Bioremediation of oil sands process affected water sourced naphthenic acid fraction compounds

by

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Abstract

Current development of Alberta's oil sands region requires large volumes of water, leading to an abundance of oil sands process affected water (OSPW). OSPW contains naphthenic acid fraction compounds (NAFCs) which have been found to contribute extensively to OSPW toxicity. Degradation of this complex group of organics is key to the remediation of OSPW and thus is often the focus of research. This remediation study conducted microcosm experiments to elucidate and characterize the capacity of OSPW sourced fungus *T. harzianum* to degrade labile commercial NAs (Merichem), and OSPW-sourced NAFCs, and two model NA compounds, cyclohexane carboxylic acid (CHCA) and 1-adamanatane carboxylic acid (ADA). This proof of concept experiment confirms that the fungal species *T. harzianum* can contribute to the biodegradation of complex dissolved organics found in OSPW, including cyclic and diamondoid structures.

As advanced oxidation processes have a proven capability for NAFCs remediation, and it has been demonstrated that microbial communities native to OSPW have the potential for biodegradation, coupling of these two processes would be an effective remediation strategy. Survivability of the native microbial community through the chemical treatment step in multi-step process is not well understood or researched with UV photocatalysis with TiO_2. A multistep treatment approach coupling biological degradation with UV photocatalytic oxidation is the focus of this research, with nutrient addition to boost the native community's degradation capacity. Step one being nutrient addition to stimulate the native microbial community, step two being UV photocatalytic oxidation, and a third step of nutrient addition to allow the

surviving native microbial community the best conditions for biodegradation of organics produced in step two. Analysis showed that OPSW is limited in phosphorus (below detection limit of study analysis), and the addition of phosphorus improves degradation of otherwise thought recalcitrant NAFCs present in OSPW. Two treatments throughout the multistep treatment received no nutrient addition at all, these bottles showed no significant ($p>0.05$) NAFC degradation post oxidative step. Indicating that phosphorus and nitrogen are critical in NAFC degradation. Recovery of microbial diversity is a key finding to demonstrate that the microbial community can withstand the harsh oxidative stress of UV photocatalytic oxidation and continue to degrade NAFCs present. This experiment confirms that the microbial community benefits NAFC degradation with the addition of nutrients and can survive oxidative stress to continue to degrade OSPW NAFCs.

Preface

Chapter 3 of this thesis research results were published as Miles, S.M., Asiedu, E., Balaberda, A., Ulrich, A.C., 2020. “Oil sands process affected water sourced Trichoderma harzianum demonstrates capacity for mycoremediation of naphthenic acid fraction compounds.” *Chemosphere,* vol. 258, November 2020. 1277281. I was responsible for experimental plan, experimental set up, data collection and analysis, as well as manuscript composition. Asiedu, E., contributed through Orbitrap-MS analysis and preliminary data compiling, and contributed to the manuscript. Balaberda, A., contributed through data processing of kinetic analysis, and contributed to the manuscript. Ulrich, A.C., was the supervisory author and was involved with concept formation, and manuscript composition.

Some of the research conducted in chapter 4 and chapter 5 for this thesis forms part of a national research collaboration of the TERRE-NET research project 5a, led by Dr. Ania Ulrich at the University of Alberta in collaboration with Dr Frank Gu at the University of Waterloo, presently at University of Toronto. The experimental plan and set up were designed by myself and Dr. Ulrich. All matters pertaining to oxidation set up and conduction of UV photocatalytic oxidation with TiO_2 was designed, and conducted by Dr Tim Leshuk of University of Waterloo, presently at University of Toronto. The data collection and analysis in chapter 4 and 5 was conducted by myself. NAFC Orbitrap-MS analysis was conducted by Kerry Peru, and Dr. John Headley at the National Water Research Institute, Environment and Climate Change Canada. Data analysis was performed by myself. Bioinformatics processing in chapter 5 was conducted by Dr. Camilla Nesbø of University of Alberta. Chapter 1, chapter 2, and chapter 6 are my original work.

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Thank you to my collaborators, Dr. Tim Leshuk and Dr. Frank Gu for their contributions to this body of work. Thank you to Dr. John Headley and Kerry Peru for their Orbitrap-MS work on chapter 5. And thank you to Dr. Camilla Nesbø for her patience and guidance processing the

bioinformatics data in chapter 5. I have been very fortunate with graduate school funding and would like to thank the National Sciences and Engineering Research Council (Postgraduate Doctoral Research Scholarship), NSERC Toward Environmentally Responsible Resource Extraction NET grant, NSERC Toward Environmentally Responsible Resource Extraction CREATE grant, the Alberta Graduate Student scholarship, the Faculty of Engineering and Department of Civil and Environmental Engineering Scholarships (several administered through the department), and the University of Alberta (Presidents Doctoral Prize of Distinction) for their financial support.

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Chapter 1: Introduction

1.1 Introduction

The Alberta oil sands comprise one of the largest proven reserves of bitumen in the world. The production of bitumen requires substantial amounts of water, and results in excessive amounts of post extraction water that must be contained and remediated before being discharged back to the environment (Giesy et al., 2010). This post extraction water is commonly referred to as oil sands process affected water (OSPW) and stored in engineered tailings ponds. The regulation and remediation of these tailings ponds and contained OSPW is of particular importance in Alberta where the Alberta Environmental Protection and Enhancement Act (1993) introduced a zero discharge policy throughout the province. This policy bars the release of tailings and OSPW into the environment (Giesy et al., 2010). The regulation of tailings ponds, their associated environmental risks, and their compliance with the zero-discharge policy falls to Alberta Energy Regulator (AER), the government regulatory body for the province.

As of 2017, the total volume of accumulated tailings stored in Alberta's oil sands tailings ponds was estimated at 1 billion m^3 and occupied an area of approximately 176 km^2 (Foght et al., 2017; Vajihinejad et al., 2017). The main purpose of this storage is to allow time for clay and sand particles to settle out of the water fraction. Companies can then access the stored OSPW for reuse in the extraction process. This recycling process acts to reduce the amount of fresh water consumed during the production of bitumen and limits the volume of OSPW that requires storage, thus reducing the overall environmental impact of the industry.(Allen, 2008b; Giesy et al., 2010). However, with the repeated reuse of OSPW in bitumen extraction, the water becomes increasingly concentrated with environmental toxins such as salts, minerals, heavy

metals, residual bitumen, and organic compounds, including naphthenic acids (NAs) (Allen, 2008b). NAs are the largest contributors to the environmental toxicity of OSPW and so are of primary importance in studies of remediation techniques. (MacKinnon and Boerger, 1986; Holowenko et al., 2002; Clemente et al., 2004; Kavanagh et al., 2013; Marentette et al., 2015a).

Remediation techniques for OSPW are ever evolving in an effort to effectively and efficiently eliminate toxicity and allow OSPW to be safely reintroduced into the surrounding watershed. The remediation and reduction of tailings ponds has become a priority as the expansion of the oil sands industry has led to increased public awareness in relation to the industry's environmental risks; and demands for cleaner and more environmentally friendly options for consumers have consequently arisen. Traditionally the remediation of OSPW relied on either physical or chemical processes, however as biological options have been increasingly investigated, they have gained traction as an effective and viable remediation option.

Using the native microbial community to degrade and remediate the toxic organic fraction within OSPW is an appealing solution, however it can present some challenges. NAs have been shown to be resistant to biological degradation (Quagraine et al., 2005; Scott et al., 2005a), however recent studies have determined that microorganisms can indeed degrade NAs, but it is often slower than the available chemical treatments (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012).

OSPW contains native microbial communities that have naturally adapted to survive the harsh environment of these ponds (Siddique et al., 2006; Foght, 2015; Foght et al., 2017;

Richardson et al., 2019). Remediation studies focusing on these communities have demonstrated that they possess some capacity to degrade the toxic organic compounds from OSPW (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). A comprehensive characterization of OSPW microbial communities revealed the substantial genetic diversity present capable of resisting the harsh OSPW environment, including fungi (Richardson et al., 2019). Historically, bacteria and algae have been the focus of remedial efforts of NAs in OSPW but no work to date has been done on fungal species (Herman et al., 1994; Quesnel et al., 2011a; Johnson et al., 2013; Demeter et al., 2014; Mahdavi et al., 2015; Paulssen and Gieg, 2019; Yu et al., 2019). The use of fungi in remediating tailings water has never been proven possible. However the Ulrich research lab has recently isolated a fungus, *Trichoderma harzianum,* which may have the capacity for bioremediation applications (Miles et al., 2019). The low cost and negligible harmful by products generated make bioremediation an attractive solution to pursue (Allen, 2008b). Understanding the composition of microorganisms in these native communities, and which microorganisms carry the capacity to degrade organics such as NAs, will be key to developing an *in situ* bioaugmentation, or biostimulation treatment plan, and may provide essential information for *ex situ* biological treatment plans (Quagraine et al., 2005).

Therefore, the best option for remediation may be a combination of biological and chemical treatments. To-date co-remediation techniques for OSPW have focused on a pre-treatment ozonation step followed with a biological polishing step to remove residual organics (Scott et al., 2008; Hwang et al., 2013b). Advanced oxidation processes such as ozonation have been shown to effectively remove NAs in OSPW (Hwang et al., 2013a; Wang et al., 2013a; Afzal

et al., 2015). UV photocatalytic oxidation which has also been investigated as a cost effective, environmentally friendly, and efficient treatment option (Leshuk et al., 2016). However, regardless of the chemical process used the majority of NAs are removed quickly at the beginning of the process, leaving a small remaining portion of organics that require substantially more treatment to remove (Leshuk et al., 2016). Developing a multistep treatment plan which first uses the chemical process of UV oxidation to remove the bulk of NAs, and then subsequently makes use of microbial activity to degrade the smaller remaining fraction, could create an option that is less costly in both an economic and energy utilization sense that UV oxidation alone. This proposed multistep process of pairing advanced oxidation with biological degradation could be an effective remediation plan for NAs in OSPW. Investigation into optimization of OSPW treatment technologies through biological methods, and through coupling of advanced oxidation processes, is key to discover a remediation strategy suitable to eliminate OSPW toxicity.

1.2 Overall Objectives

With the continual development of the oil sands region, Alberta's oil and gas industry has become heavily scrutinized both domestically and on an international scale. An increased effort to limit the future environmental impact from this industry, as well as rectify ongoing environmental concerns will contribute to the reestablishment of Canada as a leading voice on environmental stewardship. The objective of this research is to contribute to the framework needed in order to create energy efficient remediation efforts and limit the lasting impact of the oil sands industry in the region. As such, this work focuses on optimizing remediation

treatments for OSPW sourced organic fractions, NAs. The specific research questions and hypotheses are highlighted:

Objective 1. NAFC degradation characterization by Trichoderma harzianum

Research Questions: Can an OSPW sourced fungus *Trichoderma Harzianum* degrade NAFCs and decrease OSPW toxicity? Can an OSPW sourced fungus *Trichoderma Harzianum* degrade model NAFC compounds with varying structures?

Hypothesis: It is hypothesized that the aerobic microbial isolate to be tested in this research, *Trichoderma Harzianum* has the capacity to remediate NAFCs and reduce the toxicity of OSPW since it was isolated directly from OSPW and continuously exposed to NAFCs (Miles et al., 2019). Studies have also shown that fungus such as *Trichoderma asperellum* have the capacity to degrade complex organics (Zafra et al., 2014). In addition it is hypothesized that *Trichoderma Harzianum* will be able to degrade the simpler ring structure of cyclohexane carboxylic acid, but will struggle with the more complex diamondoid structure of 1-adamantane carboxylic acid. In general, the most recalcitrant NAFC to biodegradation have structures consisting of more highly branched alkyl chains or diamondoid structures (Han et al., 2008; Smith et al., 2008; Rowland et al., 2011a). Several studies have shown that structure affect microbial degradation of NAFCs, specifically location and extent of side branching & Martin, 2008; Misiti, Tezel, & Pavlostathis, 2014; Smith, Lewis, Belt, Whitby, & Rowland, 2008).

