., 2010). It is now clear that FTIR cannot be said to measure NAs in OSPW; instead, the data should be described as carboxylic acid concentrations.

Clemente and Fedorak (2005) provided a review of the most common analytical methods, including FTIR, gas chromatography (GC), negative ion electrospray ionization-mass spectrometry (ESI-MS), and high performance liquid chromatography (HPLC). Emerging, high resolution techniques are discussed by Headley et al. (2013b), such as HPLC coupled with time of flight

(TOF) MS, GCxGC/MS, LCxLC/MS, LC/MS/MS, Orbitrap MS, and ESI Fourier Transform Ion Cyclotron Resonance (FTICR) MS.

For GC analysis, NAs are derivatized to form methyl esters, which then elute as an unresolved "hump"; when integrated and compared to an internal standard, a total NA concentration may be deduced (Holowenko et al., 2002). This additional sample processing renders the analysis vulnerable to error (Bataineh et al., 2006). It is now recognized that GC paired with low resolution MS will result in substantial false high concentrations and misclassification of compounds. Figure 2-3 shows examples of three dimensional plots of individual NA isomer classes developed from two analytical methods. The dramatic differences in the two plots are the result of derivatization of the sample for GC/MS and the higher resolution of HPLC/QTOF-MS. Derivatization of hydroxylated NAs (compounds with three or four oxygen atoms rather than two), first hypothesized to be formed during microbial degradation by Clemente et al. (2004), will be misassigned by low resolution MS to be NA compounds with more than 22 carbons (Bataineh et al., 2006). Headley et al. (2013b), however, highlight that GC/MS is still a worthwhile technique for NA analysis, as it is easy to use, widely available, and at least able to detect gross changes in NA concentration and composition.

Currently, the most common tool to analyze composition of NA mixtures for research purposes is mass spectrometry (MS), whereby a profile of individual NA compounds is developed by plotting the relative response of each mass corresponding to a particular *n* and *Z* combination (Holowenko et al., 2002; Martin et al., 2008). This profile, as shown in Figure 2-3, can then be interpreted as a "signature" or composition of the NA mixture. It is hypothesized that mixtures are characteristic of a number of factors, such as petroleum source and exposure to biodegradation (Bataineh et al., 2006; Headley and McMartin, 2004).

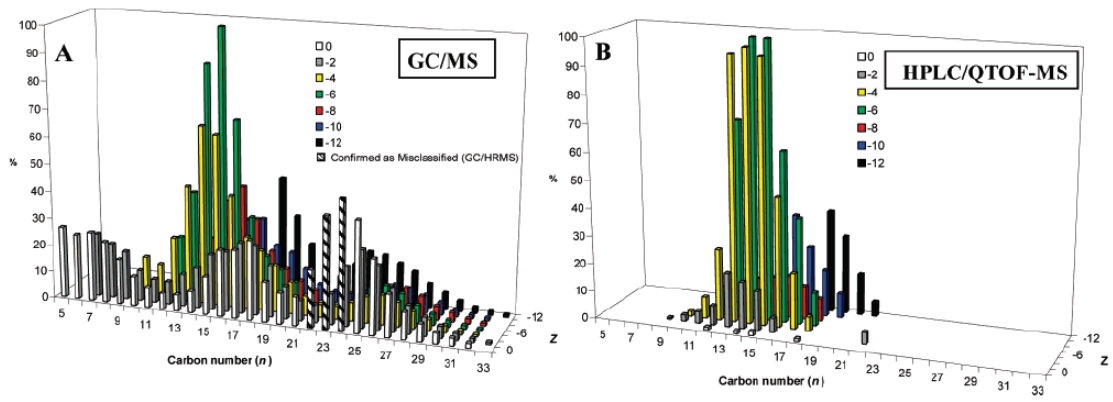


Figure 2-3: Three dimensional plots of "naphthenic acids" developed using A) GC/MS and B) HPLC/QTOF-MS for an extract of Syncrude tailings water (Bataineh et al. 2006)

A great number of high resolution MS methods have been utilized for oil sands NA analysis, furthering understanding of this complex mixture of compounds. A majority of the classically defined NAs in OSPW were polycylic (Jones et al., 2013), with Z numbers between -4 and -8 (Grewer et al., 2010; Rowland et al., 2011c; West et al., 2013), while NAs in surface waters were acyclic (Grewer et al., 2010). Grewer et al. (2010) suggest that high-pressure liquid chromatography high-resolution mass spectrometry (HPLC/HRMS) (Martin et al. 2008, Bataineh et al. 2006, Han et al. 2008, Han et al. 2009) is the best compromise between cost and access, and resolution power. An important advantage of HPLC/HRMS was highlighted by Han et al. (2009); the method is able to simultaneously detect oxidized NAs, which were suggested to indicate extent of biodegradation.

Table 2-1 summarizes published results from studies reporting NA concentrations, showing sample source, measured concentrations of NAs, and test method. Many more publications detail methodology for NA analysis, but most discuss the mixture composition without reporting absolute concentrations (Lo et al., 2006; Martin et al., 2008; Pereira et al., 2013a; Rowland et al., 2012), utilize a concentrated stock solution for toxicity studies and other analyses (Frank et al., 2008; Jones et al., 2013; Rowland et al., 2011c), or indicate the presence of NAs without providing quantification (Merlin et al., 2007).

Table 2-1: Measured NA concentrations in oil sands water samples; comparison of analytical techniques

Source of NAs	Concentration (mg/L)	Test Method	Reference
Syncrude West In Pit, sampled in 2009	25.5	FTIR	Lu et al. (2013)
	20.7	HPLC/TOFMS	
Suncor South Tailings Pond, sampled in 2009	11.0	FTIR	
	12.9	HPLC/TOFMS	
Albian External Tailings Facility Pond, sampled in 2009	8.3	FTIR	
	6.8	HPLC/TOFMS	
Athabasca River, u/s from oil sands	0.17	GC-MS	RAMP (2012); Ross et al. (2012)
	0.00491	HPLC/TOFMS	
Athabasca River, d/s from oil sands	0.1	GC-MS	
	0.016	HPLC/TOFMS	
Steepbank River	0.26	GC-MS	
	0.00582	HPLC/TOFMS	
McLean Creek	7.94	GC-MS	
	0.0807	HPLC/TOFMS	
Johnson Lake	0.22	GC-MS	
	0.0274	HPLC/TOFMS	
Syncrude West In Pit	36	GC-MS	Grewer et al. (2010)
	60	FTIR	
Syncrude Demo Pond (Big Pit)	5.9	GC-MS	
	14	FTIR	
Suncor Pond 2/3	47	GC-MS	
	63	FTIR	
Albian External Tailings Facility Pond	18	GC-MS	
	35	FTIR	
Athabasca River, Fort McMurray u/s from oil sands	<0.03	GC-MS	
	0.08	FTIR	
Athabasca River, 30 m u/s from oil sands	<0.01	GC-MS	Scott et al. (2008a)
	0.29 ± 0.08	FTIR	
Athabasca River, d/s from oil sands	<0.01	GC-MS	
	0.26	FTIR	
Consolidated tailings (CT) water	4.9	GC-MS	
	18	FTIR	
Tailings water, sampled in 2004	4	GC-MS	
	17	FTIR	
Tailings water, sampled in 2007	12	GC-MS	
	34	FTIR	
OSPW NA 0 mg/L*	0.5	FTIR	Yen et al. (2004)
	4 ± 2.5	HPLC	
OSPW NA 10 mg/L	8	FTIR	
	13 ± 1.8	HPLC	
OSPW NA 30 mg/L	24	FTIR	
	22 ± 2.1	HPLC	
OSPW NA 60 mg/L	51	FTIR	
	64 ± 2.6	HPLC	
OSPW NA 80 mg/L	75	FTIR	
	78 ± 0.5	HPLC	

*OSPW NA = naphthenic acids extracted from oil sands process-affected water (Syncrude's Mildred Lake Settling Basin) and diluted in deionized water

As is evident in Table 2-1, concentrations of NAs measured by the FTIR method were consistently higher than those measured by GC-MS (Scott et al., 2008a) and HPLC/QTOFHRMS (Lu et al., 2013). Yen et al. (2004) found, for most samples, that HPLC coupled with low resolution detection indicated higher NA concentrations than FTIR. Those methods capable of producing high resolution data tend to produce lower concentration and more precise measurements (Ross et al., 2012).

Han et al. (2009) conducted a survey of eight tailings ponds at Syncrude's site, utilizing both FTIR and HPLC/TOFHRMS to determine NA concentration. It is now recognized that FTIR typically provides falsely high measurement of NAs due to response to any carboxylic acid, but Han et al. (2009) and Martin et al. (2008) found that FTIR measurements, although higher, corresponded with HPLC/HRMS (r^2 = 0.82). It is unknown if similar correlation would be found with surface and ground water samples, as many more interfering compounds, such as fatty acids, would be present (Grewer et al., 2010; Ross et al., 2012). Ross et al. (2012) deleted mass attributed to background natural fatty acids and reported NA concentrations in regional surface waters to be one to two orders of magnitude lower than those reported from low resolution techniques (RAMP, 2011).

Less than 50% of all acid-extractable organics (the organic compounds isolated from OSPW during an acidic liquid-liquid extraction) from OSPW are compounds that fit the strict definition of NAs and their oxidized products (Grewer et al., 2010; Headley et al., 2011; Pereira et al., 2013a). Thus, it is not only important to clearly define the organic compounds that are being measured by the chosen analytical technique, but also to be mindful that the acid-extractable organic fraction of OSPW, often utilized in toxicity and treatment testing, is not comprised of only NAs. Rowland et al. (2011c) and Jones et al. (2013) both detected mostly classic NAs and some monoaromatic acids in an extensively purified acidic extract (Frank et al., 2006), highlighting the impact the chosen extraction procedure has on organic compounds captured. As reported by

Headley et al. (2013a), even choice of solvent used for liquid-liquid extraction will significantly influence the distribution and recovery of organic compounds.

An absolute analytical method for the determination of NAs does not currently exist because there is no procedure that can separate each NA compound (Scott et al., 2008a). In addition, measurements taken with even the best available methodologies are not exact, as commercial mixtures of NAs or individual compounds must be used for calibrations; thus results must be viewed as semi-quantitative, at best (Martin et al., 2008). Scott et al. (2005) found that utilisation of one commercial mixture for calibration resulted in unreliable measurement of a different commercial mixture, demonstrating that significant differences exist even amongst available commercial mixtures of NAs (Lu et al., 2013). For any analytical method to be quantitative, a standard must exist for each individual compound of interest in a sample (Headley et al., 2013b). Although a number of individual NA compounds have been identified in OSPW (Rowland et al., 2011a, 2011c, 2011f, 2011g; West et al., 2013), there have been over 3000 isomers detected in acidic extracts of OSPW (Grewer et al., 2010).

Utilization of the individual NA compounds detected in OSPW and synthesized by Rowland et al. (2011a, 2011f, 2011g) greatly simplify analysis for toxicity and biodegradation studies (as described elsewhere) as even low resolution MS techniques may be employed. The use of pure compounds also enables detailed analysis of metabolites (for biodegradation studies) (Rowland et al., 2011b) or products generated from other treatments (Pérez-Estrada et al., 2011).

Although not a robust method of NA measurement, the Microtox bacterial bioassay method is most commonly employed to monitor toxicity of oil sands process affected waters (Clemente and Fedorak 2005). Examples of studies that gathered toxicity data primarily from Microtox include Herman et al. (1994), Lai et al. (1996), Holowenko et al. (2002), Lo et al. (2006), Frank et al. (2008), and Jones et al. (2011). Clemente and Fedorak (2005) point out that Microtox, which uses the luminescent bacterium *Vibrio fischeri* as the test organism, is a popular choice as the method is quicker, easier, less expensive, and more reproducible

than other toxicity bioassays. Treatment of OSPW is actually focused on toxicity removal, versus NAs or any other organic compound, and cost effective and relevant toxicity measurement continues to present analytical challenges in the oil sands industry.

To date, universal adoption of a single analytical method for NAs, or organic compounds in OSPW, has not occurred, because all current techniques possess unique advantages and limitations, and none satisfy all analytical requirements (Headley et al., 2013b). Thus, it is essential to choose analytical techniques that suit the requirements of the project, to utilize complimentary techniques, and to clearly describe all steps of the analytical process.

2.3.2 Aerobic Biodegradation of NAs in Oil Sands Tailings Ponds

As with other organic compounds, biodegradation of NAs is considered a potential remediation technique for impacted waters. Naphthenic acids can be utilized as biomarkers indicating the extent of oil source maturation due to their recalcitrance (Headley and McMartin, 2004). Resistance to microbial degradation is also exemplified by their use, in the form of metal naphthenates, as commercial antimicrobial agents in products such as wood preservatives. Despite the toxicity of NAs to microorganisms, shown by numerous studies with the Microtox method, biodegradation by acclimated microbial communities is pursued as a potential treatment mechanism. Whitby (2010) provides an extensive review of microbial degradation of NAs, summarizing the model compounds, commercial mixtures and environmental NAs that have been biodegraded by microbial communities sourced from the Athabasca oil sands region.

Tailings ponds are a necessary element of oil sands operations; OSPW must not be released to the surrounding environment and ponds were meant to function as settling basins, allowing MFT to condense and solidify. Decades-long retention times have allowed for acclimated microbial communities to develop in tailings ponds, feeding on organic substrates that remain after the bitumen extraction process. Naphthenic acids represent a portion of the organic carbon

present in oil sands process-affected waters, suggesting that biodegradation by indigenous microorganisms is possible. A majority of the NA biodegradation research has focused on tailings, as treatment of process water in tailings ponds is an imminent industry requirement.

Aerobic biodegradation study methodologies are summarized in Table 2-2. More than any other area of research on NAs, biodegradation studies have significantly increased our understanding of oil sands NAs. Progressive advancement in knowledge has been especially aided by more sensitive analytical techniques.

The first observation made by Herman et al. (1994), that acidic extracts of oil sands process-affected water differ compositionally from commercial NA mixtures, has been confirmed by numerous biodegradation studies. Repeatedly, commercial NAs have been found to be more biodegradable than NAs extracted from OSPW (Bataineh et al., 2006; Clemente et al., 2004; Del Rio et al., 2006; Han et al., 2008; Herman et al., 1994; Scott et al., 2005; Toor et al., 2013a). For example, Clemente et al. (2004) found that aerobic tailings cultures were able to degrade more than 90% of both Kodak and Merichem refined commercial mixtures in 12 days, after enrichment with the respective commercial NA mixture. Meanwhile, no biodegradation study to date has shown almost complete biodegradation of NAs in oil sands process-affected waters. Scott et al. (2005) observed the complete depletion of commercial mixtures within 14 days while only about 20 to 25% of the oil sands NAs were biodegraded after more than a month's time. Figure 2-4 provides a visual representation of the compositional differences between commercial and oil sands NA mixtures, as well as biodegradability.

A survey of a number of tailings ponds using GC/MS enabled Holowenko et al. (2002) to hypothesize that lower molecular weight NA compounds are selectively biodegraded, as aged ponds contained a larger fraction of compounds with carbon numbers greater than 22, compared to active ponds. This fraction was later discovered to comprise oxidized NAs through the use of high resolution analyses (Bataineh et al., 2006; Han et al., 2009). Nonetheless, Clemente et al.

(2004) and Scott et al. (2005) confirmed the decreased abundance of NAs with low carbon number (n) and cyclicity (Z) due to preferential biodegradation, in both commercial and oil sands NA mixtures. Similar observations were made by Biryukova et al. (2007), Videla et al. (2009), and Toor et al. (2013b). Bataineh et al. (Bataineh et al., 2006) also attributed compound recalcitrance to carbon number, but found that branching contributed more so to the structure-persistence relationship. Han et al. (2008) found inconsistent correlation between carbon number and susceptibility to biodegradation.

Table 2-2: Microcosm incubation methodologies for aerobic biodegradation of naphthenic acids

Naphthenic acid source (Concentration - mg/L)	Analytical Technique(s)	Microbial source	Enrichment? (substrate)	Incubation length	Reference
-MLSB extract (~50, ~125) -Kodak salts (100) -Surrogate acids (1 mM)	-GC/FID -CO_2 Respirometry -Microtox	-MLSB	-MLSB extract -Kodak salts -No	24 to 70 days	Herman et al. 1994
-Surrogate acids ^{14}C labelled linear, bicyclic (45)	-$^{14}CO_2$ Respirometry -Microtox -Fish bioassay	-Tailings, reclamation ponds	-No	4 to 8 weeks	Lai et al. 1996
-MLSB extract (100) -Merichem, Kodak (100) -Surrogate acid (100)	-HPLC -CO_2 Respirometry	-MLSB	-MLSB extract -Kodak salts -Merichem	30 to 40 days	Clemente et al. 2003
-Kodak salts (100) -Merichem refined (100)	-HPLC -GC/MS -CO_2 Respirometry	-MLSB	-Kodak salts -Merichem	36 to 45 days	Clemente et al. 2004
-Kodak salts, acids, Fluka, Merichem (30-100) -WIP, CT extract (30-100)	-HPLC -GC/MS	-WIP -CT	-No	40 to 49 days	Scott et al. 2005
-Monocyclic, bicyclic surrogates (5.3 x 10^3 Bq) -Kodak (40)	-FTIR -$^{14}CO_2$ Respirometry	-Wetlands sediments -Pond 5	-Kodak	2 to 4 weeks	Del Rio et al. 2006
-WIP (~50) -Merichem refined (100)	-HPLC/QTOF-MS	-WIP	-No	28 days	Bataineh et al. 2006
-Merichem refined (2)	-HPLC -GC/MS	-Soil rhizophere	-Bitumen	42 days	Biryukova et al. 2007
-WIP (~50) -Merichem refined (100)	-HPLC/QTOF-MS	-WIP	-No	28 to 98 days	Han et al. 2008
-Synthetic surrogates (8)	-GC/MS	-Dockyard sediment	-Synthetic surrogates	30 to 42 days	Smith et al. 2008
-Merichem refined (20-50) -MLSB extract (35)	-FTIR -Stable isotope -DOC, DIC	-MLSB -Reclamation sites	-Merichem	16 to 30 days	Videla et al. 2009
-Synthetic surrogates (4) n-BPBA, iso-BPBA, sec-BPBA, tert-BPBA	-GC/MS	-Coal-tar contaminated soil	-Heavy crude oil	49 days	Johnson et al. 2011
-Synthetic surrogates (2-4) n-BPBA, t-BPBA	-GC/MS	-Isolate from dockyard sediment	-Heavy crude oil -n-BPBA	49 days	Johnson et al. 2012
-Synthetic surrogates (2-4) n-BPBA, t-BPBA	-GC/MS	-Pseudomonas putida KT2440	-No	49 days	Johnson et al. 2013

Legend: MLSB = Mildred Lake Settling Basin at Syncrude, WIP = West In-pit at Syncrude, CT = Consolidated Tailings at Suncor

A parallel observation indicates that lower molecular weight compounds represent the most toxic fraction of NAs. Holowenko et al. (2002) detected reduction in Microtox toxicity with increased age of tailings ponds, although they were unable to attribute this directly to decreased low mass NAs or simply lower overall concentrations. However, Lai et al. (1996) found that Kodak commercial NAs at representative concentrations were more toxic than OSPW, via both

Microtox and fish bioassay. Both Johnson et al. (2011) and Rowland et al. (2011b) confirmed that metabolites produced during the biodegradation of individual aromatic alkanoic NAs are less toxic in the Microtox assay than their parent compounds.

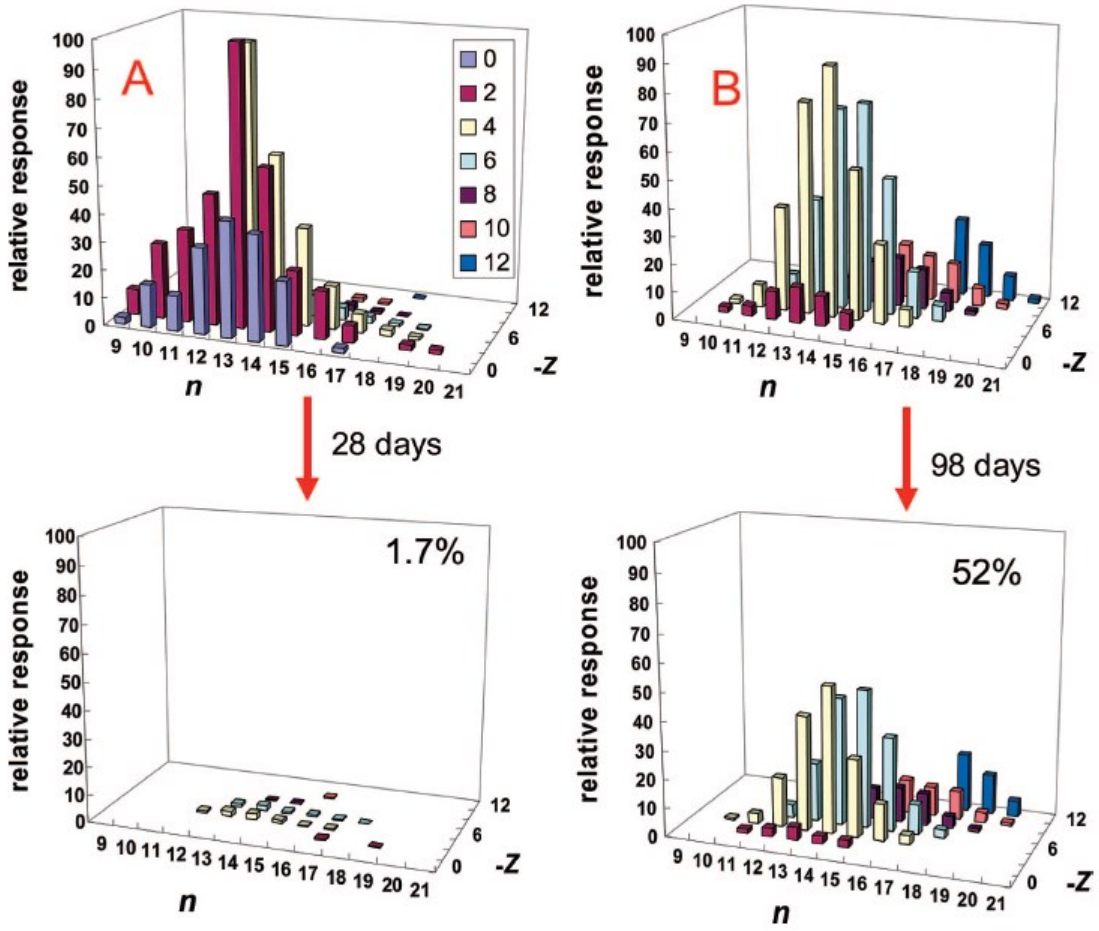


Figure 2-4: Three dimensional plots showing change in composition of A) commercial naphthenic acids and B) oil sands process affected water naphthenic acids due to biodegradation (Han et al. 2008)

Indigenous microbial populations from both Syncrude's West In-Pit and Suncor's Consolidated Tailings Pond were able to rapidly degrade Kodak commercial NAs in less than ten days under aerobic conditions, even in the presence of oil sands process-affected water (Scott et al., 2005). Herman et al. (1993) also did not observe inhibition of biodegradation of model NA compounds

in the presence of OSPW. This significant finding indicates that other constituents in OSPW are not responsible for reducing or slowing biodegradation of NAs in tailings ponds.

Utilization of high resolution analytical techniques and synthesis of individual, complex NA compounds have enabled greater inference of structural characteristics that impact biodegradability. The relationship between the extent of biodegradation and degree of branching was clearly demonstrated by Smith et al. (2008) by synthesizing model NA compounds to test this hypothesis. After 30 days, only 2.5% of the most highly branched compound was transformed, while the least branched experienced over 97% transformation (Smith et al., 2008). Likewise, the straight chain aromatic NA was completely degraded by an enriched microbial community (Johnson et al., 2011), an isolate from that community (Johnson et al., 2012), and an isolate from a culture collection (Johnson et al., 2013), while highly branched versions of the same compound were only partially degraded. Thus, an inverse relationship between biodegradation and alkyl branching appears to exist.

Naphthenic acid cyclicity has also been identified as a contributor to the structure-persistence relationship. Han et al. (2008) determined, using HPLC/HRMS, that the remaining fraction of a Merichem commercial mixture following a 28 day incubation was polycyclic. Analysis of the biodegradation kinetics of isomer classes supported increasing cyclicity correlating with greater biopersistence. Both Lai et al. (1996) and Del Rio et al. (2006) found that a bicyclic model compound was less degradable than either a linear or monocyclic compound. Toor et al. (2013b) and Hwang et al. (2013), also through HPLC/HRMS, found that increasing cyclicity rendered oil sands NA compounds more resistant to biodegradation, in a simulated wetlands environment and bioreactor, respectively.

The identification of hydroxylated, or oxidized, NA compounds by HPLC/HRMS was first made by Bataineh et al. (Bataineh et al., 2006) in oil sands process-affected water. Han et al. (2008) noted that the presence of oxidized NAs increased due to biodegradation of a commercial NA mixture. A

potentially significant trend was observed by Han et al. (2009) in comparing OSPW from active and aged (or reclamation) tailings ponds; total abundance of oxidized NAs relative to parent NA compounds was found to be a useful indicator of in-situ biodegradation. Grewer et al. (2010) also found hydroxylated NAs to be overrepresented in reclamation ponds. This supports the suggestion that mineralization of NA compounds may occur via β-oxidation (Clemente et al., 2004; Del Rio et al., 2006; Han et al., 2008). The β-oxidation pathway was directly observed in microbial degradation of individual aromatic NA compounds (Johnson et al., 2013, 2012, 2011; Rowland et al., 2011b).

Environmental factors may significantly impact a microbial community's capacity to biodegrade a substrate. Although little work has been done to optimize conditions for biodegradation of NAs, Herman et al. (1994) indicated that biodegradation was nitrogen- and phosphorus-limited. Microcosms in all studies reviewed have been supplemented with a minimal mineral medium supplying both nitrogen and phosphorus, typically modified Bushnell-Haas (Wyndham and Costerton 1981). Nitrogen and phosphorus addition to OSPW being treated in a simulated wetlands environment did not improve biodegradation of NAs, likely because sufficient nutrient loading existed (Toor et al., 2013a). Lai et al. (1996) investigated the factors that affect the degradation of model NAs, including temperature, phosphate, and dissolved oxygen. Reducing the dissolved oxygen concentration, via nitrogen gas sparging, and temperature were both shown to slow the rate of degradation while phosphate addition significantly increased biodegradation rates and also reduced the acute toxicity of the tailings pond water to a fish bioassay. However, Lai et al. (1996) suggest that the oxygen requirement for NA degradation is relatively low in the absence of phosphate. Temperature was found to have a more significant impact on microbial degradation kinetics of three model NAs in Athabasca River water, compared to pH and dissolved organic carbon; the first order rate constant increased ten-fold between 10 and 30°C (Headley et al. 2002 as referenced in Headley and McMartin 2004). The authors of this study also concluded that the persistence of the individual NA compounds in aquatic systems is structurally

dependent, whereby the trans- isomer, with more “open” geometry, was less resistant to microbial degradation than the cis-isomer.

A mixed consortium of two species of bacteria, fast growing *Pseudomonas putida* and slower growing *Pseudomonas fluorescens*, was able to degrade more than 95% of the Kodak commercial NA mixture, but inoculations with either isolate only showed about 15% degradation (Del Rio et al. 2006). This finding is not surprising as even commercial NA mixtures are complex and mixed cultures are more likely to contain organisms with the variety of metabolic pathways required for degradation. While two isolates were capable of degrading an aromatic NA completely (Johnson et al., 2013, 2012), neither have been utilized to degrade OSPW NA mixtures.

To infer capacity for in-situ biodegradation of NAs, Han et al. (2009) measured ten oil sands process-affected water samples from Syncrude’s site, using both FTIR and HPLC/HRMS, including dyke seepage, active settling ponds, and experimental reclamation ponds. Contrary to the hypothesis drawn from laboratory biodegradation experiments that cyclicity and alkyl branching render compounds biopersistent, the NA signature in reclamation ponds containing aged OSPW did not differ significantly from other, active, tailings ponds. Based on changes in concentration in the Syncrude reclamation ponds, Han et al. (2009) estimated the half-life of NAs is about 13 years, assuming first order biodegradation kinetics. Thus, depending on in-situ biodegradation is not a viable solution for OSPW treatment (Quagraine et al., 2005b).

2.3.3 Other Organics in OSPW

A vast majority of the research on treating OSPW has been focused on NAs, without much consideration for other organics found in OSPW. Indeed, as toxicity testing has shown, all chosen treatment regimes for OSPW will need to address NAs. However, since Grewer et al. (2010), Headley et al. (2011), and Headley et al. (2013a) detected the presence of many other organic compounds, including those containing nitrogen and sulphur, many of which have not yet been identified or quantified, it has become clear that there may be other

organics present in OSPW that will need to be treated prior to release. For example, Pereira et al. (2013a, 2013b) recently detected O_2 species that are not carboxylic acids in the acid-extractable fraction of OSPW, some of which may be similar in structure to hormones. Allen (2008a) suggested significant removal of benzene, BOD, COD, phenols, PAHs, and oil will be required to meet environmental guidelines for surface water.

While controlling for NA total concentration, Garcia-Garcia et al. (2011b) found that the extracted organic fraction of OSPW induced more toxic responses than commercial NA mixtures, in mammalian tissues. This could be due to either other toxic compounds (aside from NAs) being present in the organic fraction of OSPW or differences in NA composition. Two OSPW samples collected from the same tailings pond but a year apart were found to exhibit significantly different toxicity, with both acute and chronic exposures to a model aquatic invertebrate, despite similar NA concentrations; the authors postulated this may be attributed to differences in NA composition (Anderson et al., 2012b), but neither this nor the presence of other organics were measured. While Garcia-Garcia (2011b) deliberately used the entire organic content of OSPW, most toxicity testing has been conducted with the acid-extractable organic fraction of OSPW, assumed to be only NAs for many years. It cannot be said, then, that the detected toxic responses are solely due to NAs. To date, no one has been able to extract only the classical NA compounds from OSPW to perform extensive toxicity testing. While individual representative NA compounds have been synthesized for toxicity and biodegradation studies (Johnson et al., 2013; Jones et al., 2011; Rowland et al., 2011a, 2011c, 2011f), it could take many years to exhaust all the potential toxic compounds in OSPW. In addition, cumulative effects from multiple components may render OSPW more toxic as a whole than the sum of individual compounds (Kindzierski et al., 2012; Miskimmin et al., 2010).

No acute (96 hour exposure) toxicity to rainbow trout was detected in one OSPW sample, despite carboxylic acid content (as measured by FTIR, and thus including, but not limited to, NAs) (Toor et al., 2013a) and NA signature (Toor et al., 2013b) being similar to another OSPW sample that was acutely toxic. Thus,

a component of OSPW that causes toxicity, which was not measured, must have been missing from this particular sample (Toor et al., 2013a). Ozone treatment reduced mammalian immunotoxicity, but this could not be definitely attributed to the 75% removal of NAs or 40% removal of all organic matter (Garcia-Garcia et al., 2011a). Many recent treatment and toxicity experiments have focused on measuring classical NAs via high resolution techniques, yet Scarlett et al. (2013) suggested that the aromatic fraction of acid-extractable organics was similarly toxic as OSPW and moreso than classical NAs.

2.3.4 Treatment Options for OSPW

Naphthenic acids have become a priority pollutant in the oil sands industry. Marsh (2006) suggested that the NAs remaining in oil sands process-affected waters after many years are resistant to biodegradation by the indigenous microorganisms found in tailings ponds. This is supported by numerous biodegradation studies, described above, that show mineralization of commercial NA mixtures, but recalcitrance of NAs from oil sands process-affected waters. As indicated by Scott et al. (2008b), biodegradation is considered the most cost-effective treatment option for mitigating wastewater toxicity. However, biodegradation will need to be supplemented with other treatment methods to remove NAs and other organic contaminants efficiently. Two methods of treatment for OSPW that are being actively explored by industry will be discussed subsequently: chemical oxidation by ozone and constructed wetlands or end pit lake strategy.

2.3.4.1 Ozone Treatment

Advanced oxidation of NAs via ozone treatment has been pursued as a means of reducing NA recalcitrance. All studies to date using ozone to treat OSPW are summarized in Table 2-3. Scott et al. (2008b) was the first to treat fresh OSPW with dissolved ozone, a highly reactive oxygen compound. The shift in NA composition to lower molecular weight NA compounds seen in this study has been verified with high resolution analytical techniques, where ozone has

preferentially degraded NA compounds with high carbon numbers and cyclicity (Gamal El-Din et al., 2011; Pérez-Estrada et al., 2011; Wang et al., 2013). In addition, both Pereira et al. (2013b) and Gamal El-Din et al. (2011) observed an increase in low molecular weight NA compounds, and other organics (Pereira et al., 2013b), following ozone treatment. These observations indicate that ozonated OSPW NAs and other organics may be more susceptible to biodegradation (Gamal El-Din et al., 2011; Pereira et al., 2013b; Pérez-Estrada et al., 2011; Scott et al., 2008b; Wang et al., 2013). Also, biochemical oxygen demand (BOD) increased due to treatment; from 2 to 15 mg/L (Scott et al., 2008b), 8 to 25 mg/L with an ozone dose of 150 mg/L (Gamal El-Din et al., 2011), and from 3 to 15 mg/L at an ozone dose of 360 mg/L (Wang et al., 2013).

Martin et al. (2010), Hwang et al. (2013), and Wang et al. (2013) coupled ozone treatment with biodegradation to treat OSPW from an active Syncrude tailings pond, West In Pit. The authors hypothesized that ozonation would selectively oxidize the most biopersistent fraction of NAs, thereby increasing the rate and extent of biodegradation. Lower molecular weight NAs were preferentially biodegraded, and the rate of degradation of these compounds was greater in ozonated OSPW than untreated (Martin et al., 2010; Wang et al., 2013). Martin et al. (2010) monitored oxidized NAs; both ozonation and subsequent incubation for 98 days lead to an overall reduction in oxidized NAs, also observed by Wang et al. (2013) after 28 days of incubation. Hwang et al. (2013) eradicated 99% of the NAs in OSPW with an ozone dose of 80 mg/L and thus did not observe subsequent biodegradation in a continuous flow biofilm reactor after four weeks. However, 87% of the carboxylic acids, as measured by FTIR, were removed in the bioreactor with ozone pretreatment. In addition, increased biofilm thickness and reduction in microbial community diversity was observed in bioreactors fed with ozonated OSPW, versus untreated.

Table 2-3: Impact of ozone treatment on NAs and other parameters in OSPW

Ozone Dose or Exposure	NA Reduction (Method)	CA Reducation (FTIR)	Other Measurements	OSPW Source	Reference
11, 50, **130** min at 35 mg/L dissolved O_3	Shift to low MW (GC/MS)	>95% (GC/MS)	BOD: 2 to 15 mg/L TOC: 59 to 46 mg/L IC_{20}: 23% to >100%	Syncrude Recycle Water Pond	Scott et al. 2008
Dose not measured, 60 s and 90 s exposure	54% and 73% (HPLC/HRMS)	Not measured	IC_{20}: Little change	Syncrude WIP	Martin et al. 2010
Dose not measured, bubbled at 2 L/mn	24% and 85% (HPLC/HRMS)	Not measured	Various H295R cell line endpoints	Syncrude WIP	He et al. (2010)
Utilized ozone doses: 20, 40, 80, **150** mg/L	>90% (80 mg O_3/L)	64% (80 mg O_3/L)	COD: -22% $cBOD_5$: 8 to 25 mg/L IC_{20}: 23% to >100%	Syncrude WIP	Gamal El-Din et al. (2011)
Dose not measured, bubbled at 2 L/mn	Not measured Shift to low MW	Not measured	Not measured	Syncrude WIP	Pérez-Estrada et al. (2011)
As reported in Martin et al. (2010)	75% (HPLC/HRMS)	Not measured	Whole organic fraction: -37% Mouse endpoints	Syncrude WIP	Garcia-Garcia et al. (2011)
Utilized ozone dose: 80 mg/L	>90% (HPLC/HRMS)	Not measured	Various fathead minnow endpoints	Syncrude WIP	He et al. (2012)
Utilized ozone dose: 30 and 80 mg/L	49% (30 mg/L) 90% (80 mg/L) (HPLC/HRMS)	Not measured	Various *Chironomus dilutus* endpoints	Syncrude WIP	Anderson et al. (2012)
Utilized ozone dose: 80 mg/L	>99% (HPLC/HRMS)	78%	COD: -18%	Syncrude WIP	Hwang et al. (2013)
Utilized ozone dose: up to 350 mg/L	49% (30 mg/L) 90% (80 mg/L) (HPLC/HRMS)	Not measured	COD: ↓ as O_3 ↑ $cBOD_5$: 3 to 15 mg/L IC_{20}: 23 to 54% (90)	Syncrude WIP	Wang et al. (2013)
Utilized ozone dose: 20 and **50** mg/L	ESI-: -59% ESI+: -68% (HPLC/Orbitrap)	Not measured	AEO (mass): -30% (20), -73% (50)	Syncrude WIP	Pereira et al. (2013)

*Measurements from bolded and underlined dose, unless otherwise indicated

Of course, ozonation is advantageous for its ability to degrade most organic compounds, which is essential in OSPW treatment where specific contaminants have not yet been definitively linked to toxic effects. While ozone significantly degraded NAs (Anderson et al., 2012b; Gamal El-Din et al., 2011; Garcia-Garcia et al., 2011a; He et al., 2012a, 2010; Hwang et al., 2013; Martin et al., 2010; Pereira et al., 2013b; Wang et al., 2013), it has also been found to greatly reduce other organic compounds in OSPW, when measured, including carboxylic acids measured by FTIR (Gamal El-Din et al., 2011; Hwang et al., 2013), the acid-extractable organic fraction (Bataineh et al., 2006; Clemente et

al., 2003; Han et al., 2009, 2008; Quagraine et al., 2005b; Scott et al., 2005) of OSPW measured gravimetrically and with very high resolution MS (Pereira et al., 2013b), and the total organic fraction of OSPW also determined gravimetrically (Garcia-Garcia et al., 2011a). Pereira et al. (2013b) were able to identify classes of organic compounds in the acid-extractable organic fraction of OSPW that were most susceptible to degradation by ozone, including NAs, as well as organic species that were not degraded, including non-NA compounds containing O_2 (which have yet to be identified).

The relationship between organic compound structure and susceptibility to degradation by ozone and microorganims may render coupling of ozone pretreatment with biodegradation necessary for complete treatment of NAs and other organics in OSPW. Increased time of exposure to ozone has not resulted in a proportional increase in NA degradation; in fact, the reaction is most efficient in the first minute of exposure (Gamal El-Din et al., 2011; Pérez-Estrada et al., 2011; Wang et al., 2013). Thus, complete mineralization via ozonation of all organic compounds in OSPW will likely be very cost prohibitive. It is clear, however, from the work completed thus far that coupling ozonation with biodegradation will need to be optimized, particularly ozone dose and exposure time for biodegradation.

It is imperative to explore if the products of ozone treatment of OSPW would increase toxicity (Anderson et al., 2012b; Garcia-Garcia et al., 2011a; He et al., 2012a, 2010). Ozone treatment has been shown to reduce, but often not completely remove, toxicity for various endpoints, including aquatic life forms (Anderson et al., 2012b; He et al., 2012a) and mammals (Garcia-Garcia et al., 2011a; He et al., 2010; Wang et al., 2013), independent of dose. Garcia-Garcia et al. (2011a) did indicate that oxidation products may have caused toxic effects *in vivo*, as impact on some mammalian immunotoxicity endpoints were greater in ozonated OSPW than untreated. Responses of *Vibrio fischeri* to ozone treated OSPW, measured via the Microtox assay, seem less conclusive, but likely less relevant. While Wang et al. (2013) saw reduced effect on *Vibrio fischeri* after ozonation of OSPW, Martin et al. (2010) did not observe a reduction in effect until

subsequent biodegradation, where ozone pretreatment greatly improved toxicity reduction. Increasing ozone dose clearly reduced then removed toxicity to *Vibrio fischeri* (Gamal El-Din et al., 2011; Scott et al., 2008b). Interestingly, it was through ozone toxicity testing that Anderson et al. (2012b) and He et al. (2012a) were able to conclude that organics are the main cause of OSPW toxicity, versus metal compounds.

Some practical considerations of ozone treatment of OSPW have also been explored. Fortuitously, NA degradation by ozone has been found to be optimum in alkaline environments, meaning pH adjustment of OSPW will not be required for treatment (Pérez-Estrada et al., 2011). Reactor size did not impact COD removal at various ozone doses (Wang et al., 2013). Removal of particulate matter with filtration was not required for treatment of carboxylic acids (and thus, NAs) with ozone (Gamal El-Din et al., 2011). Both Wang et al. (2013) and Pérez-Estrada et al. (2011) postulate that the preferential attack by ozone of NAs with high carbon numbers and degrees of cyclicity is due to the ozone or hydroxyl radicals being better able to oxidize hydrogen atoms attached to tertiary carbons, which are introduced with more complex structural elements, such as rings and alkyl branching.