This project is novel as no research has been done on fungi degrading NAFCs.

Objective 2. Coupling solar photocatalytic oxidation and microbial degradation of NAFCs

Research Questions: Will the addition of nutrients to OSPW improve the native community's ability to degrade recalcitrant organics present? Can the native microbial community present in OSPW survive the oxidation process and continue to degrade NAFCs? Does photocatalytic oxidation treatment of OSPW breakdown organics into substrates that are biodegradable to the native community in nutrient limited or optimized conditions?

Hypothesis: It is hypothesized that a multistep treatment of OSPW will provide optimal degradation and toxicity reduction in OSPW. Addition of an UV photocatalytic oxidation step will provide more biodegradable NAFCs for the native microbial community. It has been established that solar photocatalysis can degrade NAFCs in time with 80% of degradation occurring during the first 6 hours of treatment (~9 M/m2 insolation), but 14 hours is required for total removal (25MJ/m2 insolation) (Leshuk et al., 2016). Therefore, the use of a secondary treatment that has better energy conservation, such as biological degradation, can be used to increase efficiency.

As OSPW is nutrient limiting, the addition of mineral media can increase the community's ability to degrade NAFCs. It is hypothesized that nutrient addition increases the microbial community's ability to degrade NAFCs within the untreated OSPW, therefore otherwise thought recalcitrant NAFCs will be degraded over time. Treatments lacking nutrient addition would show limited NAFCs degradation. Coupling of advanced oxidation with microbial degradation has been demonstrated possible with ozonation, with the native community surviving the oxidative stress (Brown et al., 2013). With a multistep approach, it's hypothesized that the native microbial community can survive the oxidation treatment and continue on to degrade the remaining

organics within the OSPW in step three. This multistep process will be able to degrade NAFCs more efficiently than each individual treatment alone. Step one ensures all organics left in OSPW are recalcitrant to biodegradation. Therefore, if the second step of chemical oxidation provides more biodegradable organics, then any NAFCs biodegradation in step three would therefore be evidence of step two providing more biodegradable NAFCs.

This project is novel as there has been no study done on the coupling of solar photocatalytic treatments with biological treatments targeting NAFCs within native communities in OSPW.

1.3 Organization of thesis

Chapter 1 provides a brief introduction to the topic of research, objectives and hypotheses, chapter 2 reviews relevant literature in support of this research, chapter 3 through 5 address the research questions as highlighted above, chapter 6 provides the conclusions recommendations for future work.

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Chapter 2: Literature Review

2.0 Literature Review

This chapter focuses on the background literature covering the oil sands industry, treatment technologies utilizing biological or advanced oxidation of oil sands process affected water (OSPW) sourced naphthenic acids (NAs), and commercial/model NAs. In addition, the coupling of biodegradation with advanced chemical oxidation will be explored.

2.1 Oil Sands Industry

The Athabasca, Cold Lake and Peace River oil sands in Northern Alberta, Canada, are one of the largest proven reserves of oil in the world (Alberta, 2008). There are an estimated 1.69 billion barrels (269.2 m^3) of recoverable bitumen in Alberta's oil sands region (ERCB, 2013). A portion of these deposits are quite shallow with less than 75m of overburden and are currently being produced through surface mining operations (Chalaturnyk et al., 2002). The location of mining companies, and associated tailings ponds along with surrounding surface watershed are shown in Figure 2-1. It should be noted that the main river in the region, the Athabasca river, flows from the south to the north.

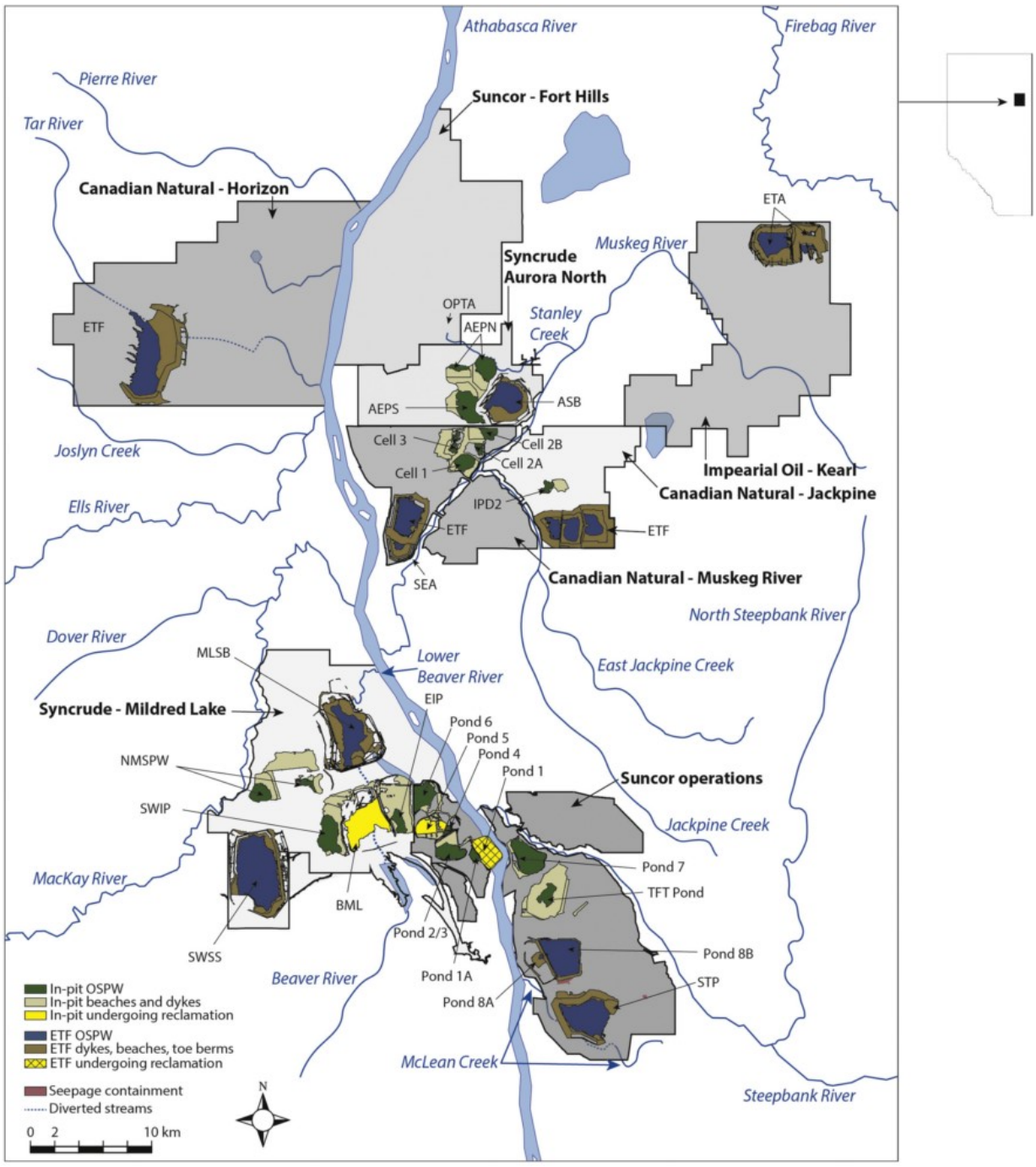


Figure 2-1: Location of oil sands mining leases and surrounding watershed, with location and names of tailings ponds in 2016. (Taken from (Fennell and Arciszewski, 2019))

With surface mining, the raw oil sand mixture is extracted using a modified Clark Hot Water extraction process. This process separates the bitumen clay from the sand particles using

a combination of heat and chemicals (Dzidic et al., 1988; Allen, 2008a). In this process, approximately 2.21 m^3 of water is used for each 1 m^3 of bitumen that is extracted (Canada, 2004). Given the production of 112 million m^3 of bitumen per year (Energy Resources Conservation Board, 2013) approximately 3.3 m^3 of raw tailings are created (Gosselin et al., 2010). This post extraction water is termed OSPW, and is stored in engineered tailings ponds in accordance with Alberta's zero discharge policy introduced by the Alberta Environmental Protection and Enhancement Act (1993) (Giesy et al., 2010). The ponds allow time for the clay and sand particles to settle out, making it possible to reuse the OSPW in future extraction processes, minimizing the need for additional fresh water use (Allen, 2008a, b). With the reuse of OSPW, the tailings ponds become increasingly concentrated with compounds from the extraction process including salts, minerals, heavy metals, bitumen and organic compounds such as NAs (Allen, 2008a, b). As of 2017, the total volume of accumulated tailings stored in these tailings ponds was estimated at 1 billion m^3 and occupied an area of approximately 176 km^2 (Foght et al., 2017; Vajihinejad et al., 2017).

Regulation of this region and oil sands development falls under the administration of Alberta Energy Regulator (AER). The purpose of this regulator is to ensure the safe, efficient and environmentally friendly development of all non-renewable resources in Alberta, Canada (AER, 2020). There are several legal regulations that are administered by AER in the oil sands region to protect the environment, specifically the Oil Sands Conservation Act, Environmental Protection and Enhancement Act and the Water Act. AER has released several specific directives for the management of oil sands tailings over time, Directive 074: Tailings Performace Criteria and Requirements for Oil Sands Mining Schemes in 2009, which set out new

requirments at the time for the regulation of tailings operations and specified performance criteria for reduction of fluid tailings. This directive was replaced with Directive 085: Fluid Tailings Management for Oil Sands Mining Project in 2016 and updated in 2017. The new directive shifted to how fluid tailings reduction is determined, measured and reported, with more emphasis on reduction on new tailings, and management of old tailings (AER, 2020). Increased government regulation on limiting the environmental impact of oil sands development, and remedating these vast stores of OSPW and tailings, are the motivation for this work.

2.2 Naphthenic Acids

Although there are many compounds of concern found in OSPW, NAs are often the focus of remediation efforts and research attention. NAs are defined as a complex mixture of cyclic and aromatic alkanes with a carboxylic acid moiety (Dzidic et al., 1988; St John et al., 1998). NAs are naturally present in bitumen and become solubilized and concentrated in OSPW during the extraction process (Frank et al., 2008; Yang et al., 2019). Natural bitumen seeps can be found in the region along the Athabasca river. NA concentrations in the river systems surrounding the tailings ponds and development therefor could have a natural source of NA exposure, not from tailings seepage. Characterization of groundwater, and surface water has found concentrations ranging from 30.1 µg/L to 190 µg/L to 2000 µg/L (Sun et al., 2017). Classical NAs are described as O_2^- species and denoted by the general formula $C_nH_{2n+z}O_2$, where *n* represents the carbon number and *Z* represents the number of hydrogen atoms in the structure which are replaced (substituted) by the presence of a ring and/or double bond (Dzidic et al., 1988; Fan, 1991; St John et al., 1998). Figure 2-2 shows the various possible structures

classical NAs can take. With advancements in high resolution analytical techniques, the definition of OSPW-sourced organics has been expanded to not only include classical NAs (O_2^- species) but also complex mixtures of oxidized organics that contain three or more oxygen-atoms, sulphur and nitrogen heteroatoms, and di-, tri-, tetra-, and pentacyclic diamondoid acids. This group of organic compounds found in OSPW is now termed naphthenic acid fraction compounds (NAFCs) (Grewer et al., 2010; Rowland et al., 2011a; Rowland et al., 2011b; Lengger et al., 2015; Wilde et al., 2015). With over 200,000 structures identified in association with NAFCs they are more chemically diverse than classical NAs, such as those found in commercial mixtures like Merichem NAs, which consist solely of O_2^- species. As a result, NAFCs have different properties and toxicity profiles than the classical NAs (Scott et al., 2005a; Grewer et al., 2010; Quesnel et al., 2011a; Hughes et al., 2017). Commercial mixtures such as Merichem NAs are often used within research as it is a focused mixture on O_2^- species, as well as model mixture that can be compared across research studies. Regardless of the NAFCs source, the majority of studies attribute the toxicity of OSPW directly to classical NAs (O_2^- species) (Morandi et al., 2017).