2.3.4.2 Constructed Wetlands and End Pit Lakes

Utilization of constructed wetlands, which are built to mimic natural wetlands systems in their ability to filter out pollutants, has been long considered a promising treatment technology in the oil sands industry (Foote, 2012). Large scale systems are in operation at a number of oil refineries, indicating promise for their use to treat OSPW (Allen, 2008b). Both Suncor and Syncrude have established experimental reclamation ponds on their leases, under various experimental conditions, to investigate the viability of the wet landscape strategies, including water capping (fresh water placed over MFT) and constructed wetlands (Quagraine et al., 2005b). Syncrude commissioned the first commercial scale demonstration end pit lake, Base Mine Lake, in 2012, consisting of fresh water capping MFT (Syncrude, 2013).

Toor et al. (2013a) utilized simulated constructed wetlands to treat fresh OSPW with sediment collected from a regional wetland. After eight months in a year long incubation period, with a hydraulic retention time of 400 days, carboxylic acids measured by FTIR had been reduced to 18 mg/L, the same concentration indicated by Quagraine et al. (2005b) as the practical limit for in-situ degradation.

Despite residence times of two decades, aged OSPW from experimental reclamation ponds continue to exhibit toxic effects on relevant species. Some, but not all, endpoints of a regional benthic invertebrate species were impacted by exposure to OSPW from three different reclamation ponds, but to a significantly lesser degree than fresh OSPW (Anderson et al., 2012a). While fathead minnows were not acutely toxic to aged OSPW, subchronic impacts were observed, including in reproduction (Kavanagh et al., 2011), and stocked fish of the same species continue to exhibit reproductive impairment and endocrine disruption, after generations of exposure (Kavanagh et al., 2013). Ponds containing both MFT and OSPW significantly impacted phytoplankton communities, while ponds containing MFT overlain with water did not (Leung et al., 2001). It remains unclear what components of aged OSPW are contributing to toxicity (Kavanagh et al., 2013, 2011; Kindzierski et al., 2012; Miskimmin et al., 2010).

Microbial communities present in OSPW-impacted wetlands environments have been shown to be different than those inhabiting natural systems in the area (Daly, 2007; Hadwin et al., 2006). Bacterioplankton communities in impacted wetlands exhibited metabolic activity five times lower than that found in reference wetlands (Daly, 2007).

Research to date indicates that depending on a constructed wet landscape environment to naturally detoxify and treat of OSPW is not wise (Anderson et al., 2012a; Quagraine et al., 2005b). That said, end pit lakes will form part of the reclamation landscape and so there may still be options for coupling pretreatment of OSPW prior to discharge and allowing in-situ processes to complete treatment may provide a cost-effective option for establishing

systems with equivalent biological viability as natural lakes in the region (Kavanagh et al., 2013).

2.3.5 Microbial Communities in Oil Sands Tailings Ponds

Despite the toxic nature of NAs and other organic components of OSPW, microorganisms are prolific in oil sands tailings ponds; only recently have efforts to profile these microbial communities been made, as next generation sequencing has enabled large scale profiling to be conducted.

Herman et al. (1994) identified microorganisms from oil sands tailings aerobic enrichment cultures that were able to biodegrade NAs. Enrichment cultures were plated and two species were isolated from the commercial NA enrichment (*Pseudomonas stutzeri* and *Alcaligenes denitrificans*) and three from the tailings extract (*Acinetobacter calcoaceticus*, *Pseudomonas fluorescens* group, and *Kurthia* sp.). Both a culture collection *Pseudomonas putida* (Johnson et al., 2013) and a *Mycobacterium* isolated from dockyard sediment in England (Johnson et al., 2012) were able to degrade model NA compounds. A co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens*, isolated from OSPW-impacted wetland sediment, were capable of degrading commercial and model NAs (Del Rio et al., 2006).

Investigation of microbial communities present in mature fine tailings was conducted by Penner and Foght (2010), identifying archaea from the *Methanosaeta* genus and diverse bacterial species, from hydrocarbon-degrading, nitrate-, iron-, and sulphate-reducing genera, using clone libraries of amplified 16S rRNA genes. Both Golby et al. (2012) and Chi Fru et al. (2013) cultured MFT samples, in a biofilm system and bioreactors, respectively, and monitored shifts in engineered systems to the original samples using next generation sequencing. The biofilm was less diverse than the inoculum and was dominated by *Pseudomonas*, *Thauera*, *Hydrogenophaga*, *Rhodoferax*, and *Acidovorax* species (Golby et al., 2012). Yergeau et al. (2012), while establishing that bacterial communities in MFT are distinctly different and less diverse than those in natural sediments in water bodies in the Athabasca oil sands region, detected

Rhodoferax, *Thiobacillus*, and *Smithella* species only in MFT. Ramos-Padrón et al. (2011) also detected these genera in the anaerobic (ie. non-surface) zone of a tailings pond.

The aerobic zone, found only at the surface of a tailings pond, was found to have a very different community profile than samples from the rest of the pond profile (Ramos-Padrón et al., 2011). One year after closure of an active tailings pond, the microbial community in OSPW had shifted from predominantly methanogenic species to aerobic hydrocarbon degraders, from the *Pseudomonas*, *Acidovorax*, and *Acinetobacter* genera (Ramos-Padrón, 2013).

While a majority of the recent microbial community profiling in tailings ponds has focused on MFT, as observed releases of methane from the surfaces of tailings ponds are investigated (Chi Fru et al., 2013; Holowenko et al., 2000; Ramos-Padrón et al., 2011; Siddique et al., 2012, 2007, 2006), understanding the microbial communities present in OSPW under aerobic conditions will be beneficial in developing appropriate treatment regimes (Ramos-Padrón et al., 2011; Yergeau et al., 2012).

3 Indigenous Microbes Survive In-Situ Ozonation Improving Biodegradation of Dissolved Organic Matter in Aged Oil Sands Process-Affected Waters

3.1 Introduction

With about one million cubic metres of fluid tailings (consisting of MFT and OSPW) currently stored in tailings ponds (Martin et al., 2008), devising efficient and cost-effective treatment technologies to remediate OSPW for eventual release to the environment is a considerable challenge facing the oil sands industry. While a significant portion of OSPW will need to be treated for release to the Athabasca River (potentially to offset future withdrawals) and to improve water quality for recycling, a portion will be used to establish reclaimed landscapes (Allen, 2008a).

Wet landscape reclamation will include end-pit lakes, whereby MFT and OSPW may be placed in mined-out areas and capped with river water (Allen, 2008a). However, aged OSPW (from tailings ponds with long retention times and no inputs of fresh OSPW) continues to contain organic compounds at concentrations greater than in regional surface and ground waters, including the Lower Athabasca River (Headley et al., 2011), and without knowledge of what impacts these compounds may have on the local ecosystem, treatment of OSPW is likely required before placement in wet landscapes. Aged OSPW samples are less acutely toxic to aquatic life after decades of retention (Holowenko et al., 2002; Anderson et al., 2012), but effects have not been completely removed (Anderson et al., 2012a; Kavanagh et al., 2013, 2011). Decades-long retention times have allowed acclimated microbial communities to develop in tailings ponds and utilize NAs and other organics as a substrate, but a fraction of oil sands NAs has proven to be recalcitrant (Quagraine et al., 2005b; Scott et al., 2005). Thus, natural attenuation is not a feasible treatment option for OSPW released to the environment via end-pit lakes.

Ozone treatment has been used for pretreatment of NAs in fresh OSPW to enhance subsequent biodegradation (Scott et al., 2008b; Martin et al., 2010; Wang et al., 2013), because the more recalcitrant NAs are preferentially attacked by molecular ozone and/or hydroxyl radicals which are formed from ozone at OSPW's alkaline pH (Pérez-Estrada et al., 2011; Wang et al., 2013). Advanced oxidation processes are often used to oxidize and thus render complex organic

compounds in wastewaters more amenable to biodegradation (Metcalf and Eddy, 2003). In groundwater remediation, survival of native microbial populations following oxidation enables more complete organic contaminant degradation leading to mineralization (Sutton et al., 2010). A similar treatment philosophy could be utilized during the establishment of end-pit lakes.

Aggressive and cost-effective technologies that address all organics in OSPW, not just NAs, are needed to transform tailings ponds environments into reclaimed aquatic environments (Quagraine et al., 2005b). Previous work by Martin et al. (2010) and Scott et al. (2008b) showed that ozone pretreatment of OSPW and subsequent inoculation with indigenous microbes improved biodegradation of NAs. Building upon these findings, here we focused on understanding the microbial response (through seeding and not seeding microbial communities indigenous to tailings ponds) to ozone pretreatment of OSPW and link this with biodegradation of not just NAs but organic compounds. Thus, the aim of this research was two-fold: (1) to explore ozonation and *in situ* biodegradation as a treatment option for aged OSPW, focusing on the dissolved organic fraction confirmed analytically to contain low NAs concentrations, and (2) to understand the capacity for indigenous oil sands tailings microbial communities to survive ozone exposure and degrade residual organics (including ozonation by-products). This is believed to be the first report of ozone pretreatment applied to aged OSPW samples to improve biodegradability of dissolved organic matter and not just classical NAs.

3.2 Methods and Materials

3.2.1 OSPW Samples and Supplies

OSPW samples were collected from Syncrude Canada in August 2009 and stored in plastic pails at 4°C until experiment establishment in April 2010. Twenty litres were collected from two experimental reclamation ponds. The “FE5” pond was constructed in 1989 to contain OSPW overlying MFT. The “Big Pit” pond was constructed in 1993 to contain fresh water (no OSPW) also over MFT

(which contains OSPW in its porewater). Both ponds were constructed with approximate 1:1 (v/v) proportions of MFT and water (Han et al., 2009).

Unless otherwise stated, all chemicals and supplies were obtained from Thermo Fisher Scientific (Fair Lawn, NJ).

3.2.2 Ozonation of OSPW

All water samples were vacuum filtered with 8 µm ashless cellulose filter paper (Whatman plc, Maidstone, UK) to remove suspended solids without the mass removal of microorganisms. Gamal El-Din et al. (2011) demonstrated, however, that filtration is not required prior to ozone treatment. Eight litres of the 20 L samples collected from each reclamation pond was not ozonated. Four litres were used as a sterile control autoclaving at 121ºC, 208 kPa, twice for 20 min with 24 h in between. Twelve litres of each aged OSPW sample were treated in a 20 L semi-batch reactor system by Nan Wang, as described previously (Gamal El-Din et al., 2011). Ozone was generated using extra dry, high purity oxygen (99.6%) fed into an ozone generator (GSO-40, Wedeco Environmental Technologies, Herford, Germany). Ozone was sparged into OSPW samples through a ceramic fine bubble gas diffuser (Cole-Parmer, Canada) located at the bottom of a PVC plastic reactor. Ozone feed and off gas were continuously monitored with inline monitors (models HC-500 and LC-500, respectively, PCI-WEDECO, Herford, Germany) and residual ozone in the reactor was measured using the Indigo colorimetric method (APHA, 2005). The calculated utilized ozone concentration was 50 mg/L for both waters. Industrial level nitrogen (99.995%) was then bubbled into the ozonated water sample for 10 min to strip any residual ozone and oxygen from the reactor after ozonation. Treated waters were stored at 4°C until microcosms were established 8 d after ozonation.

3.2.3 Establishment of Aerobic Microcosms

Biodegradation experiments were conducted to monitor reduction in the concentration of dissolved organic carbon (DOC) and OSPW NAs. NAs

speciation changes were also monitored. Incubation duration was 84 d for both oil sands reclamation pond water samples.

Four treatments were established for each water sample: Ozonated, Ozonated-Inoculated, Untreated, and Sterilized. Ozonated water, as described in Sec. 3.2.2, was split between the first two treatments. In addition, the Ozonated-Inoculated treatment received an inoculum of microorganisms indigenous to the same water sample, at the same natural microbial density. The Untreated treatment acted as a positive control to compare to previous incubation experiments (Scott et al., 2005; Han et al., 2008). The Untreated treatment could not be established for the Big Pit OSPW sample because the water sample was lost. Sterilization was utilized as a negative control to monitor any abiotic changes. Four replicates were established for each treatment. Three replicates were sampled weekly whereas the remaining replicate was only sampled at the end of the experiment. Results from the fourth replicate were comparable to that of the weekly-sampled triplicates.

Aerobic incubations were rotated on a shaker at 250 rpm at room temperature (about 20°C) in 500 mL Erlenmeyer flasks outfitted with plastic foam plugs to allow for air exchange. The initial liquid volumes of all replicates were 300 mL, consisting of 285 mL of the appropriate sample and 15 mL filter-sterilized modified Bushnell Haas medium, supplementing the microcosms with 1 mM and 0.7 mM nitrogen and phosphorus, respectively. The medium consisted of (in g/L) 1.0 KH_2PO_4, 1.0 Na_2HPO_4, 0.5NH_4NO_3, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4$-$7H_2O$, 0.02 $CaCl_2$-$2H_2O$, 0.002 $FeCl_3$, and 0.0018 $MnSO_4$-H_2O, adjusted to pH 7.0 (modified from Wyndham and Costerton, 1981). Martin et al. (2010) and Scott et al. (2005), incubated 250 mL in 500 mL flasks and aeration was adequate; in addition, sufficient oxygen for biodegradation was supplied due to long incubation time (84 d). For the Ozonated-Inoculated treatment, 300 mL of the same untreated pond water samples were centrifuged in 500 mL polypropylene containers (Kendro Laboratory Products, Hanau, Germany) for 30 min at 3000 *g* in a Heraeus Multifuge 3S-R outfitted with a swinging bucket rotor

to form a pellet of microbial cells, which was then resuspended in 15 mL modified Bushnell Haas medium.

Evaporative losses from microcosms, due to use of foam plugs, was monitored gravimetrically and found to be 0.58 mL/d. This correction factor was applied to chemical and microbial density data.

3.2.4 Chemical Analysis

Aseptic weekly sampling from three replicates included analysis for DOC, NAs, and biomass quantification. Five millilitres were collected for DOC analysis; samples were filtered with 0.45 µm Millex-LCR Millipore syringe filters prior to analysis. Sample analysis was conducted on a Shimadzu Model TOC-5000A (Shimadzu, Japan) at the University of Alberta Biogeochemical Analytical Laboratory according to EPA method 415.1. DOC concentrations were determined via external calibration. Statistical analyses of DOC data were completed using SigmaPlot Version 11.0 (Systat Software, San Jose, CA), via Two-Way ANOVA and the Holm-Sidak method for pairwise comparisons. Data were fit to an exponential decay model shown in the below equation, with the addition of an asymptote representing the recalcitrant DOC fraction, by minimizing the sum of square errors through the manipulation of k, S_n, and S_o using Excel Solver GRG nonlinear engine.

$$S = S_o\, e^{-kt} + S_n$$

Another sample of 5 mL was collected and stored with an equivalent volume of HPLC methanol at 4°C for further mass spectrometry analysis, completed by Leónidas Pérez-Estrada. To reduce NA analysis requirements, the triplicate samples were pooled, centrifuged for 15 min at 3000 *g* and the supernatant retained for NA analysis by UPLC-TOF-MS (HRMS) (Pérez-Estrada et al., 2011). Low standard deviations of DOC measurements showed low variation among replicate microcosms, supporting the use of consolidated samples for NA analysis to arrive at pseudo-averaged results. For NA analysis, briefly, an UHPLC system separated NA compounds based on retention time and exact masses corresponding to the general chemical formula $C_nH_{2n+Z}O_2$ or

$C_nH_{2n+Z}O_3$ and analyses were completed as described previously (Martin et al., 2010). Tetradecanoic acid-1-^{13}C was utilized as an internal standard.

Acid-extractable organics in ozone-treated OSPW samples were measured using Fourier transform infrared (FTIR) spectroscopy, which has a method detection limit of 1 mg/L (Scott et al., 2008a; Grewer et al., 2010). Performed in triplicate, 250 mL samples were acidified to pH 2 with HCl and exchanged three times with 50 mL HPLC grade dichloromethane (DCM). The pooled solvent extracts, containing the acid-extractable organics, were dried and reconstituted in a known amount of DCM and stored at 4 °C. Measurement was completed on a Spectrum 100 FTIR (Perkin Elmer, Waltham, MA) using a 3 mm-spaced, potassium bromide window cell (University of Alberta, Edmonton, AB). Data were collected with Spectrum software (V10, Perkin Elmer, Waltham, MA) using 32 scans. The combined peak heights at 1743 cm^{-1} (for monomers) and 1705 cm^{-1} (for hydrogen-bonded dimers) were determined. A commercial refined NA preparation (Merichem Chemicals and Refinery Services, Houston, TX) was used to prepare the calibration curve for quantification, as described by Scott et al. (2008a), and is provided in Appendix 1.

Acidic and basic extractions were completed on remaining water samples following ozone treatment and combined for gas chromatography-mass spectrometry (GC-MS) analysis to determine DOC components, but no discernible analytes were detected. The acidic and basic solvent extractions were completed as above, except that the pH was adjusted to 11 for the basic extraction. The pooled solvent extracts were run on an Agilent 7890 GC with 5975C mass spectrometer using electron ionisation. GC-MS conditions were 1 µL splitless injection 10:1 at 250°C; Phenomenex Zebron ZB-5 column (30m long, 0.25mm inner diameter and a 0.25 µm film thickness); GC temperature programme 50°C for 1 min, ramp 10°C per min to 290°C, held for 5 min, helium 1 mL/min; transfer line 250°C, the MS source 230°C, quadruple mass analyzer 150°C.

Acute toxicity analysis was conducted on OSPW samples prior to and after incubation with a Microtox 500 Analyzer (Azur Environmental, Carlsbad,

CA), using the 81.9% Basic protocol, four 2:1 dilutions, and 5 min incubation period. Measurements were expressed using EC_{20}, representing the percent volume of sample required to reduce the luminescence of the test specimen by 20%. Phenol and deionized water were used as positive and negative standards, respectively, and phenol EC_{20} was between 4 and 8 mg/L.

3.2.5 DNA Extraction

Another 1.5 mL was collected in microcentrifuge tubes from each replicate to be used for microbial community analysis. DNA was extracted using Dynabeads DNA DIRECT Universal kit (Life Technologies, Carlsbad, CA), using the provided protocol for bacterial cells. Briefly, 200 µL of Dynabeads supplied in a lysis buffer was added to 1.5 mL culture sample and incubated at room temperature for 5 min. The magnetic beads, with DNA attached, were rinsed twice with washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 0.1 mM EDTA), before being incubated in 40 µL reconstituting solution (10 mM Tris-HCl, pH 8.0) at 60 °C for 5 min to release DNA from the beads. The magnetic beads were removed from the DNA extracts prior to storage at -20 °C.

3.2.6 Microbial Density by qPCR Assay

Microbial density measurements were based on the amplification of the RNA polymerase beta subunit (*rpoB*) gene (Nava et al., 2011), utilizing *rpoB* 1698f (5'-AACATCGGTTTGCTCAAC-3') and *rpoB*2041r (5'-CGTTGCATGTTGGTACCCAT-3') primers. Quantitative PCR (qPCR) was conducted using a Bio-Rad CFX96 optical reaction module conversion of the C1000 Touch thermal cycler. A 20 µL qPCR mix contained 1 µL extracted DNA template, 10 µL Bio-Rad EvaGreen supermix 2x, 50 pmol of each primer, in sterile milliQ water. The cycle conditions were as follows: initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 10 s and annealing at 50 °C for 30 s, followed by melt curve analysis. The qPCR assay was performed on samples from Days 0, 8, 22, 34, 57, and 83 of the biodegradation experiment. Each biological replicate was measured twice (n = 6), except for Day 0 samples,

where only one biological replicate existed (n = 2). An absolute quantitative standard calibration curve was generated for each run using seven ten-fold serial dilutions of PCR amplified, purified, and measured, using NanoDrop 2000C (Thermo Scientific, Wilmington, DE), genomic DNA from *Pseudomonas putida* (n = 3). The coefficient of correlation for all standard curves was greater than 0.99 and efficiencies fell between 94 and 102%. Standard calibration curves are provided in Appendix 2. Sample gene copy number was then determined based on original culture extracted for DNA. Statistical analyses of natural log-transformed qPCR data were conducted using SigmaPlot Version 11.0 (Systat Software, San Jose, CA), via Two-Way ANOVA and the Holm-Sidak method for pairwise comparisons.

Heterotrophic plate counts were conducted initially, but no growth occurred with the ozonated treatments. After ozone treatment, 100 µL sample was spread on Lennox Agar (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 15 g agar in 1 L, adjusted to pH 7.0) and incubated for five days at 30 °C. At the same time, untreated sample was also plated.

3.3 Results and Discussion

3.3.1 Water Characterization

Two water samples were utilized in this first study on treating recalcitrant organic matter in aged OSPW. The water samples were obtained from experimental reclamation ponds, established by Syncrude Canada, to investigate remediation of tailings under steady state conditions. Both ponds were constructed with MFT, comprising approximately 70% water and 30% fine clay, and water at approximately 1:1 (v:v) ratio. As reported by Han et al. (2009), Pond “FE5” was constructed in 1989 by placing OSPW above MFT. Pond “Big Pit”, built in 1993, contains MFT capped with fresh water, and due to MFT releasing pore water and organics over time, the sample received may be viewed a mix of fresh water and OSPW.

The organic content in both ponds was stable prior to sample collection, as indicated by DOC and chemical oxygen demand internal monitoring

measurements taken by Syncrude Canada since pond establishment. These water samples represent the oldest OSPW available, because most tailings ponds on an oil sands industrial site are interconnected for water management and recycling, thus receiving fresh inputs regularly. Measured water chemistry data for cations, anions, and heavy metals are provided in Table 3-1. Despite different pond conditions (MFT with OSPW vs. fresh water), a number of parameters do not differ between Big Pit and FE5. In most cases where parameters statistically differ, the FE5 water sample exhibits higher concentrations, likely due to the initial input of OSPW at pond establishment.

Table 3-1: Water chemistry parameters for aged OSPW samples (adapted from Anderson et al., 2012)

Water Sample	pH	Anions (mg L^{-1})					Cations (mg L^{-1})			
		F^-	Cl^-	NO^-_2	NO^-_3	SO^{2-}_4	Na^+	K^+	Mg^{2+}	Ca^{2+}
Big Pit	8.7	1.7 ± 0.07	120 ± 0.84	1.1 ± 1.8	4.6 ± 1.1	210 ± 2.2	450 ± 3.4	4.6 ± 0.13	20 ± 0.38	12 ± 1.4
FE5	8.6	1.8 ± 0.1	150 ± 0.64	2.0 ± 0.86	3.9 ± 0.0	790 ± 4.1	670 ± 2.8	9.4 ± 0.072	44 ± 0.22	21 ± 1.4

Water Sample	Elements (μg L^{-1})												
	Al	As	Cd	Co	Hg	Mn	Mo	Ni	Pb	Se	Sr	U	V
Big Pit	43 ± 8.0	8.7 ± 5.0	0.4 ± 0.1	0.4 ± 0.1	0.03 ± 0.01	0.4 ± 0.1	4.0 ± 0.4	2.6 ± 0.2	0.05 ± 0.01	1.3 ± 0.2	630 ± 45	6.2 ± 0.8	6.0 ± 1.0
FE5	37 ± 4.0	4.9 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.05 ± 0.02	0.2 ± 0.04	3.0 ± 0.4	2.0 ± 0.4	0.04 ± 0.03	1.2 ± 0.2	1100 ± 42	3.1 ± 0.2	2.0 ± 0.2

Following ozone treatment, acid-extractable organics, as measured by FTIR, were reduced to 1.8 mg/L for both Big Pit and FE5. Acid-extractable organics were not measured prior to ozone treatment, but Anderson et al. (2012a) reported acid-extractable organics to be 23 and 13 mg/L for Big Pit and FE5 water samples, collected at the same time as samples used in this study. Han et al. (2009) conducted a survey of a number of tailings ponds at Syncrude's site, including the two ponds selected. Big Pit was found to have the lowest acid-extractable organic concentration of 14 mg/L, followed by FE5 at 18 mg/L, as measured by FTIR.

An indicator of the biodegradability of a wastewater, the ratio of carbonaceous biochemical oxygen demand (cBOD) to chemical oxygen demand (COD), was not monitored in this study as biodegradation was directly measured

(Metcalf and Eddy, 2003). Gamal El-Din et al. (2011) monitored change in cBOD/COD with increasing utilized ozone dose; while cBOD increased from 8 mg/L to 25 mg/L, a 22% reduction in COD resulted in cBOD/COD increasing from 0.03 to 0.13, at a utilized ozone dose of 148 mg/L.

Little acute toxicity was detected in aged OSPW samples before and after ozone treatment and incubation, as indicated by EC_{20} values greater than 100% by the Microtox bioassay. However, in reclamation environments sub-chronic and chronic toxicity will need to be remediated, and these toxicological endpoints were not tested here. Anderson et al. (2012a) found that the survival and growth of a benthic invertebrate were not significantly affected by exposure to FE5 and Big Pit, but both OSPW samples negatively impacted reproductive capability. While all toxicological endpoints tested (survival, mass, pupation, and emergence) correlated with OSPW acid-extractable organics concentration (measured by FTIR), as well as many heavy metals (Anderson et al., 2012a), other organic compounds were not measured and thus not considered. Ozone treatment reduced mammalian immunotoxicity in fresh OSPW, but 40% removal of all organic matter in addition to 75% reduction in NAs meant that toxicity could not be definitively attributed to NAs (Garcia-Garcia et al., 2011a). Ozone treatment of fresh OSPW attenuated developmental toxicity (He et al., 2012a), mice immunotoxicity (Wang et al., 2013), and endrocrine-disrupting effects in fathead minnows (He et al., 2011; He et al., 2012b) . Utilizing relevant toxicology assays will be essential when assessing OSPW treatment.

3.3.2 Naphthenic Acids

The concentrations of OSPW NAs were monitored in the current experiment because they are a major component of the OSPW toxic fraction (Frank et al., 2008; Garcia-Garcia et al., 2011b; Jones et al., 2011; Scarlett et al., 2012).

The ozone dose of 50 mg/L removed the majority of the NAs mass present in both aged water samples. Estimated total classical NAs concentrations, measured by HRMS, were reduced from 0.42 and 0.69 mg/L, in

FE5 and Big Pit water samples, respectively, to 0.008 and 0.040 mg/L. As a result, tracking changes in NAs due to biodegradation following ozone exposure was not possible in this study. This ozone dose may have enabled subsequent biodegradation and perhaps mineralization of the NA ozonation by-products (among other OSPW organics such as naturally oxidized NAs), but this could not be confirmed analytically.

Martin et al. (2010) reduced NAs in fresh OSPW by 50 and 70% through ozone treatment, thereby reducing the biodegradation half-life of the remaining NAs to 55 and 48 d, respectively, from 83 d. Hwang et al. (2013) removed more than 99% of the NAs concentration (50 mg/L) in fresh OSPW by ozonation, to less than 0.5 mg/L and saw no further change in NAs in a biofilm reactor. Despite removal of more than 90% of NAs from fresh OSPW at a utilized ozone dose of 80 mg/L, the $cBOD_5$ to COD ratio increased with higher ozone doses (Gamal El-Din et al., 2011), indicating improved biodegradability of organics. Thus, it is possible that the remaining NAs in the current study are more biodegradable than before ozone treatment, but that NAs concentrations were too low in relation to other labile organics in OSPW for preferential biodegradation to occur.

Three dimensional profiles of the relative responses of each NA class are used to visualize a NA fingerprint signature (Holowenko et al., 2002). Untreated (ie. not ozonated) FE5 NAs fingerprint signatures before and after 84 d of incubation are shown in Figure 3-1. No observable reduction in NAs occurred after 84 d of aerobic incubation. A previous study found that simply incubating OSPW aerobically allowed for a 52% reduction in NAs after 98 d (Han et al., 2008). However, in the present study, the OSPW source has been stagnant for over 20 years (aged OSPW); it is expected that the extent to which the acclimated indigenous microbial community would have degraded NAs had already been reached.

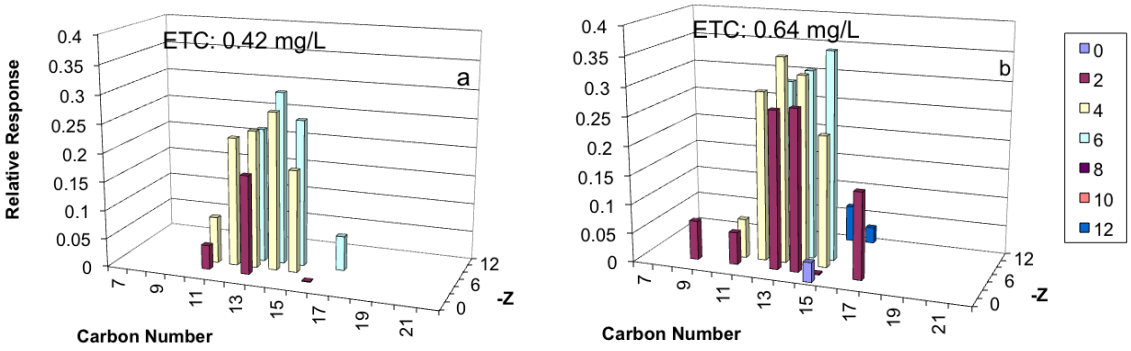


Figure 3-1: Response of naphthenic acid isomer classes of (a) untreated (non-ozonated) FE5 at Day 0 and (b) untreated FE5 at Day 83, relative to internal standard. Data are corrected for evaporative effects (Refer to Sec. 2.3). ETC = estimated total concentration. Legend indicates Z number.

The NAs profile for untreated FE5 does not differ greatly from that of a fresh OSPW sample after 98 d of incubation (Han et al., 2008), indicating again that those NAs in the aged OSPWs (Figure 3-1a) are recalcitrant to further aerobic biodegradation. However, Han et al. (2009) found no significant difference between NAs profiles of fresh and aged OSPW samples. Thus, recalcitrance of particular NA isomer classes may not be the reason for no observable NAs degradation in aged water samples in this study. A NAs concentration threshold may exist, whereby below this value, indigenous microbial communities at low biomass concentrations will find other, more labile carbon substrates to metabolize in OSPW (Quagraine et al., 2005b).

Han et al. (2009) suggested the ratio of oxidized NAs (ie. NAs with one or two additional oxygen atoms) to classical NAs may be an indicator of biodegradation. Oxidized NAs were monitored in this study, but, as with NAs, the oxidized NAs were removed by more than 85% due to the ozone treatment, and no appreciable change was observed in their concentration as well as their profile following the 84 d of incubation. The estimated total concentration of oxidized NAs increased, by a factor of 1.6, in the Untreated FE5 sample in 84 d shown in Figure 3-2, but without replicates, it is not possible to determine if this

change, or the ratio of oxidized to classical (i.e., parent or unoxidized) NAs, is significant.

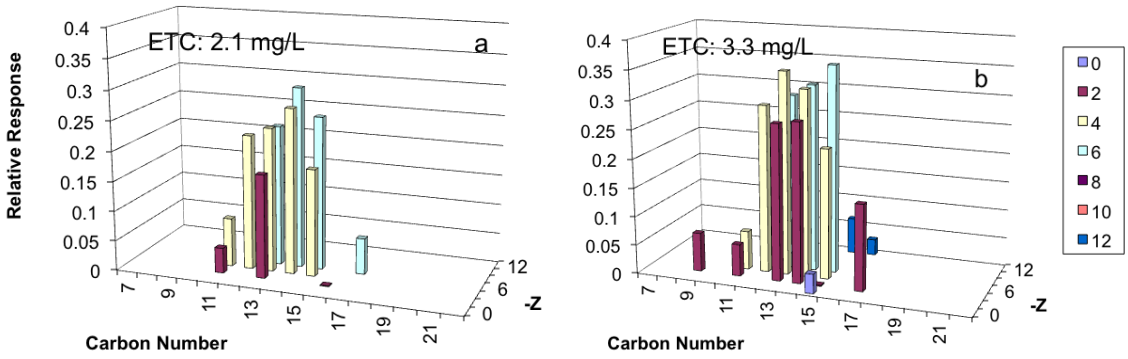


Figure 3-2: Response of oxidized naphthenic acid isomer classes of (a) untreated (non-ozonated) FE5 at Day 0 and (b) untreated FE5 at Day 83, relative to internal standard. Data are corrected for evaporative effects (Sec. 2.3). ETC = estimated total concentration. Legend indicates Z number.

3.3.3 Dissolved Organic Carbon

A majority of the acid-extractable organic compounds in oil sands process-affected waters do not fit the strict formula for NAs (Headley et al., 2009; Grewer et al., 2010) and NAs are not the only organic compounds in OSPW which could contribute to chronic toxicity of fresh OSPW (Garcia-Garcia et al., 2011a; Garcia-Garcia et al., 2011b). Measurement of DOC assisted in capturing these other organic compounds in aged OSPW samples.

DOC measurements indicate a reduction in organic matter during incubation in all treatments, except the Sterilized controls, for both FE5 and Big Pit water samples, as shown in Figure 3-3. For both OSPW samples, DOC reduction occurred in the first 35 days of incubation, with the greatest rate of DOC reduction occurring between 14 and 35 days, as indicated by these data differing significantly from later timepoints ($p < 0.05$). Days 35, 43, 50, 57, 64, 70, 78, and 83 do not differ from one another ($p > 0.05$) for all treatments. Thus, after 35 days of incubation, the microcosms have stagnated. Stagnation may be

caused by recalcitrance of remaining organic compounds or depletion of required nutrients provided to the inocula at inception of the incubation experiment.

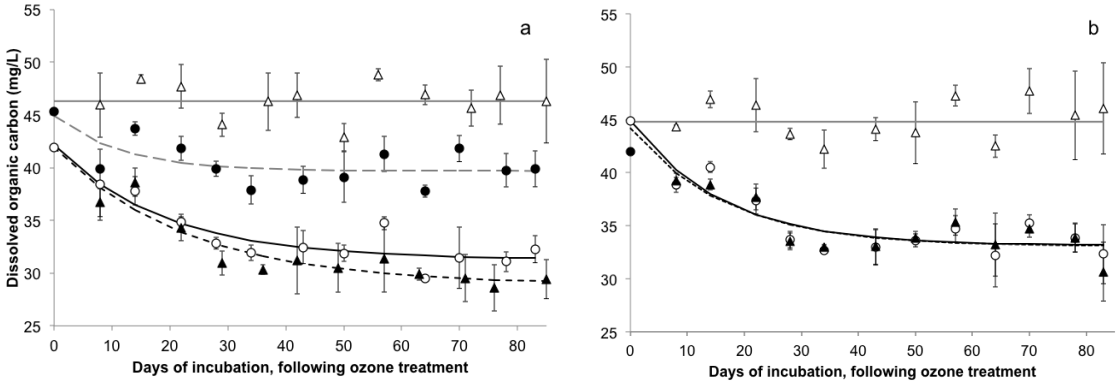


Figure 3-3: Dissolved organic carbon concentration in aerobic microcosms of (a) FE5 and (b) Big Pit water samples from Syncrude Canada Ltd., corrected for evaporative effects (Refer to Sec. 3.1.3). Treatments were Ozonated (○), Ozonated-Inoculated (▲), Untreated (●) and Sterilized (△). Exponential decay models for each treatment are shown; model parameters are provided in Table 3-2. Error bars indicate one standard deviation (n=3, n=1 for Day 0, n=4 for Day 83). Ozonated Day 0 sample indicates DOC measurement prior to incubation.

The DOC concentrations in both Ozonated treatments were significantly lower than Untreated, in the FE5 water sample ($p < 0.001$). As indicated by exponential decay modeling, shown in Figure 3-3 and summarized in Table 3-2, the labile fraction of the ozone-treated DOC in both water samples was double that of the Untreated DOC, indicating improved biodegradability of organic matter. Improved removal of DOC in both Ozonated treatments is attributed to biodegradation as no change was seen over 84 days of incubation to an ozonated and sterilized treatment, presented in Appendix 3. As expected, the Sterilized control differs from the Untreated (in FE5), Ozonated, and Ozonated-Inoculated treatments (in FE5 and Big Pit) significantly ($p < 0.05$).

The Ozonated and Ozonated-Inoculated treatments were not significantly different from one another ($p > 0.05$) in both water samples. The indigenous microbial community was not eliminated after the ozone treatment and was able to re-establish itself to degrade the residual ozone-treated DOC equally as well as the same microbial community not impacted by ozone (Ozonated-Inoculated

treatment). It was expected that ozone exposure would compromise the microbial community so that, even if recovery occurred, subsequent biodegradation would be hampered, but this was not observed. Ozone has been utilized for drinking water disinfection for the last century and more recently for wastewater (Metcalf and Eddy, 2003). However, full inactivation of microorganisms will not occur when ozone is utilized for pretreatment due to ozone consumption for the oxidation of organic matter (Camel and Bermond, 1998).

Table 3-2: Exponential decay modeling ($S= S_o e^{-kt}+S_n$) of DOC biodegradation following treatment of two aged OSPW samples, where S_o represents the labile DOC and S_n the recalcitrant DOC

Water Sample	Treatment Prior to Incubation	Model Parameter			R^2
		k	S_o	S_n	
		/day	*mg/L*	*mg/L*	
Big Pit	Ozonated	0.063	11.8	33.1	0.93
	Ozonated-Inoculated	0.060	11.1	33.0	0.93
	Sterilized	-	0.0	44.8	0.00
FE5	Ozonated	0.052	10.9	31.3	0.94
	Ozonated-Inoculated	0.044	13.0	28.9	0.96
	Untreated	0.086	5.2	39.7	0.69
	Sterilized	-	0.0	46.3	0.00

Little change was seen in DOC concentration immediately following ozone exposure, in both water samples, as seen in Figure 3-3. Ozonation caused a small increase in DOC in the Big Pit sample, but a small decrease in FE5. This variation may be attributed to treatment with different water chemistry. However, because only one replicate was taken at Day 0, it is not possible to determine if these differences between Untreated and Ozonated treatments are significant. In contrast to NAs, where more than 90% of the mass was removed by ozone treatment, no reduction in DOC is likely due to organic compounds being oxidized and not mineralized, resulting in no change in the carbon mass balance. Gamal El-Din et al. (2011) also observed low DOC reduction even with high

ozone exposure, seeing only 22% DOC consumption at a utilized ozone doze of 150 mg/L.

3.3.4 Microbial density

Microbial density was monitored with the conservative, single copy polymerase beta subunit (*rpoB*) gene (Dahllöf et al., 2000; Nava et al., 2011) using quantitative PCR as an indicator of bacterial growth during incubation and is shown in Figure 3-4 for both water samples. The Ozonated-Inoculated treatment for both water samples showed significant growth throughout incubation ($p < 0.05$ against sterilized control). However, because this treatment received an inoculum of the same microbial community following ozonation, theoretically it would have the same biomass potential as Ozonated and Untreated samples combined.

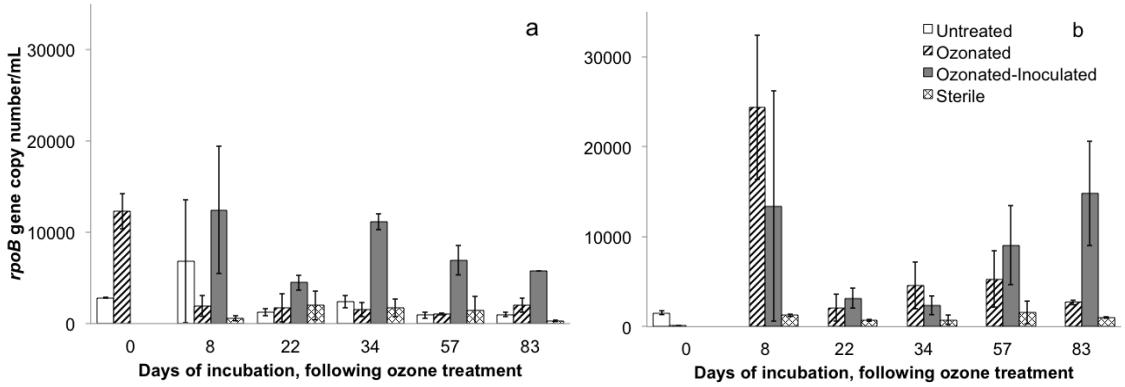


Figure 3-4: Copy number of the *rpoB* gene in aerobic microcosms of (a) FE5 and (b) Big Pit water samples from Syncrude Canada Ltd., corrected for evaporative effects (Refer to Sec. 2.3). Error bars indicate one standard deviation (n=6, n=2 for Day 0).

Visual observation of turbidity indicated that the Ozonated-Inoculated treatment in both water samples experienced greater growth than other treatments (data not shown). For the FE5 water sample, inoculation resulted in higher gene copy numbers compared to Untreated and Ozonated treatments ($p < 0.05$), but inoculation increased gene copy numbers in ozone-treated Big Pit water only on Day 83 ($p < 0.05$). However, as previously discussed, this may be

irrelevant as the Ozonated-Inoculated treatment would have more cells to be counted.

Gene copy number in the Ozonated treatment was greater than the Untreated sample on Day 0 for the FE5 water sample and greater than the inoculated treatment on Day 8 for the Big Pit sample ($p < 0.05$). It is unclear why this data would be this high following ozone exposure. However, this assay would include DNA from live, compromised and dead cells.

Based on these data, it would seem that ozonation did not impede microbial growth, as the Ozonated treatments, in both waters (all timepoints for Big Pit, Days 8 and 83 for FE5), showed evidence of higher gene copy number, compared to the Sterilized control ($p < 0.05$). Despite clear evidence of improved biodegradability of organic carbon in ozone-treated waters, this measurement technique did not clearly show increased gene copy number in ozonated waters relative to the same microbial community not exposed to ozone-treated organics (untreated). Meanwhile, Hwang et al. (2013) observed greater biofilm thickness and density in a reactor receiving ozonated fresh OSPW, versus unozonated OSPW, in response to the increased labile organic loading.