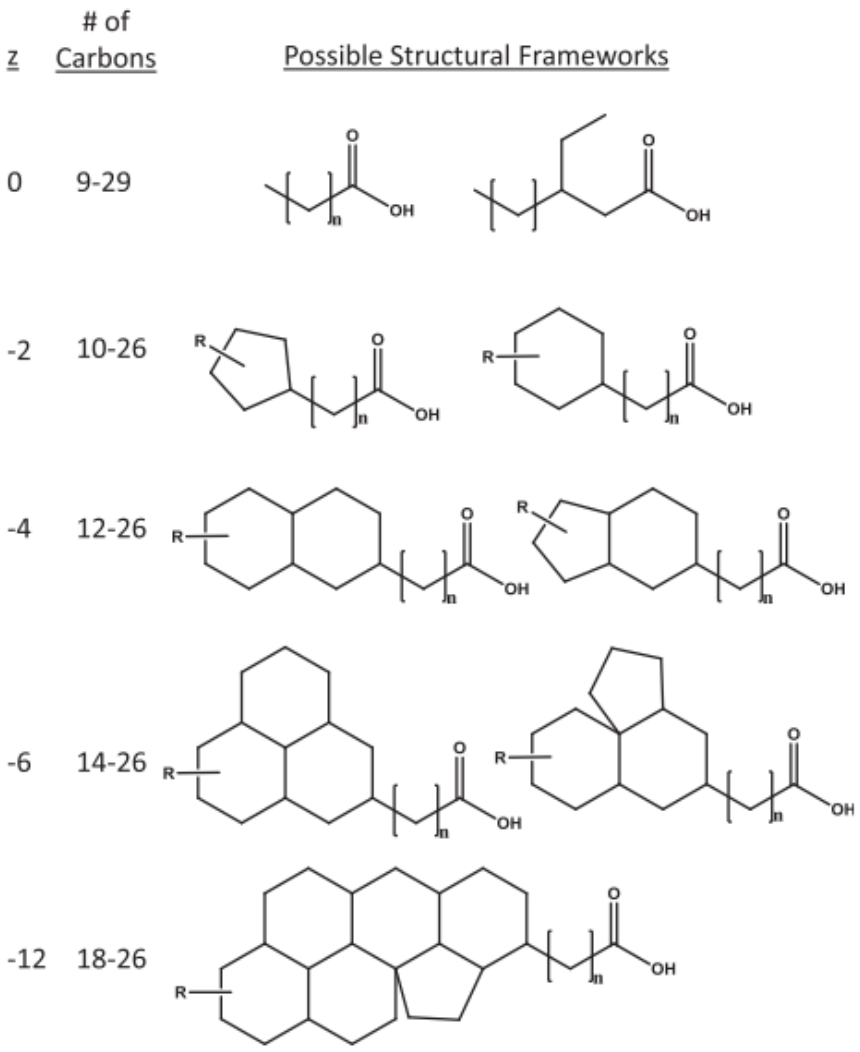


Figure 2-2: Examples of classical NAs found in OSPW, where Z- represents the hydrogen loss due to ring formation, *n* is carbon number, and R is the functional group. Non classical NAs contain nitrogen, sulfur and oxygen in the R group (Goff et al., 2014).

NAFCs are polar organic carboxylic acids that are a natural fraction found in the bitumen reserves and are solubilized during the extraction process (MacKinnon and Boerger, 1986). The concentration of NAFCs found in tailings ponds varies greatly (<1 mg/L to 120 mg/L) depending on the operator, the age of the tailings pond and the type of extraction process used (Holowenko et al., 2002; Scott et al., 2005a; Frank et al., 2008). Age of the tailings water is a

particularly important factor as degradation of NAFCs occurs naturally over time, leaving a larger proportion of high molecular weight organics in the Z=-4, Z=-6 species (Grewer et al., 2010; Whitby, 2010). This presents a unique challenge for remediation as each tailings pond has a distinctive NAs profile and associated toxicity (Holowenko et al., 2002).

2.2.1 NAFC Toxicity

NAFCs are the principle group of compounds within OSPW that have been determined to contribute extensively to toxicity (MacKinnon and Boerger, 1986). As OSPW contains a multitude of potentially toxic components, like high salt and heavy metal concentrations, determining whether toxicity is attributed to the organic or inorganic factors is important. Qin et al. (2019) separated the inorganic and organic fractions within OSPW and assessed *in-vitro* bioassays with mouse macrophage cells lines. They found the organic fraction within OSPW contributed significantly to toxicity whereas the inorganic fraction displayed no significant cytotoxicity or effect on metabolic activities of the cell (Qin et al., 2019). Although Qin et al. (2019) found that the organic fraction was the main contributor to toxicity, Miles et al. (2019) found that OSPW on a whole was more toxic (via Microtox™ bioassay) than the NAFC fraction alone, suggesting a synergistic effect was occurring to compound the toxic effects of the NAFCs. The mechanism of toxicity was also investigated by measuring the effect of NAFCs on the membrane fluidity of several microbial isolates from OSPW. Little to no membrane disruption occurred, indicating that the method of toxicity is not reliant on membrane disruption as previously speculated (Miles et al., 2019).

Measuring toxicity is often difficult and expensive, but the use of Microtox bioassay is a relatively inexpensive and quick option to test the toxicity of a sample against a single bioluminescent bacteria, *Vibrio fisheri* (Holowenko et al., 2002; Frank et al., 2008; Gamal El-Din et al., 2011; Wang et al., 2013b; Leshuk et al., 2016). This testing gives quick and easy results to allow for comparisons to the literature and to compare the relative shifts in toxicity for any given treatment. However, it is still important to measure the toxicity on both invertebrates and vertebrates to give a full characterization of the toxic impact of a compound in the environment. Kavanagh et al. (2013) determined that both high (> 25 mg/L) and low (~10 mg/L) concentrations of NAFCs adversely affect the reproductive physiology of fathead minnows in the oil sands area. Several other studies show that mammals, trees, fish and microorganisms are all susceptible to NAFC toxicity (MacKinnon and Boerger, 1986; Holowenko et al., 2002; Clemente et al., 2004; Marentette et al., 2015b). A review paper by Li et al. (2017) summarizes the extensive OSPW toxicity studies done on in vitro (bacterial, single cells, etc.), and *in vivo* (invertebrates and vertebrates like fish, amphibians, mammals (rats and mice)). Overall the review determined that toxic effects of OSPW are species specific, and toxicity responses in prokaryotic organisms may not be applicable to eukaryotic organisms (Li et al., 2019). Route of entry (dermal sorption, ingestion, inhalation) is also a vital factor, as exposure types will vary between species which can lead to potential differences in toxicity values. *In vitro* tests only expose single cell types to the NAFCs and so the results may not necessarily relate directly to living or complex biological systems. However, *in vitro* tests such as Microtox bioassay hold the advantage of providing a quick and inexpensive way to measuring relative toxicity changes.

Most studies investigate the overall toxicity of OSPW, and while it is important to determine toxicity of the whole, determining which portions of the OSPW organics fraction contributes to toxicity is crucial to understanding targeted remedial efforts. Scarlett et al. (2013) found that although alicyclic acids are toxic with an LC_{50} of 13.1 mg/L, the higher molecular weight aromatic acids are more toxic with a LC_{50} 8.1 mg/L. Hughes et al. (2017) compared the toxicity of different fractions of OSPW organics to rainbow trout (*Oncorhynchus mykiss*) and found that the O_2^- species were primarily responsible for toxicity. Although the majority of classical NAs present were compounds with a carbon number ≤16, the toxicity mainly corresponded to the larger (i.e. higher molecular weight), more complex compounds with ≥17 carbons indicating a higher priority for remediation (Hughes et al., 2017). These results are supported by a recent study by Morandi et al. (2015) that determined classically defined NAs (O_2^-) contribute most significantly to toxicity, but non-acidic species (O^+, O_2^+, SO^+, NO^+) also contribute. Based on this study, remediation methods must include addressing all NAFCs species, not just classical NAs or (O_2^- species). Clearly, the unique components in NAFCs play a role in toxicity, and therefore remediation methods must aim to address the complete mixture of organic compounds within OSPW. Due to the complex nature of NAFCs in OSPW, currently no environmental guidelines for concentration are set for these compounds. As remediation technologies are developed, it will be critical for engineers and scientist to have a guideline to use in designing treatment programs. When these guidelines are determined, the enforcement and administration of the policy will fall to AER.

2.3 Remediation Techniques

Several different remediation approaches have emerged to treat OSPW and its associated organic toxicity. Treatment technologies have traditionally utilized a chemical approach such as ozonation(Wang et al., 2013a), UV photocatalysis (Leshuk et al., 2016), and potassium ferrate (VI) (Wang et al., 2016), or physical processes through the use of adsorbents such as activated carbon (Niasar et al., 2019). While these treatments are fast acting and reliable, they may create unwanted by-products and be cost prohibitive for the large-scale treatment needed for OSPW. Alternatively, with the abundance of organic compounds found in tailings ponds, microbial communities are very active and diverse, indicating that *in situ* bioremediation is a plausible method for treating OSPW toxicity (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). The potential for coupling of biological and chemical treatments would potentially yield the most efficient and effective treatment option for the vast volumes of OSPW present.

2.3.1 Microbial Degradation

Several studies have focused on the process of microbial degradation of NAFCs in OSPW and have suggested that in situ bioremediation of organic acids is a plausible method for the treatment of OSPW (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007). However, studies can use NAFCs from varying sources which will impact results, for example degradation of NAFCs is much slower than that of model or commercial NAs (Clemente et al., 2004; Scott et al., 2005a). It has been suggested that only a fraction of NAFCs are biodegradable, with an enrichment of high molecular weight and ringed

compounds over time as the lighter compounds are degraded (Holowenko et al., 2002). Direct analysis of NAFCs in tailings ponds of various ages show very slow degradation profiles, with a reduction of lower carbon number (< 22 carbons) NAFCs and an increase of compounds containing greater than 22 carbons (Holowenko et al., 2002). Numerous studies therefore use model NAs and commercial NAs to determine the effect of NA structure on the capacity for biodegradation (Han et al., 2008; Smith et al., 2008; Johnson et al., 2011). Recalcitrant compounds have been found in OSPW containing a higher degree of alkyl branching, making them less biodegradable for microbial alteration (Han et al., 2008; Smith et al., 2008; Toor et al., 2013c). With bioremediation being a practical and economical option for treatment of OSPW, many studies have focused on the ability of various microbial cultures and isolates to degrade NAs and model compounds. Table 2-1 gives a summary of several key studies using microorganisms, whether communities naturally found in OSPW or soil; or isolates, either purchased or isolated directly from OSPW/contaminated soils.

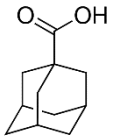
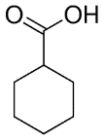
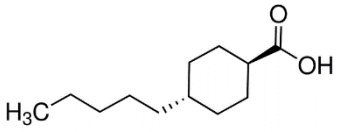
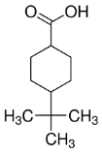
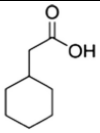
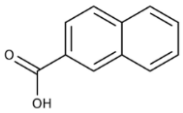
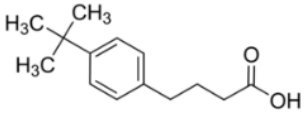
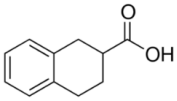
Table 2-1: Summary of select aerobic NAFC/ model NAFC biodegradation studies

Substrate	Microbial source	Citation
Four butylcyclohexylbutanoic acids: n-BCHBA, sec-BCHBA, iso-BCHBA, tert-BCHBA	Petroleum exposed sediments	Smith et al. (2008)
Merichem	Rhizosphere sourced microorganisms, 5 plants used, Paper birch used for profile	(Biryukova et al., 2007)
Cyclohexane Carboxylic acid, Cyclohexane acetic acid, Cyclohexane propionic acid, Cyclohexane butyric acid, 1,2,3,4-tetrahydro-2-naphthonic acid	*Dunaliella tertiolecta* (purchased)	(Quesnel et al., 2011a)
Kodak Naphthenic acid sodium salts, MLSB extract, hexadecane, palmitic acids, cyclohexane carboxylic acid, (±)2-methyl-1-cyclohexane carboxylic acid, cyclohexane pentanoic acid), trans-4-pentylcyclohexane carboxylic acid	MLSB sourced culture, two additional cultures either enriched on Kodak NAs, or MLSB extract	(Herman et al., 1994)
Cyclohexane carboxylic acid, and cyclohexane acetic acid	Culture from OSPW, Biofilm culture, as well as a planktonic culture	(Demeter et al., 2014)
cyclohexanecarboxylic acid, cyclohexaneacetic acid, and cyclohexanebutyric acid	OSPW sourced microbial culture, algae isolates *Chlorella kessleri* and *Botryococcus braunii* and in co-culture	(Yu et al., 2019)
Suncor OSPW (drainage adjacent to Pond 2/3) Syncrude OSPW (seepage dyke adjacent to MLSB) Merichem used as positive control	Sediment from non-oil sands impacted wetland	(Toor et al., 2013a)
4 Commercial mixes: Kodak naphthenic acids, Kodak naphthenic acids sodium salt, Merichem, and Fluka naphthenic acids	Suncor OSPW culture (consolidate tailings pond) or Syncrude OSPW (West in Pit)	(Scott et al., 2005a)

Commercial NAs: Kodak naphthenic acids sodium salts, and Merichem	MLSB OSPW culture	(Clemente et al., 2004)
Model compounds: (4'-n-butylphenyl)-4-butanoic acid, (4'-iso-butylphenyl)-4-butanoic acid, (4'-sec-butylphenyl)-4-butanoic acid, and (4'-tert-butylphenyl)-4-butanoic acid. (4'-n-butylphenyl)ethanoic acid, (4'-iso-butylphenyl)ethanoic acid, and (4'-tert-butylphenyl)ethanoic acid	Coal tar exposed sediment mix culture	(Johnson et al., 2011)
Model Compounds: (4'-n-butylphenyl)-4-butanoic acid, and 4'-t-butylphenyl)-4-butanoic acid	*Mycobacterium aurum* (isolated from hydrocarbon contaminated soil)	(Johnson et al., 2012)
Model Compounds: (4'-n-butylphenyl)-4-butanoic acid and 4'-t-butylphenyl)-4-butanoic acid	*Pseudomonas putida* (purchased)	(Johnson et al., 2013)
Commercially available: Kodak naphthenic acids Extract from wetland sites with seepage from Syncrude and Suncor tailings ponds. Model compounds: ^{14}C-labelled cyclohexane carboxylic acid, and decahydro-2-naphthoic acid-8-^{14}C	Culture enriched from wetland sediment exposed to OSPW *Pseudomonas putida*, and *Pseudomonas fluorescens*	(Del Rio et al., 2006)
adamantane-1-carboxylic acid, and 3-ethyladamantane-carboxylic acid	OSPW sourced microbial community	(Folwell et al., 2019)

Model compounds are often used in degradation studies as an analog of compounds found in OSPW, providing a clear single compound to determine the possible effect of structure on biodegradation. A few commonly used model compounds and structure are given in Table 2-2, and commonly used model compounds are summarized in Table 2-1 in association with degradation studies. Cyclohexane carboxylic acid (CHCA) is frequently used as an organic substrate that is a simpler carbon source and has demonstrated to be readily degradable by microorganisms native to OSPW (Herman et al., 1994; Del Rio et al., 2006; Demeter et al., 2014; Yu et al., 2019). Conversely, 1-adamantane carboxylic acid (ADA) has frequently been demonstrated to be recalcitrant to biodegradation (Demeter et al., 2015; Frankel et al., 2016). With the surge of interest in utilizing bioremediation for reduction of OSPW toxicity, greater emphasis needs to be placed on finding microorganisms capable of degrading the more complex organics in OSPW, such as ADA.

Table 2-2: Commonly used model NAFCs used in degradation studies.

Model Compound	Formula	Structure	Reference
Adamantane-1-Carboxylic Acid	$C_{11}H_{16}O_2$		(Rowland et al., 2012, 2011)
Cyclohexane Carboxylic Acid	$C_7H_{12}O_2$		(Demeter et al., 2014; Herman et al., 1993)
Trans-4-Pentylcyclohexane-1-Carboxylic Acid	$C_{12}H_{22}O_2$		(Pérez-Estrada et al., 2011)
4-Tert-Butylcyclohexane Carboxylic Acid	$C_{11}H_{20}O_2$		(Pérez-Estrada et al., 2011)
Cyclohexane Acetic Acid			(Demeter et al., 2014)
decahydro-2-naphthoic acid	$C_{11}H_{18}O_2$		(Del Rio et al., 2006)
4-(4-tert-butylphenyl) butanoic acid	$C_{14}H_{20}O_2$		(Johnson et al., 2011)
1,2,3,4-tetrahydro-2-naphthoic acid	$C_{11}H_{12}O_2$		(Quesnel et al., 2011a)

Few studies have investigated the biodegradation potential of ADA with microbial communities native to OSPW. Paulssen et al. (2019) evaluated the ability of a native photosynthetic microbial community found in OSPW to degrade ADA. They found that

microbial communities dominated by algae of the order *Chlorella/Chlorellales* and genus *Actuodesmus (Scenedesmus)* (~90% relative abundance) removed approximately 80% of ADA in 90 days, demonstrating that biodegradation of diamondoid NAFCs is possible by photosynthetic communities native to OSPW (Paulssen and Gieg, 2019). A study by Folwell et al. (2019) also found that communities sourced from OSPW could degrade ADA and 3-ethyl adamantane carboxylic acid. During degradation, two metabolites were found to be produced: 2-hydroxyadamantane-1-carboxylic acid and 2-ethyladamantane-2-ol. However, accumulation was less than predicted indicating the community was also degrading the metabolites (Folwell et al., 2019). The advantage of microbial community dynamics is in the synergistic relationships amongst microorganisms, whereas an isolate must either be provided with or create all co-factors needed for efficient degradation of substrates and prevent dead end metabolites (Demeter et al., 2015).

Studies using microbial isolates found in OSPW can often enhance degradation of NAFCs or model compounds by using a co-culture. Yu et al. (2019) studied the ability of algae isolates *Chlorella kessleri*, *Botryococcus braunii* and OSPW microbial community, both as a co-culture and on their own to degrade three model compounds cyclohexane carboxylic acid, cyclohexeneacetic acid and cyclohexanebutyric acid. It was found that degradation of cyclohexanebutyric acid and cyclohexane carboxylic acid was improved with co-culture of *C. kessleri* with the OSPW microbial community. Additional benefits found were the microbial diversity in the OSPW culture was vastly improved, enriching known hydrocarbon degraders like *Brevundimonas* sp. (Rochman et al., 2017), *Hydrogenophaga, Parvibaculum , Pseudofulvimonas* and *Hyphomonas* (Song et al., 2018). With the added biodiversity and an

effective isolate capable of degradation, removal was improved further supporting that microbial communities provide a synergistic environment for optimizing degradation of recalcitrant organic compounds.

2.3.2 Mycoremediation

Bioremediation strategies generally focus on the bacteria or algae present, often overlooking the potential remediation power of fungi. Numerous studies in different fields have indicated that fungal species are often present at sites of contaminated with complex organic compounds and possess the capacity for degradation, however no studies have been done with NAFCs (Obuekwe et al., 2005; Mohsenzadeh et al., 2009; Zafra et al., 2014; Lee et al., 2015; Andreolli et al., 2016; Marchand et al., 2017).

Marchand et al. (2017) investigated the microbial diversity at a highly petroleum contaminated soil from a former petrochemical plant and characterized degradation potential of 95 bacterial and 160 fungal isolates identified. *Fusarium oxysporum* and *Trichoderma tomentosum* significantly degraded all polycyclic aromatic hydrocarbons (PAH) compounds tested (anthracene, phenanthrene, fluorene, and pyrene. *Sordariomycetes* has often demonstrated high affiliation with hydrocarbon degradation, fungi species studied belonging to *Sordariomycetes* class, *Trichoderma* and *Fusarium* were found to be more efficient degraders than those of other classes studied (Hong et al., 2010; Wu et al., 2010; Argumedo-Delira et al., 2012). A study conducted by Argumedo-Delira et al. (2012) tested the tolerance of 11 strains of *Trichoderma* to naphthalene, phenanthrene, and benzo(α)pyrene. Several strains of *Trichoderma* tested (species not described) were capable of tolerating concentrations of

phenanthrene and naphthalene above 250 mg/L, and 100 mg/L of benzo(α)pyrene. Although established potential for PAH remediation by fungi is present, enzymatic activity and pathways are not well understood.

Andreolli et al. (2016) isolated *Trichoderma longibrachiatum* from uncontaminated forest soil through selective enrichment for hydrocarbon degraders. In a diesel contaminated soil microcosm, *Trichoderma longibrachiatum* inoculated soil demonstrated the fastest removal of $C_{12\text{-}40}$ hydrocarbon fraction at 54.2±1.6% in 30 days, compared to 7.3±6.1% removal in controls (Andreolli et al., 2016). Additionally, Andreolli et at. (2016) characterized the potential for PAH removal, with 69-71% removal of phenanthrene, anthracene, pyrene and fluoranthene, indicating that *Trichoderma longibrachiatum* was a strong potential hydrocarbon degrader found in a non-hydrocarbon contaminated environment.

Fungal species are found in all environments, a comprehensive study by Richardson et al. (2019) revealed a substantial genetic diversity of the microbial community present in OSPW. Analysis of 18S rRNA revealed that, although limited in classification below the phylum level, two of the most abundant operational taxonomic units (OTU's) of the entire dataset were fungi (Richardson et al., 2019). This major presence of fungal activity within the water fraction of the tailings pond indicates that fungi possess the capacity to resist the harsh OSPW environment and as such there is an opportunity to understand their potential for mycoremediation. Repas et al. (2017) isolated *Trichoderma harzianum* from plant roots growing in coarse tailings, and found it had the capacity to remediate complex petrochemical residues present in the tailings, indicating the potential for novel fungal remediation of NAFC within OSPW. The fungal isolate *T.*

harzianum was isolated in OSPW by Miles et al. (2019) and demonstrated the ability to withstand high salinity conditions (≥60 g/L), a pH range of 2-9, and a NAFC inhibitory concentration of 2400 mg/L. Further testing revealed this OSPW sourced isolate was able to grow on a single pure drop of Merichem NAs as its sole source of carbon on an agar plate, indicating a strong potential for NAFC remedial efforts through mycoremediation (Miles et al., 2019). With vast fungal diversity and ubiquitous presence in the environment, in situ mycoremediation is a compelling method for organic contaminants such as NAFCs.