It is clear that a better understanding of microbial response to ozone exposure is needed. A more robust bioassay that distinguishes between live, damaged and dead microbial cells would be required to elucidate how oxidant exposure would impact a microbial community indigenous to an oil sands tailings pond. The presence of a well-adapted indigenous microbial community able to survive ozonation and achieve bioremediation of residual organics may accelerate *in situ* treatment of aged OSPW prior to or during the establishment of wet reclamation landscapes.

3.4 Conclusions

Ozone pretreatment of OSPW has been shown to improve the biodegradability of NAs as well as other organics, indicated by increased BOD after treatment. The response of the indigenous microbial communities in OSPW to ozone exposure and incubation with ozonation by-products had not yet been

explored, nor had the treatment of aged OSPW, which continues to have toxic effects on relevant endpoints. NAs contributed less than 1 mg/L to total DOC content in two aged OSPW samples, and therefore, DOC, at approximately 45 mg/L was used to assess ozonation coupled with biodegradation for treating OSPW. While ozone treatment did not reduce DOC, subsequent biodegradation was significantly improved by ozonation such that about 15% more DOC was removed in ozone treated samples than Untreated OSPW. Finally, this study has shown a capacity for microbial communities indigenous to aged oil sands tailings ponds to resist a moderate ozone dose (50 mg/L) exposure and biodegrade 11-13 mg/L DOC content, as indicated by no significant difference in organic matter degradation between Ozonated and Ozonated-Inoculated treatments. A biomass measurement technique capable of distinguishing live, dead, and compromised cells would improve understanding of microbial response to ozone exposure.

In groundwater treatment, low doses of chemical oxidants are injected to oxidize and desorb organic materials from solids. Low oxidant dosage ensures microbial survival, and biodegradation is utilized to further reduce organic matter at low cost. Coupling oxidation pretreatment, such as ozonation, with biodegradation mediated by microorganisms indigenous to oil sands tailings ponds is a potential remediation scheme for OSPW being placed in wet landscape reclamation environments. As the oil sands industry moves towards closure of portions of their active mining sites, there is pressing need to not only devise efficient water treatment schemes for OSPW, but also to understand the behaviour of end pit lakes and the potential for cost-effective ongoing remediation of residual organic contaminants and toxic effects.

4 Ozone Treatment for Oil Sands Tailings Remediation: Impacts on Native Microbial Communities in Aged Process-Affected Waters

4.1 Introduction

Establishment of successful constructed wetlands and end pit lakes will enable remediation of OSPW, storage of MFT, and reclamation of disturbed landscapes. Microorganisms have a, potentially conflicting, role to play in all three; bioremediating organics in OSPW (Hadwin et al., 2006; Scott et al., 2005), influencing solids settling and release of methane or hydrogen sulphide under anaerobic conditions (Penner and Foght, 2010; Ramos-Padrón et al., 2011; Siddique et al., 2012, 2007, 2006), and as an integral component of a healthy food web in aquatic ecosystems (Yergeau et al., 2012).

Biodegradation continues to be sought for the treatment of organic compounds found in OSPW; in-situ treatment represents a cost-effective option (Brown et al., 2013; Quagraine et al., 2005b). Understanding microbial communities present in OSPW and reclamation environments, in terms of both structure and metabolic capacity, will be essential in designing bioremediation strategies (Hadwin et al., 2006; Quagraine et al., 2005b).

An abrupt shift from the surface of a tailings pond to the next sampling point 1.5 m below surface was observed, where both chemistry data and microbial community profiling indicated a change from aerobic to anaerobic conditions (Ramos-Padrón et al., 2011). Ozone treatment has emerged as a method for at least reducing NA recalcitrance (Gamal El-Din et al., 2011; Pereira et al., 2013b; Pérez-Estrada et al., 2011; Scott et al., 2008b). Ozone, like many other oxidants, raises the redox condition of a system and is likely to stimulate aerobic microorganisms in OSPW. To date, profiling aerobic microbial communities in OSPW has been minimal, especially in aged OSPW.

A number of studies have coupled ozone pretreatment with biodegradation (Hwang et al., 2013; Martin et al., 2010; Wang et al., 2013), and the impact of exposure to products of ozone treatment of OSPW on the microbial community inhabiting a bioreactor has been explored (Hwang et al., 2013). However, the ability of microbes to survive ozone exposure and then degrade OSPW organics has not been considered. Thus, the aims of this work were to understand how indigenous microbial communities in aged OSPW respond to a)

ozone exposure, and b) increased availability of more labile organic compounds following ozonation. In addition, this work has provided an opportunity to explore, for the first time, the microbial communities that inhabit aged OSPW from two different experimental reclamation ponds.

4.2 Materials and Methods

Descriptions of water samples, ozone treatment, microcosm establishment, sampling, and DNA extraction are found consecutively in Sections 3.2.1 to 3.2.5.

4.2.1 PCR-DGGE for Microbial Community Analysis

DNA extracts from biological triplicates from Weeks 0, 2, 6 and 11 were combined to arrive at pseudo-average results. A fragment of the16S rRNA gene was amplified with primers 341F (5'-CCTACGGGAGGCAGCAG -3') and 534R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). A GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGGGGG-3') was added to the forward primer. The 50 µL PCR mixture contained 1µL DNA template, 5 µL AmpliTaq Gold 10x coloured buffer (Life Technologies, Paisley, UK), 1.5 mM $MgCl_2$ (Life Technologies), 0.2 mM each deoxynucleoside triphosphate (Fermentas, Vilnius, Lithuania), 0.4 µM each primer, and 1.25 U AmpliTaq Gold polymerase (Life Technologies), in sterile milliQ water. PCR amplification was carried out using 2720 Thermocycler (Applied Biosystems, Foster City, CA, USA) with the following protocol: denaturing at 95°C for 5 min, then 38 cycles of denaturing for 30 s at 95°C, annealing for 30 s at 57°C, and a 1.5 min extension at 72°C. The final extension step was held for 10 min at 72°C. PCR products were checked on a 1.5% agarose gel, stained with ethidium bromide, prior to running on DGGE, to allow for loading of approximately equal amounts in each sample lane.

The 16S rRNA PCR products were loaded in approximately equal concentration and run on 8% polyacrylamide gels, cast using 40 to 60% denaturing gradient, where 100% denaturant contained 40% formamide and 7 M urea. To improve gel handling during staining and to enable long-term storage, Gelbond PAG film (Lonza, Basel, Switzerland) was used. The DCode Universal

Mutation Detection System (Bio-Rad, Hemel Hampstead, UK) was operated for 18 h at 60V and a constant temperature of 60°C. The DGGE gels were silver stained (Nicol et al., 2005) and photographed.

A DGGE pattern was reduced to a binary matrix by manual identification of presence or absence, as well as position, of bands. Total number of bands in each DGGE lane was analyzed statistically with SigmaPlot Version 11.0 (Systat Software, San Jose, CA), using Two-Way ANOVA and the Holm-Sidak for pairwise comparison. Band intensity was estimated using GelComparII (Applied Maths, Austin, TX). Cluster analysis was conducted with Primer 6 (PRIMER-E Ltd., Lutton, UK), using both the Dice/Sorenson and Jaccard similarity indices to build the distance matrix. Dendrograms were then generated from the unweighted pair group method with arithmetic averages (UPGMA) clustering method, in addition to non-metric multi-dimensional scaling (nMDS) (Clarke, 1993). Statistical analysis was completed with Primer 6 on the similarity matrix, with the one-way ANOSIM package to determine influence of treatment on samples and with the two-way crossed ANOSIM2 package (Spearman rank correlation coefficient), used for samples with no replication, to influence of treatment and time on separation of samples. Diversity analysis was conducted with the DIVERSE package, also in Primer 6, calculating the Shannon-Weaver (Shannon, 1948) and Simpson (Simpson, 1949) indices.

4.2.2 Band Sequencing

Once the DGGE gels were photographed, bands of interest were excised with razor blades sterilized with ethanol. Bands were placed in 50 µL sterile milliQ water for approximately one month at 4°C and vortexed briefly prior to use to release DNA. Four microliters of this water was then used as the DNA template for PCR, with 5 µL TopTaq 10x buffer (Qiagen, Crawley, UK), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.4 µM each primer, and 1.25 U TopTaq polymerase (Qiagen), in sterile milliQ water. PCR amplification was carried out using a 2720 Thermocycler (Applied Biosystems) with the following protocol: denaturing at 95°C for 5 min, then 35 cycles of denaturing for

30 s at 95°C, annealing for 30 s at 57°C, and a 1 min extension at 72°C. The final extension step was held for 10 min at 72°C. PCR products were checked on a 1.5% agarose gel, stained with ethidium bromide, prior to purification with QIAquick PCR Purification Kit (Qiagen). Amplified DGGE bands (54) were Sanger sequenced (GATC Biotech, Konstanz, Germany) using the 534R primer, with approximately 80% success rate.

Sequences were edited with Chromas Lite 2.0 (Technelysium, South Brisbane, Australia) and are provided in Appendix 6. The sequences were then aligned manually using SeaView 4.0 (Gouy et al., 2010) with type strain sequences obtained from NCBI GenBank using BLAST, as well from species of interest from the literature. The final amplicon length was 100 bp. Phylogenetic analysis was conducted using MEGA 5.0 (Tamura et al., 2011) with Jukes-Cantor DNA distance and neighbour joining methods (Jukes and Cantor, 1969; Saitou and Nei, 1987). Bootstrap analysis was replicated 1000 times. A maximum parsimony tree, bootstrapped 100 times, is provided in Appendix 5.

4.3 Results

4.3.1 DOC Removal

Dissolved organic carbon was measured to track potential degradation of organic compounds present in aged OSPW during aerobic incubation. A detailed analysis of the DOC results can be found in Chapter 3. Here, DOC removal is provided to enable comparison to microbial community dynamics data.

Figure 4-1 shows percent DOC remaining over twelve weeks of aerobic incubation, normalized against sterilized controls. The data show no statistical difference between Ozonated and Ozonated-Inoculated treatments, and that Untreated aged OSPW experienced significantly less DOC degradation ($p < 0.001$). Ozonation enabled up to 40% DOC content to be degraded in the FE5 water sample and about 30% in the Big Pit sample, compared to 20% removal in the Untreated sample (FE5 only). Meanwhile, the Ozonated-Sterilized treatment showed no DOC removal, as seen in Appendix 3. As indicated in Chapter 3, significant degradation did not occur after the fifth week of incubation ($p < 0.05$).

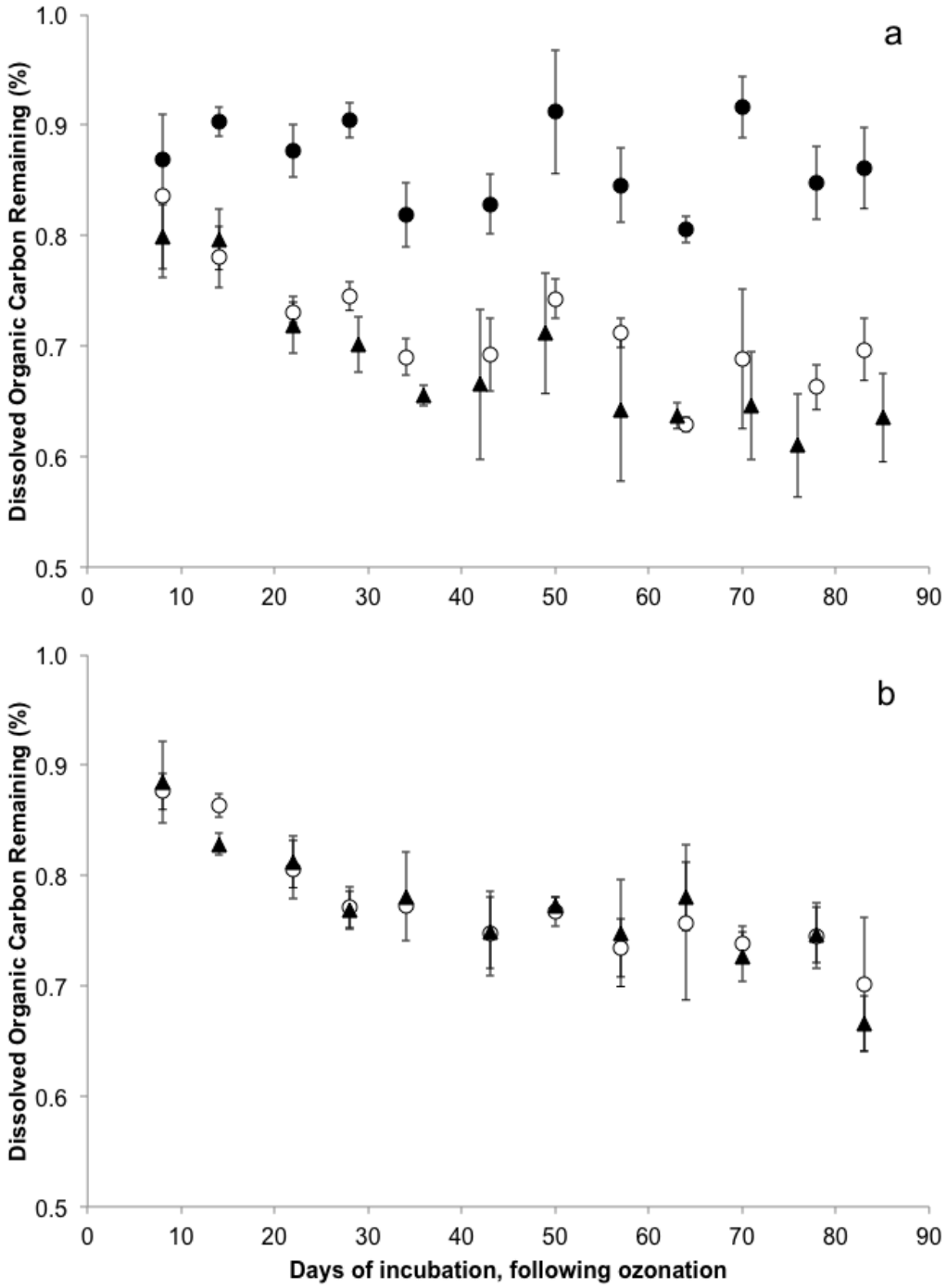


Figure 4-1: Remaining dissolved organic carbon, normalized against sterilized controls, in aerobic microcosms of (a) FE5 and (b) Big Pit water samples from Syncrude Canada Ltd., corrected for evaporative effects (Refer to §3.2.3). Treatments were Ozonated (○), Ozonated-Inoculated (▲), and Untreated (●). Error bars indicate one standard deviation (n=3, n=4 for Day 83).

4.3.2 Community Dynamics Analysis with DGGE

In order to monitor changes in the bacterial communities present in samples from two experimental reclamation ponds upon exposure to ozone and during aerobic incubation, PCR-DGGE utilizing the 16S rRNA gene was conducted. PCR-DGGE is a rapid molecular fingerprinting tool that is particularly useful for monitoring changes in microbial community structure in response to environmental stress or time (McCaig et al., 2001; Muyzer et al., 1993; Neilson et al., 2013). It was very clear, upon visual assessment of the DGGE images obtained, that treatment and incubation time impacted the indigenous bacterial communities found in two aged OSPW samples. Numerous bands were identified that varied in intensity over time and across treatments; a number of these bands of interest were excised for amplicon sequencing. To quantitatively assess the changes seen in the bacterial communities due to treatment and incubation time, numerically based similarity analyses were undertaken.

Both aged OSPW samples contained rich bacterial communities through this experiment, as indicated by the number of unique bands detected during DGGE analysis; 55 distinct bands were identified in FE5 16S rRNA DGGE, shown in Figure 4-2, and 46 in Big Pit, shown in Figure 4-3. Each band is assumed to represent a predominant, nominally greater than 1% of the community, species of bacteria in the community (Muyzer et al., 1993) and thus the number of bands detected may be likened to species richness (Nübel et al., 1999). The number of bands identified in each lane was compared via two-way ANOVA analysis, for both OSPW samples. The Sterilized control at all time points exhibited a significantly lower number of bands than all other treatments for FE5 ($p < 0.05$), but contained significantly greater numbers over the Ozonated treatment for Big Pit ($p < 0.05$). However, the number of bands detected in the Week 0 time point for the Big Pit Ozonated treatment was four to five times lower than other time points. When this outlier was removed, the Ozonated treatment exhibited significantly greater richness than all other treatments ($p < 0.05$).

In order to explore diversity in more detail, estimated DGGE band intensity was utilized for determination of the Shannon-Weaver biodiversity index, most

common in ecology (Nübel et al., 1999), and the Simpson index, which places more weight on species with greater dominance (Ampe and Miambi, 2000). The Shannon-Weaver index ranged from 2.0 to 3.1 for FE5 and 2.1 to 3.2 for Big Pit, while the Simpson index ranged from 0.057 to 0.23 for FE5 and 0.049 to 0.17 for Big Pit. Two way ANOVA analysis determined there was no significant difference in either index due to treatment or time, for FE5, and only a significant difference in the Simpson index between Weeks 0 and 6 was detected for Big Pit, likely due to the Week 0 ozonated sample, as highlighted previously.

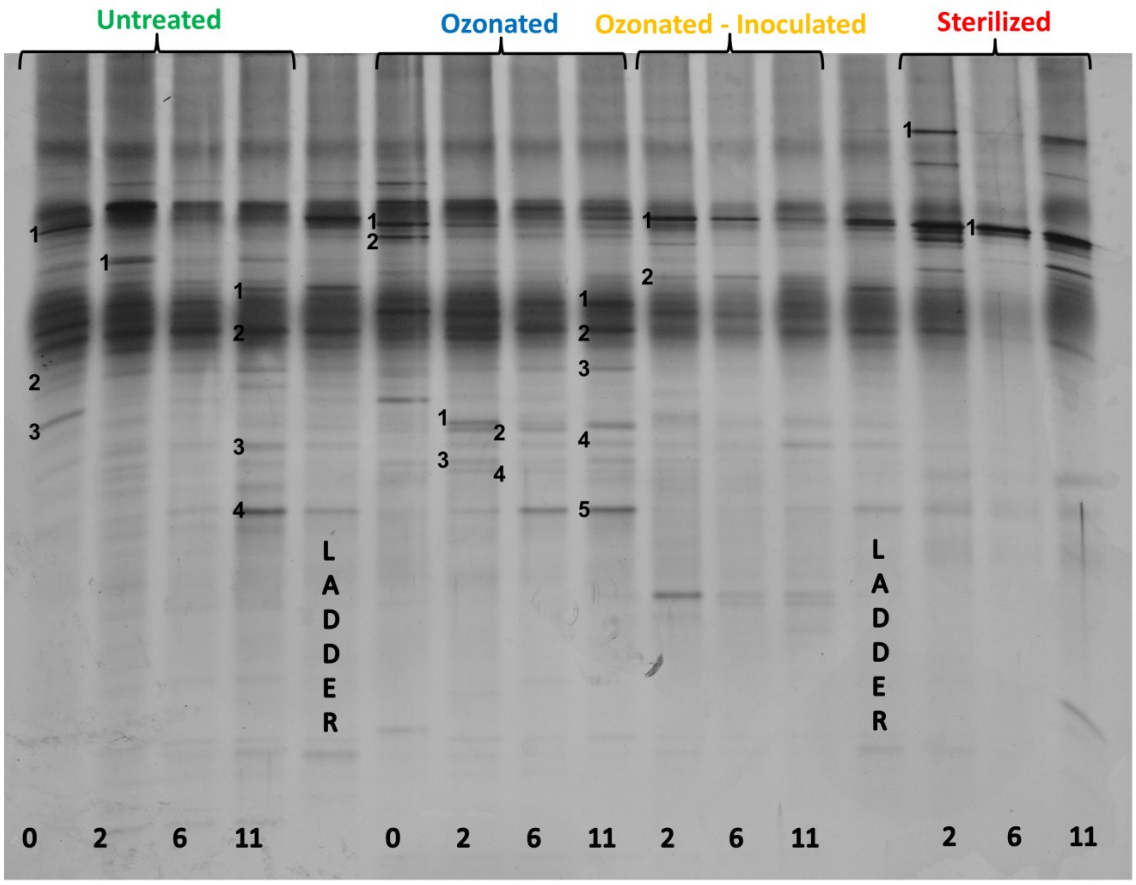


Figure 4-2: DGGE profile for FE5 water sample. Labels indicate treatment and weeks of incubation, following ozone treatment. The ladder is an environmental sample from a different tailings pond. Successfully excised and sequenced bands are numbered by lane.

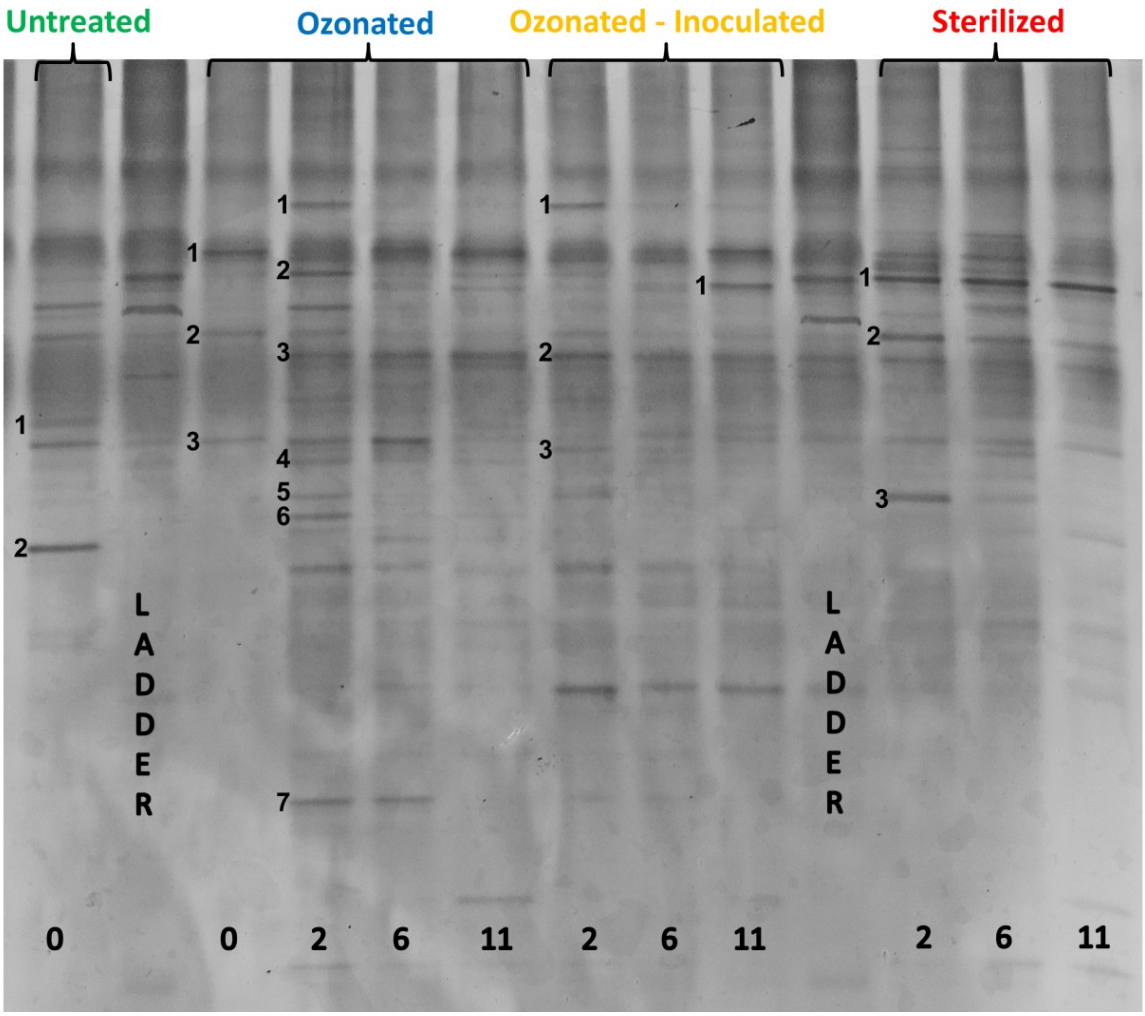


Figure 4-3: DGGE profile for Big Pit water sample. Labels indicate treatment and weeks of incubation, following ozone treatment. The ladder is an environmental sample from a different tailings pond. Successfully excised and sequenced bands are numbered by lane.

Similarity analysis of individual samples on each DGGE was completed and interpreted with three methods: cluster analysis (UPGMA dendrogram), nMDS, and ANOSIM, using both Jaccard (provided in Appendix 4) and Dice similarity indices. The nMDS were also overlain with appropriate contours from the relevant cluster analysis, to aid in interpretation.

For the FE5 water sample, the Sterilized controls very strongly grouped together and separate from other treatments, with only 40% similarity to all other

treatments, as seen in Figure 4-4. The other samples also grouped within their respective treatments, but were found to be more than 60% similar to one another. Evident in the nMDS with the Sterilized treatment removed, shown in Figure 4-5, the Ozonated samples group together at 75%, as do some of the Ozonated-Inoculated and Untreated samples. Both treatment (ρ = 0.93, p = 0.003) and time (ρ = 0.67, p = 0.04) were significant causes of separation of samples into these respective factors. However, when the Sterilized controls were removed from the data set, the strength of separation due to treatment was reduced below significance, but time continued to be a significant cause of sample grouping (treatment: ρ = 0.33, p = 0.3; time: ρ = 0.76, p = 0.05). Both the nMDS and cluster analysis do indicate separation of individual samples into their respective treatment groups, though.

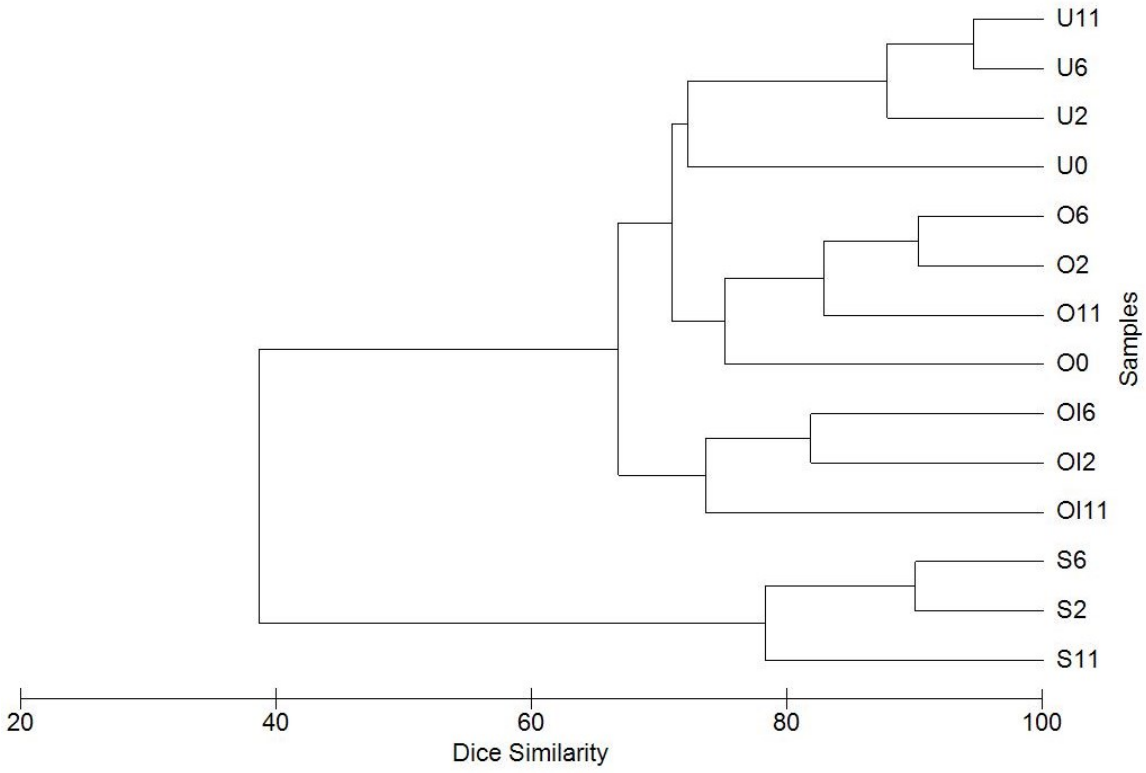


Figure 4-4: UPGMA Dice dendrogram of 16S rRNA PCR-DGGE for FE5 water sample. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

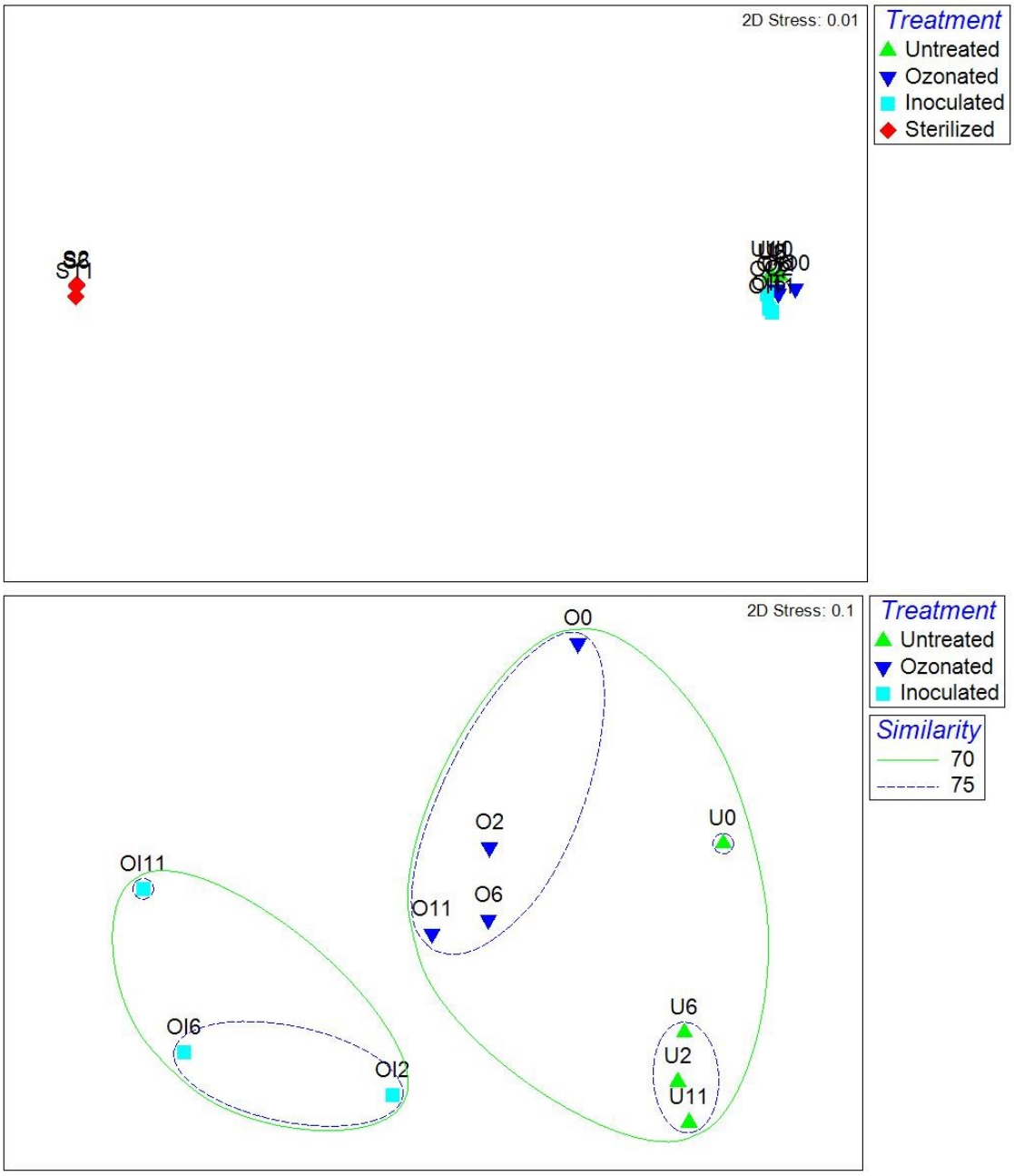


Figure 4-5: Non-metric multi-dimensional scaling analysis of 16S rRNA PCR-DGGE for FE5 water sample, including similarity contours from UPGMA Dice cluster analysis. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

Both the cluster analysis and nMDS, shown in Figure 4-6 and

Figure 4-7, for Big Pit water sample, show the Week 0 time points separate from all other samples with less than 40% similarity and are also less

than 50% similar to one another. As with the FE5 sample, the Sterilized controls grouped tightly together and separately from the other treatments. The Ozonated and Ozonated-Inoculated treatments grouped together at the 60% similarity threshold. When the single Untreated sample was removed from the data set, ANOSIM2 analysis indicated separation due to treatment (ρ = 0.67, *p* = 0.2) was not significant and insignificant separation due to time occurred (ρ = 0.17, *p* = 0.3).

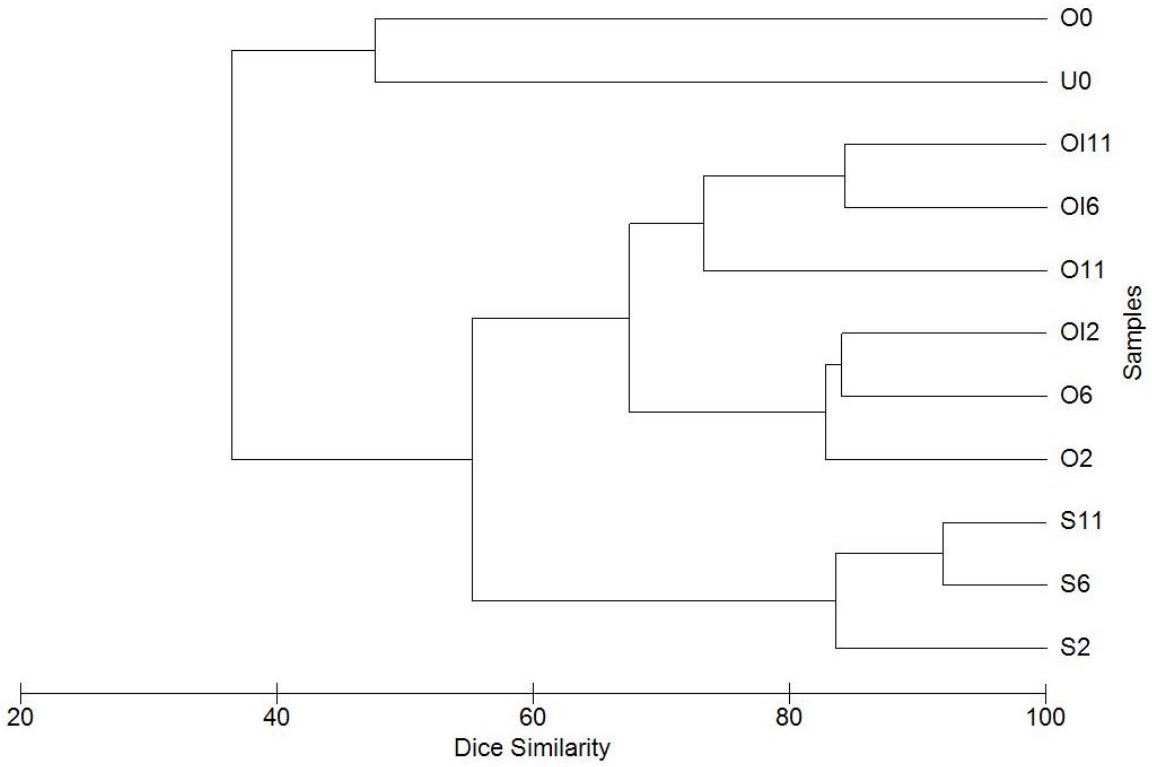


Figure 4-6: UPGMA Dice dendrogram of 16S rRNA PCR-DGGE for Big Pit water sample. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

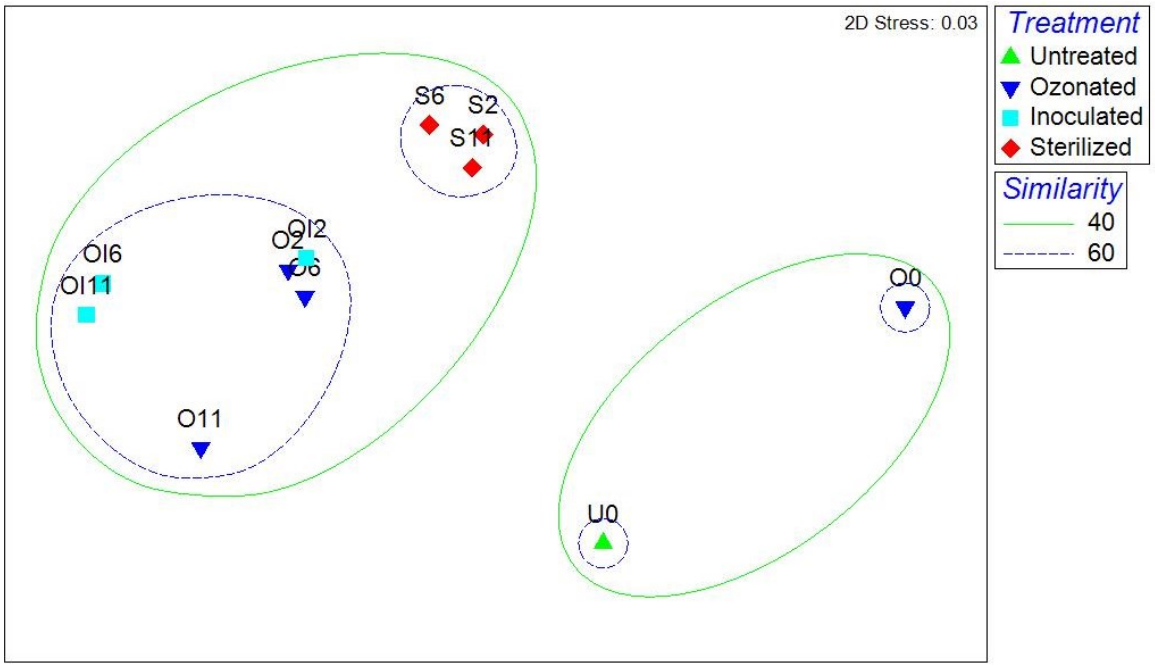


Figure 4-7: Non-metric multi-dimensional scaling analysis of 16S rRNA PCR-DGGE for Big Pit water sample, including similarity contours from UPGMA Dice cluster analysis. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

4.3.3 Microbial Community Characterization

Of the 54 excised 16S rRNA DGGE bands from both the FE5 and Big Pit water samples, 42 were successfully aligned, followed by Jukes-Cantor DNA distance and neighbour joining analyes to generate a phylogenetic tree, shown in Figure 4-8. Since the sequences from these 42 bands contained minimal background interference and most exhibited similarity to known bacterial species, each excised band likely represents a unique amplicon (Juck et al., 2000). These bands are labelled on the DGGE images, in

Figure 4-2 and Figure 4-3. To construct the phylogenetic tree, type strains from the NCBI GenBank nucleotide database were selected based on similarity to the excised DGGE band sequences according to the BLAST algorithm. In addition, select species that represent genera identified previously in OSPW samples have been included. A majority of the DGGE band sequences cluster closely to known strains.

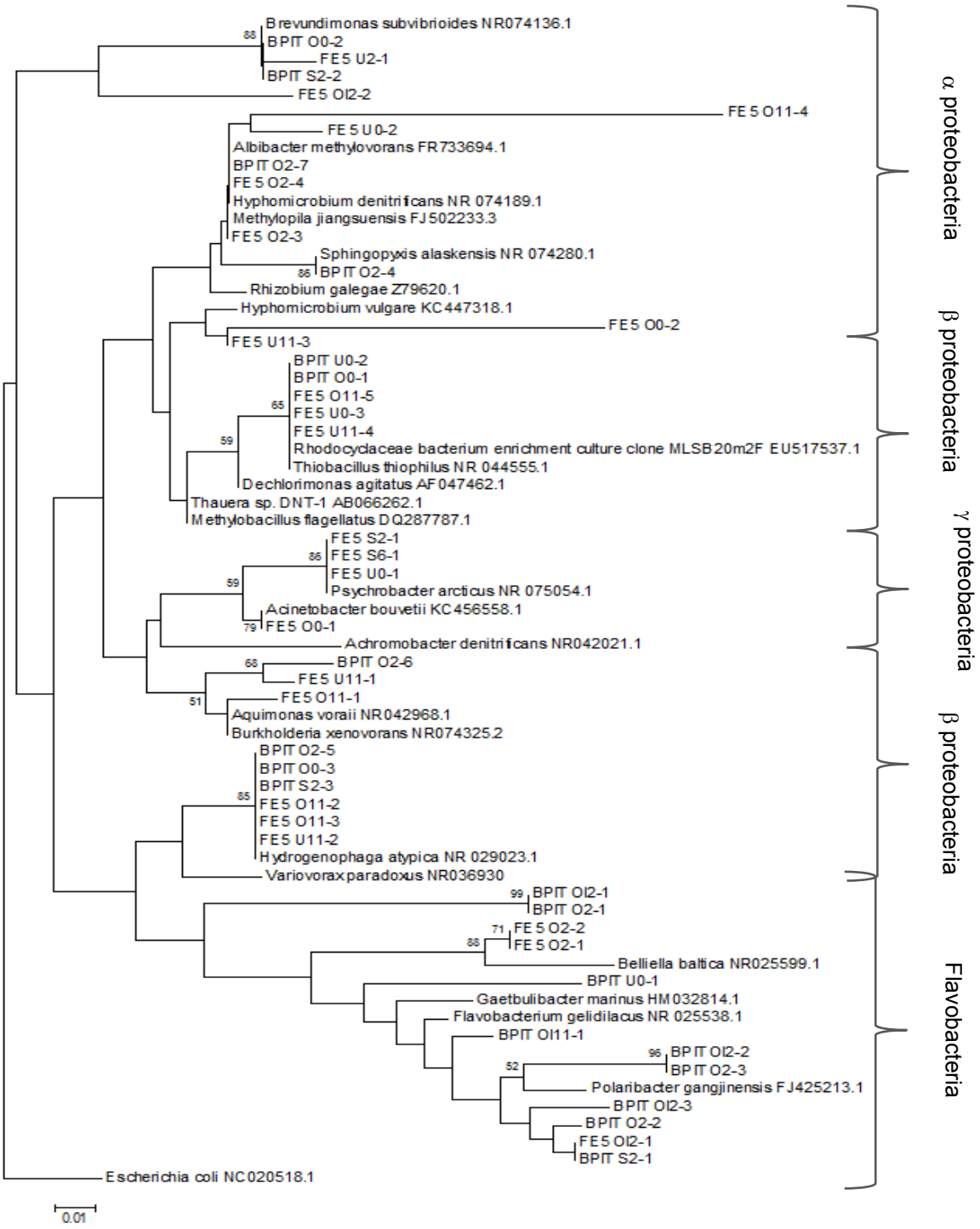


Figure 4-8: Phylogenetic analysis of 16S rRNA gene sequences from excised DGGE bands. Type strains from NCBI GenBank are included. Aligned sequences (100 bp) were analyzed using Jukes-Cantor DNA distance and neighbour-joining methods. Bootstrap values greater than 50% are shown, based on 1000 replicates. The scale bar indicates 0.01 substitutions per nucleotide base. Labels indicate water sample (FE5, BPIT=Big Pit), treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized), and weeks of incubation, following ozone treatment.