2.3.3 Advanced Oxidation Treatment

A reliable and relatively quick method for the remediation of compounds in a water matrix is the use of chemical treatment through advanced oxidation processes. It has been found that advanced oxidation treatment of NAFCs in OSPW is especially effective method of treatment (Liang et al., 2011; Afzal et al., 2012; Drzewicz et al., 2012; Afzal et al., 2015; Livera et al., 2018). This method degrades target compounds through a series of ionic or radical reactions, involving an oxidant that either accepts electrons or donates electron-accepting groups (Allen, 2008b). The most frequently used oxidants are hydrogen peroxide (Fenton based reaction) (H_2O_2), ozone (O_3), and persulfate (Allen, 2008b). A strong pathway for oxidation is the formation of hydroxyl radicals; however, it is difficult to maintain this in complex systems of compounds due to reactivity, so additional enhancements (for example metal catalysts) are required to sustain the redox potential. Under appropriate conditions, the end products of advanced oxidation processes consist of CO_2 and H_2O (complete mineralization). However low molecular weight carboxylic acids and other organics can remain depending on the strength and type of oxidation process (Xu et al., 2017). Oxidation of NAFCs is a complex system of

multiple reactions involving radical species, target organics, and intermediates of the reaction pathway.

Ozonation is a prevalent treatment researched for treatment of OSPW. Ozonation reactive species come from the O_3, creating $O_3^{\bullet -}$ and $HO^{\bullet}$ radicals which attack the organics in OSPW (Xu et al., 2017). Studies have found that ozonation will oxidize higher molecular weight NAs in OSPW. Several studies have concluded that ozonation will degrade a higher proportion of higher molecular weight NAFCs, while also creating lower molecular weight NAFCs (Scott et al., 2008; Perez-Estrada et al., 2011; Wang et al., 2013a).

Another common well-known oxidation process is Fenton-based reactions, which us H_2O_2 as a reagent with Fe^{2+} as a metal catalyst. The reaction of this process produces Fe^{3+} and $HO^{\bullet}$ radicals, and a secondary reaction regenerates Fe^{2+} through reaction of H_2O_2 with Fe^{3+} to form $HOO^{\bullet}$ (hydroperoxyl radical). These radicals then go one to oxidize target NAFCs in OSPW. Applications of Fenton reactions requires a pH around 3 to proceed, however the addition of iron chelates have been used for the activation of H_2O_2 and prevent iron precipitation (Xu et al., 2017). Zhang et al. (2016) determined that chelation by nitrilotriacetic acid with H_2O_2 degraded model NAFC cyclohexane carboxylic acid. It was found that hydroxyl radicals were the main species responsive for oxidation.

The use of active persulfate has also been proposed as an advanced oxidation treatment of NAFCs in OSPW (Drzewicz et al., 2012; Xu et al., 2016). The ability of persulfate to generate radicals is an important factor for its treatment potential. There are a several ways to activate persulfate such alkaline activation (elevating the pH above 10) (Tsitonaki et al., 2010).

Activation is possible through hydrogen peroxide addition, thermal activation (above 35°C) and metal activation (addition of Fe, Cu or Ag) is also possible (Tsitonaki et al., 2010). Alternatively un-activated persulfate has recently been investigated in the treatment of NAFCs in OSPW, however this is not an advanced oxidation process as no radicals are produced or responsible for oxidation (Sohrabi et al., 2013).

2.3.4 UV photocatalysis with TiO_2

Advanced oxidation has been studied extensively as a remediation technology for inorganic and organic compounds in both wastewater and contaminated groundwater (Oturan and Aaron, 2014). Advanced oxidation processes discussed above are effective treatments however un-intended by-products are possible, more chemicals or chelating agents may be required to effectively overcome drawbacks of OSPWs properties like high pH (Zhang et al., 2016). UV photocatalysis with TiO_2 is an effective and powerful advanced oxidation treatment that is emerging as a process to treat NAFCs (Headley et al., 2009; Leshuk et al., 2016). Benefits of this process over other advanced oxidations processes are that TiO_2 itself is chemically stable and biologically inert for easy transport and application, TiO_2 can be recovered and reused, UV can be passive treatment using solar radiation, TiO_2 is relatively low cost (Leshuk et al., 2016).

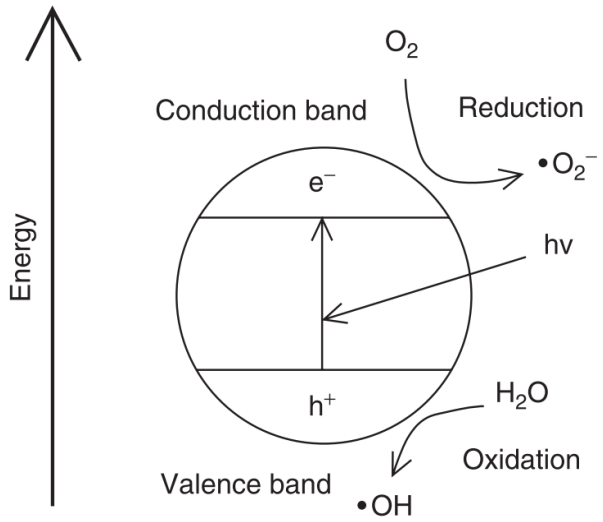


Figure 2-3 : Schematic representation of photopromotion of an electron, reduction of oxygen and oxidation of water during photocatalysis with TiO2 (taken from (Castellote and Bengtsson, 2011)).

As TiO_2 is a semiconductor, its surface can be described with band theory to have a conduction band and a valence band, with a small gap between (Figure 2-3). For photocatalysis to occur, light (*hv*) provides energy to the bands, and once the energy threshold is met (3.2eV) for anatase, corresponds to photons with wavelength of 388 nm), an electron is promoted from the valence band to the conduction band leaving behind a hole (h^+). As shown in Figure 2-3 there are two sites possible for oxidation and reduction where a hydroxyl radical can be formed (equation 1), and a superoxide (equation 2) (Castellote and Bengtsson, 2011).

$$H_2O + h^+ \rightarrow \cdot OH + H^+ \qquad \text{(Equation 1)}$$

$$O_2 + e^- \rightarrow \cdot O_2^- \qquad \text{(Equation 2)}$$

Based on these two reactions, the hydroxyl radical and superoxide can then go on in solution to oxidize the target compound. Additionally, any compound on the surface of the TiO_2 can also be directly oxidized (Castellote and Bengtsson, 2011; Oturan and Aaron, 2014). Although UV photocatalysis with $Ti0_2$ is an emerging treatment option, a few studies have indicated that photocatalysis with TiO_2 is a very promising treatment for NAFCs (Headley et al., 2009; Leshuk et al., 2016). Leshuk et al. (2016) has shown to completely mineralize target organics (OSPW, 43.3mg/L NAFC content) within a 14h period with 25 Mj/m^2 exposure and a TiO_2 loading rated of 0.5g/L. This treatment eliminated toxicity as measured through Microtox® bioassay. Leshuk et al. (2016) found that this process follows apparent first order kinetics. Approximately 80% of the organics mineralized within the first 6 hours of treatment exposure, with the remaining 20% taking 8h to remove. Therefore, the use of a secondary treatment that has better energy conservation, such as biological degradation, can be used to increase efficiency of this potential treatment option (Leshuk et al., 2016). Much like ozonation, UV photocatalysis using TiO_2 has been found to effectively target the larger higher molecular weight NAFCs in OSPW. Livera et al. (2018) found that UV photocatalytic oxidation was significantly more efficient in oxidizing NAFC compounds with greater structural complexity, which are often recalcitrant to biodegradation. Increased carbon number, aromaticity, and the degree of cyclicity in NAFCs are properties that often decrease biodegradability but increase the chemical oxidation potential. Using UV photocatalytic oxidation with TiO_2 to degrade recalcitrant heavy NAFCs into lighter less complex carboxylic acids that are amenable to biodegradation, and then coupling with a secondary biological remediation step may be an effective method of NAFC treatment.

2.4 Coupling of Oxidation and Bioremediation Treatments

Oxidation processes are typically used to disinfect industrial wastewater and groundwater *in situ* where microbial communities are already present. Coupling of oxidation and bioremediation recently has become a more attractive treatment option than oxidation alone due to lower cost and enhanced removal efficiency. As a fraction of NAFCs within OSPW have been shown to be recalcitrant, chemical oxidation pre-treatment processes have been proposed to break down the complex organics into smaller and more biodegradable compounds (Quagraine et al., 2005; Scott et al., 2005a). Several studies in other fields have determined that combining oxidation processes with biological treatments is more efficient for removing contaminants and is also more cost effective (Chen et al., 2016). Table 2-3 presents a summary of studies that found microbial treatment coupled with an oxidation process was more effective and cost efficient than oxidation alone.

Table 2-3: Summary of studies the positively combine oxidation processes with microbial degradation treatments.

Contaminant	Oxidation Process	Source of microorganisms	Citation
Diesel	Persulfate, permanganate, hydrogen peroxide	Native to soil	(Chen et al., 2016)
Polycyclic aromatic hydrocarbons	H_2O_2/Fe^{2+}	Native to soil	(Kulik et al., 2006)
OSPW NAFCs	Ozone	Native to OSPW	(Wang et al., 2013a)
OSPW NAFCs	Ozone	Native to OSPW	(Dong et al., 2015)
OSPW NAFCs	Ozone	Native to OSPW	(Zhang et al., 2019)
Pendimetalin (pesticide)	H_2O_2/Fe^{2+}	Native to soil	(Miller et al., 1996)
NAFCs and Merichem NAs	Ozone	Native to OSPW	(Martin et al., 2010)
Polycyclic aromatic hydrocarbons	H_2O_2/Fe^{2+}	Native to soil	(Valderrama et al., 2009)
NAFCs	Ozone	Native to OSPW	(Hwang et al., 2013b)
Commercial NA mixture (TCI chemicals) & 5 model NAs (Octanoic acid, 2-ethylhexanoic acid, 1-methyl-1-cyclo-hexane carboxylic acid, 4-methyl-1-cyclo-hexane carboxylic acid, 2,2-dicyclo-hexylacetic acid)	Ozone	Unstressed NA enriched mixed culture	(Vaiopoulou et al., 2015)
OSPW NAFCs	Ozone	Native to OSPW	(Shi et al., 2015)
OSPW NAFCs	Ozone	Native to OSPW (stressed in ozone process)	(Brown et al., 2013)

Although studies have shown it is beneficial to couple chemical oxidation and biodegradation treatments, limited studies have been conducted on the microbial response to the oxidation treatments. Valderrama et al. (2009) found that the maximum efficiency of removal of PAH by Fenton's reaction was reached with two different treatments options, first with Fenton's reaction alone, or secondly a moderate reagent dose combined with the native community. This indicates that combination of chemical and bioremediation treatments are a more attractive option for PAH removal for both cost and environmental impact (Valderrama et al., 2009). Additionally Valderrama et al. (2009) found that an overly heavy oxidation pre-step did not lead to higher biological degradation, therefore a lower dose of oxidant can be used to promote the same level of biodegradation. Although no microbial community analyses were conducted during this experiment, the microorganisms survived the oxidation stress as evident by the continued degradation of the more biodegradable oxidation by-products.

Brown et al. (2013) combined the use of ozonation and microbial degradation by the native microbial community of the OSPW to degrade NAFCs. This study found that even with 50mg/L ozone dose the native community survived oxidation and was able to re-establish itself to the same microbial density and same NAFC degradation capacity as the non-ozonated community. This study removed the majority of NAFCs present, tracking remaining NAFCs specifically during bioremediation step was therefore not possible using high resolution techniques. Remaining organics present were produced during oxidation and were quantified via dissolved organic carbon (DOC) measurement. DOC reduction was determined to be greatest between 14d and 35d, stagnating after this point in time (either due to recalcitrant nature of remaining organics, or depletion of nutrients present). These findings indicate that

the native community can resist moderate oxidative stress and recover to degrade organics made more biodegradable by the oxidation process. Although Brown et al. (2013) did not find a change microbial density with the increase in biodegradable compounds after ozonation, Hwang et al. (2013b) found biofilms grown on ozonated OSPW grew thicker than biofilms grown on untreated OSPW indicating more biodegradable oxidation by products increased biological activity. Additionally, Mahour (2016) found that a *Pseudomonas* sp. native to OSPW could withstand varying doses of persulfate, recover and degrade organics to the same degree as those who were not exposed to the persulfate. However, the lag time in growth varied, indicating recovery time of the cells depends on the level of oxidant exposure.