While the sequences were trimmed to 100 base pairs for phylogenetic analysis, complete, but edited, sequences, ranging in length from 118 to 157 base pairs were used to conduct the BLAST algorithm against the greengenes taxonomy database (DeSantis et al., 2006). Those sequences that could be classified to the genus or species level, at similarity greater than 97%, are shown in Table 4-1.

Table 4-1: Classification of sequences with greater than 97% similarity to excised DGGE bands, using the greengenes database. Labels indicate water sample (FE5, BPIT=Big Pit), treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized), and weeks of incubation, following ozone treatment.

Band	Length (bp)	Closest Relative at Identified Taxonomic Level	Similarity (%)
FE5 U0-1	143	*Psychrobacter* (genus)	100
FE5 U0-3	148	*Thiobacillus thioparus* (species)	97
FE5 U2-1	118	*Brevundimonas* (genus)	98
FE5 U11-2	156	*Hydrogenophaga palleronii* (species)	99
FE5 U11-3	121	*Hyphomicrobium* (genus)	98
FE5 U11-4	147	*Methyloversatilis universalis* (species)	97
FE5 O0-1	152	*Acinetobacter johnsonii* (species)	99
FE5 O2-3	122	*Rhizobium* (genus)	98
FE5 O2-4	122	*Methylopila* (genus)	98
FE5 O11-2	154	*Hydrogenophaga palleronii* (species)	99
FE5 O11-3	151	*Hydrogenophaga palleronii* (species)	99
FE5 OI2-1	148	*Polaribacter* (genus)	97
FE5 S2-1	147	*Psychrobacter* (genus)	98
FE5 S6-1	157	*Psychrobacter* (genus)	99
BPIT U0-2	147	**Thiobacillus thioparus* (species)	97
BPIT O0-2	126	*Brevundimonas* (genus)	100
BPIT O0-3	149	*Hydrogenophaga palleronii* (species)	100
BPIT O2-4	123	**Sphingomonas* (genus)	99
BPIT O2-5	151	*Hydrogenophaga palleronii* (species)	99
BPIT O2-7	120	**Methylobacterium* (genus)	97
BPIT S2-1	149	*Polaribacter* (genus)	97
BPIT S2-2	120	*Brevundimonas* (genus)	100
BPIT S2-3	149	*Hydrogenophaga palleronii* (species)	99

*Indicates sequences that could not be classified against the greengenes database. Taxonomic identity assigned based on NCBI BLAST results.

4.4 Discussion

Two OSPW samples were collected from experimental reclamation tailings ponds, established by Syncrude two decades ago, and subjected to ozone treatment prior to aerobic incubation with the indigenous microbial communities present in the samples. Discussion of the use of this treatment regime for NAs and other organic compounds in OSPW may be found in Chapter 3 and Brown et al. (2013), as well as Martin et al. (2010), Hwang et al. (2013), and Wang et al. (2013). This work focused on understanding community dynamics of indigenous microorganisms in response to ozonation and subsequent bioremediation of OSPW, in addition to characterization of those communities in aged OSPW. As highlighted in Chapter 3, this is the first time treatment of aged OSPW, which continues to exhibit toxic effects on a variety of aquatic organisms (Anderson et al., 2012a; Kavanagh et al., 2013), has been conducted.

Chemistry analysis indicated that inoculating OSPW with unexposed indigenous microorganisms following ozone treatment did not improve DOC removal, as there was no significant difference between the Ozonated and Ozonated-Inoculated treatments (Brown et al., 2013). Although not as strong an indicator as the DOC removal results, PCR-DGGE analysis also suggested less difference between the Ozonated and Ozonated-Inoculated treatments than expected, given that ozone treatment would likely compromise microorganisms (Gehr et al., 2003). Numerical similarity analyses based on PCR-DGGE indicated that there was little significant difference between bacterial communities in the Ozonated, Ozonated-Inoculated and Untreated (for FE5) groups. However, there are bands seen on the DGGE images that only occur in a particular treatment, such that the bacterial communities in the Ozonated and Ozonated-Inoculated treatments are about 65% similar, whereas the Untreated group was 70% similar to the Ozonated group (for FE5).

With both OSPW samples, the Ozonated-Inoculated treatment exhibited some prominent DGGE bands that were not observed in other treatments. The dissimilarity between the Ozonated and Ozonated-Inoculated bacterial communities is likely due to ozone exposure affecting community structure in the

Ozonated treatment, as both treatments would have similar water chemistry and thus the same potential to enrich for the same microorganisms. Most of the prominent bands observed in only the Ozonated-Inoculated samples decrease in intensity over time, which may indicate that these species do not contribute to observed DOC degradation, as their populations decline over time, in addition to not surviving ozone exposure, as they do not occur in high numbers in the Ozonated treatment. While observed dissimilarities between the Untreated and Ozonated communities may have contributed to the difference in DOC removal, as there are bands that appear in the Ozonated treatment that are absent from the Untreated samples, it is also possible that the differences in organic matter between these two treatments enabled more DOC degradation after ozone treatment without a significant shift in the microbial community.

Another component of this research was monitoring shifts in the indigenous microbial communities from experimental reclamation ponds over time during aerobic incubation. For all treatments, similarity analyses and visual assessment of the DGGE images indicated the bacterial communities present did change over time. For each treatment, the community at the outset of the experiment and after 11 weeks were least similar, except in the Untreated sample (for FE5). It was previously concluded that the extent of DOC degradation possible under these experimental conditions had occurred after five weeks of incubation (Brown et al., 2013), and so the microbial communities observed at 11 weeks (the final time point in the PCR-DGGE analysis) may represent stationary or decline phases of growth. As expected, there are a number of bands that are most intense at Week 0 or 2. Interestingly, though, there are some bands that are most intense at this final time point; these may represent bacteria that utilized the biomass in the system as a source of carbon.

With the Big Pit OSPW sample, the samples from the Week 0 time points were less than 40% similar to all other samples, and only about 40% similar to each other. If the Untreated control had not been lost for this water sample, it may have been seen that Ozonation significantly altered the microbial community present; without more samples over time from the Untreated control, it is difficult

to make this assertion. It is not unexpected for the Week 0 Ozonated sample to differ from later time points; it is likely that ozone exposure disrupted the community, but recovery of the microorganisms occurred in the following weeks. Indeed, the Ozonated treatment at Week 2 shows the highest band number and greatest total intensity, demonstrating a revived and thriving community.

Hwang et al. (2013) detected a noticeable change in the biofilm-forming microbial community from fresh OSPW when pretreatment with ozone was performed. The enrichment of some species and an overall reduction in diversity, also monitored by PCR-DGGE, was observed and attributed to changes in OSPW chemistry and tolerance of certain microbes to ozone exposure. That biofilm communities formed in ozonated OSPW were more similar to the unexposed biofilm communities than to the community detected from the ozonated OSPW presents a strong case of recovery of microbial communities after ozone exposure, as observed in this experiment.

Those bands that were excised and successfully sequenced revealed diverse microbial communities present in aged OSPW, even without exhaustive DGGE band excisation. Unfortunately, not all bands of interest, identified by change in intensity over time or between treatments, were successfully sequenced. Thus, there is much unresolved diversity and bacterial community dynamics, which would be better captured with next generation sequencing (Roh et al., 2010). Despite the limitations of the PCR-DGGE method used here, some interesting observations can be made about these bacterial communities. Sequences divided into two phyla (*Proteobacteria* and *Bacteroidetes*) and six classes (*Alpha-*, *Beta-*, and *Gamma-proteobacteria*, *Flavobacteria*, *Cytophaga*, and *Sphingobacteria*).

Of the 42 bands successfully sequenced, 23 were classified to the genus or species level based on at least 97% similarity with the closest relative. A number of these have been identified in other OSPW samples, including *Brevundimonas* (Hwang et al., 2013; Ramos-Padrón, 2013), *Hydrogenophaga* (Golby et al., 2012), *Rhizobium* (Golby et al., 2012; Hwang et al., 2013), *Acinetobacter* (Golby et al., 2012; Herman et al., 1994; Hwang et al., 2013; Ramos-Padrón, 2013;

Ramos-Padrón et al., 2011), *Thiobacillus* (Ramos-Padrón et al., 2011), and *Methyloversatilis* (Ramos-Padrón et al., 2011).

Three sequenced bands closely identified with the genus *Brevundimonas*, which is capable of PAH degradation (Chaîneau et al., 1999; Xiao et al., 2010), tolerant of heavy metals (Xiao et al., 2010), and has been found in crude oil stockpiles (Yoshida et al., 2005). This band was present in all treatments for Big Pit, with greater intensity at early time points, and more prominent in Untreated samples compared to Ozonated and Ozonated-Inoculated treatments for FE5. Hwang et al. (2013) found this genus in biofilms formed from unozonated OSPW as well as newly ozone treated OSPW. The organic matter utilized by *Brevundimonas* may have been greatly reduced by ozonation and thus were not found or as prevalent in those samples pretreated with ozone. *Hydrogenophaga* was the most common genus sequenced; these autotrophic bacteria oxidize hydrogen (Willems et al., 1989) and thus it is possible their occurrence in these samples is due to utilization of free hydrogen that has been cleaved off organic compounds during dehydrogenation (Gibson et al., 1968). Multiple band positions, found in all treatments for both OSPW samples, associated with *Hydrogenophaga palleronii*. At one band position identified as *Hydrogenophaga palleronii*, intensity was greatest at the final time point, while other positions showed greater intensity at Week 0 or 2. Golby et al. (2012) enriched for *Hydrogenophaga* in biofilms formed in OSPW and reported the first detection of this genus in OSPW. *Acinetobacter*, on the other hand, has been repeatedly detected in OSPW, and is capable of degrading NAs (Herman et al., 1994) in addition to crude oil in both soil and liquid media (Hanson et al., 1997). The band associated with *Acinetobacter* is most intense at Weeks 0 and 2 of the Ozonated treatment and faint at Week 2 of Ozonated-Inoculated, suggesting contribution to DOC degradation. The most interesting of the genera identified through DGGE band sequencing was *Sphingomonas*, which was enriched in a sediment exposed to crude oil and then incubated with model aromatic NA compounds (Johnson et al., 2011). *Sphingomonas* isolates were capable of degrading aromatic compounds at low temperatures and without nutrient addition

(Baraniecki et al., 2002; Ye et al., 1996), conditions that microbial communities in end pit lakes would experience. The relevant DGGE band was present in both Untreated and Ozonated treatments and most intense at Week 0 of the Ozonated sample, and thus may be involved in DOC degradation observed.

Numerous other sequences showing similarity of at least 97% to classified genera were methylotrophic [*Methylopila* (Doronina et al., 1998), *Methylobacterium* (Khongkhaem et al., 2011), *Methyloversatilis* (Kalyuzhnaya et al., 2006), *Hyphomicrobium* (Moore, 1981)]. The two former were most prevalent in Ozonated samples and early time points, while the latter two were most intense at Week 11 in all treatments. *Methylobacterium* has been isolated from oil contaminated soils and is capable of phenol degradation (Dourado et al., 2012; Khongkhaem et al., 2011); once again, as this band was most intense in the Ozonated and Ozonated-Inoculated treatments at Weeks 2 and 6, during which DOC degradation occurred, contribution of this genus to the observed DOC biodegradation is likely. *Hyphomicrobium* are found in numerous environments, including fresh, sea and brackish waters, soil and nutrient deficient habitats (Moore, 1981). Increased relative abundance of this genus may be opportunistic; the single carbon compounds utilized by methylotrophs (Moore, 1981) must be present in these aged OSPW samples during aerobic incubation, and *Hyphomicrobium* would be able to survive in the nutrient depleted environment in the final weeks of incubation.

Finally, *Thiobacillus*, *Psychrobacter* and *Polaribacter* were prominent at Week 0 and in Sterilized controls. The presence of these genera may be indicative of the storage of OSPW samples at 4˚C prior to microcosm establishment. *Thiobacillus* is a sulphur bacterium that will oxidize sulphide in oxygen limiting conditions (Ende and Gemerden, 1993), which is likely during storage. The intensity of these bands in the Sterilized controls, where no significant degradation of DOC for the duration of aerobic incubation was detected, highlight a substantial limitation of PCR-based molecular analyses. Without the use of an intercalating agent like ethidium monoazide to bind DNA from dead or damaged cells prior to DNA extraction, all downstream analyses will

encapsulate both live and dead cells (Nocker and Camper, 2006; Pisz et al., 2007).

To date, microbial communities present in the decades-old experimental reclamation ponds have not been profiled, and yet, these communities have been subjected to long-term exposure to residual OSPW contaminants (Quagraine et al., 2005b). The existence of viable microbial communities in these environments will not only potentially demonstrate the potential for biodegradation of NAs and other organic compounds in end pit lake environments, but healthy microbial communities are essential for sustainable aquatic ecosystems (Yergeau et al., 2012). While this work is not sufficient for an ecology-based study, as samples were stored and then treated prior to molecular characterization, it provided some insight into the bacterial communities that populate two different aged OSPW samples. Of course, this survey was not extensive, as only select DGGE bands were excised for sequencing. In addition, a number of important bands were not successfully sequenced, likely due to little resolution of different sequences at these positions. The use of DGGE limited amplicon length, as well, which reduces success of taxonomic classification during BLAST searches.

Finally, it is recognized that PCR-DGGE for microbial community characterization is plagued by many limitations (Neilson et al., 2013). The use of PCR will introduce preferential amplification bias and artefacts such as chimeric sequences (von Wintzingerode et al., 1997). Amplification bias translates to inaccurate abundance determination based on DGGE band intensity (Neilson et al., 2013). Interestingly, more distinct bands were detected in the FE5 samples than Big Pit, despite FE5 likely containing more contaminants because it contains OSPW while Big Pit was constructed with fresh water over MFT. Unfortunately, the diversity indices calculated from estimated band intensity did not reveal differences due to treatment or time. The use of DGGE patterns to determine these parameters has certainly been contested, as artefacts introduced during PCR (although other molecular techniques would suffer from this) and DGGE resolution (prominence of species, multiple bands per species, multiple

sequences in one band position) render the biodiversity measures inaccurate (Neilson et al., 2013). Reduced diversity in microbial communities could result in stalled or slow biodegradation (Yergeau et al., 2012), so understanding the impact of ozone exposure on the diversity of indigenous microorganisms is essential before adopting ozonation for pretreatment of OSPW prior to discharge into end pit lakes or constructed wetlands.

4.5 Conclusions

PCR-DGGE was utilized to evaluate microbial community dynamics in response to ozone pretreatment and subsequent aerobic incubation for the treatment of OSPW organic compounds. Up to 40% removal of DOC was observed in Ozonated OSPW, while incubation resulted in 20% reduction of DOC in Untreated samples. Similarity analyses with the Dice index, based on PCR-DGGE fingerprints, indicated that both ozone exposure and incubation with ozonated OSPW did influence microbial community structure. Untreated, Ozonated, and Ozonated-Inoculated treatments were no more than 70% similar to one another. For both FE5 and Big Pit OSPW samples, Ozonated and Ozonated-Inoculated treatments were about 65% similar to one another. The detectable difference between the Ozonated and Ozonated-Inoculated treatments may be due to microorganisms that did not survive ozone exposure, as indicated by visual assessment of DGGEs. Despite this, both treatments resulted in the same overall DOC removal due to aerobic biodegradation. Incubation with ozonated OSPW also influenced community structure, as indicated by Untreated and Ozonated-Inoculated treatments being equally dissimilar to one another (65% in FE5 and less than 40% in Big Pit) as to the Ozonated treatment. The significant difference in DOC removal due to ozone pretreatment did not result in an equally stark difference in microbial community structure between Untreated and Ozonated treatments. The microbial communities in all treatments did shift over time, due to aerobic incubation, with similarity indices ranging from 70% to 90%. Weeks 0 and 11 were typically least similar within each treatment. Both aged OSPW samples exhibited rich bacterial

communities, as indicated by 55 distinct PCR-DGGE bands identified in FE5 and 46 in Big Pit. Genera previously identified in OSPW samples were obtained through excised DGGE band sequencing, including *Brevundimonas*, *Hydrogenophaga, Rhizobium, Acinetobacter, Thiobacillus*, and *Methyloversatilis*. In addition, genera attributed with hydrocarbon degradation were discovered, including *Sphingomonas* and *Brevundimonas*.

Ozonation did influence the microbial communities indigenous to two aged OSPW samples. Overall, microbial community structure and dynamics proved too complicated to thoroughly assess with PCR-DGGE, and characterization of both structure and dynamics over time and treatment will improve with use of next generation sequencing. A more extensive understanding of the organic compounds present in OSPW and their degradation under this treatment regime would aid identification of metabolic functions of the microorganisms inhabiting reclamation ponds. The current reclamation ponds are potential models for future end pit lakes, a final reclamation strategy for MFT and/or OSPW being aggressively pursued by the oil sands industry. This study showed that, while microbial community structure is influenced by ozone exposure, degradation capacity is retained. Delineating how ozone pretreatment of OSPW impacts the survival of indigenous microorganisms is essential, both to identify capacity for biodegradation of remaining organic matter and to capture influence on microbial community structure and thus overall health of the reclamation environment ecosystem.

5 Ozone Pretreatment of Model Naphthenic Acids Improves Biodegradation

5.1 Introduction

The recognized resistance of OSPW-sourced NAs to biodegradation by microorganisms indigenous to oil sands tailings ponds (Bataineh et al., 2006; Del Rio et al., 2006; Han et al., 2008; Herman et al., 1994; Scott et al., 2005; Toor et al., 2013a) has driven the exploration of other treatment techniques that could improve and accelerate NA biodegradation, including ozone pretreatment (Gamal El-Din et al., 2011; Hwang et al., 2013; Martin et al., 2010; Scott et al., 2008b; Wang et al., 2013). While a number of studies, summarized in 2.2.4.1, have established the capability of ozone treatment for reducing or removing NAs and other organics from OSPW, thereby improving biodegradation of the remaining compounds, only one study utilized model NA compounds (Pérez-Estrada et al., 2011). Model NA compounds enable proof-of-concept in treatment of NAs, as the analytical challenges in measuring OSPW-NAs are greatly reduced (Martin et al., 2008; Pereira et al., 2013a) and metabolite tracking is possible (Johnson et al., 2011; Rowland et al., 2011b; Smith et al., 2008). The identification, and synthesis when authentic compounds are not available commercially, of individual NA compounds in OSPW was a substantial progression in NA research (Rowland et al., 2011a, 2011c, 2011f, 2011g). Not only has this facilitated greater understanding of mechanisms of toxicity (Jones et al., 2011; Scarlett et al., 2012), because these tend to be dependent on specific structure (Rowland et al., 2011c), but it has also allowed for delineation of structure-biological reactivity relationships of OSPW-relevant NA compounds (Johnson et al., 2013, 2012, 2011; Smith et al., 2008). For the first time, NA model compounds were subjected to ozone pretreatment combined with aerobic biodegradation to further investigate this coupled regime for the treatment of OSPW.

A bacterial isolate, tentatively identified as *Acidovorax* sp., was obtained from OSPW and able to completely degrade single ring NA compounds (Ramos-Padrón, 2013). Ramos-Padrón (2013) suggested that this isolate should be utilized to study the biodegradation of more complex NA compounds. Both Rowland et al. (2011c) and Martin et al. (2008) found tricyclic NAs (*Z*=-6 in

$C_nH_{2n+Z}O_2$) to be the most abundant in OSPW. Aromatic acid compounds have also been found to contribute significantly to the acidic fraction of OSPW, with some individual compounds being identified (Jones et al., 2012; Rowland et al., 2011f). Adamantane-1-carboxylic acid has been identified in OSPW (Rowland et al., 2011c); both tetrahydro-2-naphthoic acid and decahydro-2-naphthoic acid are metabolites in anaerobic degradation of naphthalene (Annweiler et al., 2002) and decahydro-2-naphthoic acid has been detected in oil sands ore (Aitken et al., 2004).

Thus, the aim of this work was to utilize model NA compounds that are representative of OSPW-NAs to 1) further explore the treatment scheme of ozone pretreatment followed by aerobic biodegradation for OSPW through testing with model NAs, and 2) examine the capability of a bacterial isolate, *Acidovorax* sp., indigenous to oil sands tailings, to biodegrade more complex and OSPW-representative model NAs.

5.2 Methods and Materials

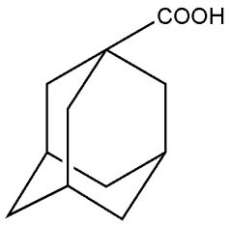
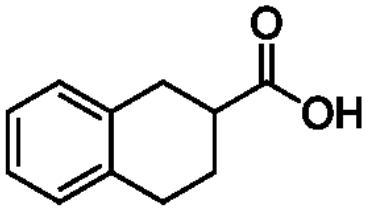
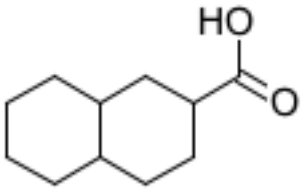
Unless otherwise stated, all materials were obtained from Fisher Scientific (Waltham, MA and Loughborough, UK).

5.2.1 Ozonation of Model NAs

Prior to ozone pretreatment, model NA compounds were added to 80 mL autoclaved minimal salts medium (MSM, in g/L: 0.2 $MgSO_4$, 0.5 $(NH_4)_2SO_4$, 0.5 KH_2PO_4, 1.5 K_2HPO_4, 0.02 NaOH, 0.12 Na_2EDTA, 0.004 $ZnSO_4$, 0.001 $CuSO_4 \cdot 5H_2O$, 0.0001 Na_2SO_4, 0.001 $Na_2MoO \cdot 2H_2O$, 0.0004 $MnSO_4$, 0.0001 $CoCl_2 \cdot 6H_2O$, pH 7.0), via a concentrated stock solution made in 0.1 M NaOH, to a final concentration of 5 mg/L. Three model NAs were utilized: adamantane-1-carboxylic acid (Sigma Aldrich, Dorset, UK), 1,2,3,4-tetrahydro-2-naphthoic acid (Sigma Aldrich), and decahydro-2-naphthoic acid (synthesized, Rowland et al., 2011b), shown in Table 5-1. Each 125 mL serum bottle containing MSM with one acid was ozonated individually in triplicate. Ozone was generated with an OZO-1VTT ozonator (Ozomax, Shefford, QC). Medical grade oxygen feed gas

was converted to ozone at a maximum rate of 5 g/h (10% ozone). The outgoing gas flow rate was maintained at two cubic feet per hour. Gas was bubbled into MSM using a glass Pasteur pipet. Bottles were exposed for 0, 30, and 60 s then left uncovered at room temperature (20ºC) overnight to allow for residual ozone to dissipate prior to inoculation.

Table 5-1: Chemical structures of three model NA compounds used for ozone pretreatment biodegradation trials.

adamantane-1-carboxylic acid	1,2,3,4-tetrahydro-2-naphthoic acid	decahydro-2-naphthoic acid
		

In an attempt to quantify ozone concentration, the indigo colorimetric method was followed (APHA, 2005). In brief, 0.3 mL of Indigo Reagent II (in g/L: 0.077 $C_{16}H_7K_3N_2O_{11}S_3$ (Sigma Aldrich, St. Louis, MO), 10 NaH_2PO_4, 7.1 mL H_3PO_4) was added to 2.7 mL sample and measured on a Nanodrop 2000C (Thermo Scientific, Wilmington, DE) at 600 nm. Prior to experimental setup, ozone dosage was tested with 4-phenylbutanoic acid (Acros Organics, Geele, Belgium) in MSM, at a concentration of 10 mg/L. Samples were taken after 0, 1, 5, 10 and 15 min of exposure, for GC-MS measurement and residual ozone concentration. In addition, MSM was exposed at the original test conditions (0, 30, and 60 s) to enable measurement of ozone residual in the absence of an organic compound.

5.2.2 NA Degradation Experiment and Microcosm Sampling

The inoculant used in this experiment was an isolate obtained from Suncor's Pond 5 sampled in 2009, by Esther Ramos-Padrón (Ramos-Padrón,

2013), possessing 99% identity with *Acidovorax* sp., obtained from comparing the 16S rRNA gene sequence to the NCBI GenBank database through BLAST. Prior to establishment of the biodegradation experiment, the inoculant was incubated in the dark at 28ºC for 24 h in LB broth (in g/L: 10 Bacto-tryptone, 5 yeast extract, 10 NaCl, pH 7.5) and then centrifuged four times at 3000 *g* for 15 min using a Heraeus Multifuge 3S-R table top centrifuge outfitted with a swinging bucket rotor, rinsing with sterile MSM in between to remove all traces of broth. The pellets were resuspended in MSM and divided amongst the live bottles (36 total). In addition, both abiotic (36) and killed (36) controls, containing 0.5 g $HgSO_4$ in addition to cell suspension, were established to monitor for abiotic losses. Killed controls were verified by plating on LB agar. Bottles were crimped with polyisobutylene seals and incubated statically in the dark for ten days at 28ºC.

On Days 0, 4, and 10, a 20 mL sample was removed and acidified for liquid-liquid extraction; samples were either taken to University of Calgary by Dr. Corinne Whitby (Day 0) or shipped to the University of Essex (Days 4 and 10) at this stage. A 1 mL sample was also removed to monitor growth via optical density measurement at 600 nm on a Nanodrop 2000C, and these data were statistically analyzed with One-Way ANOVA at each time point using SigmaPlot Version 11.0 (Systat Software, San Jose, CA). On Day 10, 1 mL was retained from live cultures for Microtox analysis; aliquots from three replicates were combined.

5.2.3 NA Extraction and GC-MS Analysis

All glassware utilized for NA extraction was soaked overnight in 5% (v/v) Decon-90 (Decon, Hove, UK), rinsed three times with milli-Q water, and dried at 110°C prior to use, to remove any hydrocarbon contamination. Samples (20 mL) were acidified with a few drops of concentrated hydrochloric acid. Prior to liquid-liquid extraction, 50 µL of the internal standard stock solution, consisting of 1 mg of 4-phenylbutanoic acid dissolved in 1 mL methanol (high-performance liquid chromatography grade), was added (final concentration 2.5 mg/L).

Samples were exchanged with 20 mL ethyl acetate (HPLC grade) three times in 250 mL glass separatory funnels. The ethyl acetate fractions were combined and passed through anhydrous sodium sulphate to remove any traces of water. The volume of ethyl acetate was reduced using a rotary evaporator, and after transfer to GC-MS vials, dried in a heat block.

In sets of 30 (enough for approximately 24 h run), samples were derivatized with 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Aldrich) and 950 µL dichloromethane, both added quantitatively. Samples were heated to approximately 60˚C in a metal heat block for at least 20 min to form trimethylsilyl derivatives. Day 0 samples were run by Dr. Corinne Whitby at the University of Calgary on an Agilent 7890A GC combined with an Agilent 5975C MS (Aglient Technologies, Santa Clara, CA), operating with an injector on split mode 50:1 at 270ºC and a 30 m x 250 µm x 0.25 µm HP-5MS column. The GC program was as follows: hold at 45ºC for 5 min, ramp to 270ºC at 4ºC/min, hold for 5 min. Day 4 and 10 samples were run at the University of Essex on a Thermo Finnigan Trace 7890 gas chromatograph with Thermo Finnigan Trace 5975C DSQ mass spectrometer (Agilent, Wokingham, UK), operating with 1 mL splitless injection at an injector temperature of 250ºC and a 30 m x 250 µm x 0.25 µm HP-5MS column. The GC program was as follows: ramp from 40ºC to 250ºC at 10ºC/min, hold at 300ºC for 10 min. The flow rate of the carrier gas, helium, was 1 mL/min. Standard calibration curves for each model NA compound are provided in Appendix 7. Because Day 0 samples were analyzed on a different GC-MS unit from Day 4 and 10 samples, calculation of degradation was based on abiotic and killed control data at each time point. In addition, gross outliers were removed from the data set. All data are provided in Appendix 8.

All statistical analyses of the NA data were completed using SigmaPlot Version 11.0 (Systat Software), except for the student's t-test, which was conducted in Office Excel (Microsoft, Redmond, WA). Ozone degradation comparisons were completed with Two-Way ANOVA and the Holm-Sidak method for pairwise comparisons. Biodegradation data were also analyzed via

Two-Way ANOVA when the data passed the requirements for normality and equal variance.

5.2.4 Microtox Analysis

Acute toxicity analysis was conducted on a single sample of each compound prior to ozonation and on pooled samples of three replicates from live cultures at Day 10 with a Microtox 500 Analyzer (Azur Environmental, Carlsbad, CA), using the 81.9% Basic protocol, four 2:1 dilutions, and 5 min incubation period. Measurements were expressed using EC_{20}, representing the percent volume of sample required to reduce the luminescence of the test specimen, *Vibrio fischeri*, by 20%. Phenol and deionized water were used as positive and negative standards, respectively, and phenol EC_{20} was between 4 and 8 mg L^{-1} (Ghosh and Doctor, 1992).

5.3 Results

5.3.1 Ozone Pretreatment of NAs

Ozonation of the NA solutions was performed for 0, 30, and 60 s, to ensure that a portion of the original NA compounds remained for aerobic incubation with *Acidovorax* sp.. Measured NA concentrations following ozone pretreatment are shown in Table 5-2.

Table 5-2: Measured NA concentration at Day 0 following ozone pretreatment. Values are averaged from live, killed and abiotic samples (n=9). Error provided is one standard deviation.

	NA Compound (mg/L)		
Ozone Exposure (s)	adamantane-1-carboxylic acid	1,2,3,4-tetrahydro-2-naphthoic acid	decahydro-2-naphthoic acid
0	5.1 ± 0.5	4.7 ± 0.2	3.7 ± 0.3
30	1.7 ± 0.2	0.8 ± 0.1	0.7 ± 0.1
60	0.7 ± 0.1	1.6 ± 0.4	0.6 ± 0.2

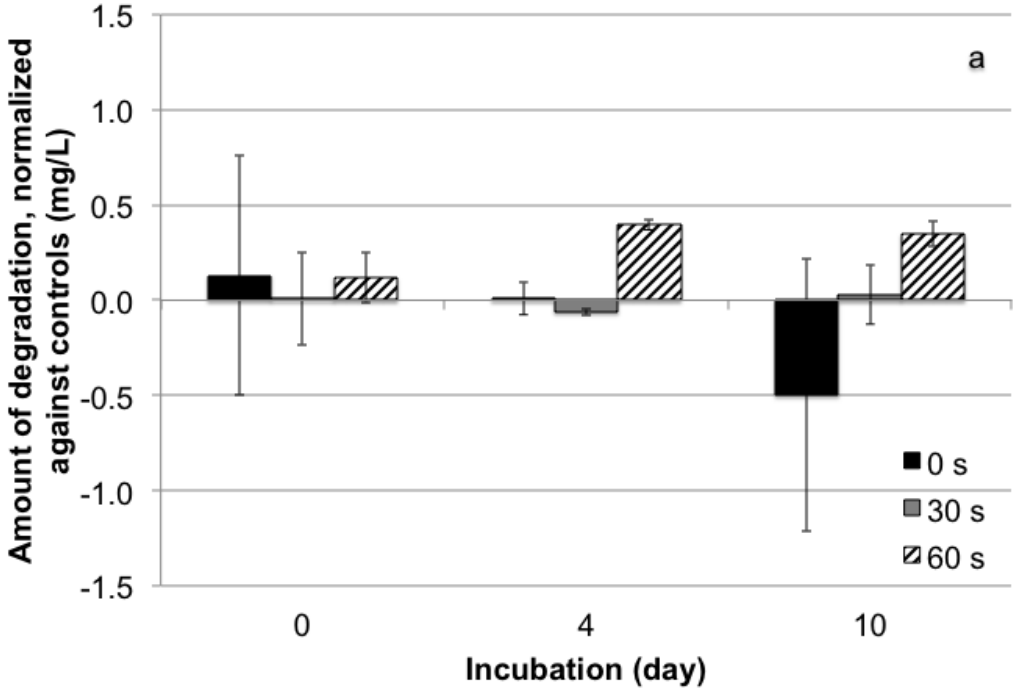
Ozonation reduced the NA concentrations by 70-85%. Significant reduction in all three compounds occurred after 30 s and 60 s of ozone exposure ($p < 0.001$), compared to no ozone exposure, from a starting concentration of approximately 5 mg/L. There was no statistically significant difference in decahydro-2-naphthoic acid concentration between 30 and 60 s ($p > 0.05$) and, interestingly, the measured concentration of 1,2,3,4-tetrahydro-2-naphthoic acid was greater after 60 s of ozone exposure versus 30 s ($p < 0.001$). Adamantane-1-carboxylic acid significantly reduced in concentration from 0 to 30 s of ozone exposure and again from 30 to 60 s ($p < 0.001$). Both 1,2,3,4-tetrahydro-2-naphthoic acid and decahydro-2-naphthoic acid experienced significantly greater reduction than adamantane-1-carboxylic acid after 30 s ozone exposure ($p < 0.001$), but no significant difference between adamantane-1-carboxylic acid and decahydro-2-naphthoic acid was observed after 60 s of ozonation ($p > 0.05$).

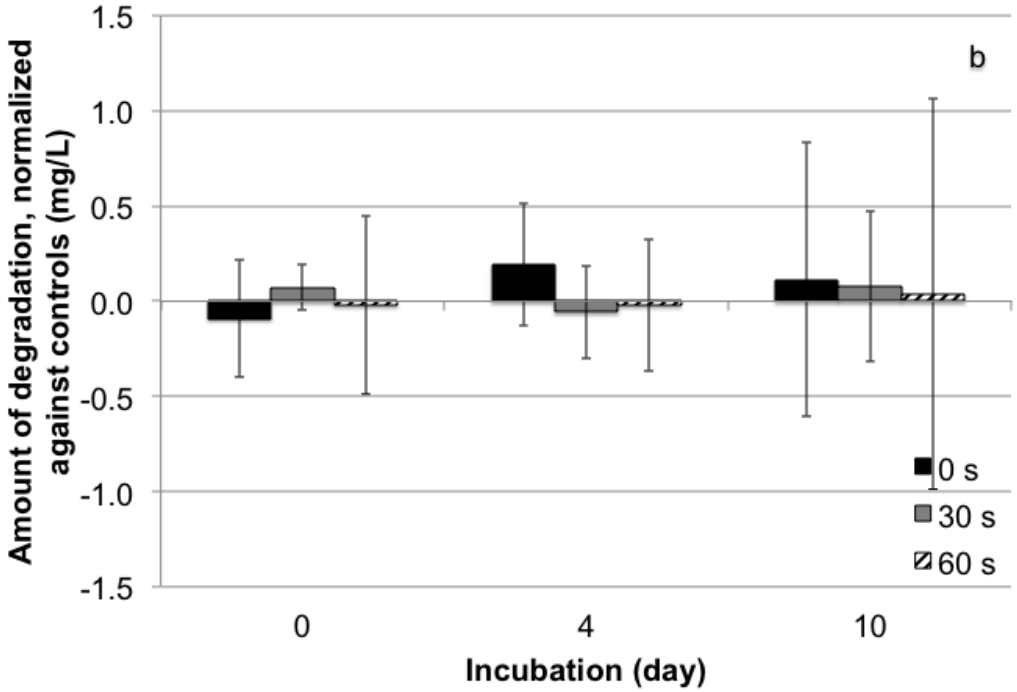
Measurement of utilized ozone dosage was limited by lack of inlet ozone concentration and release of ozone from the reactor during exposure. No residual ozone was detected during the 4-phenylbutanoic acid trial until 10 min of ozone exposure had elapsed, at which time no 4-phenylbutanoic acid could be detected via GC-MS analysis. Thus, it is likely that ozone was completely consumed during the 30 and 60 s exposures due to oxidation of the model NA compounds. Ozone residual in MSM was 0.04 and 0.05 mg/L after 30 and 60 s of exposure, but these concentrations do not represent utilized ozone dose, as inlet and offgas ozone concentrations are required for calculating utilized ozone (Gamal El-Din et al., 2011).

5.3.2 Degradation of Model NA Compounds

Degradation of three model NA compounds, adamantane-1-carboxylic acid, 1,2,3,4-tetrahydro-2-naphthoic acid, and decahydro-2-naphthoic acid, over 10 d of aerobic incubation with an *Acidovorax* sp. isolate was monitored with GC-MS. For this experiment, in part because Day 0 samples were analyzed on a different GC-MS unit than Day 4 and 10 samples, degradation in the live cultures

was normalized against the killed and abiotic controls at the same time point. Amount of degradation is presented in Figure 5-1.





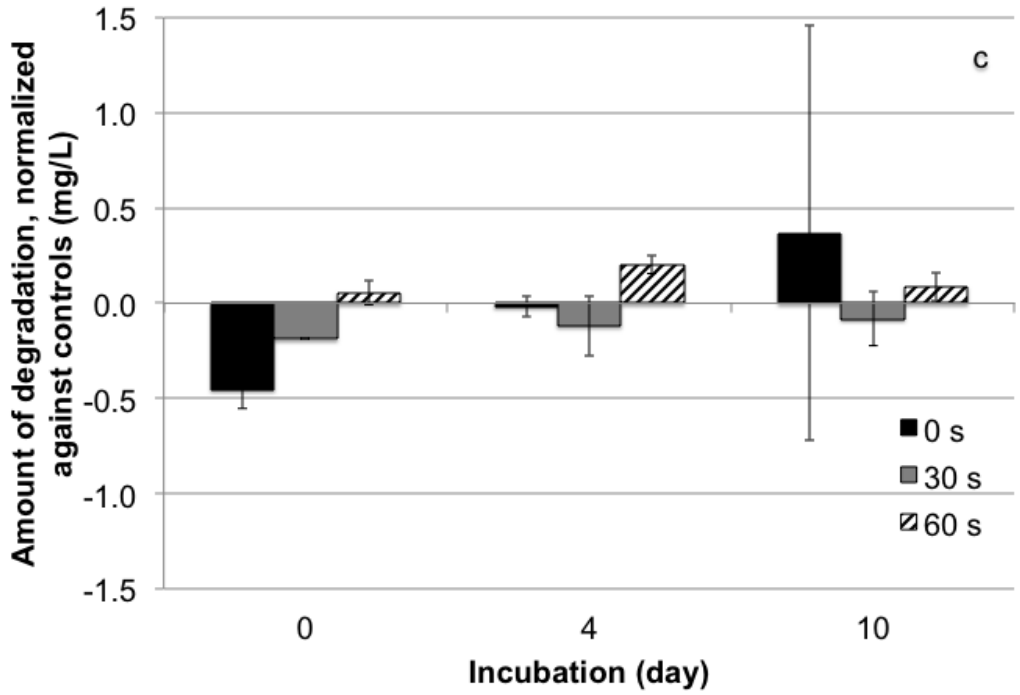
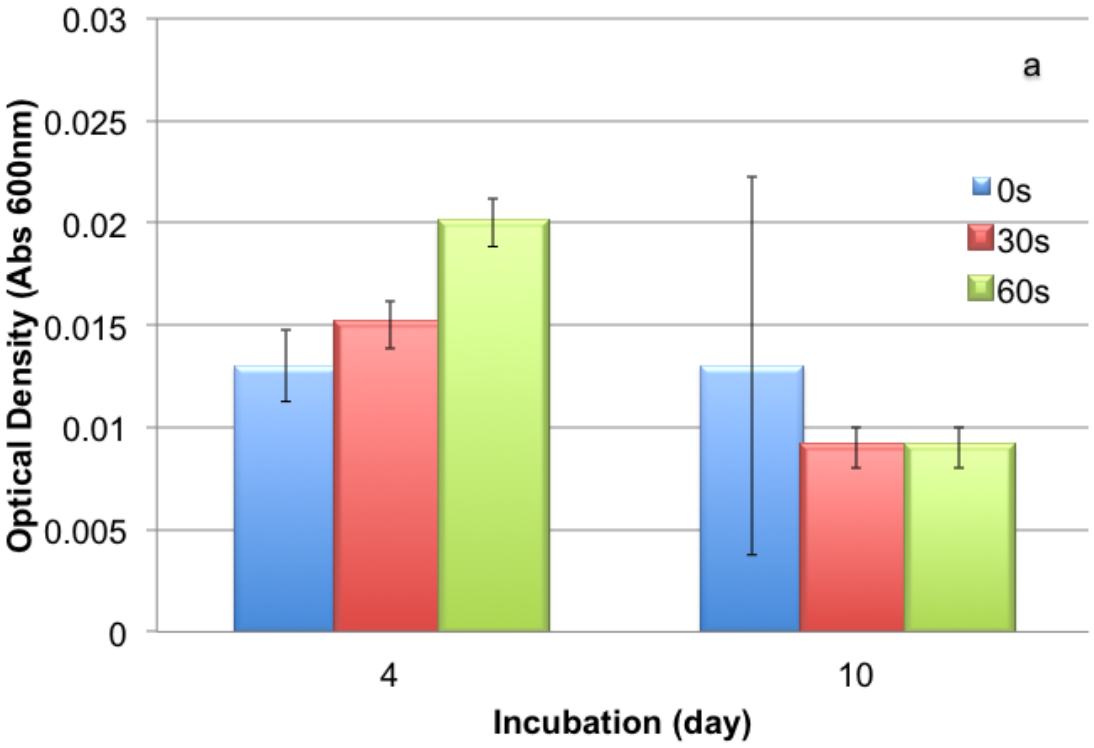


Figure 5-1: Amount of degradation of a) adamantane-1-carboxylic acid, b) 1,2,3,4-tetrahydro-2-naphthoic acid, and c) decahydro-2-naphthoic acid, normalized against killed and abiotic controls, at Days 0, 4, and 10 of aerobic incubation with *Acidovorax* sp., following ozone exposure for 0, 30, and 60 s. Error bars indicate one standard deviation (n=3).

In the 60 s ozonation treatment, adamantane-1-carboxylic acid significantly decreased in concentration over ten days of aerobic incubation (student's t-test, $p < 0.1$). In four days, 40% of the compound had been degraded, from a starting concentration of 0.7 mg/L, with no additional removal in the subsequent six days. Also in the 60 s ozonation treatment, decahydro-2-naphthoic acid experienced 35% degradation, also from 0.7 mg/L, in four days (student's t-test, $p < 0.05$), but the measured concentration at Day 10 was not statistically significantly different from Day 0 (student's t-test, $p > 0.05$). No degradation of 1,2,3,4-tetrahydro-2-naphthoic acid was observed in ten days ($p > 0.05$).

5.3.3 Biomass Growth Monitoring

Biomass growth of *Acidovorax* sp. was monitored using optical density on Days 4 and 10 of aerobic incubation with three model NAs and is shown in Figure 5-2.



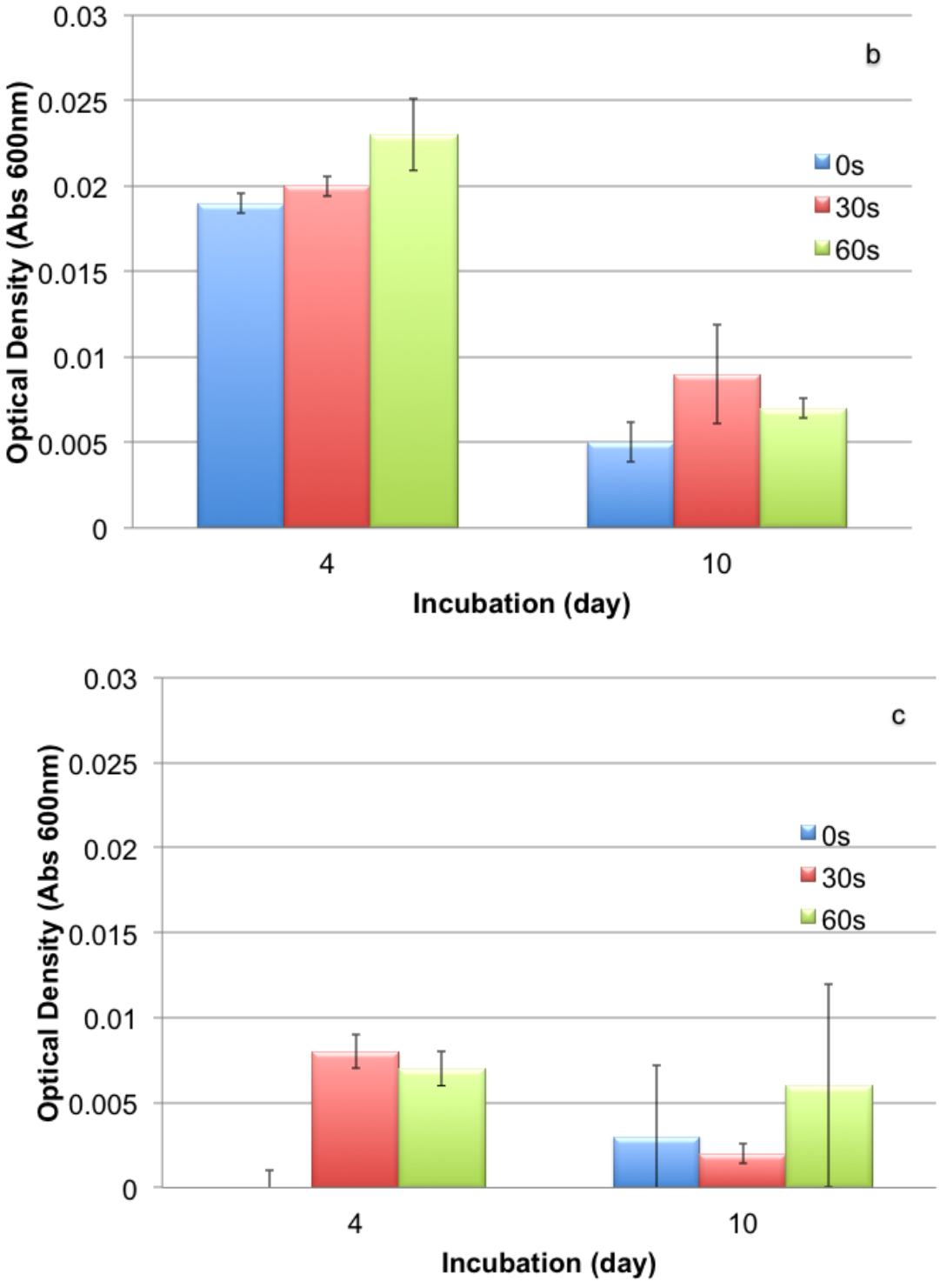


Figure 5-2: Growth of *Acidovorax* sp. over ten days of aerobic incubation, as measured by optical density (at 600 nm), on a) adamantine-1-carboxylic acid, b) 1,2,3,4-tetrahydro-2-naphthoic acid, and c) decahydro-2-naphthoic acid. Each NA was pretreated with ozone for 0, 30, or 60 s, prior to incubation with *Acidovorax* sp.. Error bars indicate one standard deviation (n=3).

In general, optical density was greater on Day 4 than Day 10, indicating that the *Acidovorax* sp. isolate was no longer experiencing growth at the conclusion of the experiment. There tended to be higher optical density in those samples that had been pretreated with ozone for 60 s than 0 or 30 s. Optical density was significantly greater, at Day 4, in the ozonated samples (both 30 and 60 s) than unozonated ($p < 0.05$) for both 1,2,3,4-tetrahydro-2-naphthoic acid and decahydro-2-naphthoic acid, but only the 60 s ozone exposed treatment was significantly higher for adamantane-1-carboxylic acid ($p < 0.05$). The *Acidovorax* sp. isolate experienced the highest growth at Day 4 on 60 s ozone exposed 1,2,3,4-tetrahydro-2-naphthoic acid.

5.3.4 Microtox Testing

As NAs have long been identified as the primary source of acute toxicity of OSPW to aquatic life (MacKinnon and Boerger, 1986), Microtox, an analytically simple and cost-effective bioassay for monitoring aquatic toxicity (Johnson, 2005), was utilized. The effective inhibitory concentration that resulted in 20% reduction of function of *Vibrio fischeri* (EC_{20}) is given in Table 5-3 as percentage of the original sample, at Day 0 and Day 10.

Table 5-3: EC_{20} (in percent of original sample) of *Vibrio fischeri* after 5 min exposure to model NA compounds. Measurement conducted on a pooled sample from three replicates (n=1). Lower value indicates greater toxicity. Shaded values indicate that EC_{20} could not be determined.

			Day 10 (Live)		
	Day 0		Ozone Exposure (s)		
NA Compound		**(mM)**	**0**	**30**	**60**
adamantane-1-carboxylic acid	16%	0.0048	34%	>80%	176%
1,2,3,4-tetrahydro-2-naphthoic acid	38%	0.0102	>80%	71%	49%
decahydro-2-naphthoic acid	31%	0.0064	23%	33%	85%

Ozone pretreatment reduced the overall toxicity of the samples originally containing adamantane-1-carboxylic acid and decahydro-2-naphthoic acid, but actually increased the toxic response of *Vibrio fischeri* when ozone exposure of

1,2,3,4-tetrahydro-2-naphthoic acid was increased. However, these observations are based on data from Day 10 live samples, and thus it cannot be ruled out that biodegradation products may be influencing the toxicity of these samples. In comparing data from Day 0 and Day 10 at 0 s of ozone exposure, incubation with the Acidovorax sp. isolate reduced the toxicity of the sample containing adamantane-1-carboxylic acid and 1,2,3,4-tetrahydro-2-naphthoic acid, but increased toxicity of the sample with decahydro-2-naphthoic acid. Finally, adamantane-1-carboxylic acid was found to be the most toxic to *Vibrio fischeri*, followed by decahydro-2-naphthoic acid, then 1,2,3,4-tetrahydro-2-naphthoic acid.

As a pooled sample from three replicate samples from the live cultures at Day 10 were used for the Microtox analysis due to restricted sample volume availability, statistical analyses could not be completed. The measured EC_{20} values at Day 0 were calculated to molar concentration with measured mass concentration based on GC-MS and theoretical molar mass for each model NA compound. This calculation could not be performed for the Day 10 samples as compounds other than the model NAs would be present, such as ozone byproducts and biodegradation metabolites.

5.4 Discussion

Model NA compounds that have been identified in OSPW or the oil sands deposit (Aitken et al., 2004; Jones et al., 2012; Rowland et al., 2011c) were subjected to both ozone exposure and aerobic incubation with an isolate, *Acidovorax* sp., to determine the capacity of this isolate to degrade these compounds and whether ozone would further improve biodegradation.

Even short term ozone exposure significantly reduced the concentration of all three model NAs. More than 80% of both 1,2,3,4-tetrahydro-2-naphthoic acid and decahydro-2-naphthoic acid were consumed after 30 s of ozone exposure, while it took 60 s to consume more than 80% of adamantane-1-carboxylic acid. Pérez-Estrada et al. (2011) observed that the highest rate of NA degradation by ozone occurred within the first minute of exposure and resulted in more than 70%

of the observed total degradation, when testing the commercially available Merichem NA mixture at 40 mg/L. As the NA compounds are oxidized, it is possible that the ozone preferentially reacts with these ozone products, resulting in reduced reaction with the original compounds of interest with increasing exposure time (Pérez-Estrada et al., 2011), resulting in a residual concentration that may be difficult to oxidize. Thus, if biodegradation is able to treat the ozone products and any remaining original organic matter, the coupling of these two treatment mechanisms will minimize the ozone dose required, thereby reducing costs without compromising treatment (Hwang et al., 2013; Martin et al., 2010; Scott et al., 2008b).

Adamantane-1-carboxylic acid, a tricyclic NA, required longer ozone exposure than the other two model NA compounds, both containing two rings. This contrasts with the observation that compounds with greater rings, while controlling for carbon number as in this case, are more susceptible to attack by ozone or the hydroxyl radical (Gamal El-Din et al., 2011; Pérez-Estrada et al., 2011; Wang et al., 2013). However, in this experiment, only two ozone exposure times were utilized, which is insufficient to draw conclusions on reactivity kinetics.

In general, the isolate *Acidovorax* sp. was not able to metabolize the three chosen representative model NA compounds over 10 days. The only statistically significant degradation occurred at Day 4 (but not Day 10) for decahydro-2-naphthoic acid and at Days 4 and 10 for adamantane-1-carboxylic acid, both in the 60 s ozone treatment. Forty percent removal of adamantane-1-carboxylic acid coincided with significantly higher biomass and removal of toxicity to *Vibrio fischeri*. Both adamantane-1-carboxylic acid and decahydro-2-naphthoic acid are cyclic aliphatic compounds, while 1,2,3,4-tetrahydro-2-naphthoic acid contains an aromatic ring. Aromatic hydrocarbons are more recalcitrant than aliphatic compounds (Atlas, 1981; Head et al., 2006). Interestingly, adamantane-1-carboxylic acid was relatively more biodegradable than decahydro-2-naphthoic acid, despite it containing one more ring. Without examination of metabolites produced during degradation, it is difficult to surmise the cause of greater degradation of a more complex structure.

No degradation of the three model NAs by the *Acidovorax* sp. isolate was observed between Days 4 and 10. Since optical density was lower at Day 10 than Day 4, it is unlikely that longer incubation time would have resulted in additional degradation. This same isolate completely metabolized single ring NA compounds within six days (Ramos-Padrón, 2013). Thus, these preliminary results indicate that the *Acidovorax* sp. isolate may not be capable of degrading these model bi- and tri-cyclic NAs. Del Rio et al. (2006) observed up to 25% degradation of decahydro-naphthoic acid in 14 days by wetland sediment microbial communities previously exposed to OSPW; the rates of degradation, though, were 2-60 times lower than degradation of a single ring NA compound.

The only observed degradation of the model NAs in this experiment occurred when ozone pretreatment occurred for 60 s. More than 80% of both adamantane-1-carboxylic acid and decahydro-2-naphthoic acid were consumed during 60 s of ozone exposure and it is expected that this led to the production of more labile products (Gamal El-Din et al., 2011; Martin et al., 2010; Scott et al., 2008b). The isolate may have been utilizing these ozone degradation products as the primary carbon source, and cometabolism of the original NA compounds occurred (Atlas, 1981; Horvath, 1972). *Pseudomonas* sp. have been found to cometabolize organic compounds (Horvath, 1972), and *Acidovorax* was separated from the genus *Pseudomonas* (Willems et al., 1990). Co-metabolism is now a recognized method of bioremediation and is used to treat recalcitrant classes of compounds such as chlorinated organics and polycyclic aromatic hydrocarbons (Atlas, 1981; Nzila, 2013). It would have been advantageous with this work to investigate the production of ozone by-products from the three model NA compounds utilized here. However, this was limited by lack of authentic standards that would be used for verification of mass spectrometry signals of the compounds. Removal of both the original NA compound and ozone by-products would provide strong evidence for the occurrence of co-metabolism (Horvath, 1972). Cometabolism is a mechanism of bioremediation that has not been investigated for NA treatment in OSPW (Ahad and Pakdel, 2013; Whitby, 2010).

Higher measured growth in the ozone pretreated samples, especially in the 60 s treatment, suggest the presence of more labile ozone degradation products providing organic carbon substrates for growth of the *Acidovorax* sp. isolate. Strains from the *Acidovorax* genus are capable of growing on a wide range of organic compounds, including simple sugars, hydroxybenzoate, fumarate (Willems et al., 1990), and polycyclic aromatic hydrocarbons such as naphthalene, phenanthrene, benzo[a]anthracene, and benzo[a]pyrene (Singleton et al., 2009). *Acidovorax* sp. have been enriched in soils exposed to hydrocarbons and were implicated in aerobic naphthalene (Eriksson et al., 2003) and monoaromatic hydrocarbon (Tischer et al., 2013) degradation. Thus, it is likely that the isolate would have been capable of utilizing the ozone degradation products as substrates for growth. Given that the genus *Acidovorax* is known for aerobic aromatic hydrocarbon degradation, it is strange that the model NA compounds were not appreciably degraded here, especially 1,2,3,4-tetrahydro-2-naphthoic acid, as it is an aromatic compound.

Treatment of NAs in OSPW is ultimately driven by the need to remove toxicity, mainly attributed to the acid extractable content of OSPW (MacKinnon and Boerger, 1986) of which NAs contribute (Grewer et al., 2010; Headley et al., 2011). Of the three model NAs used, adamantane-1-carboxylic acid was found to be the most toxic to *Vibrio fischeri*, which agreed with Scarlett et al.'s (2012) findings using toxicity models but contrasted with Jones et al. (2011), who found decahydro-2-naphthoic acid to be more toxic to *Vibrio fischeri*. Ozone treatment, and possibly aerobic incubation with *Acidovorax* sp., reduced the toxicity of samples containing adamantane-1-carboxylic acid and decahydro-2-naphthoic acid, which is required for this treatment scheme to successfully remediate OSPW, but samples containing 1,2,3,4-tetrahydro-2-naphthoic increased in toxicity with increased ozone exposure, even after aerobic incubation. While ozone treatment of OSPW has generally reduced toxic responses in various endpoints (Anderson et al., 2012b; Gamal El-Din et al., 2011; He et al., 2012a, 2010; Scott et al., 2008b), ozonation has also been found to impact toxicity very little (Martin et al., 2010) or even increase toxicity for some endpoints (Garcia-

Garcia et al., 2011a). Thus, it will be essential for model NA compounds to be utilized in future ozone treatment studies to further elucidate the potential toxicity of NA ozone degradation products (Rowland et al., 2011d).

5.5 Conclusions

The main objectives of this work were to utilize three model NA compounds that are representative of the classes of compounds found in OSPW to investigate coupling ozone pretreatment with biodegradation and to test the capability of an OSPW-sourced bacterial isolate, *Acidovorax* sp., to biodegrade complex NAs. In this experiment, it was found, after 10 days, that the *Acidovorax* sp. isolate was not capable of degrading the three model NA compounds chosen, a tricyclic aliphatic compound, adamantane-1-carboxylic acid, a bicyclic aliphatic compound, decahydro-2-naphthoic acid, and an aromatic compound, 1,2,3,4-tetrahydro-2-naphthoic acid. Pretreatment of the model NA compounds with 60 s of ozone exposure enabled 40% removal of adamantane-1-carboxylic acid in four days, but since no biodegradation was observed in the untreated sample, it is possible that adamantane-1-carboxylic acid was cometabolized while labile ozone degradation products were used as substrates for *Acidovorax* growth. Cometabolism has not yet been investigated as a potential bioremediation treatment mechanism for NAs or other organics in OSPW. While it appears the *Acidovorax* sp. isolate was not capable of biodegrading the chosen NA compounds over 10 d, it would still be advantageous to look for metabolites in those samples with evidence of degradation.

Meanwhile, ozone exposure significantly reduced the concentration of all three model NA compounds in 60 s or less, which also resulted in greatly reduced toxic response in *Vibrio fischeri* for adamantane-1-carboxylic acid and decahydro-2-naphthoic acid. Ozone exposure greatly increased the toxicity of the samples containing 1,2,3,4-tetrahydro-2-naphthoic acid. Thus, it is essential for ozone degradation products from the treatment of relevant and OSPW-representative model NAs be identified to further understanding of ozone

pretreatment of OSPW. The toxicity and potential metabolic pathways may then be elucidated and linked to structural characteristics.

Ozone pretreatment coupled with biodegradation remains a promising treatment technology for organic compounds in OSPW, especially NAs, but prior to adoption by industry, it would be beneficial to expand the body of knowledge on ozone treatment of NAs through the use of model NA compounds. In addition, this body of work emphasizes the need for extensively exploring the capabilities of either indigenous microorganisms or those used for bioaugmentation with representative organic compounds, and ultimately whole OSPW.

6 Enriched Microbial Cultures from an Oil Sands Tailings Pond are Capable of Degrading Complex Model Naphthenic Acids

6.1 Introduction

Because biological treatment of NAs and other organic compounds is the most cost-effective option for OSPW (Scott et al., 2005), much work has focused on delineating the capacity for biodegradation of NAs, in particular, by indigenous microbial communities in tailings ponds (Bataineh et al., 2006; Clemente et al., 2004; Han et al., 2008; Herman et al., 1994, 1993; Scott et al., 2005; Videla et al., 2009). Others have isolated bacteria, from OSPW (Del Rio et al., 2006; Ramos-Padrón, 2013) or unrelated sites (Johnson et al., 2013, 2012), with varying degrees of success in degrading NAs.

Enrichment and isolation of microorganisms capable of degrading recalcitrant organic compounds, to be used for bioaugmentation in in-situ treatment regimes or as seed for ex-situ treatment facilities, is common in wastewater treatment and has been explored for biodegradation of hydrocarbons in groundwater and soil systems (El Fantroussi and Agathos, 2005). The addition of well-adapted microorganisms to a system, or bioaugmentation, is necessary when the indigenous microbial community lacks the metabolic ability to degrade the organic compounds of interest. Bioaugmentation can reduce the need for the expensive addition of nutrients and terminal electron acceptors (biostimulation), shorten treatment times, and produce predictable results (El Fantroussi and Agathos, 2005). Utilizing microorganisms from the same environment reduces acclimation times and promotes survival, as they are likely more acclimated to the particular abiotic and biotic pressures in that environ (Thompson et al., 2005). In tailings ponds, acclimated microbial communities have developed after years, sometimes decades, of OSPW storage, enriched on the very compounds needing to be treated.

Microorganisms of both bacterial and fungal origin were isolated from OSPW enriched on acid-extractable organics (Hofstetter et al., 2014). Isolates exhibited high tolerance to extreme conditions found in OSPW; a wide range of pH, salinity and AEO concentrations were tolerated. A fungal isolate most closely related to *Trichoderma harzianum* was capable of growing directly on pure commercial NAs placed on agar plates.

To test the metabolic capabilities of these microbial isolates, and the enriched community from which they were obtained, representative complex model NA compounds that have been identified in OSPW were chosen. Tricylic NAs are the most abundant in OSPW (Martin et al., 2008; Rowland et al., 2011c), of all NA compounds, and so two tricyclic, or diamondoid, NAs, adamantane-1-carboxylic acid and 3-ethyl-adamantane-1-carboxylic acid that have been explicitly identified in OSPW (Rowland et al., 2012, 2011c) were selected. In addition, one naphtheno-aromatic acid, dehydroabietic acid, also explicitly detected in OSPW, was chosen (Jones et al., 2012; Rowland et al., 2011d) as the aromatic acid fraction has been found to contribute 30% to the acid extractable fraction of OSPW (Jones et al., 2012).

The main objective of this body of work was to determine the capability of three microbial cultures from Hofstetter et al. (2014) that have been enriched on OSPW-sourced organic compounds to degrade three model NA compounds, representing the more recalcitrant fractions of NAs found in OSPW, diamondoid and aromatic carboxylic acids.

6.2 Methods and Materials

Unless otherwise stated, all materials were obtained from Fisher Scientific (Waltham, MA and Loughborough, UK).

6.2.1 Enrichment and Isolation of NA-degrading OSPW-sourced Cultures

OSPW collected from the surface of Suncor's South Tailings Pond (STP) in 2009 was aerobically incubated with progressively increasing concentrations of OSPW acid-extractable organics (AEO). In order to obtain sufficient quantities for this enrichment procedure, AEO from three OSPW samples, collected in 2009, were combined: Suncor South Tailings Pond, Syncrude West In-Pit, and Albian Tailings Pond. AEO were obtained by liquid-liquid extraction with dichloromethane (DCM) (Stabilized/Certified ACS grade) by Andrea Ewanchuk (Ewanchuk, 2011). Briefly, OSPW samples, 1 L at a time, were brought above pH 10 with concentrated NaOH and exchanged three times with 50 mL DCM, to

remove neutral and basic organic compounds. The aqueous phase was retained and acidified with concentrated HCl to less than pH 2 prior to exchange three times with 50 mL DCM. The DCM fractions were retained, dried, and measured gravimetrically.

Five hundred millilitres of STP OSPW were aerobically incubated at 200 rpm for about six weeks at room temperature (~21ºC), to stimulate the microbial community after long-term storage at 4˚C. A 10% transfer into 125 mL modified Bushnell-Haas medium (MBH, in g/L: 1.0 KH_2PO_4, 1.0 Na_2HPO_4, 0.5NH_4NO_3, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4$-$7H_2O$, 0.02 $CaCl_2$-$2H_2O$, 0.002 $FeCl_3$, and 0.0018 $MnSO_4$-H_2O, adjusted to pH 7.0) (Wyndham and Costerton, 1981) containing 50 mg/L AEO was then performed. Subsequent 10% transfers occurred every two weeks to progressively increase the exposure concentration, to 100, 150 and both 200 and 300 mg/L. The enrichment culture was established by Elena Dlusskaya and then maintained in MBH with commercially produced Refined NAs (Merichem Company, Houston, TX, gifted to P.M. Fedorak) until the below isolation procedure commenced, due to insufficient quantities of OSPW AEO.

Isolation of NA-degrading cultures was completed by Simmon Hofstetter and Timothy Edwards (Hofstetter et al., 2014). Isolates were obtained by plating the enrichment culture on various general growth media, including Brain Heart Infusion (Difco, Becton, Dickinson and Company, Sparks, MD), Nutrient Broth (Difco), and a general growth medium (in g/L: 10 malt extract, 5 tryptone, 1 dextrose, 1 yeast extract, 5 NaCl). Spread plates of each medium, in triplicate, were made with 100 µL aliquots of the enrichment culture. Unique colonies were triple streaked on the same medium, in duplicate; this procedure was repeated six times for every colony. All plates were incubated at room temperature (21ºC) for no more than 144 hours. Purity and unique morphology were visually assessed with light microscopy (Zeiss AXIOVision Imager, Toronto, ON). Selected colonies were incubated in the corresponding broth medium at room temperature for 24 to 72 hours prior to storage at -80ºC in 60% glycerol. Two of the frozen isolates were shipped to the University of Essex (Colchester, UK),

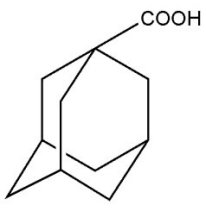
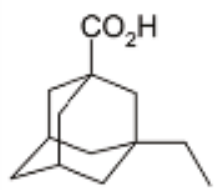
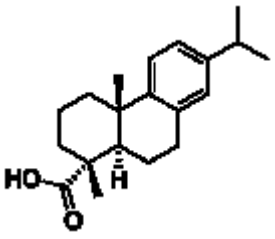
where the experiment was performed, in addition to the original enrichment culture in MBH.

6.2.2 NA Degradation Experiment and Microcosm Sampling

Inocula for this experiment were taken from glycerol stocks (stored at -80°C) of the above cultures. Inocula were incubated in the general growth medium, except that dextrose was replaced with glucose, statically in the dark at 20°C for six days prior to establishment of the biodegradation experiment. To rinse all general growth medium from the pellets, inocula were centrifuged at 5000 x *g* with a Sorvall Heraeus Biofuge Stratos centrifuge (Thermo Scientific) for 15 min three times, rinsing with mineral salts medium (MSM, in g/L: 0.2 $MgSO_4$, 0.5 $(NH_4)_2SO_4$, 0.5 KH_2PO_4, 1.5 K_2HPO_4, 0.02 NaOH, 0.12 Na_2EDTA, 0.004 $ZnSO_4$, 0.001 $CuSO_4 \cdot 5H_2O$, 0.0001 Na_2SO_4, 0.001 $Na_2MoO \cdot 2H_2O$, 0.0004 $MnSO_4$, 0.0001 $CoCl_2 \cdot 6H_2O$, pH 7.0) in between. The pellets were suspended in 5 mL MSM.

Three NA compounds were utilized: adamantane-1-carboxylic acid (Sigma Aldrich, Dorset, UK), 3-ethyl-adamantane-1-carboxylic acid (Sigma Aldrich), and dehydroabietic acid (Helix Biotech, Vancouver, BC), shown in Table 6-1. Stock solutions were made using 10 mg NA in 0.1 M NaOH.

Table 6-1: Chemical structures of three model carboxylic acids used for biodegradation trials

adamantane-1-carboxylic acid	3-ethyl-adamantane-1-carboxylic acid	dehydroabietic acid
		

All serum bottles were soaked overnight in 5% (v/v) Decon-90 (Decon, Hove, UK), rinsed three times with milli-Q water, dried at 110°C, rinsed with acetone, and autoclaved prior to use. Each serum bottle contained 75 mL MSM, 0.5 mL suspended inoculant (live or none, for abiotic controls), and 37.5 µL stock NA (final concentration 5 mg/L). Bottles were crimped with polytetrafluoroethylene seals incubated statically in the dark for 33 days at 20°C.

On Days 0, 11, and 33, 22.5 mL sample was removed and centrifuged at 9000 x *g* for 10 m. The retained pellet was stored at -80°C and later freeze-dried for shipping from the University of Essex to University of Alberta. The supernatant was divided and stored at -20°C: 2.5 mL for Microtox and 20 mL for liquid-liquid extraction and GC-MS measurement. Another 1 mL sample was removed to monitor growth via optical density on a Cary 500 UV-VIS-NIR spectrophotometer (Varian, Palo Alto, CA) at 600 nm/min on double beam mode.

6.2.3 NA Extraction and GC-MS Analysis

All glassware utilized for NA extraction was soaked overnight in 5% (v/v) Decon-90 (Decon, Hove, UK), rinsed three times with milli-Q water, and dried at 110°C prior to use, to remove any hydrocarbon contamination. Samples (20 mL) were acidified with a few drops of concentrated hydrochloric acid. Prior to liquid-liquid extraction, 50 µL of the internal standard stock solution, consisting of 1 mg of 4-phenylbutanoic acid (Acros Organics, Geele, Belgium) dissolved in 1 mL methanol (high-performance liquid chromatography grade), was added (final concentration 2.5 mg/L).

Samples were exchanged with 20 mL ethyl acetate (HPLC grade) three times in 250 mL glass separatory funnels. The ethyl acetate fractions were combined and passed through anhydrous sodium sulphate to remove any traces of water. The volume of ethyl acetate was reduced using a rotary evaporator, and after transfer to GC-MS vials, dried in a heat block.

In sets of 30 (enough for approximately 24 h run), samples were derivatized with 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 950 µL dichloromethane, both added quantitatively. Samples were heated to

approximately 60˚C in metal heat block for at least 20 min to form trimethylsilyl derivatives. Samples were run at the University of Essex on a Thermo Finnigan Trace 7890 gas chromatograph with Thermo Finnigan Trace 5975C DSQ mass spectrometer (Agilent, Wokingham, UK), operating with 1 mL splitless injection at an injector temperature of 250ºC and a 30 m x 250 µm x 0.25 µm HP-5MS column. The GC program was as follows: ramp from 40ºC to 250ºC at 10ºC/min, hold at 300ºC for 10 min. The flow rate of the carrier gas, helium, was 1 mL/min.

Standard calibration curves for each model NA compound are provided in Appendix 7.

6.2.4 Microtox Analysis

Acute toxicity analysis was conducted on NA samples at time points Day 0 and 33 with a Microtox 500 Analyzer (Azur Environmental, Carlsbad, CA), using the 81.9% Basic protocol, four 2:1 dilutions, and 5 min incubation period. Measurements were expressed using EC_{20}, representing the percent volume of sample required to reduce the luminescence of the test specimen by 20%. Phenol and deionized water were used as positive and negative standards, respectively, and phenol EC_{20} was between 4 and 8 mg L^{-1}.

6.2.5 Sequencing for Identification

The freeze-dried pellets were resuspended in 1 mL sterile deionized water, transferred to 1.5 mL microcentrifuge tubes, and subjected to three freeze-thaw cycles. Samples remained at -80˚C until frozen, then were placed in a boiling water bath until completely thawed. DNA extraction was completed with DNeasy blood and tissue kit (QIAGEN Sciences, Gaithersburg, MD) following the procedure for Gram positive bacterial cells. Extraction of fungal DNA from samples was completed by Sophie Dang from the Molecular Biology Service Unit, University of Alberta. The DNeasy plant mini kit (QIAGEN Sciences) was utilized with the protocol for plant tissue, aside from the following modifications. In lieu of steps 1-6, 400 µL of the lysis buffer AP1 was added to the freeze-dried pellets, vortexed, and centrifuged. The buffer containing the cell mass was

transferred to 2 mL screw cap tube containing 3 mm tungsten carbide beads; bead beating was conducted three times for 30 s at full speed. Buffer RNase A was then added and vortexed, as in step 7, and the protocol was followed from step 8 onwards.

The V6-8 region of the 16S rRNA gene of original enrichment culture, at Days 0 and 33 of the incubation, was amplified for massively parallel sequencing analysis using primers 926F (aaa ctY aaa Kga att gac gg) and 1392R (acg ggc ggt gtg tRc), designed to target both bacteria and archaea (Ramos-Padrón et al., 2011). Both the forward and reverse 16S rRNA gene primers were fitted with adaptors for use with the 454 platform (Roche Diagnotics, Branford, CT); the forward primer also included a unique barcode for each sample, to enable multiplexing. PCR amplification was performed in triplicate, with each 25 µL reaction containing 1x Phusion HF Buffer (Thermo Scientific, Waltham, MA), providing 1.5 mM $MgCl_2$, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 10 mM each dNTP, 0.4 mM each primer, and 1 µL genomic DNA, on a MyCycler (Bio-Rad, Hercules, CA). The amplification protocol was as follows: denaturing at 98°C for 30 s, then 10 cycles of denaturing for 10 s at 98°C, annealing for 30 s with touchdown starting at 63°C and reducing 0.5ºC/cycle, and a 15 s extension at 72°C. This was followed by 20 cycles of denaturing for 10 s at 98°C, annealing for 30 s 58°C, and a 15 s extension at 72°C. The final extension step was held for 10 min at 72°C. Once PCR products were confirmed on a SYBR Safe (Life Technologies, Carlsbad, CA) stained 1.5% agarose gel, triplicates were pooled and purified by mixing Agencourt AMPure XP (Beckman Coulter, Indianapolis, IN) at a 1:1 ratio with amplicons, following the protocol. Purified product was eluted in 40 µL 10 mM Tris-HCl, pH 8.0, quantified using a Nanodrop 2000C (Thermo Scientific, Wilmington, DE), and diluted to 20 ng/µL. Twenty microlitres of each product was sent to the McGill University and Genome Quebec Innovation Centre (Montreal, QC), where equimolar pooling was completed via measurement by the Quanti-iT PicoGreen dsDNA kit (Life Technologies). The pooled sample was sequenced on a quarter plate of a

Genome Sequencer FLX Instrument using GS FLX Titanium chemistry (Roche Diagnostics Corporation, Fishers, IN).

454 sequencing data were processed using both the Phoenix pipeline developed by the Sun Center of Excellence for Visual Genomics in Calgary (Ramos-Padrón et al., 2011) and mothur Version 1.33.0 (Schloss et al., 2009). The Phoenix pipeline included removal of sequences shorter than 200 bp and longer than 450 bp, containing chimeras and ambiguous bases, and for primer mismatching; final number of sequence reads for each sample ranged from 2167 to 3716. Sequences were clustered into operational taxonomic units (OTUs) based on a 3% dissimilarity average neighbour distance cut-off and aligned with the RDP classifier to the SILVA database based on a consensus sequence for each OTU (Quast et al., 2013). The mothur standard operating procedure for 454 data analysis (http://www.mothur.org/wiki/454_SOP) included denoising, removal of sequences with homopolymers longer than 8 bp or ambiguous bases, sequence trimming based on 95% retention after meeting minimum length requirement of 200 bp, and removal of chimeras with the uchime tool. Sequences were aligned with the SILVA reference alignment, and binned into OTUs based on 3% dissimilarity average neighbour distance cut-off. Taxonomy was assigned based on the greengenes taxonomy database and Wang classifier.

Three samples from each isolate (UA STP-6 and UA STP-8), at Day 11 of the incubation, were selected for bidirectional sequencing, with eubacterial primers 27F (aga gtt tga tcM tgg ctc ag) and 1492R (tac ggY tac ctt gtt acg act t) (Lane, 1991) for both isolates and fungal primers ITS1 (tcc gta ggt gaa cct gcg g) and ITS4 (tcc tcc gct tat tga tat gc) (White et al., 1990) for UA STP-8. PCR amplification of the eubacterial samples was performed with a MyCycler (Bio-Rad) with the following protocol: denaturing at 98°C for 30 s, then either 30 or 35 (for UA STP-8 and UA STP-6, respectively) cycles of denaturing for 10 s at 98°C, annealing for 30 s at 54°C, and a 1.5 min extension at 72°C. The final extension step was held for 10 min at 72°C.and each 50 µL reaction contained 1x Phusion HF Buffer (Thermo Scientific) containing 1.5 mM $MgCl_2$, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 10 mM each dNTP, 0.4 mM each

primer, and 1 µL genomic DNA. For fungal amplification with ITS primers, each 15 µL reaction contained 1x Buffer (Invitrogen) containing 1.5 mM $MgCl_2$, 0.5 U Platinum DNA Polymerase (Invitrogen), 10 mM each dNTP, 0.4 mM each primer, and 5 µL genomic DNA. Cycle conditions were as follows: denaturing at 94°C for 2 min, then 35 cycles of denaturing for 30 s at 94°C, annealing for 30 s at 60°C, and a 1.5 min extension at 72°C, and final extension step for 10 min at 72°C. Once PCR products were verified on SYBR safe stained 1.5% agarose gel, they were purified with the QIAquick PCR Purification Kit (QIAGEN) and quantified on a NanoDrop 2000C. Sanger sequencing was completed with the same forward and reverse primers on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) at the Molecular Biology Service Unit, University of Alberta. Sequences were aligned and assembled into contigs using the online version of CAP3 Sequence Assembly Program (available from http://doua.prabi.fr/software/cap3, Huang and Madan, 1999).

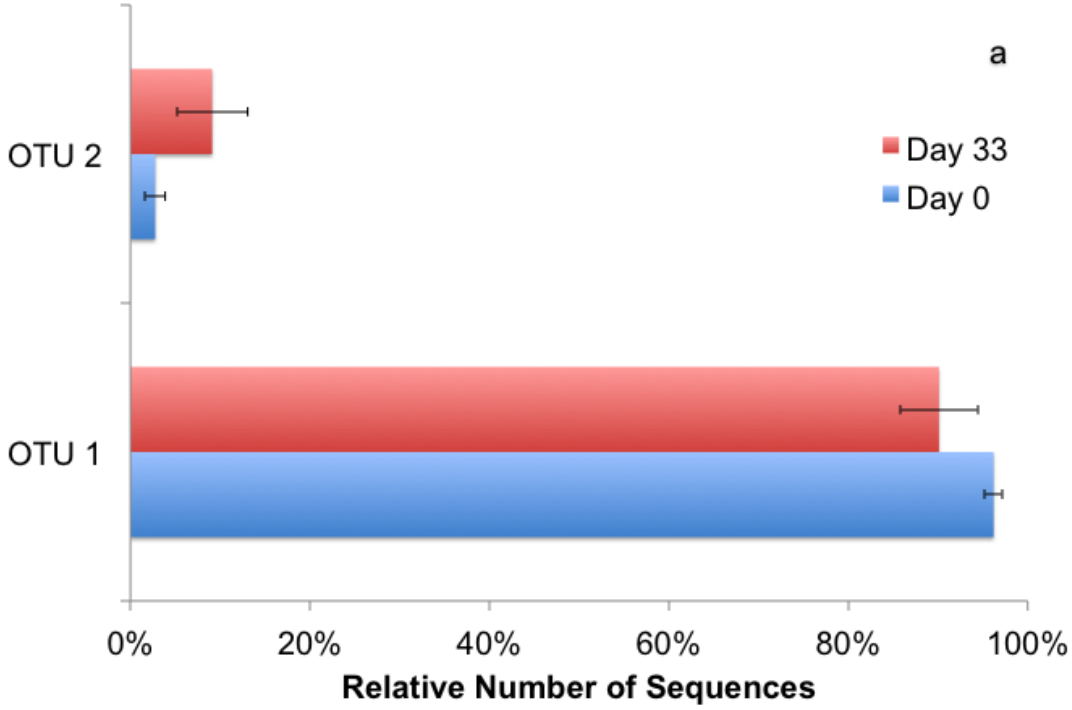
Quantitative PCR was conducted using the same protocol provided in 3.2.6. Samples from Day 11 of incubation of the cultures UA STP-6 and UA STP-8 and samples from Day 0 of incubation of the enrichment culture were utilized. Each biological replicate was measured three times (n=9). The standard calibration curve is provided in Appendix 10.

6.3 Results

6.3.1 Characterization of Microbial Enrichment Cultures

A microbial culture from a recently established (in 2006, sampled in 2009) oil sands tailings pond, Suncor South Tailings Pond, was enriched on progressively increasing concentrations of OSPW-sourced AEO and then maintained on commercial NAs until isolation of NA-degrading microorganisms was attempted. In this degradation study, the original enrichment culture, C300, and two additional cultures obtained during the isolation procedure, UA STP-6 and UA STP-8, were examined for their capabilities of metabolizing three model NAs with complex structures.