As coupling of advanced oxidation steps with microbial degradation is proving to be a more economical remediation plan, pairing more environmentally friendly, economical oxidation such as solar photocatalytic oxidation with microbial degradation has potential to be highly effective in OSPW remediation. Understanding the native microbial communities' ability to withstand the harsh oxidative stress and recover to degrade NAFCs is poorly understood for UV photocatalytic oxidation with TiO_2. As UV photocatalytic oxidation with TiO_2 targets the more recalcitrant NAFCs with an increased carbon number, aromaticity, and the degree of cyclicity, coupling of this oxidation process with biological degradation of the oxidation by-products could be an effective treatment coupling.

In OSPW, NAFCs are known to be recalcitrant to biodegradation and a major contributor to toxicity. As NAFCs are the primary source of toxicity, effective remediation of this complex mix of organic compounds is the primary focus of my PhD research. Several studies have

focused on microbial degradation of NAFCs in OSPW however none focus on remedial potential of fungal isolates. Additionally, chemical oxidation of NAFCs has been proven effective in degrading NAFCs and removing toxicity. However, coupling of bioremediation and solar photocatalytic oxidation to degrade NAFCs as a remediation strategy has never been studied.

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Chapter 3: Oil sands process affected water sourced *Trichoderma harzianum* demonstrates capacity for mycoremediation of naphthenic acid fraction compounds

3.1 Introduction

The Albertan oil sands are one of the largest proven reserves of bitumen in the world. The production of this resource requires a substantial amount of water, which leads to an abundance of post extraction water that must be contained and remediated before discharge (Giesy et al., 2010). This post extraction water is termed oil sands process affected water (OSPW) and becomes concentrated with compounds including salts, minerals, heavy metals, residual bitumen, and organic compounds such as naphthenic fraction compounds (NAFC) (Defined in Section 2.2) (Allen, 2008b). NAFCs are the principle group of compounds within OSPW that contribute extensively to its toxicity (Described in Section 2.2.1) (MacKinnon and Boerger, 1986; Holowenko et al., 2002; Clemente et al., 2004; Kavanagh et al., 2013; Marentette et al., 2015a). More recently, the definition of OSPW-sourced organics has been expanded to not only include classical NAs (O_2^- species), but also a complex mixture of oxidized organics that contain three or more oxygen-atoms, sulphur and nitrogen heteroatoms and is termed naphthenic acid fraction compounds (NAFCs) (Grewer et al., 2010; Rowland et al., 2011a; Rowland et al., 2011b). NAFCs are more chemically diverse than classical NAs, such as those found in commercial mixtures like Merichem NAs, which consist solely of O_2^- species. As a result, NAFCs have different properties and toxicity profiles than the classical NAs (Scott et al., 2005a; Grewer et al., 2010; Quesnel et al., 2011a; Hughes et al., 2017). Regardless of the source, the majority of studies attribute toxicity directly to classical NAs (O_2^- species) (Morandi et al., 2017).

Treatment technologies for NAFCs have traditionally utilized chemicals, through the process of advanced oxidation such as ozonation (Wang et al., 2013b), UV photocatalysis (Leshuk et al., 2016), and potassium ferrate (VI) (Wang et al., 2016), or physical processes through the use of adsorbents such as activated carbon (Niasar et al., 2019). Alternatively, with the abundance of organic compounds found in tailings ponds, microbial communities are very active and diverse, indicating that *in situ* bioremediation is a plausible method for treating toxicity within OSPW (Described in Section 2.3) (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012).

A comprehensive sampling study by Richardson et al. (2019) revealed substantial genetic diversity of the microbial community present in OSPW. Analysis of 18S rRNA revealed that, although limited in classification below the phylum level, two of the most abundant operational taxonomic units (OTU's) of the entire dataset were fungi (Richardson et al., 2019). This major presence of fungal activity within the water fraction of the tailings pond indicates that fungi possess the capacity to resist the harsh OSPW environment and as such there is an opportunity to understand their potential for mycoremediation.

Previous work from the Ulrich research group found 6 microbial isolates within OSPW with the capacity for growth at high salinity, pH and NA concentration, including the fungal isolate *Trichoderma harzianum,* which was the first report of a fungus being isolated from OSPW (Miles et al., 2019). *T. harzianum* demonstrated the ability to withstand high salinity (≥60 g/L), pH range (2-9) and NA concentration (inhibitory concentration of 2400 mg/L of OSPW NAs (Miles et al., 2019). In addition, this isolate was also able to grow on a pure drop of Merichem

NAs as a sole source of carbon on an agar plate (Miles et al., 2019). These results indicated that *T. harzianum* could be a prime candidate for mycoremediation of the complex and toxic organic fraction found in OSPW. It is hypothesized that the aerobic microbial isolate to be tested in this research, *Trichoderma Harzianum* has the capacity to remediate NAFCs and reduce the toxicity of OSPW since it was isolated directly from OSPW and continuously exposed to NAFCs (Miles et al., 2019). In addition it is hypothesized that *Trichoderma Harzianum* will be able to degrade the simpler ring structure of cyclohexane carboxylic acid, but will struggle with the more complex diamondoid structure of 1-adamantane carboxylic acid The aim of this study was to: 1) determine if the OSPW sourced isolate *T. harzianum* possess the capacity to degrade complex OSPW sourced NAFCs; 2) use Merichem NAs to determine the specific impact of classical NAs (O_2^- species) on degradation; and, 3) use model NA compounds to determine how the complexity of chemical structure effected degradation.

3.2 Materials and Methods

3.2.1 Fungal Isolate

The microbial isolate used in this study was obtained through selective enrichment of OSPW for NA degrading microorganisms. Details of this enrichment, confirmation of isolate purity and subsequent tolerance testing of *T. harzianum* are described previously by Miles et al. (2019) (GenBank database, 2015/03/25, accession #KR011318). Biomass for microcosms was obtained by cultivating *T. harzianum* on Lysogeny broth (LB) nutrient agar plates. Frozen stock (0.100mL) of *T. harzianum* was inoculated into 250mL of LB liquid media containing 200mg/L Merichem NAs and incubated for 48 hours at room temperature (20±2°C). Approximately

100µL of LB media was then transferred to each LB nutrient agar plate and incubated for 48 hours at room temperature. Dry cell mass was carefully scraped off plates to ensure no agar was selected and weighed before inoculating each individual microcosm.

3.2.2 NA Sources

NAFCs were extracted as described previously (Grewer et al., 2010). Concentration of the final extract was analyzed using Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (see Section 3.2.4). A 100mg/mL stock was prepared by volume in 1N NaOH and stored at 4˚C until use.

Merichem NAs (gifted from Merichem Chemicals and Refinery Services LLC) were chosen as a positive control as previous research has demonstrated this mixture is comparable to the O_2^- species found in OSPW, and is readily biodegradable (Clemente et al., 2004; Scott et al., 2005a; Toor et al., 2013b).

Model NAs cyclohexane carboxylic acid ($C_7H_{12}O_2$) (CHCA) (99%; Sigma-Aldrich; St. Louis, MO, USA) was chosen as a single ring compound analogous to simple structures naturally found in OSPW, while 1-adamantane ($C_{11}H_{16}O_2$) (ADA) (99%; Sigma-Aldrich; St. Louis, MO, USA), was chosen as a diamondoid (three-ring) model compound to represent the more complex ring structures found in OSPW (Rowland et al., 2011a).

3.2.3 Microcosm Set Up

1L Fisherbrand™ reusable glass media bottles with cap (modified with a 20mm blue butyl septum for periodic gas and liquid sampling) were filled aseptically with 500mL of

modified Bushnell Haas media (compound list can be found in the Appendix). All bottles were incubated at room temperature (20±2°C) with light restriction (covered in foil wrapping) on the benchtop. Unless otherwise stated, all materials were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Bottles were fed 60mg/L of NAFCs, 80 mg/L of commercial NAs (Merichem), or 40mg/L of either ADA or CHCA. All microcosms were inoculated with 35mg (dry cell weight, off agar medium) of *Trichoderma harzianum*. These biotic bottles are herein referred to as "Live" bottles (denoted with an L) and were conducted with four (Merichem) or five replicates (all other live tests). Abiotic controls were set-up (with four replicates) identically to each live test bottle condition with the addition of 0.04% (w/v) sodium azide to kill *T. harzianum* and are referred to as "Killed" bottles (denoted with a K). Dissolved oxygen was monitored periodically to ensure aerobic conditions were maintained, and it was found that the 50/50 liquid/headspace set up provided sufficient oxygen throughout the experiment (data not shown). Liquid samples (5mL-25mL) were taken periodically, filtered with 0.22µm nylon filters and stored at 4˚C until analysis.

3.2.4 Merichem NAs Analysis

Merichem was extracted under methods previously described (Grewer et al., 2010) using 5mL of sample, derivatized by 50µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (Agilent J&W 122-5512 column: 15 m x 250 µm x 0.25 µm). The temperature gradient was as follows: 50 °C for 2 min, then increasing at a rate of 30 °C/min to 280 °C which was maintained for 8 min. Helium was used as carrier gas

with the following flow program: 0.15 mL/min for 2 min, then decreased to 0.063 mL/min which was maintained until the end of the separation. Total run time was 17.7 min. The detector was maintained at 300 °C. Column flow plus makeup gas (helium) was set to 32.1 mL/min, air flow 450 mL/min and H_2 flow 40 mL/min. A typical liquid injection volume was 1 µL. Merichem NAs were used as standards for quantification (Calibration curves given in Appendix A2), and fluorene-9-carboxylic acid (97% FCA) was used as an internal standard to determine extraction efficiency.

3.2.5 O_2^- Orbitrap- MS Analysis

Instrumental analysis. Reversed-phase High Performance Liquid Chromatography (HPLC) was paired with a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose) and operated using a modified version of the method of (Pereira et al., 2013). HPLC separation was performed on a Hypersil Gold C18 Selectivity Column (Thermo Scientific, Edmonton, AB, 50 × 2.1 mm, particle size 1.9 µm), using an Accela HPLC system (Thermo Scientific, San Jose, CA). The column was maintained at 40°C and a flow rate of 0.5 mL/min was used with 20 µL injection volumes. Initial mobile phase composition was 95% A (0.1% acetic acid in water) and 5% B (100% methanol) for 30 seconds followed by linear gradient ramp to 90% B over 10 min, and a final ramp to 99% B over 5 min. Percent composition of B was then decreased to 5% over 2 min 30 s and held for 2 min for column re-equilibration. Although electrospray ionization (ESI) is commonly used , matrix effects were reduced using atmospheric pressure chemical ionization (Headley et al., 2011; Peru et al., 2019). All analyses were performed in negative (-) ionization mode. Nominal resolution was set

to 240 000 at m/z 400 and mass spectral data was acquired in full scan mode between 100 and 500 m/z. Capillary temperature was 300 °C, needle voltage was set to 2.25 kV, and sheath, auxiliary and capillary gas flow was set to 20, 15, and 3 (arbitrary units), respectively.