Identification of bacteria present in the original enrichment culture was completed using massively parallel sequencing, as simultaneous characterization of shifts in the microbial communities in response to incubation with recalcitrant NA compounds could also be monitored. The relative abundance of the two most dominant operational taxonomic units (OTUs) at Days 0 and 33 is shown in Figure 6-1, processed with the Phoenix pipeline (Ramos-Padrón et al., 2011). Taxonomy was assigned based on the Silva database to genus level; OTU 1 was *Brevundimonas* and OTU 2 was *Rhodococcus*. These two OTUs accounted for 99% of all sequences; the next abundant OTU contained 0.4% of all sequences and was assigned taxonomically to the genus *Shinella*. Analysis with mothur (Schloss et al., 2009) also indicated these three genera as the most dominant.



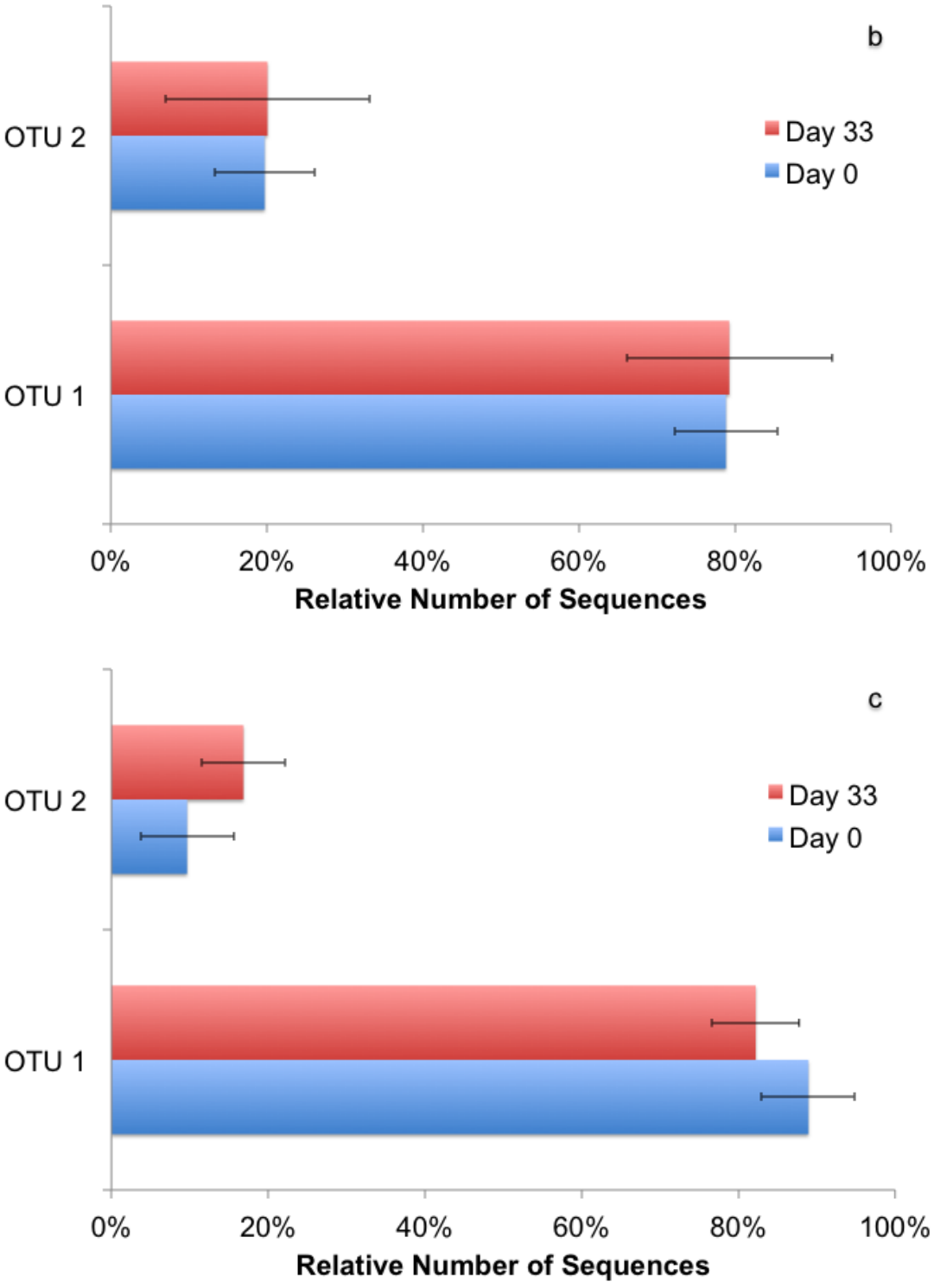


Figure 6-1: Relative number of sequences binned in two most abundant operational taxonomic units (OTU) at 0.03 cutoff, accounting for 99% of all sequence reads, in the enriched community C300 incubated on a) adamantane-1-carboxylic acid, b) 3-ethyl-adamantane-1-carboxylic acid, and c) dehydroabietic acid, at Days 0 and 33. Error bars indicate one standard deviation (n=3).

The community shifted in response to incubation with both adamantane-1-carboxylic acid and dehydroabietic acid, but not 3-ethyl-adamantane-1-carboxylic acid. An increase in the relative number of sequences in OTU 2 over 33 days corresponded with an equivalent decrease in OTU 1. However, these changes were not significant ($p > 0.05$), as the amova package in mothur indicated neither day nor compound influenced community structure.

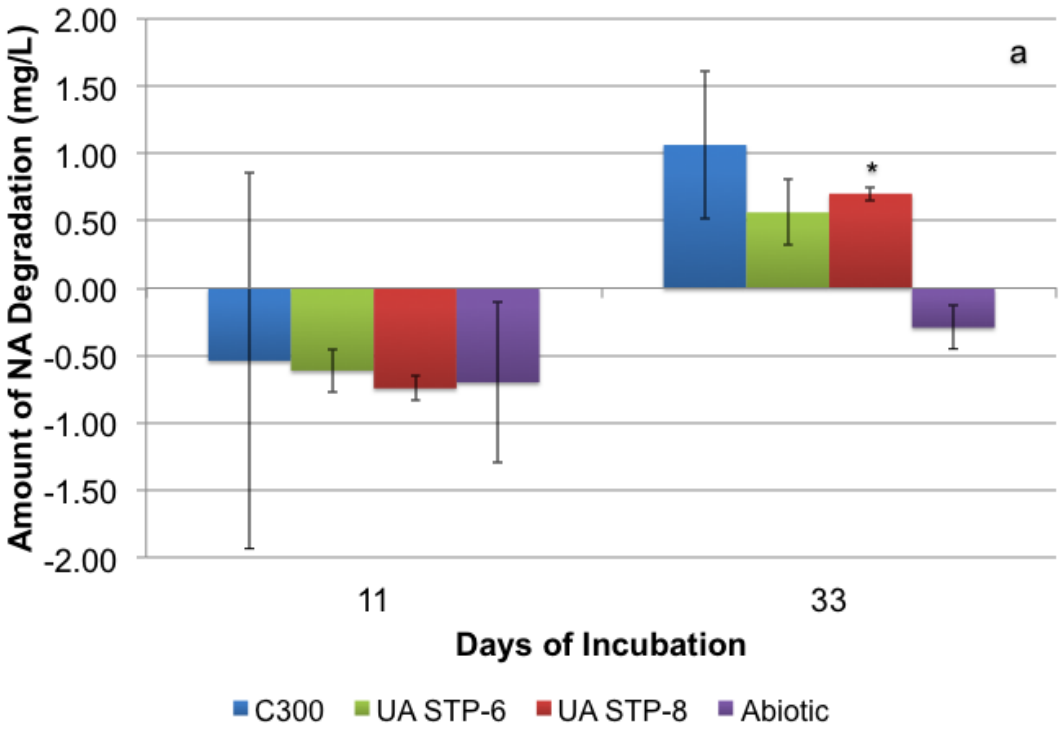
The two microbial cultures obtained from the enriched community were characterized using Sanger sequencing, shown in Table 6-2. The culture UA STP-6 contained a single isolate, identified as *Shinella granuli*. UA STP-8 was a co-culture, containing both *Trichoderma harzianum* and a *Brevundimonas* sp. Sequences are provided in Appendix 9.

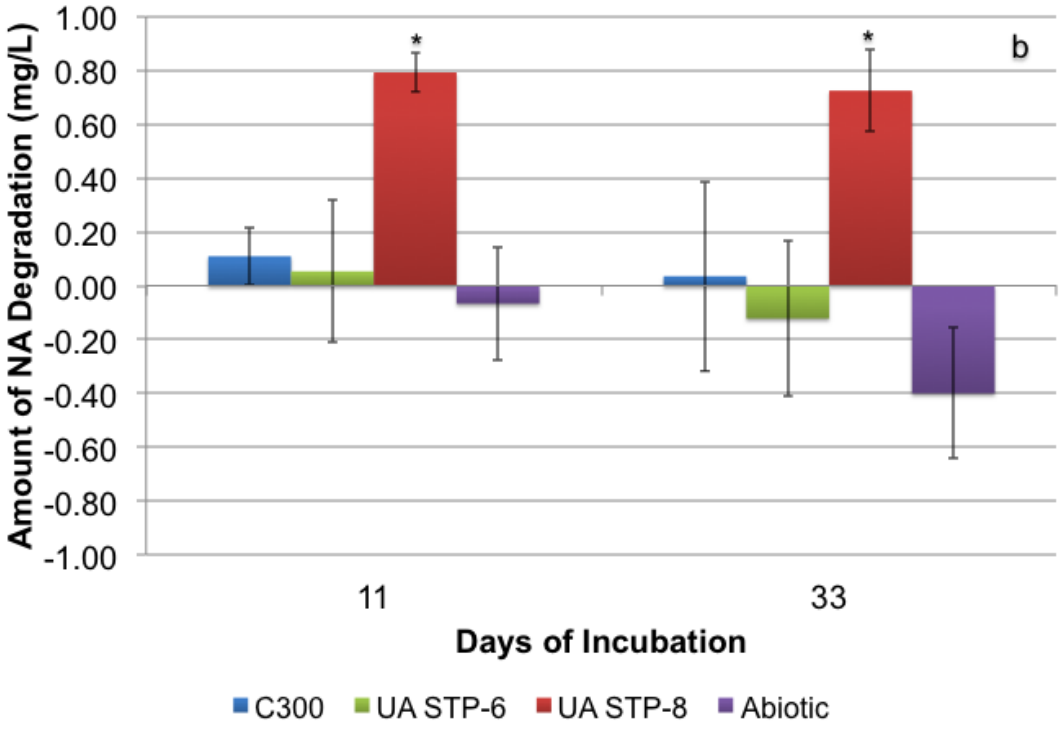
Table 6-2: Identification of a microbial isolate and a co-culture obtained from enriched community C300. Taxonomy assigned based on NCBI BLAST algorithm.

Culture	Identification	Percent Identity	Primers Used
UA STP-6	*Shinella granuli*	99	27F / 1492R
UA STP-8	*Brevundimonas* sp.	99	27F / 1492R
	Trichoderma harzianum	100	ITS1 / ITS4

6.3.2 Degradation of Model NA Compounds

To explore the metabolic capabilities of three microbial enrichments from OSPW to degrade NAs, the microbial cultures were incubated with two diamondoid NA compounds (Rowland et al., 2011c) and one aromatic NA compound (Jones et al., 2012; Rowland et al., 2011d) for 33 days. The amount of degradation of each compound at Days 11 and 33 is shown in Figure 6-2.


a
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
Amount of NA Degradation (mg/L)
*
11
33
Days of Incubation
C300
UA STP-6
UA STP-8
Abiotic


b
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
Amount of NA Degradation (mg/L)
*
*
11
33
Days of Incubation
C300
UA STP-6
UA STP-8
Abiotic

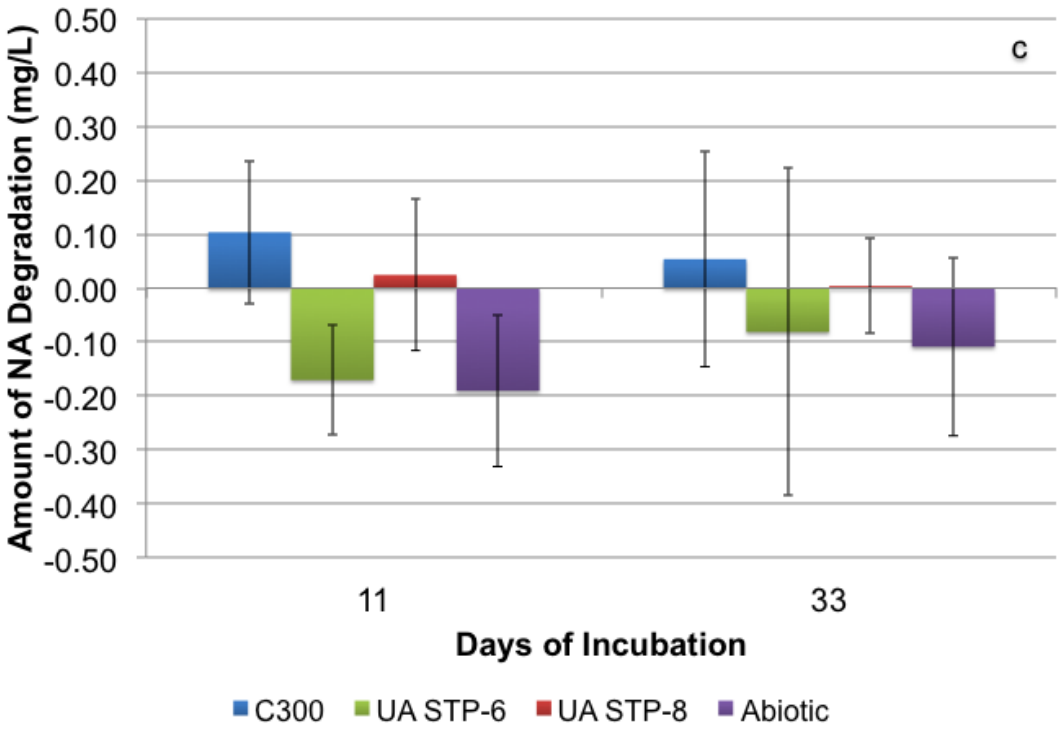
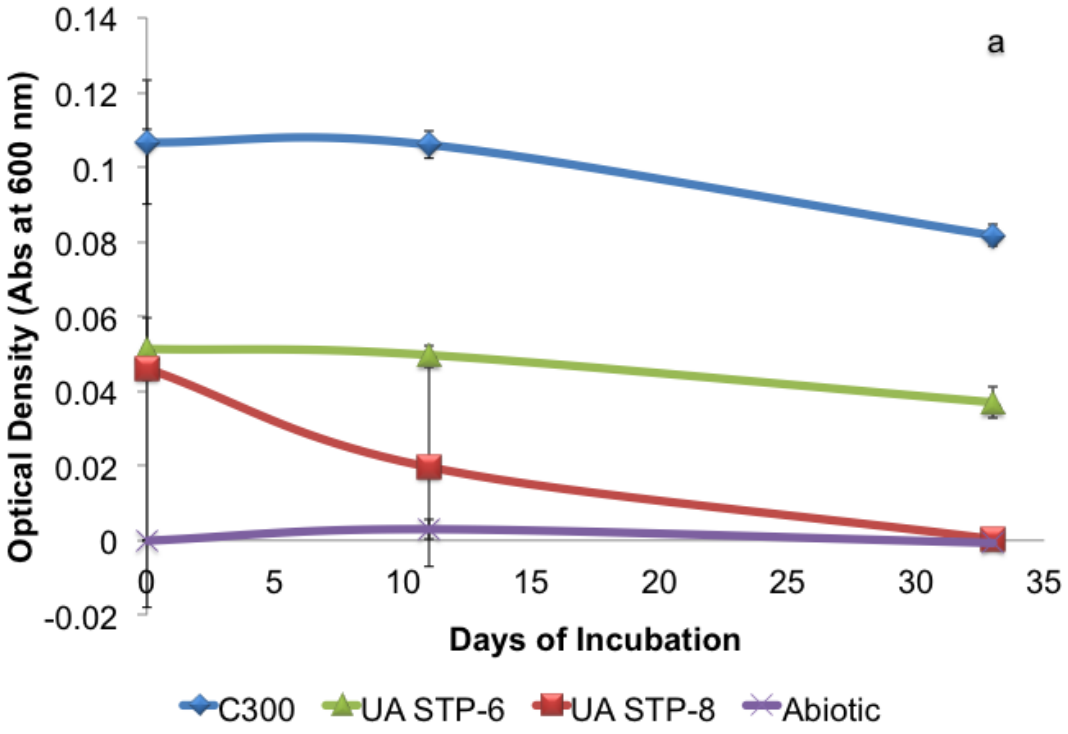


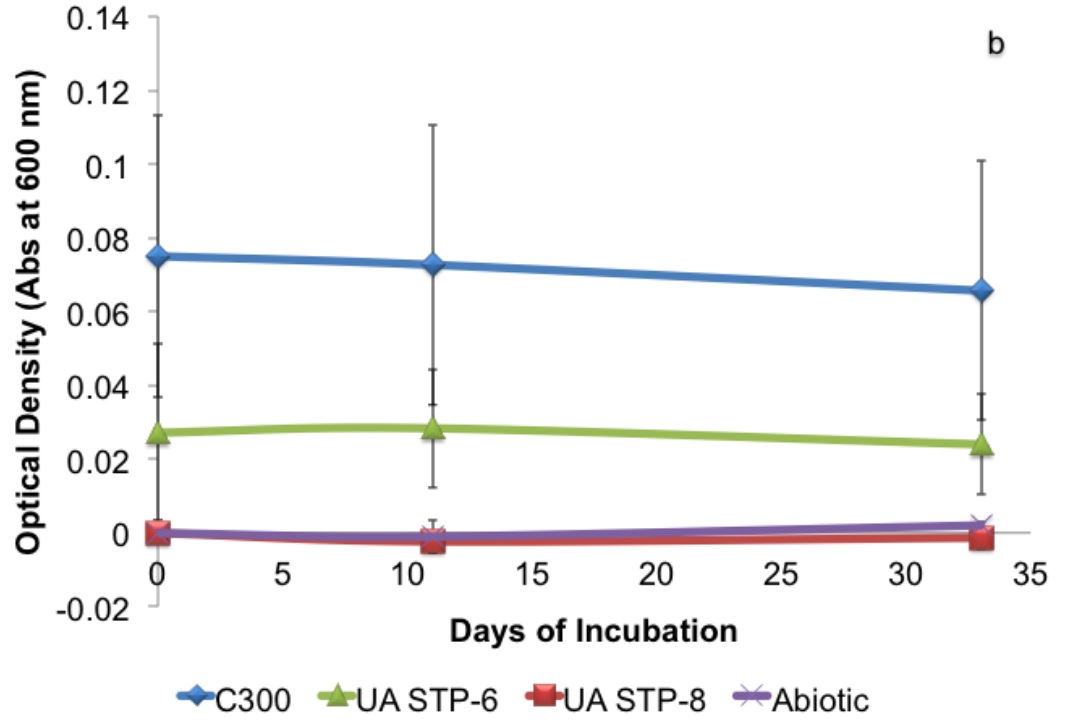
Figure 6-2: Amount of degradation of a) adamantane-1-carboxylic acid, b) 3-ethyl-adamantane-1-carboxylic acid, and c) dehydroabietic acid, in live samples, after 11 and 33 days of aerobic incubation with enriched community C300 and isolates UA STP-6 and UA STP-8. Asterisks indicate value is significantly greater than 0 (student's t-test, $p < 0.05$). Error bars indicate one standard deviation (n=3).

Both ANOVA and the student's t-test verified that significant degradation of 3-ethyl-adamantane-1-carboxylic acid by UA STP-8 occurred ($p < 0.05$). Approximately 30% of this compound was degraded in 33 days, from a starting concentration of 2.7 mg/L. The co-culture UA STP-8 also significantly degraded adamantane-1-carboxylic acid between days 11 and 33 ($p < 0.05$), reducing the concentration by 16%, from 4.4 mg/L. While it appeared that both the enriched community C300 and the isolate UA STP-6 also degraded adamantane-1-carboxylic acid, the amount of degradation was not significant (student's t-test, $p > 0.05$). No degradation of dehydroabietic acid was observed in any of the microbial cultures after 33 days of aerobic incubation.

6.3.3 Growth Monitoring

Biomass growth of the three cultures was monitored with optical density at each time point of the experiment and is shown in Figure 6-3.


a
Optical Density (Abs at 600 nm)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
0
5
10
15
20
25
30
35
Days of Incubation
C300
UA STP-6
UA STP-8
Abiotic


b
Optical Density (Abs at 600 nm)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
0
5
10
15
20
25
30
35
Days of Incubation
C300
UA STP-6
UA STP-8
Abiotic

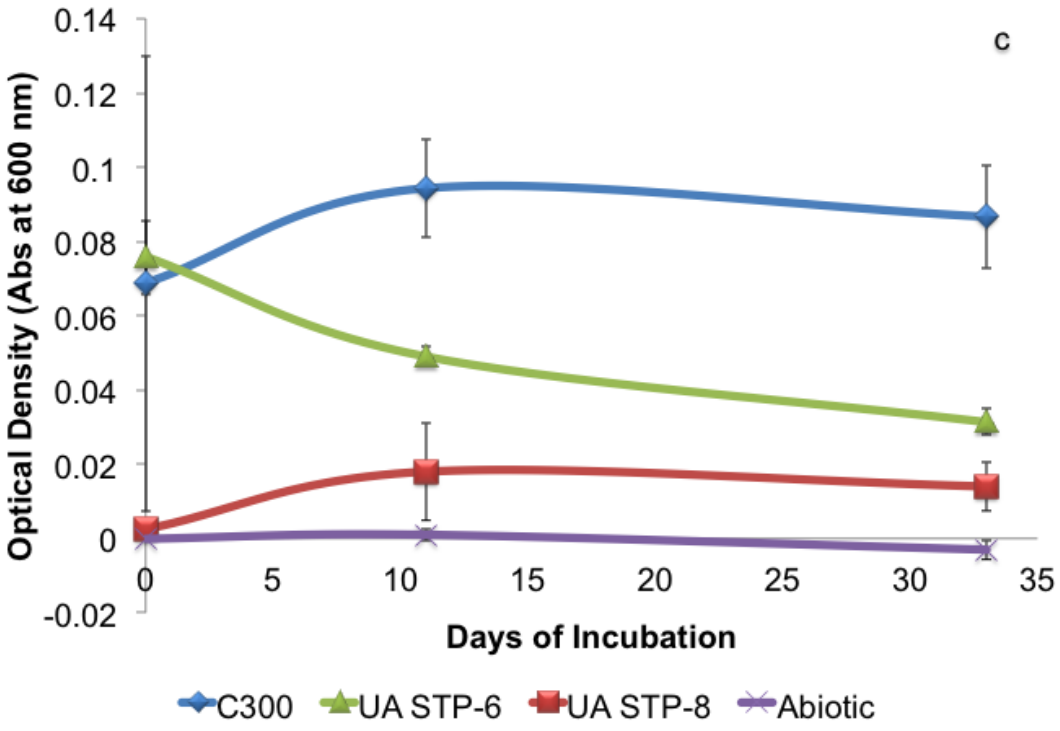


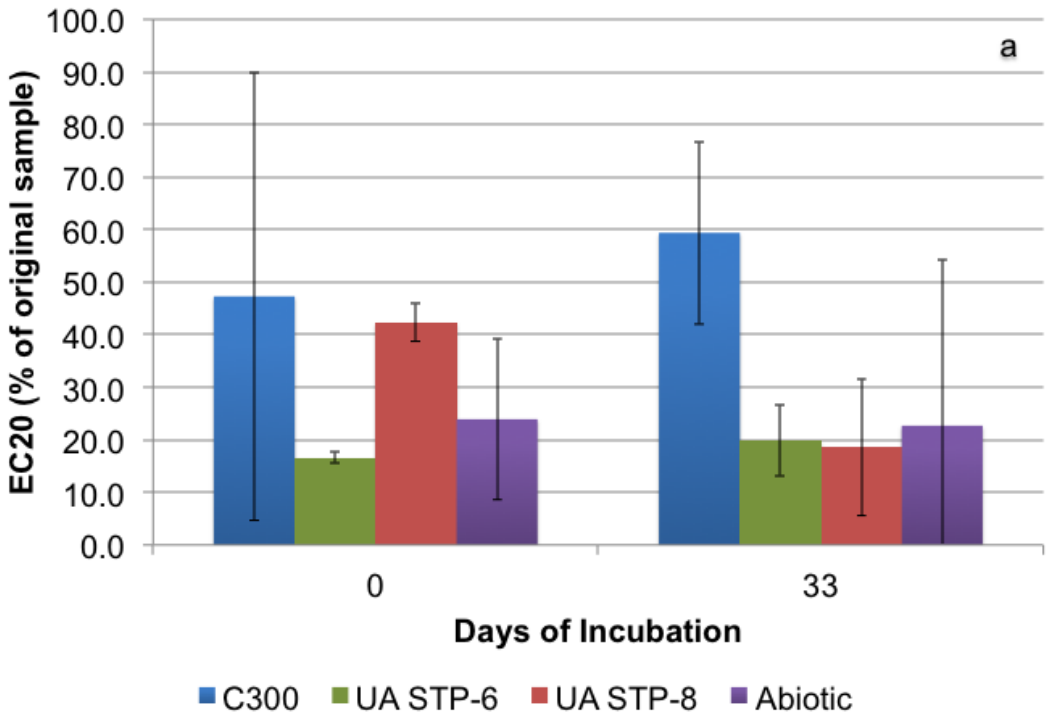
Figure 6-3: Growth of enriched community C300 and isolates UA STP-6 and UA STP-8 over 33 days of aerobic incubation, as measured by optical density (at 600 nm), on a) adamantane-1-carboxylic acid, b) 3-ethyl-adamantane-1-carboxylic acid, and c) dehydroabietic acid. Error bars indicate one standard deviation (n=3).

Overall, optical density indicated that growth was only seen in the enriched community C300 at Day 11 and UA STP-8 at Days 11 and 33 when incubated with dehydroabietic acid. Optical density measurements were much lower for UA STP-8 compared to C300 or UA STP-6, but this culture was visually assessed to flocculate and therefore optical density measurements were less relieable.

Quantitative PCR of the single copy *rpoB* gene was conducted to determine how much the bacterium contributed to the co-culture UA STP-8. As a point of reference, both the enriched community C300 and the isolate UA STP-6 were also measured. All samples at Day 11 were measured in triplicate and averaged; UA STP-6 had 28000 ± 27000 (n=27) copy numbers per mL of culture, and UA STP-8 had 12000 ± 11000 (n=27). The community had 780000 ± 160000 (n=9) copy numbers per mL at Day 0.

6.3.4 Microtox Testing

Microtox analysis was conducted to supplement NA degradation and is shown in Figure 6-4.



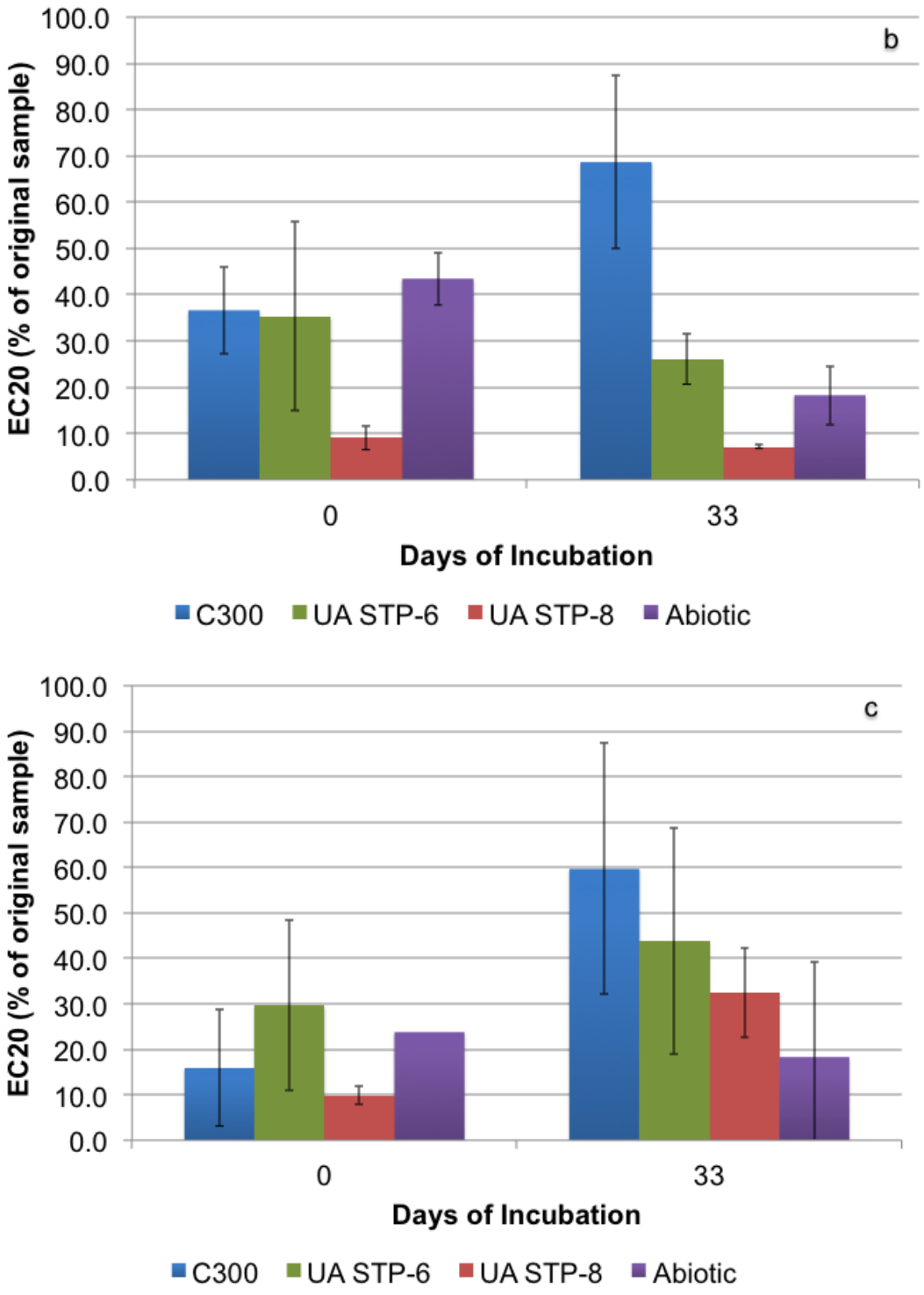


Figure 6-4: EC_{20} (in percent of original sample) of *Vibrio fischeri* after 5 min exposure to a) adamantine-1-carboxylic acid, b) 3-ethyl-adamantane-1-carboxylic acid, and c) dehydroabietic acid, at Days 0 and 33 of incubation with enriched community C300 and isolates UA STP-6 and UA STP-8. Error bars indicate one standard deviation (n=3). Lower value indicates greater toxicity.

Overall, measured EC_{20} of *Vibrio fischeri* were inconsistent, evidenced by differences in measurements from Day 0 and abiotic controls, and with large sample errors. No statistically significant change in EC20 was observed in cultures containing adamantane-1-carboxylic acid ($p > 0.05$). However, a significant increase in EC_{20}, which corresponds to a reduction in toxicity, was measured after 33 days for both 3-ethyl-adamantane-1-carboxylic acid and dehydroabietic acid, due to incubation with C300 ($p < 0.05$).

6.4 Discussion

An enrichment culture was established from an OSPW sample through exposure to progressively increasing concentrations of OSPW-AEO in order to obtain both a microbial community and isolates capable of degrading NAs and other organics found in OSPW (Hofstetter et al., 2014). Purification of this enriched microbial community resulted in isolation and identification of microbial isolates indigenous to OSPW. Two of these microbial cultures, and the original enrichment culture from which they were obtained, were incubated with three complex model NA compounds to assess their metabolic capabilities.

Sequencing of the microbial cultures UA STP-6 and UA STP-8 were completed to confirm identification. UA STP-6 was confirmed to be *Shinella granuli*. The first type strain of *S. granuli* was isolated from granules in an anaerobic sludge blanket reactor in a brewery wastewater treatment plant (An et al., 2006). Species from this genus are capable of metabolizing organic compounds such as pyridine (Bai et al., 2009) and dehydroabietic acid (Mohn, 1995).

The culture UA STP-8, as utilized in this experiment, was a co-culture containing both a *Brevundimonas* sp. and the fungus *Trichoderma harzianum*. Hofstetter et al. (2014) further isolated the fungus from the bacterium through the use of antibiotics. *Trichoderma harzianum* is an abundant and ubiquitous soil fungus and easily culturable (Harman et al., 2004). It has been shown to be capable of degrading polychlorinated biphenyl compounds (Mouhamadou et al., 2013), polycyclic aromatic hydrocarbons (Giraud et al., 2001; Ravelet et al.,

2000; Saraswathy and Hallberg, 2002), and total hydrocarbons (Colombo et al., 1996). Fungi are excellent candidates for bioaugmentation due to their capability of thriving in various environments and degrading a wide variety of compounds (Harman et al., 2004).

The enriched community C300 was dominated by bacterial sequences that assigned to the genera *Brevundimonas*, *Rhodococcus*, and *Shinella*, each of which were isolated by Hofstetter et al. (2014). Members of the genus *Brevundimonas* have been previously identified in OSPW (Hwang et al., 2013; Ramos-Padrón, 2013) and are capable of saturated and aromatic hydrocarbon degradation (Chaîneau et al., 1999; Xiao et al., 2010). Meanwhile, *Rhodococcus* species have not been explicitly identified in OSPW, yet it is a well-known genus for bioremediation of numerous classes of organic compounds (Kuyukina and Ivshina, 2010), including hydrocarbons such as alkanes (Lee et al., 2010; Paje et al., 1997), monoaromatic hydrocarbons such as benzene and toluene (Kim et al., 2002; Lee et al., 2010), polycyclic aromatic hydrocarbons (Dean-Ross et al., 2001), and diesel and oil (Chaîneau et al., 1999; Lee et al., 2010).

The co-culture UA STP-8 was capable of degrading 30% of 3-ethyl-adamantane-1-carboxylic acid within the first 11 days of incubation and 16% of adamantane-1-carboxylic acid between 11 and 33 days. It is possible that the alkyl branch on 3-ethyl-adamantane-1-carboxylic acid rendered this compound more susceptible to attack. Analogous to these diamondoid NAs containing a ring structure, ethylbenzene was nearly exhausted when incubated with a different deuteromycete fungus, while benzene was not degraded; the first step in the metabolic pathway is oxidation of the side chain (Prenafeta-Boldu et al., 2002). Without searching for metabolites, though, the presence of the side chain on 3-ethyl-adamantane-1-carboxylic acid resulting in preferential degradation is speculative. A lack of authentic standards for GC-MS analysis of potential degradation products hampered this search for metabolites.

While there was no change in total degradation of 3-ethyl-adamantane-1-carboxylic acid in the last 22 days of incubation, this is when adamantane-1-carboxylic acid degradation began. Colombo et al. (1996) observed that *T.*

harzianum experienced progressively increasing rates of hydrocarbon degradation with time and suggested that incubation times greater than the 90 days tested would have resulted in further degradation. As this species may be slow growing, longer incubation periods should be utilized in future NA degradation studies with *T. harzianum*.

UA STP-8 was the most metabolically capable of the three cultures tested. Neither C300 nor UA STP-6 significantly degraded either diamondoid NA compound. The two microorganisms in the co-culture UA STP-8, *Trichoderma harzianum* and *Brevundimonas* spp., are known for hydrocarbon degradation. Testing both of these organisms separately for degradation of diamondoid and other NA compounds to determine the metabolic capabilities of each microorganism would help determine if a symbiotic relationship exists in this co-culture, enabling degradation of the two adamantane compounds. Bacterial-fungal co-cultures have been shown to improve extent and rate of degradation of polycyclic aromatic hydrocarbons (Boonchan et al., 2000; Chávez-Gómez et al., 2003). Once again, analysis of metabolites in samples would help indicate if the co-culture is necessary for degradation, especially since it is unknown if the portion of the NA compound degraded was mineralized or if the process stalled at a degradation product. Mineralization in the co-culture may be indicative of each microorganism handling a portion of the metabolic pathway (Boonchan et al., 2000). While it is possible that *T. harzianum* did not contribute to the observed degradation of the diamondoid NA compounds, *Brevundimonas* spp. accounted for about 90% of the biomass in C300 and yet no degradation was observed with this culture. Thus, it is likely that this is the first report of a fungal species degrading NAs.

No degradation of the complex aromatic compound, dehydroabietic acid, occurred over 33 days in any of the three cultures tested. Dehydroabietic acid, recently identified in OSPW (Jones et al., 2012; Rowland et al., 2011d), is a resin acid produced by trees and concentrated in pulp mill effluents, causing acute toxicity to aquatic life (Ali and Sreekrishnan, 2001). Although aromatic hydrocarbons are generally considered more recalcitrant than aliphatics (Atlas,

1981; Colombo et al., 1996), resin acids are readily biodegraded in conventional aerobic biological wastewater treatment of pulp mill effluents (Liss et al., 1997). All three cultures tested here contained microorganisms from genera known for hydrocarbon degradation; *Shinella granuli*, of which UA STP-6 was identified, has even degraded dehydroabietic acid (Mohn, 1995). Thus, it is possible that the experimental conditions, such as nutrients or temperature, were not adequate to enable degradation of this compound. The *S. granuli* isolate capable of degrading dehydroabietic acid was isolated from a biological reactor treating paper mill effluent operating at 30ºC, but later incubated in medium with similar nutrient concentrations as utilized here (Mohn, 1995).

Community dynamics in enriched community C300 did not significantly change over the time course of the experiment, which was not unexpected given that no significant degradation of the three model NA compounds was observed.

Growth, as measured by optical density, was only seen in C300 and UA STP-8 when incubated with dehydroabietic acid, and yet degradation of this compound was not observed. No growth, as measured with optical density, was observed in the cultures where NA degradation occurred, namely UA STP-8 incubated with 3-ethyl-adamantane-1-carboxylic acid and adamantane-1-carboxylic acid. Optical density is a poor indicator of growth of a fungal culture; gravimetric analysis of filtered sample is the preferred method (Giraud et al., 2001; Mouhamadou et al., 2013; Ravelet et al., 2000; Saraswathy and Hallberg, 2002). However, this method was not possible with this experimental set-up due to insufficient volumes of sample. Future study of the NA degradation potential of *T. harzianum* should allow for appropriate quantification techniques. Meanwhile, quantitative PCR, which was used to approximate bacterial density in UA STP-8, indicated that the number of *rpoB* gene copies in UA STP-6 and STP-8 were the same order of magnitude. This may indicate that the *Brevundimonas* sp. in UA STP-8 was not growing on the NA compounds when degradation occurred, signifying that the *T. harzianum* may have been mostly responsible for degradation. That said, the range between biological replicates was very large,

leading to very high errors (the errors for technical replicates were typically less than 20% relative standard deviation).

The Microtox assay results contributed little to understanding the metabolic capabilities of the cultures. The only significant reduction in toxicity, occurring in 3-ethyl-adamantane-1-carboxylic acid and dehydroabietic acid incubated with C300, did not correspond with a reduction in concentration of these compounds. Microtox has successfully been used previously with model NA compounds (Johnson et al., 2011; Jones et al., 2011). Increasing the number of serial dilutions during the assay may improve data quality, although this is likely to reduce the error only.

6.5 Conclusion

Three microbial cultures that were enriched on OSPW-sourced organic compounds were assessed for their metabolic capabilities to degrade three model NA compounds, representing the more recalcitrant fractions of NAs found in OSPW, diamondoid and aromatic carboxylic acids. A co-culture containing *Trichoderma harzianum* and a *Brevundimonas* sp. degraded 30% of 3-ethyl-adamantane-1-carboxylic acid within 11 days and 16% of adamantane-1-carboxylic acid after 33 days of aerobic incubation. This is the first report of a fungus isolated from OSPW potentially degrading NAs. Neither the enriched community dominated by the genera *Brevundimonas* and *Rhodococcus* nor the bacterial isolate *Shinella granuli* exhibited significant degradation of the three model NA compounds over 33 days. No degradation of dehydroabietic acid occurred. Degradation of the diamondoid NA compounds did not correspond with a reduction in toxicity, as measured by the Microtox assay, nor with microbial growth, monitored by optical density.

Further assessment of the co-culture is recommended to determine how much each microorganism is contributing to overall metabolic capability and whether a symbiotic relationship is improving degradation, or if the fungus *Trichoderma harzianum* is the predominant degrader. Further exploration of metabolic capability of *T. harzianum* to degrade additional NAs and other organic

compounds in OSPW should be pursued. Bioaugmentation of OSPW with this fungus to enhance rate and extent of NA degradation is a promising concept, as fungi are known to proliferate in a wide variety of environments and have been shown to degrade numerous organic compounds.

7 Final Conclusions and Recommendations

7.1 State of Knowledge

The purpose of this research was to further investigate biological treatment options for OSPW, focusing on NAs and other organics. Naphthenic acids have long been attributed with OSPW acute toxicity (Headley and McMartin, 2004; Kindzierski et al., 2012; MacKinnon and Boerger, 1986; Miskimmin et al., 2010), but it is now recognized that other organic compounds in OSPW contribute to toxic effects observed in various relevant endpoints, including mammals and aquatic life (Garcia-Garcia et al., 2012, 2011b; Kavanagh et al., 2013, 2011; Scarlett et al., 2012).

Establishment of oil sands NA resistance to biodegradation by indigenous microorganisms inhabiting tailings ponds (Bataineh et al., 2006; Han et al., 2009, 2008; Herman et al., 1994; Quagraine et al., 2005b; Scott et al., 2005) has driven exploration of more aggressive treatment regimes. Chemical oxidation with ozone to render NAs more amenable to subsequent biodegradation has been shown to significantly improve treatment (Hwang et al., 2013; Martin et al., 2010; Wang et al., 2013). Here, aged OSPW, which continues to exhibit toxicity after decades of storage (Anderson et al., 2012a; Kavanagh et al., 2013, 2011), was treated with ozone coupled with aerobic biodegradation for the first time (Chapters 3 and 4). The influence of ozonation and subsequent incubation with ozone by-products on microbial communities indigenous to OSPW had not yet been considered (Chapters 3 and 4). Delineating the microbial communities in OSPW and reclamation environments is essential for designing successful bioremediation strategies (Hadwin et al., 2006; Quagraine et al., 2005b), as is understanding impacts of a pretreatment solution such as ozonation on community structure. In addition, microbial communities in aged OSPW had not been studied (Chapter 4).

Coupling ozone pretreatment with biodegradation was tested further by subjecting three model NA compounds representative of the abundant classes of NAs found in OSPW to this treatment regime (Chapter 5). The model NA compounds were incubated with an OSPW-sourced bacterial isolate, *Acidovorax* sp., which had previously been shown to completely mineralize single ring model

NA compounds (Ramos-Padrón, 2013), to determine capability of this isolate to biodegrade more complex NAs (Chapter 5). Three model NA compounds were also utilized to assess the metabolic capabilities of three microbial cultures obtained from OSPW enriched on OSPW-AEO. These cultures had exhibited high tolerance for environmental conditions in oil sands tailings ponds, including high pH, salinity and AEO concentration (Hofstetter et al., 2014) (Chapter 6). As bioremediation is the most cost effective treatment option for organics in OSPW (Scott et al., 2008b), seeding OSPW reclamation environments or treatment facilities with microorganisms capable of improving upon the rate and extent of biodegradation of organic compounds occurring with indigenous microbial communities in OSPW, or bioaugmentation, represents another biological treatment option (Johnson et al., 2013, 2012).

7.2 Summary of Findings

A summary of the contributions of this research is provided below.

Chapter 3

1. *Aged OSPW samples* from Syncrude's experimental reclamation ponds, FE5 and Big Pit, *contained low NA concentrations* (<1 mg/L), and total dissolved organic carbon concentrations were approximately 45 mg/L. Thus, *NAs contributed very little to DOC content in aged OSPW*.
2. A moderate dose of *ozone* (50 mg/L) *did not remove DOC from aged OSPW*, but *ozonation significantly improved the biodegradation of DOC in aged OSPW*. Subsequent incubation with indigenous microbes resulted in removal of 11-13 mg/L DOC from samples pretreated with ozone and 5 mg/L from untreated OSPW. Ozonation coupled with biodegradation removed up to 40% DOC, while biodegradation alone resulted in less than 20% reduction in DOC.
3. The moderate dose of *ozone* used (50 mg/L) *did not compromise the indigenous microbial communities' capability to degrade DOC*. The labile fraction of DOC was similar in Ozonated (11.8 and 10.9 mg/L, for Big Pit and

FE5) and Ozonated-Inoculated (11.1 and 13.0 mg/L, for Big Pit and FE5) treatments.

4. *DOC removal from ozone-pretreated aged OSPW* due to aerobic incubation with the indigenous microbial communities *was well represented by the exponential decay model* ($S= S_oe^{-kt}+S_n$, where S_o represents the labile DOC and S_n the recalcitrant DOC) (R^2 = 0.93-0.96).
5. A quantitative PCR assay using single copy gene *rpoB* indicated *ozone exposure did not impede microbial growth*, as significantly greater copy numbers were measured in the Ozonated treatments (1000-25000) versus the Sterilized control (300-2000).

Chapter 4

6. *Both aged OSPW samples exhibited rich bacterial communities* in this experiment. Across all treatments, 55 distinct PCR-DGGE bands were identified in FE5 and 46 in Big Pit.
7. *Ozone exposure influenced microbial community structure*. Cluster analysis completed on PCR-DGGE fingerprints, calculated with the Dice similarity index, indicated Ozonated and Ozonated-Inoculated treatments were approximately 65% similar to one another for both FE5 and Big Pit OSPW samples. Visual inspection of DGGEs indicated that *ozone removed some organisms from the Ozonated treatment and enabled the growth of others, not seen in the Ozonated-Inoculated treatment*. Despite differences in structure, both Ozonated and Ozonated-Inoculated microbial communities were able to remove DOC to the same degree.
8. *Incubation with ozonated OSPW influenced microbial community structure*. Untreated and Ozonated-Inoculated treatments theoretically contained the same microbial communities at the onset of incubation, but showed only 65% similarity in FE5 and less than 40% in Big Pit.
9. *Microbial community structure shifted over three months of aerobic incubation*. Similarity indices between time points ranged from 70% to 90%. In general, Weeks 0 and 11 were least similar within treatments.

10. Sequencing of excised PCR-DGGE bands of interest led to *identification of genera in aged OSPW previously identified in other OSPW samples*, including *Brevundimonas*, *Hydrogenophaga, Rhizobium, Acinetobacter, Thiobacillus*, and *Methyloversatilis*.
11. The genus *Sphingomonas*, known for hydrocarbon degradation, was observed for the first time in OSPW. This sequence came from a DGGE band that was most intense at Week 2, when degradation of DOC was occurring, of the ozonated treatment in Big Pit.

Chapter 5

12. Ozone treatment of three model NA compounds, a tricyclic aliphatic compound, adamantane-1-carboxylic acid, a bicyclic aliphatic compound, decahydro-2-naphthoic acid, and an aromatic compound, 1,2,3,4-tetrahydro-2-naphthoic acid, *reduced the NA concentrations by 70-85% within 60 s of exposure*, from a starting concentration of approximately 5 mg/L. Adamantane-1-carboxylic acid required 30 s more ozone exposure to reach the same concentration (0.7 mg/L) as decahydro-2-naphthoic acid.
13. *Ozone treatment greatly reduced toxicity*, as measured by the Microtox assay, of 10 day live cultures containing *adamantane-1-carboxylic acid and decahydro-2-naphthoic acid, but increased toxicity of 1,2,3,4-tetrahydro-2-naphthoic acid*. The EC_{20} (in percent of original sample) shifted from 34% to 176%, 23% to 85%, and >80% to 49%, in adamantane-1-carboxylic acid, decahydro-2-naphthoic acid, and 1,2,3,4-tetrahydro-2-naphthoic acid, due to 60 s of ozone exposure
14. *The isolate Acidovorax sp. was not capable of degrading any of the three model NA compounds tested*. No degradation was observed in 10 days of aerobic incubation with the untreated NA compounds.
15. *Sixty seconds of ozone pretreatment enabled 40% removal of adamantane-1-carboxylic acid*, from a starting concentration of 0.7 mg/L. It was *hypothesized that adamantane-1-carboxylic acid was cometabolized during*

biodegradation of more labile ozone by-products, as no degradation of non-ozonated adamantane-1-carboxylic acid was observed.

Chapter 6

16. A microbial co-culture containing *Trichoderma harzianum* and a *Brevundimonas* sp. degraded 30% of 3-ethyl-adamantane-1-carboxylic acid within 11 days and 16% of adamantane-1-carboxylic acid after 33 days of aerobic incubation. This is the *first report of a fungus isolated from OSPW degrading NAs*.
17. No degradation of the three model NA compounds was observed in the original OSPW enrichment culture, dominated by *Brevundimonas*. This provides additional evidence that the fungus *Trichoderma harzianum* in the co-culture was responsible for NA degradation.
18. No degradation of the naphtheno-aromatic acid, dehydroabietic acid, was observed with the three microbial cultures tested.
19. *Microbial growth*, as measured by optical density, *was not seen in cultures with observed biodegradation of NA compounds*.
20. *Degradation of NAs did not correspond with reduction in toxicity*, as measured by the Microtox assay.

7.3 Recommendations

7.3.1 Future Research

This research provided additional evidence in support of ozone pretreatment for improving the biodegradability of organic matter in OSPW. Determining if ozone pretreatment of OSPW prior to placement in reclamation environments results in adequate removal of toxicity and contaminants of concern will assist industry in assessing the need to build wastewater treatment facilities. Future research on optimization of ozone dosage should take into consideration the ability of indigenous microorganisms in OSPW to survive and degrade remaining organic compounds after ozone exposure, especially if in-situ treatment of OSPW is feasible. A biomass assessment able to distinguish live,

dead and compromised microbial cells will be essential in resolving the impacts of ozone exposure on microbial communities in OSPW (Brown et al., 2013). Utilization of next generation sequencing technologies to monitor microbial community dynamics through proposed treatment processes will provide better understanding of metabolic capabilities of indigenous microorganisms in OSPW and the surrounding environment. Delineation of the microbial communities in aged OSPW from decades-old experimental reclamation ponds is essential for predicting viability of end pit lakes and constructed wetlands, both to treat OSPW and evolve into viable ecosystems (Yergeau et al., 2012).

While concerted efforts have already begun to demarcate the entire organic fraction of OSPW (Frank et al., 2014; Grewer et al., 2010; Headley et al., 2013a, 2013b, 2011; Pereira et al., 2013a; Ross et al., 2012; Rowland et al., 2012, 2011c), it is essential that this work be completed, as it greatly impacts water quality monitoring efforts in the oil sands region (Schindler, 2010), elucidation of toxic effects (Garcia-Garcia et al., 2012, 2011b), and design of treatment technologies (Allen, 2008a, 2008b). In addition, the use of model NA compounds when validating treatment regimes for OSPW (Johnson et al., 2011; Pérez-Estrada et al., 2011; Rowland et al., 2011b) should continue in parallel with utilization of whole OSPW. The identification and determination of toxic effects of both specific metabolites of biodegradation and by-products of ozone treatment will assist in the design of effective OSPW treatment technologies. (Anderson et al., 2012b; Garcia-Garcia et al., 2011a; Pérez-Estrada et al., 2011; Rowland et al., 2011b).

This work has potentially shown that a fungus isolated from OSPW, *Trichoderma harzianum*, was more effective in degrading NAs than bacteria obtained under the same enrichment conditions. Fungi are prolific and resilient to hostile environments and known to degrade a wide variety of organic compounds (Harman et al., 2004). Future research should thoroughly investigate the metabolic capabilities of this species of fungus, and other fungi, for bioremediation of organics in OSPW.

7.3.2 To The Oil Sands Industry

With mounting pressure on the oil sands industry to reduce the large volumes of MFT and OSPW (ERCB, 2013), concerted efforts are required at this time to develop cost-effective and efficient treatment strategies. In addition, treatment of OSPW may offset withdrawals from the Athabasca River (Allen, 2008a). Natural attenuation of priority pollutants such as NAs and associated toxicity in OSPW has been established to be ineffective (Kavanagh et al., 2013, 2011; Quagraine et al., 2005b) and more aggressive treatment technologies will need to be adopted (Allen, 2008b). As in-situ treatment techniques, such as constructed wetlands, are both more cost-effective and able to form part of the final reclamation environment, pretreatment of OSPW, by ozonation, prior to placement in constructed wetlands or end pit lakes should be adopted, to enhance, both in terms of rate and extent, biodegradation of remaining organic matter in OSPW by the indigenous microbial communities inhabiting both OSPW and the reclamation environments.

Oil sands companies are actively researching end pit lake technologies; Syncrude commissioned the first commercial scale demonstration end pit lake, Base Mine Lake, in 2012 (Syncrude, 2013). It is recommended that industry monitor microbial systems in these experimental reclamation environments in addition to chemistry, toxicity, and biology of larger lifeforms. Use of next generation sequencing is feasible, as costs of molecular methodologies come have been greatly reduced. Microorganisms can be used as an indicator of the health of an ecosystem (Yergeau et al., 2012) and thus should not be ignored when monitoring the evolution of systems during the establishment of these first full scale facilities.

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Appendices

Appendix 1: FTIR Calibration Curve

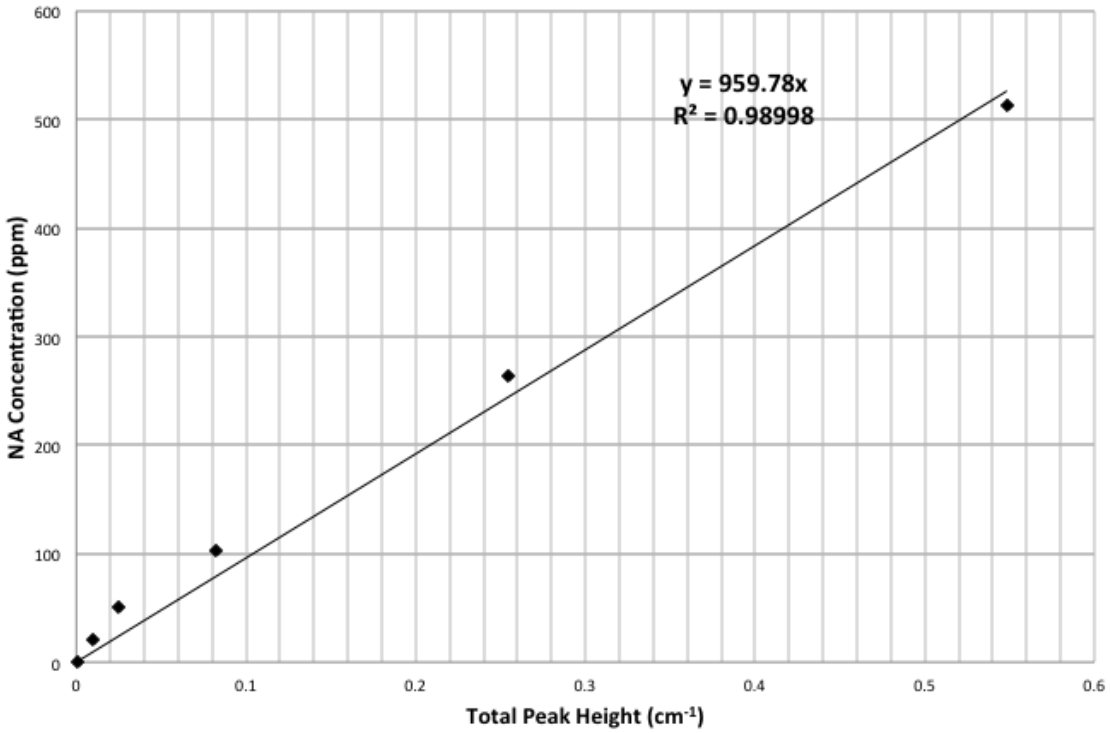
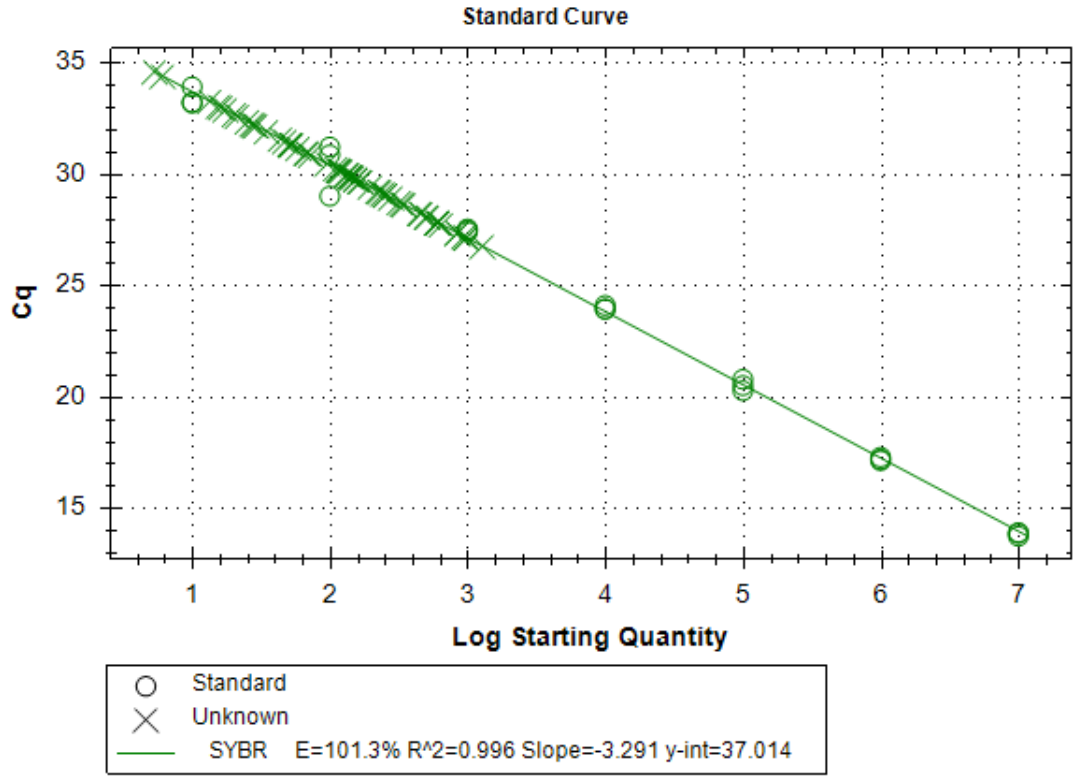
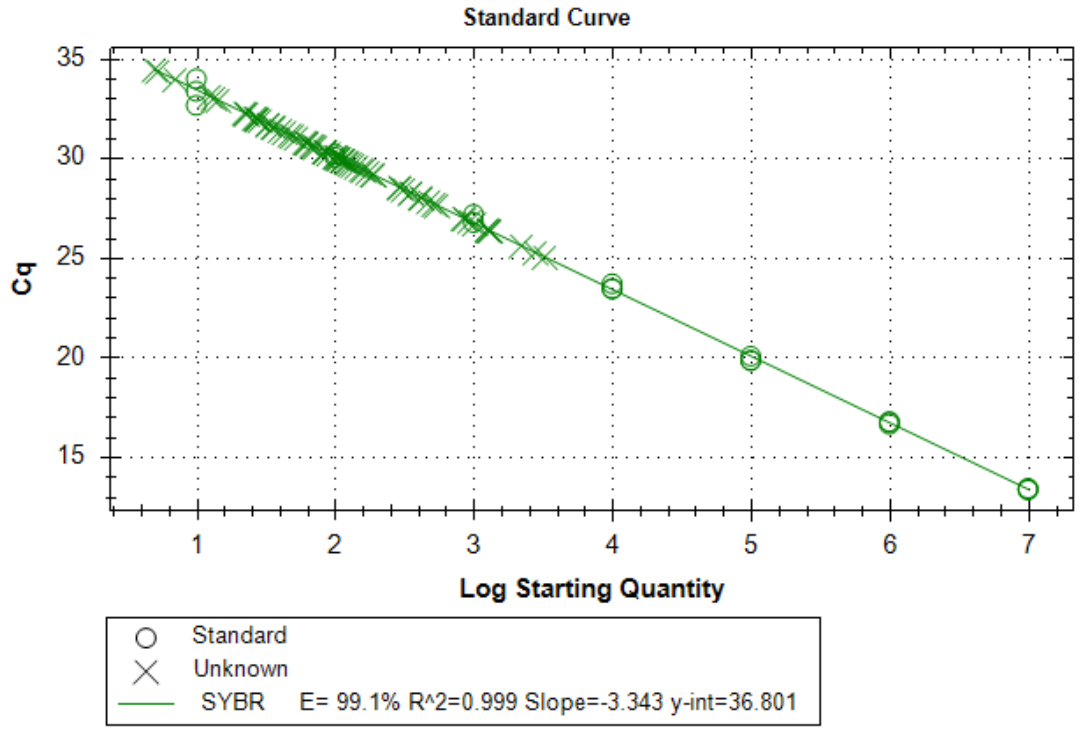
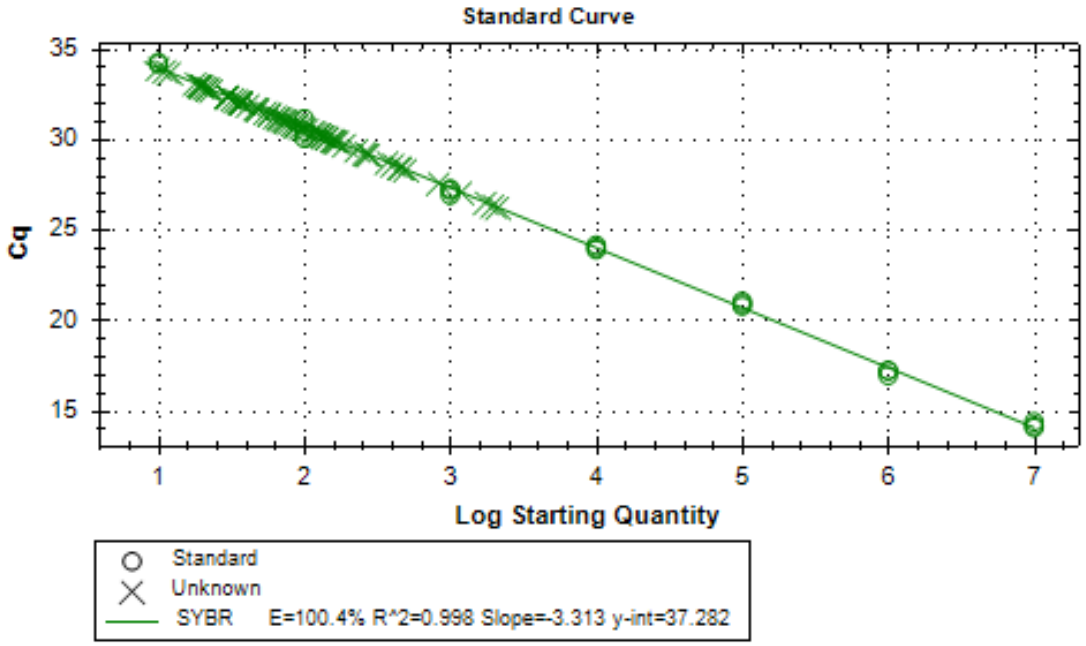


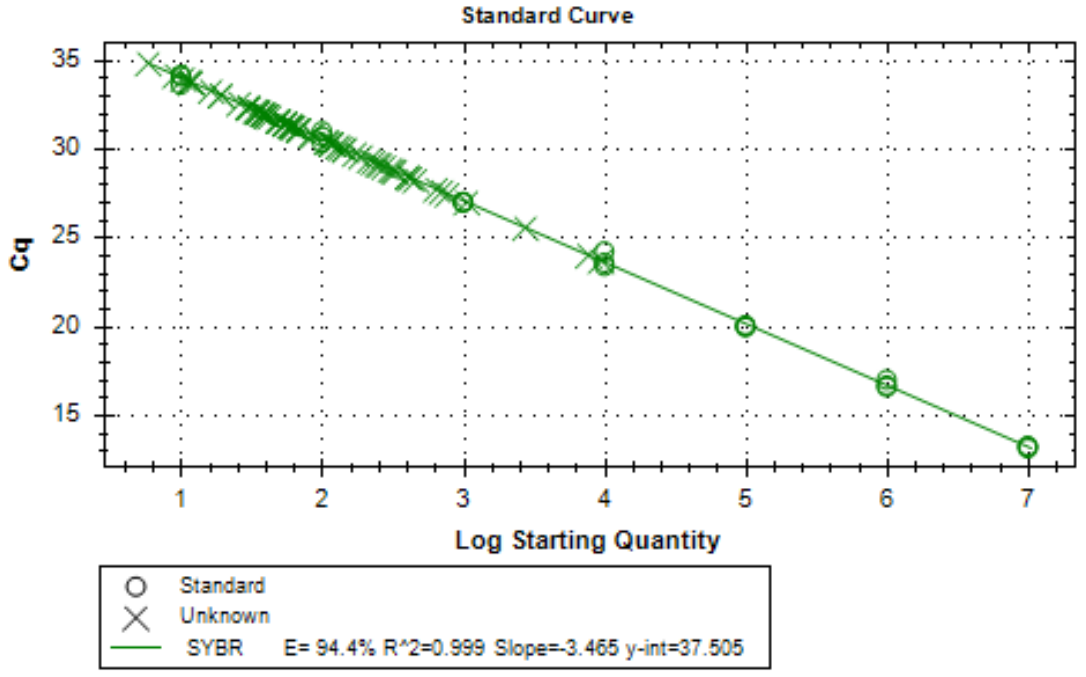
Figure A-1: FTIR Standard Calibration measuring carboxylic acid groups based on combined total peak height at 1743 cm^{-1} (for monomers) and 1705 cm^{-1} (for hydrogen-bonded dimers) using a commercial refined NA preparation (Merichem NAs) at six concentrations.

Appendix 2: Quantitative PCR Calibration Curves for Chapter 3






Standard Curve
Cq
35
30
25
20
15
1
2
3
4
5
6
7
Log Starting Quantity
Standard
Unknown
SYBR E=100.4% R^2=0.998 Slope=-3.313 y-int=37.282


Standard Curve
Cq
35
30
25
20
15
1
2
3
4
5
6
7
Log Starting Quantity
Standard
Unknown
SYBR E= 94.4% R^2=0.999 Slope=-3.465 y-int=37.505

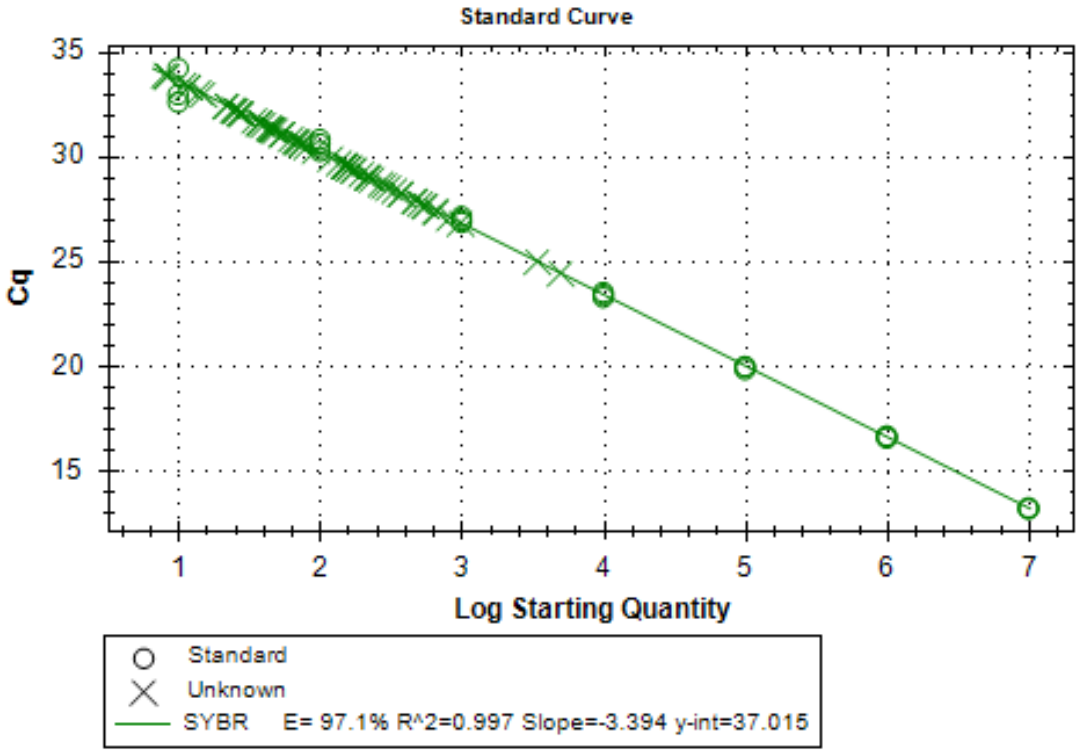


Figure A-2: Standard calibration curves for quantitative PCR of the *rpoB* gene. Calibration completed with 10-fold serial dilutions of *P. putida* PCR product. Calibration curves generated with Bio-Rad CFX Manager software.

Appendix 3: DOC Degradation in Ozonated-Sterilized and Ozonated-Sterilized-Inoculated Controls

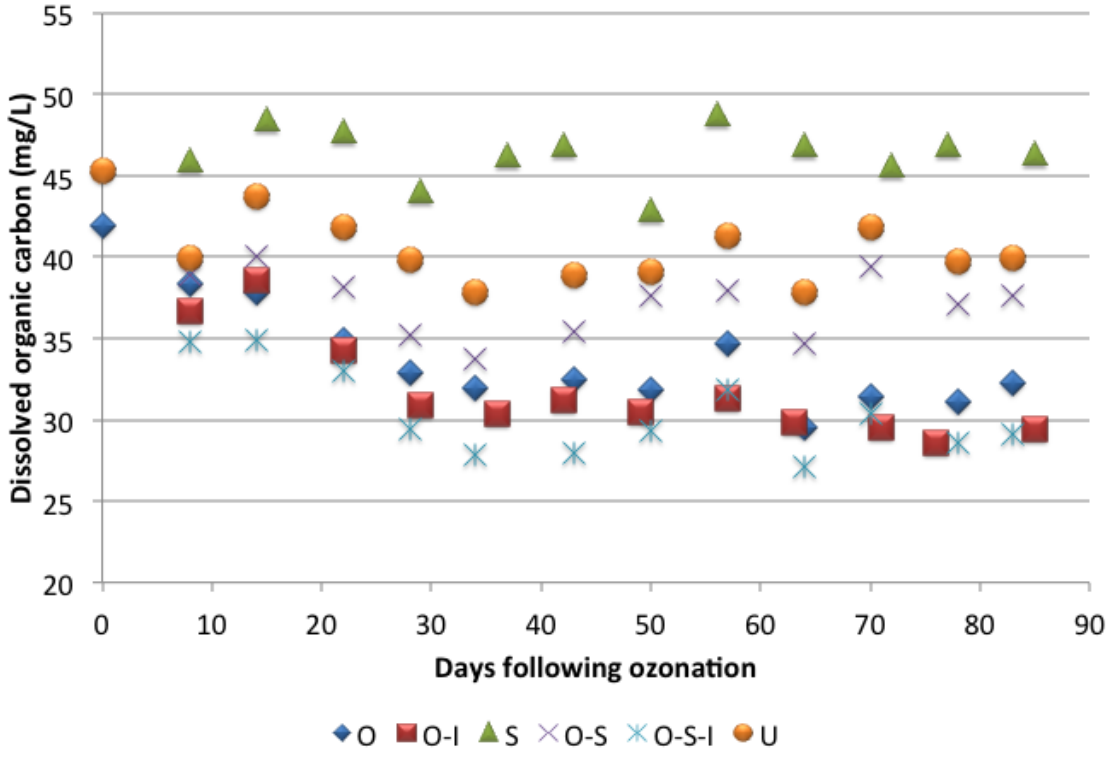


Figure A-3: Dissolved organic carbon concentration in aerobic microcosms of FE5 water sample from Syncrude Canada Ltd., corrected for evaporative effects (Refer to Sec. 3.1.4). Treatments were Ozonated (O), Ozonated-Inoculated (O-I), Untreated (U), Sterilized (S), Ozonated-Sterilized (O-S), and Ozonated-Sterilized-Inoculated (O-S-I). Ozonated Day 0 sample indicates DOC measurement prior to incubation.

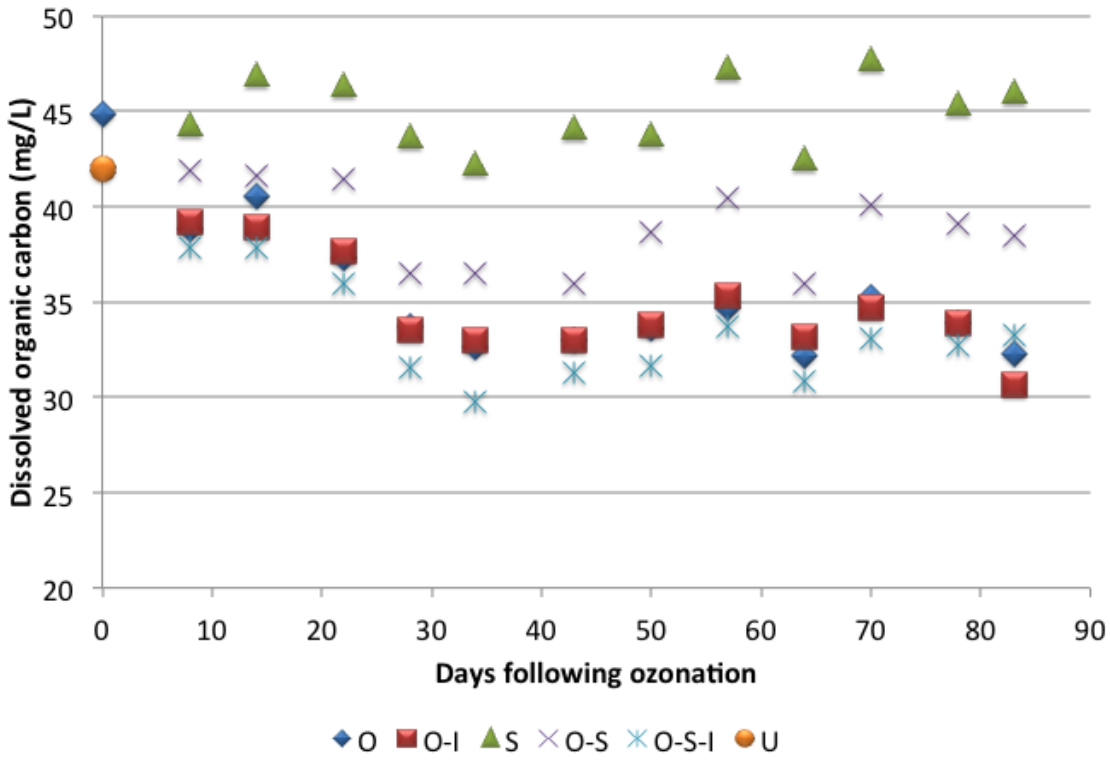


Figure A-4: Dissolved organic carbon concentration in aerobic microcosms of Big Pit water sample from Syncrude Canada Ltd., corrected for evaporative effects (Refer to Sec. 3.1.3). Treatments were Ozonated (O), Ozonated-Inoculated (O-I), Untreated (U), Sterilized (S), Ozonated-Sterilized (O-S), and Ozonated-Sterilized-Inoculated (O-S-I). Ozonated Day 0 sample indicates DOC measurement prior to incubation.

Appendix 4: Similarity Analyses for PCR-DGGE based on Jaccard Index

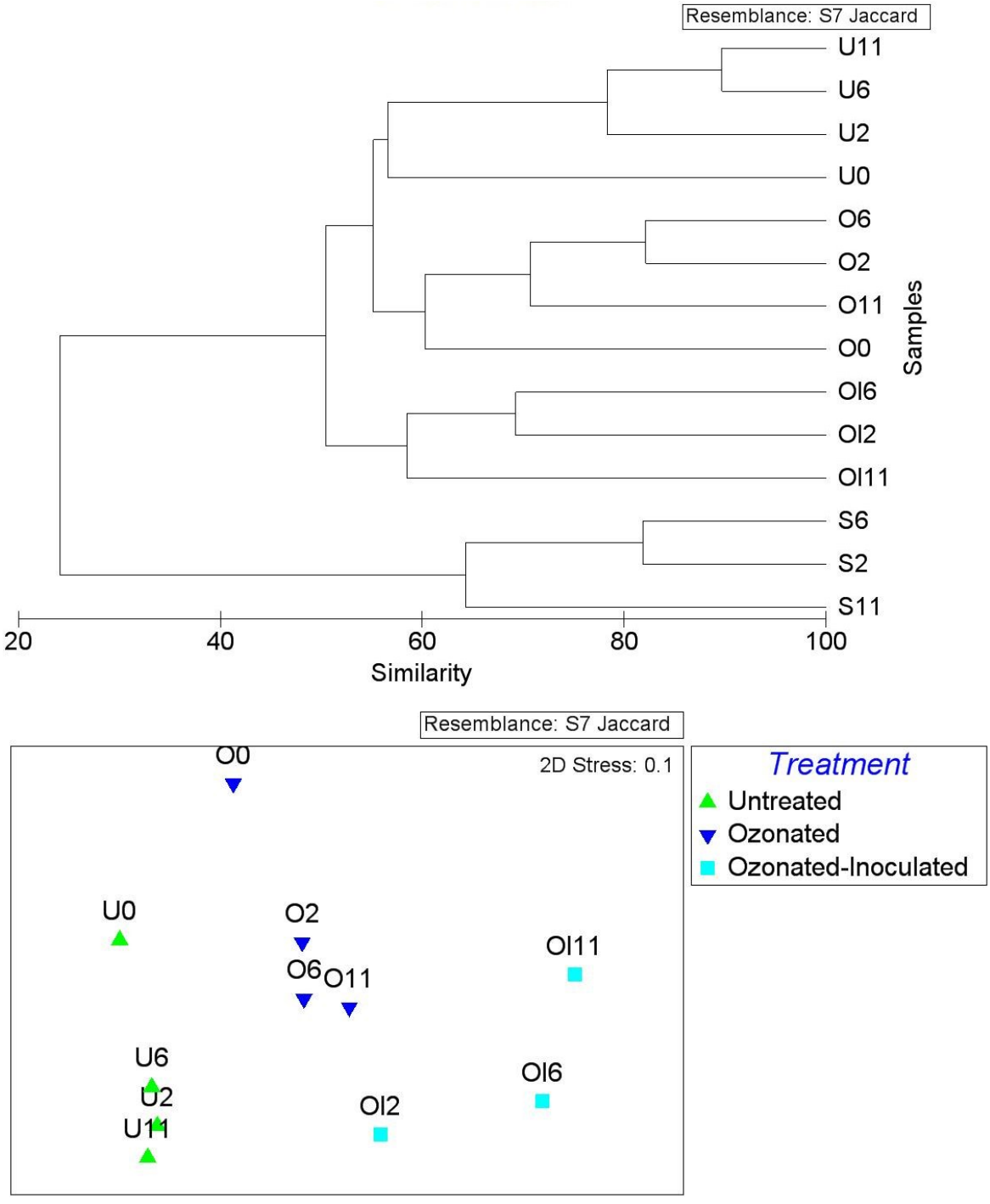


Figure A-5: UPGMA dendrogram and non-metric dimensional scaling of 16S rRNA PCR-DGGE for FE5 water sample, based on the Jaccard similarity index. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

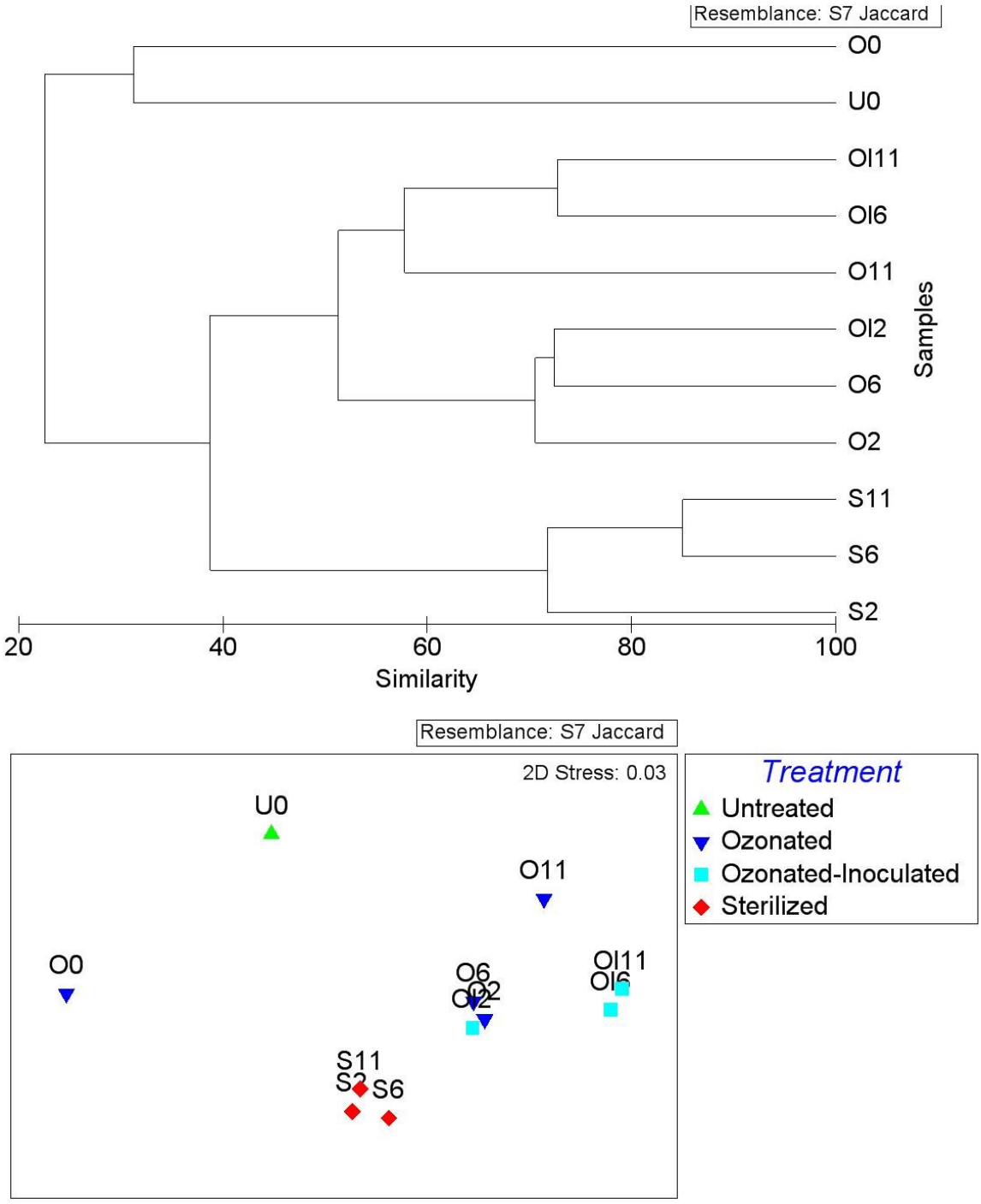


Figure A-6: UPGMA dendrogram and non-metric dimensional scaling of 16S rRNA PCR-DGGE for Big Pit water sample, based on the Jaccard similarity index. Labels indicate treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized) and weeks of incubation, following ozone treatment.