Qualitative and quantitative analysis. Xcalibur ™ software was used for data acquisition. Mass spectral peak intensities were used to quantify relative concentrations by taking total spectra over the window during which most species eluted (7-13 min). Empirical formulas were assigned using the exact mass of all detected ions using the following restrictions: minimum (C-5, H-10), maximum (N-1, S-1, O-6) and the maximum error tolerance for formula prediction was 5 ppm. By this method, NAs are those species with two oxygens detected in negative ion mode (i.e. O_2^- species). Data analyses were based exclusively on mass spectra rather than chromatographic peaks, so chromatograms were only examined for the purposes of review separation of isomers for a given species.

3.2.6 Model NAs Analysis

Concentrations of ADA and CHCA were analyzed by HPLC. The HPLC was an Agilent 1200 Infinity Series HPLC with an autosampler, thermostated column compartment and UV-visible diode array detector. The HPLC analytical column ThermoScientific Acclaim Organic LC (DX062902902) (Thermo Scientific, Waltham, Massachusetts, USA) was kept at 30°C. Mobile phase was a programmed mix of 40:60 acetonitrile:2mM phosphate solution, pH adjusted to 2.5 (KH_2PO_4, Fisher Scientific, Fair Lawn, NJ, USA) with a flow rate of 1mL/min. Total run time was 7.002min for CHCA, and 18.002min for ADA with injections of 25µL and 100µL respectively. Mobile phase and samples were filtered at 0.22µm nylon filters and sonicated for 30min to remove all particles and air. Stock solutions of respective model NAs (1000mg/L in water) were used for standards and development of calibration curves (Calibration curves given in Appendix

A2). The detector was set to detect wavelengths between 200nm and 260nm, and 210nm was most accurate for the model compounds. Software for operation was ChemStation for LC. Integration was done using baseline hold.

3.2.7 Kinetics Analysis

The reaction rate constants for Merichem and model NA live bottles were determined by comparing the coefficient of determinations for zero order, first order and second order plots. Biodegradation of organics followed first order kinetics, as described by Equation (1).

$$ln\left(\frac{C_t}{C_o}\right) = -Kt \qquad \text{Equation (1)}$$

Where: *t* refers to the time of expeirmental measurement (days); C_o and C_t are the concentration (mg/L) of organics at time zero and a given day *t*; and *K* is the first order reaction rate constant (day^{-1}). Values of *K* were obtained from the slope of plots of the natural log of the concentration of organics versus time.

3.2.8 COD

Chemical oxygen demand (COD) was measured with HACH COD High Range (HR; limits of 20 – 1500 mg/L) digestion solution vials (product 2125925, Fisher Scientific, Fair Lawn, NJ, USA) following method 8000, using a HACH digestor reactor and UV-Vis Hach DR/4000 spectrophotometer (Hach, Loveland, CO, USA).

3.2.9 Fungal Growth Analysis

Headspace $CO_{2(g)}$ was measured to track CO_2 production as a metabolic byproduct of biodegradation with an Agilent 7890A gas chromatograph equipped with a thermal conductivity detector (GC-TCD) (Agilent HP-PLOT/Q column: 30 m x 320 µm x 0.2 µm). The oven temperature gradient was as follows: 50 °C for 2 min, then increased at a rate of 30 °C/min to 150 °C which was maintained for 2 min. Helium was used as carrier gas with the following flow program: 8.83 mL/min for 2 min, decreasing to 5.67 mL/min until the end of the separation. Total run time was 7.33 min. The detector was maintained at 200 °C, and the injection port at 300 °C. The makeup gas (helium) was set to 5 mL/min. The injector split ratio was set to 5:1 (no gas saver), with a column flow of 8.89 mL/min, split vent flow of 44.4 mL/min, and a septum purge flow of 58.3 mL/min under a pressure of 30 psi. A typical injection volume was 100 µL. Gas standards were created using various concentrations of CO_2 and N_2 gas mixes (Calibration curves given in Appendix A2).

3.2.10 Microtox™ assay

Microtox™ is routinely used to assess the toxicity of OSPW and NAFCs (Herman et al., 1994; Lo et al., 2006; Frank et al., 2008; Toor et al., 2013a). Toxicity of samples at the beginning and end of the experiment was determined using the Microtox™ toxicity assay (Osprey Scientific, Edmonton, Alberta, Canada). Day 0 samples were taken within 2 hours of set up, filtered at .22µm to sterilize and stored at 4°C until analysis. The 81.9% Basic Test protocol for 5 min and 15 min acute exposure toxicity assay were performed on a Microbics model M500 analyzer (AZUR Environmental Corporation, Fairfax, California, USA) according to the manufacturer's recommendations. Both 5 and 15 minute acute toxicity was measured, however

there was no significant difference in toxicity measured between tests and therefore only the 5 min data is described. The IC_{50} values were recorded representing percentage of the sample resulting in a 50% decrease in bioluminescence of the target microorganism *Vibrio fischeri*. Toxicity units (TU) were then derived from IC_{50} ($TU=100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

3.3 Results and Discussion

3.3.1 NAFCs and Merichem NAs

NAFCs are a mixture of organic acids with structures of varying carbon numbers and degrees of unsaturation in the form of double bonds, aliphatic and aromatic rings. Due to their complexity, characterization of NAFCs requires methods with high resolving power (Rowland et al., 2011a; Headley et al., 2013; Peru et al., 2019). The use of commercial NA mixtures, such as Merichem NAs, allows for a more focused comparison of changes within the mixture, as they are comprised of solely of O_2^- species (Scott et al., 2005b). Focusing on O_2^- species, or classical NAs, allows focused efforts on remediation of this fraction which is known to be the major contributor to toxicity in OSPW (Marentette et al., 2015a; Hughes et al., 2017; Morandi et al., 2017).

In addition to difficulties characterizing NAFC degradation, fungal growth is challenging to track and measure. However, consistent monitoring of CO_2 production in the headspace can be used to estimate mineralization of organics and serve as a proxy indicator of *T. harzianum* growth. Figure 3-1 shows CO_2 production linked to substrate degradation of Merichem NAs (3-

1A), and CO_2 production for NAFCs (3-1B). GC-FID was used to monitor Merichem NAs concentration; however, this method is limited in that it combines all carboxylic acids within a sample together. Consequently, specific changes in the profile of organics cannot be assessed and thus quantification of complex NAFCs is inaccurate. Therefore, for NAFCs, only CO_2 production was regularly monitored, and Orbitrap-MS was used to determine organic profile shifts. NAFCs-Live bottles L1, L3, and L4 performed similarly and averaged 3mg/L of CO_2 production in 250 days, indicating that a portion of the NAFCs were completely mineralized. NAFCs-Live bottle L2 produced 30mg/L of CO_2 in 250 days, a considerable difference to the other replicate treatments, likely due to variability in the inoculated mass or other inherent biological ambiguity.

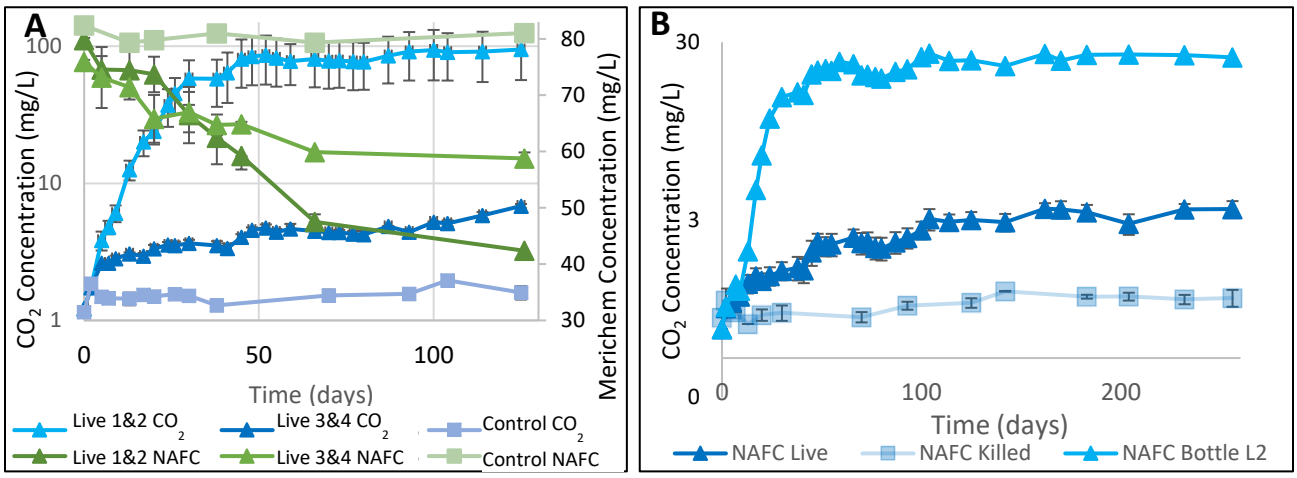

Figure 3-1: Degradation of Merichem NAs and NAFCs with subsequent CO2 production by T. harzianum. (A) Merichem NAs degradation (green; as per GC-FID) with CO_2 headspace production (blue) *by T. harzianu*m. (B) NAFCs CO_2 headspace production by *T. harzianum.* Results are presented as average ± one standard error of replicates.

Microcosms with Merichem NAs showed variable growth of *T. harzianum* over time, with pairs of replicate treatments growing differently from each other (Figure 3-1A). Therefore, Merichem-Live bottles that performed most similarly have been combined for discussion (Mer-

L1&L2 and Mer-L3&L4). Largely, all four live treatment replicates produced CO_2 and demonstrated removal of Merichem NAs over time, indicating mineralization of a portion of Merichem NAs. Conversely, all replicates of the killed controls remained consistent throughout the time series, indicating the production of CO_2 and removal of Merichem NAs was due to microbial activity, not abiotic processes. Live bottles Mer-L1&L2 produced 93±38 mg/L of CO_2 in 183 days and removed 47±1% of Merichem NAs (as per GC-FID) in 126 days, while Mer-L3&L4 produced 6±0.5 mg/L of CO_2 in 183 days and removed 23±1% of Merichem NAs (as per GC-FID) in 126 days. Merichem NA degradation was found to follow first order kinetics, with reaction rate constants (K) found from the slope of the graph shown in Figure 3-2. Mer-L1&L2 was found to have a faster reaction rate constant of 5.3×10^{-3} d^{-1} (r^2=0.92) compared to that of Mer-L3&L4. Albeit slower, the first order reaction rate constant of $3.3.0 \times 10^{-3}$ d^{-1} (r^2=0.91) for Mer-L3&L4 indicates that microorganisms in these replicates were also actively degrading the Merichem NAs present.