Appendix 5: Parsimony Tree for 16S rRNA PCR-DGGE Bands

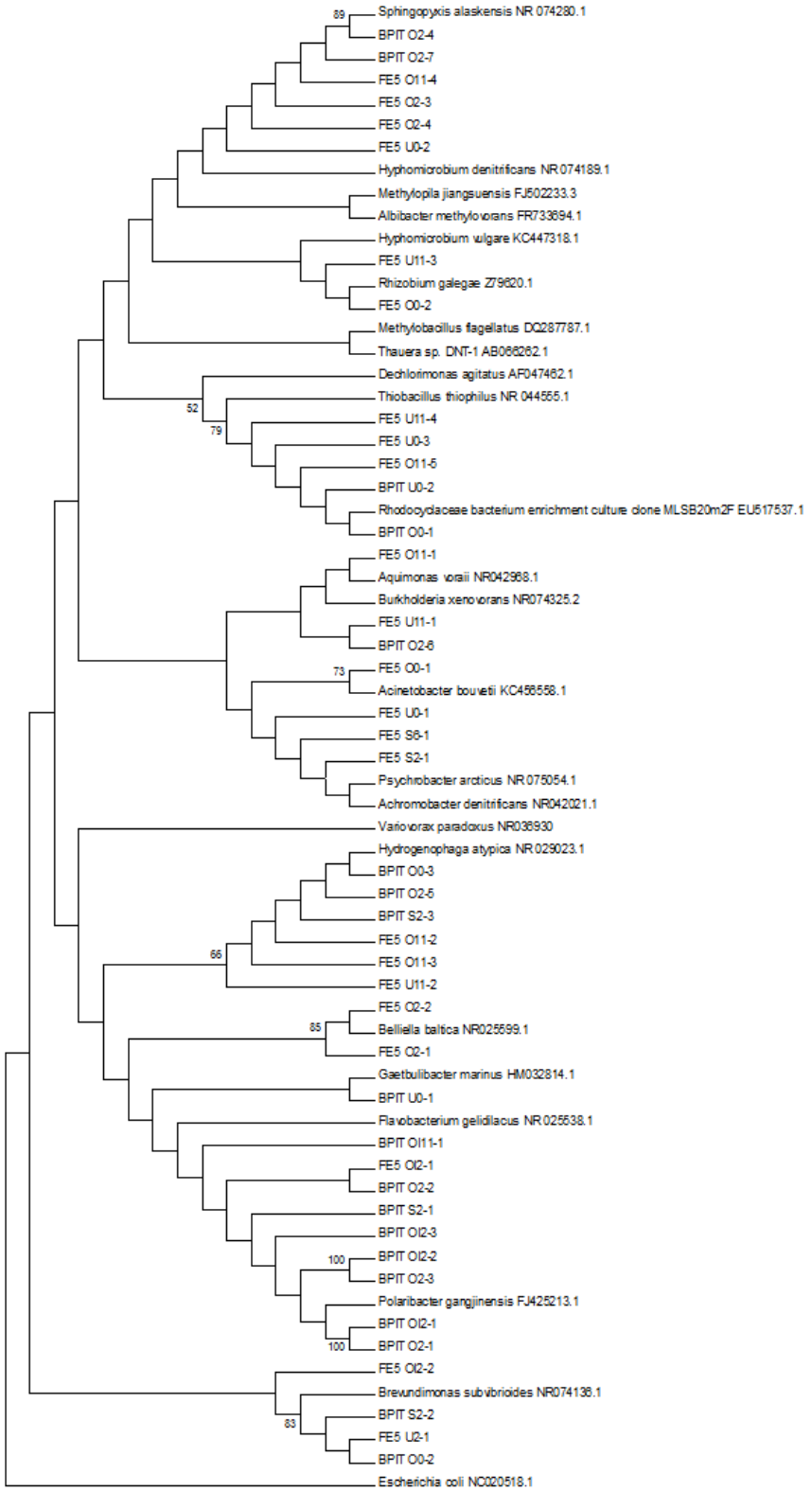


Figure A-7: Maximum parsimony tree, bootstrapped 100 times.

Appendix 6: Sequences for Excised PCR-DGGE Bands

Table A-1: 16S rRNA sequences from excised PCR-DGGE bands for two water samples. Labels indicate water sample (FE5, BPIT=Big Pit), treatment (U=Untreated, O=Ozonated, OI=Ozonated-Inoculated, S=Sterilized), and weeks of incubation, following ozone treatment.

Band	Sequence
FE5 U0-1	AGCATCGTCATGGGTATTAACCATGGAGTCTTCTTCACTGC TTAAAGTGCTTTACAACCAAAAGGCCTTCTTCACACACGCG GCATGGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCC CACTGCTGCCTCCCGTAGGA
FE5 U0-2	GGTACGTCATTATCTTCCGNTGAAGAGCTTTACAACCCTAA GGCCGTCATCACTCACGCGGCATGGCTGGATCAGGCTTGC GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
FE5 U0-3	GTACGTCATTAACACGCTATGTTAGAGCGTGCCGTTTCGTT CCTGCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTC ACGCGGAATGGCTGGATCAGGGTTGCCCCCATTGTCCAAA ATTCCCCACTGCTGCCTCCCGTAGGA
FE5 U2-1	GTCATTATCTTCACCGGTGAAGTATTTTACAATCCTAAGACC TTCATCATACACGCGGCATGGCTGCNTCANGCTTTCGCCCA TTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGA
FE5 U11-1	TACGTCATCNCACCAGGTAGTAACCAGTATCGATCTTCCGT CCCGAACAAAATGCTTTACAACCCGCAGGCCTTCTTCACAC ACGCNGCATGGCTGGATCACGCTTGCCCCCATTGTCCAATA TTCCCCACTGCTGCCTCCCGTAGGA
FE5 U11-2	TCTTACGGTACGTCATTAGCCCCAGGTATTAACCAGGTACC GTTTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTT CTTCCTGCACGCGGCATTGCTGGATCAGGCTTGCGCCCATT GTCCAAAATTCCCCACTGCTGCCTCCCGTAGGA
FE5 U11-3	TACGTCATTATCGTCCCTGGCAAAGAGCTTTACAATCCGAA GACCTTCTTCACTCACGCGGCATGGCTGGATCAGGCTTGC GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
FE5 U11-4	GTACGTCATTACGCGCTATGTTANAGCGCGCCGTTTCGTTC CTGCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCA CGCGGAANGGCTGGATCAGGGTTGCCCCCATTGTCCAAAA TTCCCCACTGCTGCCTCCCGTAGGA
FE5 O0-1	GCGAGTACGTCACTATCCTAGAGTATTAATCCAAGTACCCT CCTCCTCGCTTAAAGTGCTTTACAACCAAAAGGCCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCC AATATTCCCCACTGCTGCCTCCCGTAGGA
FE5 O0-2	CGTCNAGTGTGACTTTCCACTCTCACAGCTCGTTCTTCNCTT ACAACAGAGCTTTACGATCCGAAANCCTTCTTCACTCACGC GGCGTGGCTNGGTCAGACTTCCGTCCATTGCCGAAGATTC CCTACTGCTGCCTCCCGTAGGA

Band	Sequence
FE5 O2-1	CTACGGTACGGCATGTCCCCGCATGGGCATTTTCTTCCCGT ATAAAAGCAGTTTACAACGCATAACGCCGTCTTCCTGCACG CGGCATGGCTGGGTCAGGCTTCCGNCCATTGCCCAATATT CCCTACTGCTGCCTCCCGTAGGA
FE5 O2-2	CTACGGTACGGCATGTCCCCGCATGGGCNTTTTCTTCCCGT ATAAAAGCAGTTTACAACGCATAACGCCGTCTTCCTGCACG CGGCATGGCTGGGTCAGGCTTCCGACCATTGCCCAATATTC CCTACTGCTGCCTCCCGTAGGA
FE5 O2-3	TACGTCATTATCTTCCCGGTGAAAGAGCTTTACAACCCTAAG GCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCG CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTANGAA
FE5 O2-4	TACGTCATTATCNTCCCAGTGAAAGAGCTTTACAACCCTAAG GCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCG CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTANGAA
FE5 O11-1	CTTCGGTACGTCATNCCCTAACGATATTAACCTCCGGATTTC TTCCCGAACAAAAGTGCTTTACAACCCGAAGGCCTTCTTCC CACACGCGGCATTGCTGGATCAGGCTTGCCCCCATTGTCC AATATTCCCCACTGCTGCCTCCCGTAGGA
FE5 O11-2	CTTACGGTACGTCATTAGCCCCAGGTATTAACCAGGACCGT TTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCT TCCTGCACGCGGCATTGCTGGATCAGGCTTGCGCCCATTG TCCAAAATTCCCCACTGCTGCCTCCCGTAGGA
FE5 O11-3	ACGGTACGTCATTAGCCCCGGGTATTAACCAGGACCGTTTC GTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCTTCC TGCACGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCC AAAATTCCCCACTGCTGCCTCCCGTAGGA
FE5 O11-4	GTACGTCATTATCTTCCGGGTAAGAGCTTTACTTCCCTAANG CCTTCATCACTCACACAGCATGGCGGCATCATGCTTGCGCC GNTTGTNCAAGATTCCGCNCTGCTGCCTNGCNTAAGATTCC CCACTGCTGCCTCCCGTAGGA
FE5 O11-5	GTACGTCATTACGCGCTATGTTAAGCGCGCCGTTTCGTTCC NGCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCAC GCGGAANGGCTGGATCAGGGTTGCCCCCATTGTCCAAAAT TCCCCACTGCTGCCTCCCGTAGGA
FE5 OI2-1	TCTACGTACCGTCAGTCCCTACACGTAGGGAGGTTTCTTCC TGTATAAAAGCAGTTTACAATCCATAGGACCGTCTTCCTGCA CGCGGCATGGCTGGGTCAGAGTTGCCTCCATTGCCCAATA TTCCTCACTGCTGCCTCCCGTAGGA
FE5 OI2-2	ACGTCATTATCTTCCCGAGTGAAGGAGTTTACAATCCTAAG GCCTTCATCACTCACGCAGCATGGCTGCATCATGCTTGCGC CGATTGGGCAAGATTCCCCACTGCTGCCTCCCGTANGA

Band	Sequence
FE5 S2-1	CTATGTCATCGTCCCGGGTATTAACCANGGAGTCTTCTTCA CTGCTTAAAGTGCTTTACAACCAAAAGGCCTTCTTCACACAC GCGGCATGGCTGGATCAGGGTTTCCCCCATTGTCCAATATT CCCCACTGCTGCCTCCCGTAGGA
FE5 S6-1	CTTCTGCAGCTATGTCATCGTCCATGGGTATTAACCATGGA GTCTTCTTCACTGCTTAAAGTGCTTTACAACCAAAAGGCCTT CTTCACACACGCGGCATGGCTGGATCAGGGTTTCCCCCATT GTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
BPIT U0-1	TCCGAGGGAAGTTTGCTCCGGTGTAAAAGCAGTTTACGACC CATAGGCCTTCTTCCTGCACGCGGCATGGCTGGATCACCCT TGCGGGCATTGTCCAATATTCCTTACTGCTGCCTCCCGTAG GA
BPIT U0-2	TACGTCATTAACACGCTATGTTAGAGCGTGCCGTTTCGTTC CTGCCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCA CGCGGAATGGCTGGATCAGGGTTGCCCCCATTGTCCAAAA TTCCCCACTGCTGCCTCCCGTAGGA
BPIT O0-1	GTACCGTCATTAACATAAGCTATTCACTTACATCGTTTCTTC CCTTGCGAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTC ACGCGGAATGGCTGGATCAGGGTTGCCCCCATTGTCCAAA ATTCCCCACTGCTGCCTCCCGTAGGA
BPIT O0-2	CGGCTACGTCATTATCTTCACCGGTGAAAGTATTTTACAATC CTAAGACCTTCATCATACACGCGGCATGGCTGCGTCAGGCT TTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAG GA
BPIT O0-3	GGTACGTCATTAGCCCCAGGTATTAACCAGGACCGTTTCGT TCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCTTCCTG CACGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAA AATTCCCCACTGCTGCCTCCCGTAGGA
BPIT O2-1	ACTTCAGCTTAGATGAATCCAAGTTTTTATTCCCAGAGAAAA GAAGTTTACAATCCATAGGACCTTAATCCTTCACGCGGGAT GGCTGGATCAGAGTTGCCTCCATTGTCCAATATTCCCTACT GCTGCCTCCCGTAGGA
BPIT O2-2	CGTCAGTCCCTACACGTANGGAGGTTTCTTCCTGTATAAAA GCAGTTTACAATCCATACGACCGTCTTCCTGCACGCGGCAT GGCTGGGTCAGAGTTGCCTCCATTGCCCAATATTCCTCACT GCTGCCTCCCGTAGGA
BPIT O2-3	CGGTACGTCAGACAGGTCGAAACCCGTTTGTTCTTTCCGAA TAAAAGCAGTTTACAATCCATAGGACCTTCTTCCTGCACGC GGCGTGGCTGCGTCAGAGTTTCCTCCATTGCGCAATATTCC TCACTGCTGCCTCCCGTAGGA
BPIT O2-4	TACTGTCATTATCATCCCGGGTAAAAGAGCTTTACAACCCTA AGGCCTTCATCACTCACGCGGCATTGCTGGATCAGGCTTTC GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTANGA

Band	Sequence
BPIT O2-5	TCGGTACGTCATTAGCCCCGGGTATTAACCAGGACCGTTTC GTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCTTCC TGCACGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCC AAAATTCCCCACTGCTGCCTCCCGTAGGA
BPIT O2-6	GTACGTCATCCGCCCAAGGTATTAACTCGGACTATTTCTTTC CGGACAAAATTGCTTTACAACCCGCANGCCTTCTTCACACA CGCGGCATTGCTGGATCANGCTTGCNCCCATTGTCCAANAT TCCCCACTGCTGCCTCCCGTAGGA
BPIT O2-7	TACGTCATTATCGTCCCGCTAAAGAGCTTTACAACCCTAAG GCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCC CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
BPIT OI2-1	GGTACTTCAGCTTAGATGAATCCAAGTTTTTATTCCCAGAGA AAAGAAGTTTACAATCCATAGGACCTTAATCCTTCACGCGG GATGGCTGGATCAGAGTTGCCTCCATTGTCCAATATTCCCT ACTGCTGCCTCCCGTAGGAA
BPIT OI2-2	TCGGTACGTCAGACAGGTCGAAACCCGTTTGTTCTTTCCGA ATAAAAGCAGTTTACAATCCATAGGACCTTCTTCCTGCACGC GGCGTGGCTGCGTCAGAGTTTCCTCCATTGCGCAATATTCC TCACTGCTGCCTCCCGTAGGA
BPIT OI2-3	GTACGTCATTCCCCTCCGAGGGAGGTGTTTCTTCCCCTATA AAAGCANTTTACAATCCATAGGACCGTCTTCTTGCACGCGG CANGGCTGGGTCAGATTTGCCTCCATTGCCNAATATTCCTC ACTGCTGCCTCCCGTAGGATAAGG
BPIT OI11-1	TACGGTACCGTCAGTCACTACACGTAGTGGTGTTTCTTCCC GTATAAAAGCAGTTTACAATCCATAGGACCGTCTTCCTGCA CGCGGCATGGCTGGGTCAGGCTTGCGCCCATTGCCCAATA TTCCTCACTGCTGCCTCCCGTAGGAAGGA
BPIT S2-1	CGTACCGTCAGTCCCTACACGTAGGGAGGTTTCTTCCTGTA TAAAAGCAGTTTACAATCCATAGGACCGTCTTCCTGCACGC GGCATGGCTGGGTCAGAGTTGCCTCCATTGCCCAATATTCC TCACTGCTGCCTCCCGTAGGATAGGA
BPIT S2-2	CGTCATTATCTTCACCGGTGAAAGTATTTTACAATCCTAAGA CCTTCATCATACACGCGGCATGGCTGCGTCAGGCTTTCGCC CATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGA
BPIT S2-3	GGTACGTCATTAGCCCCGGGTATTAACCAGGACCGTTTCGT TCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCTTCCTG CACGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAA AATTCCCCACTGCTGCCTCCCGTAGGA

Appendix 7: Calibration Curves for GC-MS Analysis of Model NA Compounds

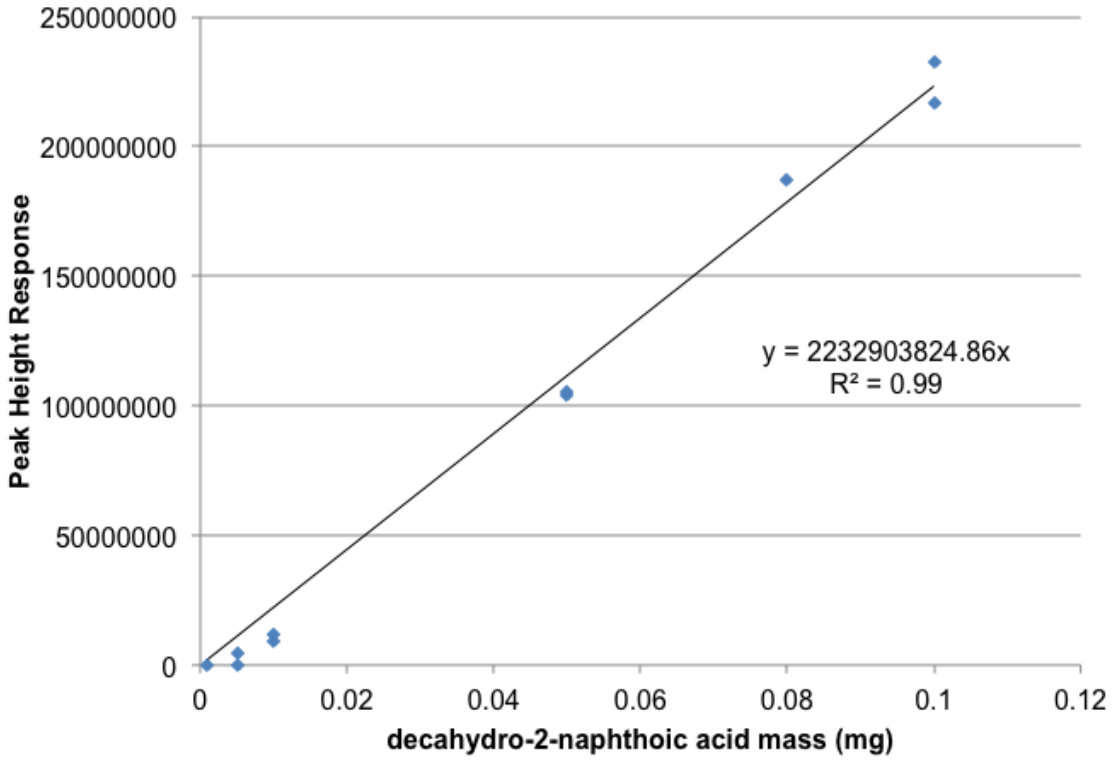


Figure A-8: Standard calibration curve for decahydro-2-naphthoic acid (Chapter 5)

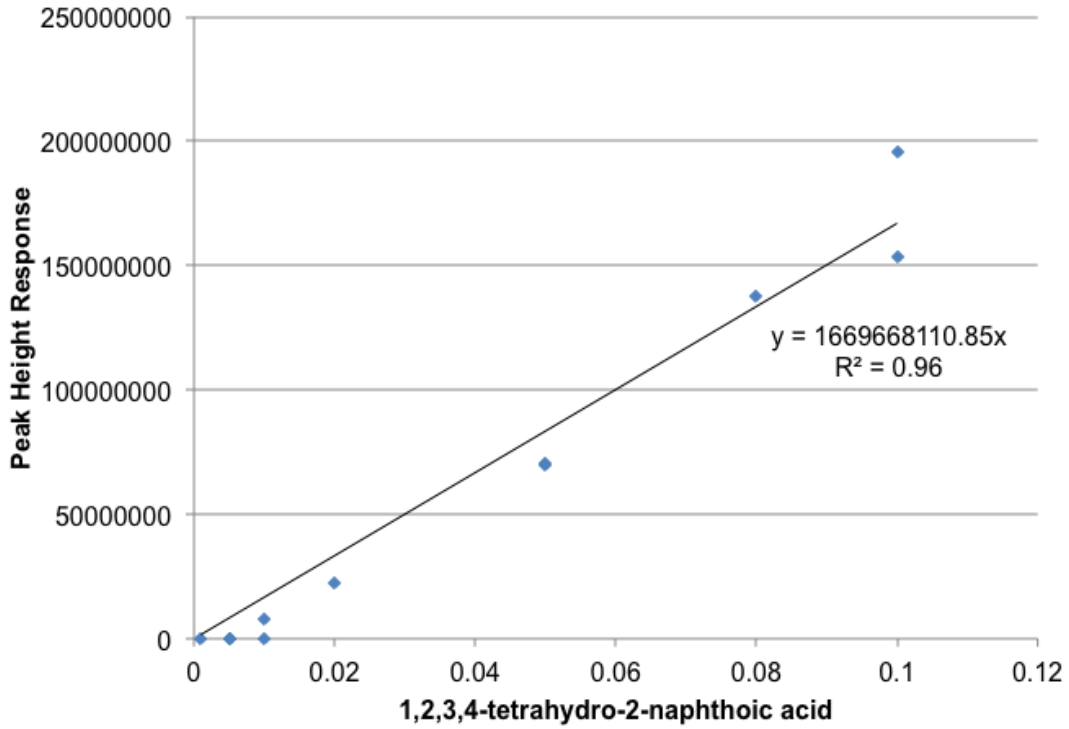


Figure A-9: Standard calibration curve for 1,2,3,4-tetrahydro-2-naphthoic acid (Chapter 5)

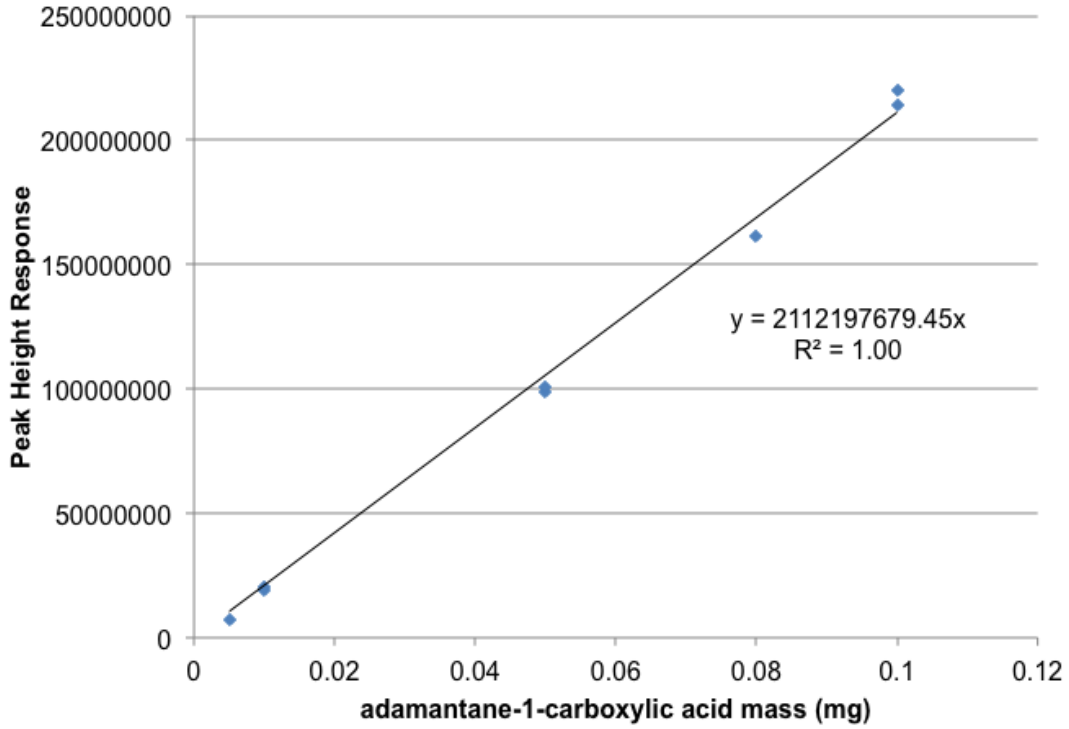


Figure A-10: Standard calibration curve for adamantane-1-carboxylic acid (Chapters 5 and 6)

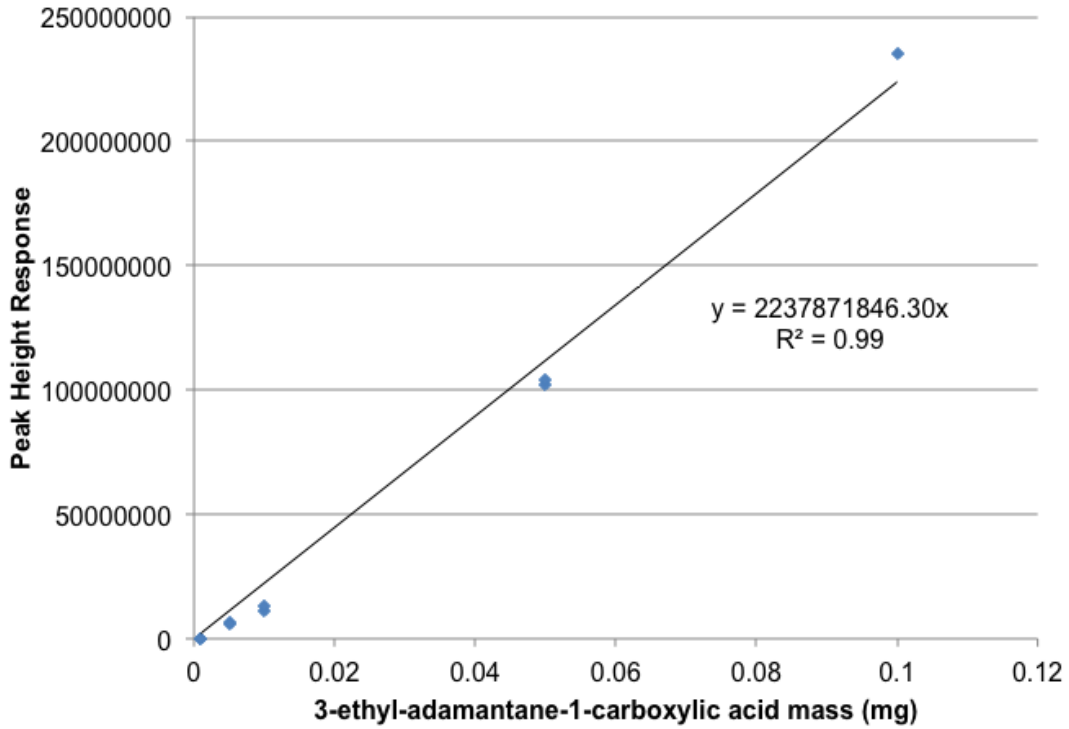


Figure A-11: Standard calibration curve for 3-ethyl-adamantane-1-carboxylic acid (Chapter 6)

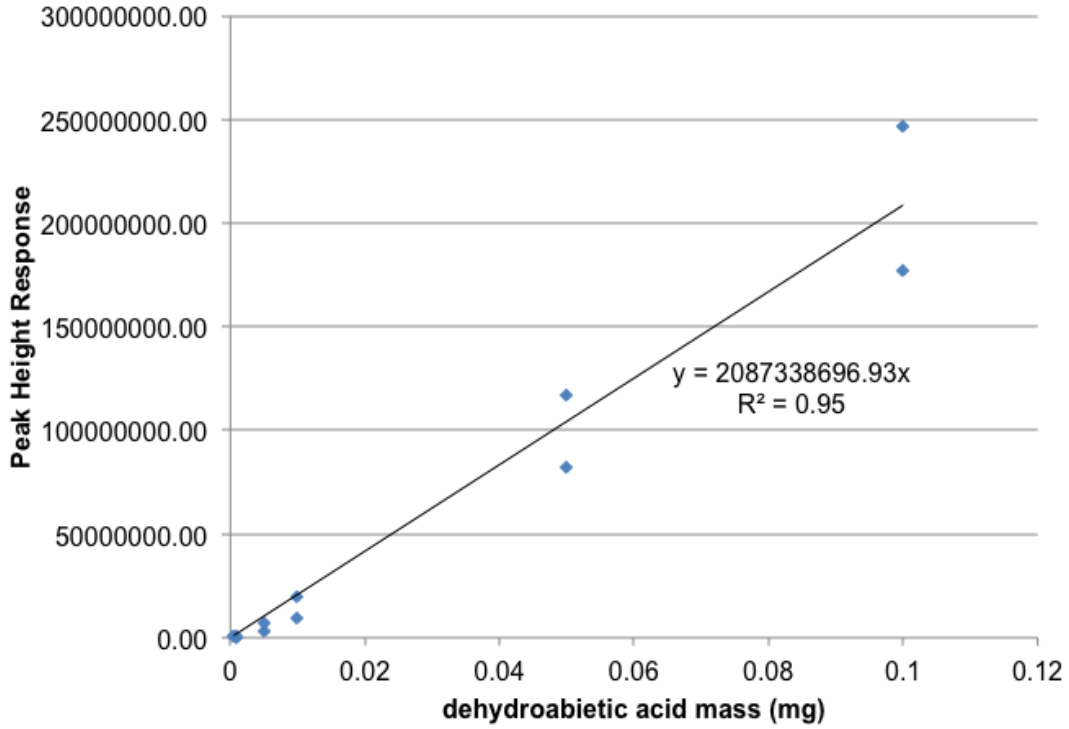


Figure A-12: Standard calibration curve for dehydroabietic acid (Chapter 6)

Appendix 8: Raw GC-MS Data for Chapter 5

Table A-2: GC-MS Data for adamantane-1-carboxylic acid, with values removed from analyses highlighted in red.

	Ozone Exposure for 0 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	4.75	5.07	12.4%	4.99	5.06	6.2%	6.46	6.02	7.1%
	4.66			5.41			5.60		
	5.79			4.79			6.01		
4	3.24	3.28	2.6%	3.15	3.28	3.8%	3.24	3.30	1.6%
	3.38			3.31			3.33		
	3.22			3.39			3.33		
10	4.66	4.04	17.8%	3.42	3.55	11.5%	3.45	3.53	3.9%
	3.25			4.01			3.69		
	4.21			3.22			3.46		

	Ozone Exposure for 30 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	1.60	1.67	14.5%	1.72	1.53	11.1%	2.06	1.82	13.8%
	1.47			1.39			1.56		
	1.94			1.48			1.85		
4	1.10	0.80	67.9%	1.06	1.00	5.8%	1.25	1.11	13.1%
	0.17			0.98			0.96		
	1.13			0.95			1.12		
10	1.21	1.17	13.2%	1.51	1.33	16.2%	1.07	1.07	4.1%
	1.00			1.39			1.12		
	1.30			1.09			1.03		

	Ozone Exposure for 60 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0		0.66	20.5%	0.70	0.78	13.7%	*1.06*	1.88	38.2%
	0.75			0.85			*2.40*		
	0.56						*2.17*		
4	0.56	0.56	5.1%	0.63	0.94	42.3%	0.63	0.99	51.4%
	0.59			0.79			1.35		
	0.53			1.39					
10		0.60	11.2%	0.64	1.05	48.7%	0.68	0.85	22.3%
	0.65			0.89			0.82		
	0.55			1.62			1.05		

Table A-3: GC-MS Data for decahydro-2-naphthoic acid, with values removed from analyses highlighted in red.

	Ozone Exposure for 0 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	4.12	4.05	2.4%	3.64	3.65	0.3%	3.74	3.52	8.8%
				3.64					
	3.98			3.66			3.30		
4	1.89	1.92	2.8%	2.29	1.92	18.9%	1.80	1.88	4.2%
	1.89			1.91			1.89		
	1.98			1.57			1.96		
10	3.11	2.34	46.7%	2.61	2.68	5.9%	3.14	2.74	16.3%
	203.90			2.57			2.26		
	1.57			2.86			2.81		

	Ozone Exposure for 30 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0		0.84	#DIV/0!	0.70	0.65	17.9%	0.52	0.50	59.5%
	0.84			0.52			*0.19*		
				0.74			0.78		
4	0.36	0.45	34.9%		0.30	19.2%		0.24	97.3%
	0.63			0.26			0.07		
	0.36			0.34			0.40		
10	0.29	0.45	31.3%	0.54	0.37	41.9%	0.21	0.37	61.4%
	0.52			0.28					
	0.54			0.27			0.53		

	Ozone Exposure for 60 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	0.62	0.61	10.7%	0.45	0.67	49.9%		1.20	#DIV/0!
	0.67			1.05			*1.20*		
	0.54			0.50					
4	0.34	0.39	12.3%		0.53	36.1%	0.80	0.63	27.3%
	0.43			0.66			0.64		
	0.38			0.39			0.45		
10	0.46	0.49	15.6%		0.66	#DIV/0!	0.77	0.55	36.3%
	0.43			0.66			0.38		
	0.58						0.49		

Table A-4: GC-MS Data for 1,2,3,4-tetrahydro-2-naphthoic acid, with values removed from analyses highlighted in red.

	Ozone Exposure for 0 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	5.11	4.76	6.4%	4.42	4.67	6.4%	4.59	4.66	3.4%
	4.56			4.59			4.84		
	4.60			5.00			4.55		
4	4.55	4.32	7.4%	4.59	4.48	9.8%	4.82	4.55	6.5%
				4.86			4.59		
	4.10			4.00			4.23		
10	5.64	4.92	20.7%	4.13	4.76	12.9%	6.40	5.25	20.1%
	4.83			4.79			4.32		
	4.20			5.36			5.03		

	Ozone Exposure for 30 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	0.90	0.76	16.0%	0.76	0.83	9.7%	2.13	2.03	13.5%
	0.68			0.82			1.72		
	0.70			0.92			2.24		
4	1.22	0.94	25.8%	0.82	0.89	14.8%	2.03	2.20	16.0%
	0.77			0.81			1.96		
	0.84			1.04			2.60		
10	1.08	1.05	37.6%	1.19	1.13	15.5%	2.59	2.49	25.9%
	0.64			0.93			1.80		
	1.43			1.26			3.09		

	Ozone Exposure for 60 s								
Day	Live	Ave	RSD	Killed	Ave	RSD	Abiotic	Ave	RSD
0	1.84	1.58	29.6%	0.69	0.80	13.2%	1.20	1.56	32.6%
	1.86			0.80					
	1.04			0.90			1.92		
4	1.54	1.22	28.5%	0.49	0.54	24.7%	0.73	1.20	69.2%
	1.25			0.70			0.01		
	0.85			0.45			1.66		
10	2.09	1.15	89.2%	0.49	0.64	24.9%	0.95	1.18	61.0%
	1.29			0.80			0.00		
	0.06			0.61			1.42		

Appendix 9: Complete Sequences for Microbial Cultures Isolated from OSPW

UA STP-6 16S rRNA: *Shinella granuli* (99% maximum identity)

TGCAGTCGAACGCATCGCAAGATGAGTGGCAGACGGGTGAGTAACGCGTG
GGAACGTACCCTTTACTACGGAATAACTCAGGGAAACTTGTGCTAATACCGT
ATGTGCCCTTCGGGGGAAAGATTTATCGGTAAAGGATCGGCCCGCGTTGGA
TTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGT
CTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGC
CATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGT
GAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAG
CAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGT
AAAGCGCACGTAGGCGGGTATTTAAGTCAGGGGTGAAATCCCGGAGCTCAA
CTCCGGAACTGCCTTTGATACTGGGTACCTAGAGTATGGAAGAGGTAAGTG
GAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTG
GCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATG
TTAGCCGTCGGCATGCATGCATGTCGGTGGCGCAGCTAACGCATTAAACAT
TCCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGA
ACCTTACCAGCCCTTGACATGTCGGTCGCGGTTTCCAGAGATGGATACCTT
CAGTTAGGCTGGACCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTT
GCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGA
GGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTAC
ACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACAGCGATGTCGAG
CTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCA
TGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCC
GAAGGCGATGCGCTAACCGCAAGGAGGCAGTCGACC

UA STP-8 16S rRNA: *Brevundimonas* sp. (99% maximum identity)

TGCAGTCGAACGAACTCTTCGGAGTTAGTGGCGGACGGGTGAGTAACACGT
GGGAACGTGCCTTTAGGTTCGGAATAACTCAGGGAAACTTGTGCTAATACC
GAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCCGCGTCT
GATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTG
GTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGACGCA
GCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCAGTAG
GGACGATAATGACGGTACCTACAGAAGAAGCCCCGGCTAACTTCGTGCCAG
CAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGT
AAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCGGGGCTCA
ACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTGTGAGAGAGGTATGT
GGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAACACCAGT
GGCGAAGGCGACATACTGGCTCATTACTGACGCTGAGGCTCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATT
GCTAGTTGTCGGGATGCATGCATTTCGGTGACGCAGCTAACGCATTAAGCA
ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGA
ACCTTACCACCTTTTGACATGCCTGGACCGCCAGAGAGATCTGGCTTTCCCT
TCGGGGACTAGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCATTAGTTGCCA
TCATTTAGTTGGGAACTCTAATGGGACTGCCGGTGCTAAGCCGGAGGAAGG
TGGGGATGACGTCAAGTCCTCATGGCCCTTACAGGGTGGGCTACACACGT
GCTACAATGGCGACTACAGAGGGTTAATCCTTAAAAGTCGTCTCAGTTCGG
ATTGTCCTCTGCAACTCGAGGGCATGAAGTTGGAATCGCTAGTAATCGCGG
ATCAGCATGCCGCGGTGAATACGTTCCCTGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTTGGTTCTACCCGAAGGCGCTGCGCTGACCGCAAGGGG
GCAGGCGACCGC

UA STP-8 ITS: *Trichoderma harzianum* (100% maximum identity)

CTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCAATGTGAACGT
TACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCC
CGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTATTGTATACCCC
CTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTT
CGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCC
TGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGG
GGATCGGCCCTCCCTTAGCGGGGTGGCCGTCTCCGAAATACAGTGGCGGT
CTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCG
CGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGA
TCAGGTAGGAATACCCGCTGAACTTAAGCATATC

Appendix 10: Quantitative PCR Standard Calibration Curves for Chapter 6

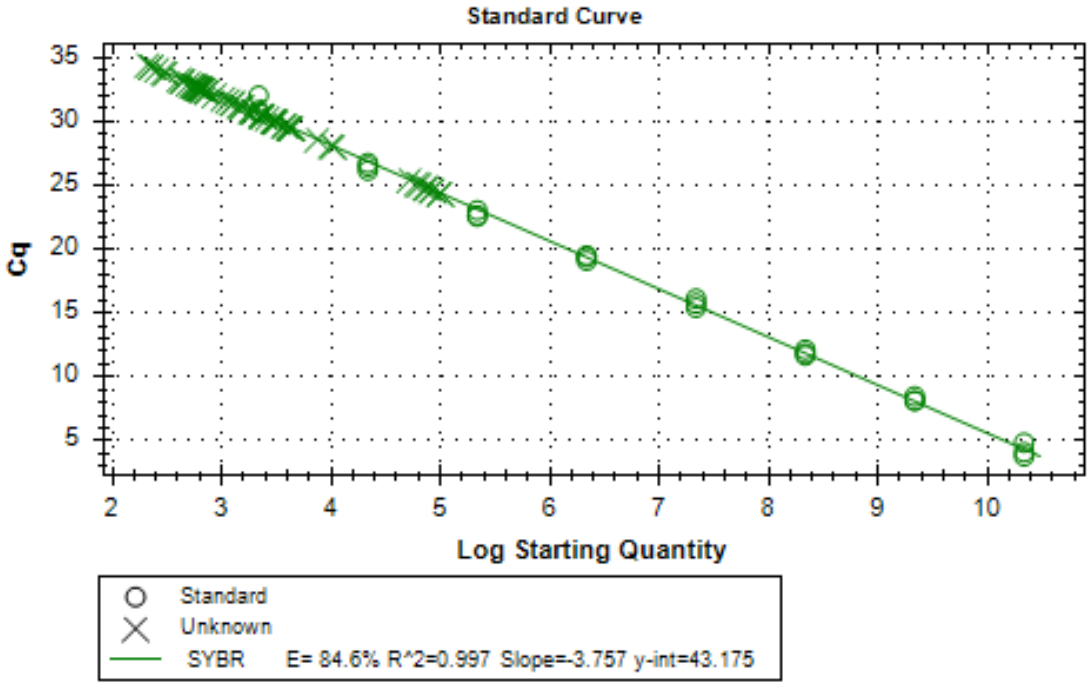


Figure A-13: Standard calibration curves for quantitative PCR of the *rpoB* gene. Calibration completed with 10-fold serial dilutions of *P. putida* PCR product. Calibration curves generated with Bio-Rad CFX Manager software.