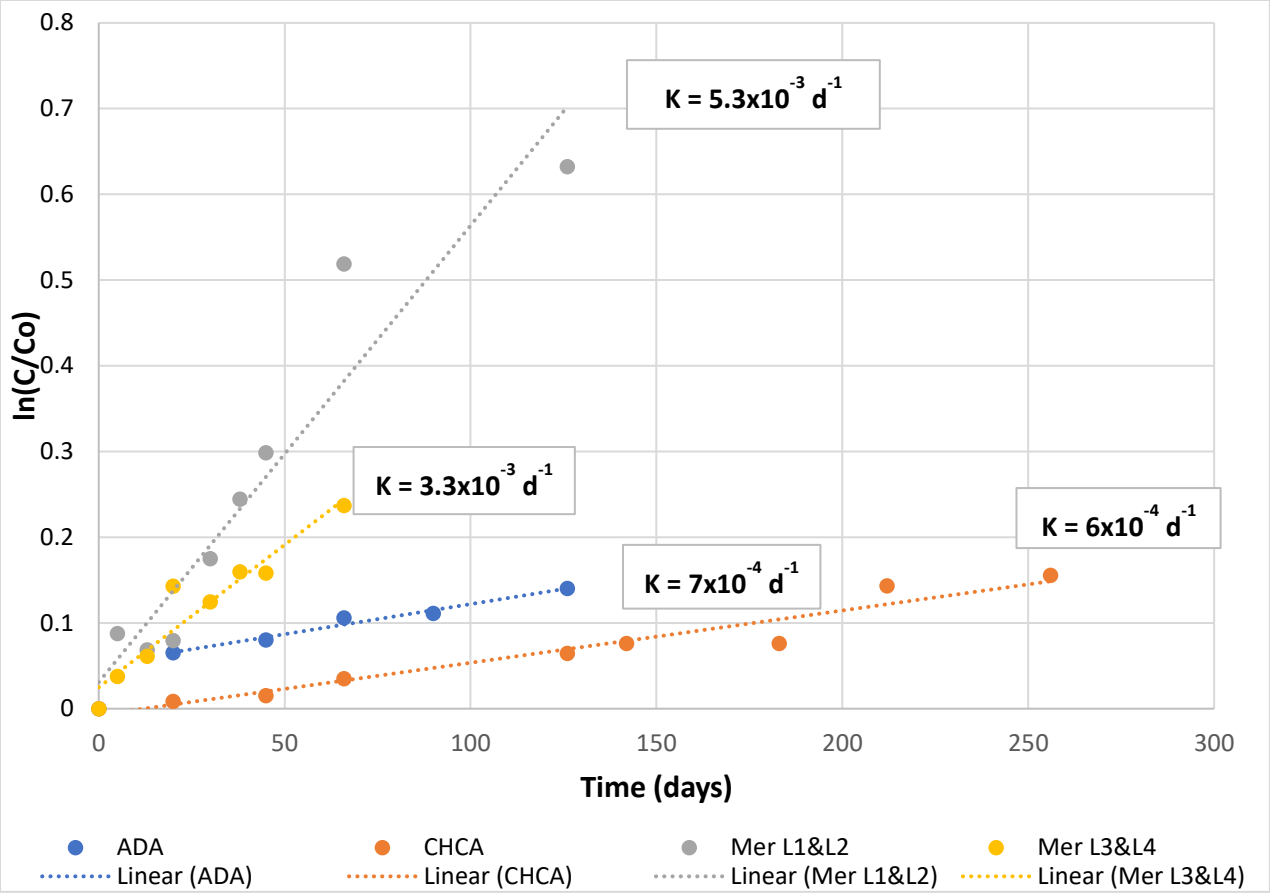

Figure 3-2: First order reaction kinetics for Merichem NAs and Model NAs (CHCA and ADA). The natural log of NA concentration at each time point relative to initial concentration was plotted over time and the slope of the line of best fit was established to give reaction rate constants.

Although there was variability within live treatments, overall, *T. harzianum* grew and degraded Merichem NAs as a sole source of carbon, which a fungus has never demonstrated before. *T. harzianum* exhibited a period of faster growth and degradation within the first 105 and 50 days for both NAFCs and Merichem NAs respectively. Considering the structural diversity of NAFCs, it is likely that the fungus could only degrade a portion, after which their growth slowed and plateaued, and/or a intermediate product built up which inhibited further growth (Sanchez and Demain, 2008).

A common method for determining the quantity of oxidizable organics within a water sample is to measure the chemical oxygen demand. One limitation to using COD is that it only measures organics that are oxidizable by the reagent (dichromate ions). While dichromate ions are considered the best available oxidizing agent for measuring COD, there are still exceptions of organics that cannot be oxidized (Sawyer et al., 2003). Figure 3-3A shows NAFCs with 3 replicates (NAFC-L1-L3) producing 36±4mg/L over 256 days, a 17% increase in COD. However, one replicate (NAFC -L4) was removed from this analysis as COD increased by 435mg/L, a 67% increase in oxidizable organics in 256 days. Killed controls remained constant throughout the experiment. It should be noted the initial measurements of COD in killed controls were higher than live treatments, as the *T. harzianum* cells lysed at death and released measurable organics. Measurements of COD for Merichem NAs shows the continuing trend of two pairs of replicates with significant differences between each other (Mer-L1&L2 vs Mer-L3&L4). Over the course of 256 days, there was an increase of 249±6mg/L and 120±10mg/L of measurable COD, a 172% and 81% increase for Mer-L1&L2 and Mer-L3&L4 respectively.

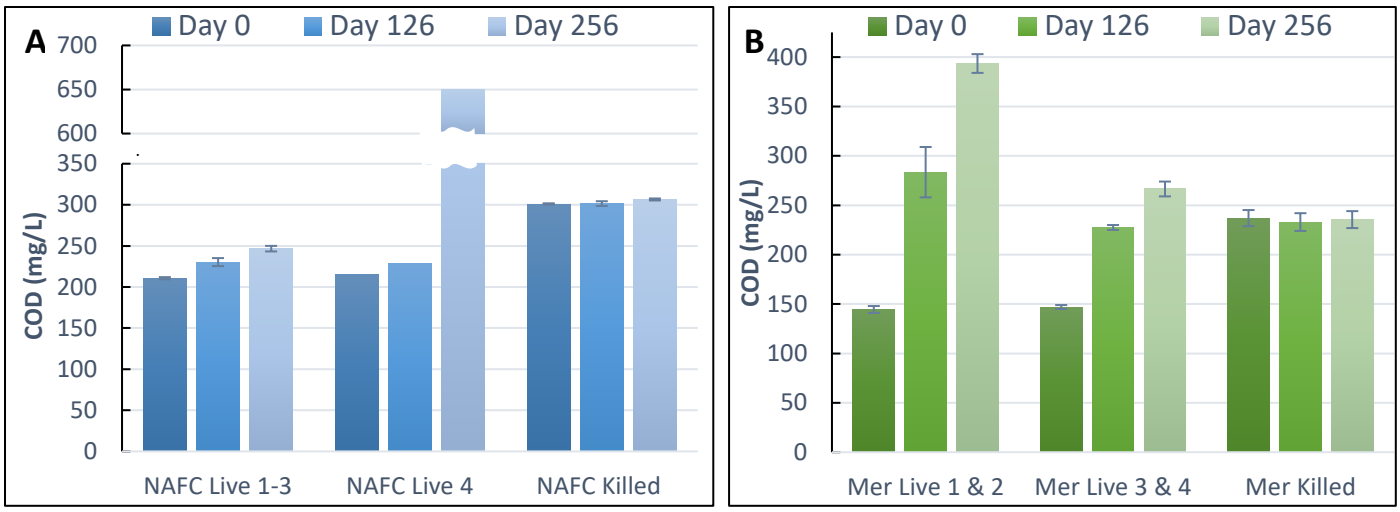

Figure 3-3: COD analysis of *T. harzianum* live treatments and killed controls for NAFC and Merichem NAs. (A) NAFCs COD change over time, bars represent averaged data ± standard error of replicates. A single bottle NAFC-Live 4 is shown outside the averaged replicates as a notable outlier on day 256. (B) Merichem NAs COD change over time, bars represent averaged data ± standard error of replicates.

As this is the first study to demonstrate NAs degradation by a fungus, the mechanism or pathway *T. harzianum* used to degrade and consume the NAs is not yet fully understood, and the uncommon increase in COD should be explored in more depth in future studies. The initial decrease in Merichem NA concentration and subsequent plateau combined with the increase in COD indicates that a metabolite or intermediate is being produced and is accumulating within the microcosm. Therefore, it is possible that *T. harzianum* is utilizing a substrate that was not measurable by COD and producing intermediates that are measurable, and/or a metabolite such as an exopolysaccharide (EPS) is being produced (Mahapatra and Banerjee, 2013). Further characterization of organics measured by COD is needed to determine what exactly is being generated. Despite the large difference of COD production between the Mer-L1&L2 and Mer-L3&L4 replicates, the fraction of COD increase to Merichem NA removal is approximately the same, 3.7 and 3.5 respectively. This suggests that the two parameters are correlated and as *T.*

harzianum degrades Merichem NAs, it is consistently producing the same fraction of metabolite, measured as COD.

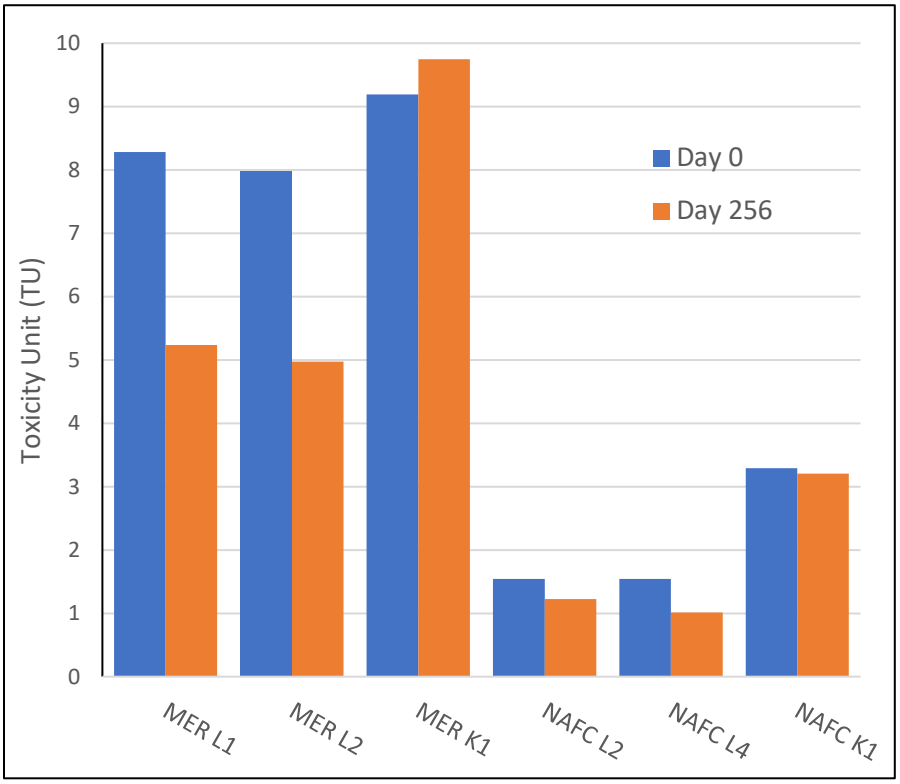

Figure 3-4: 5-minute acute toxicity Microtox™ analysis of NAFCs and Merichem NAs at Day 0 and Day 256. Data represents beginning and end of *T. harzianum* degradation treatment. Toxicity units were derived from IC_{50} ($TU=100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

Vaiopoulou et al., (2015) demonstrated that with chemical oxidation or biodegradation using a mixed microbial community there was no residual metabolite retention over time or correlated COD increase (Vaiopoulou et al., 2015). However, these treatment options involve chemical agents which oxidize metabolites produced during the treatment, or a mixed microbial community where syntrophic relationships exist between microorganisms that allow for any produced metabolites to be consumed. Mycoremediation of Merichem NAs showed a reduction of approximately 59% in toxicity, with very little variation between replicates.

However, the live *T. harzianum* treatments of NAFCs showed variability between replicates ranging from 26% to 52% reduction of total toxicity in NAFC-L2 and NAFC-L4 respectively. As discussed previously, NAFC-L4 showed a greater increase in COD production compared the the other treatment replicates. This trend of greater toxicity reduction paired with the increased COD production also suggests that the more toxic and complex NAFCs were degraded into simpler, less toxic metabolites. Initial toxicity measurements indicate commericial Merichem NAs are almost 5 times more toxic than NAFCs. This is reasonable as Merichem NAs are composed almost exclusively of O_2^- species, which has been determined to be the main fraction of organics within OSPW that contribute to toxicity (Morandi et al., 2017). Merichem NAs potentially could be more bioavailable than OSPW sourced NAFCs, thus enter the microbial cell more easily.

Characterization of NAFCs has been evolving for the last 20 years, and the advent of powerful mass spectrometers, like the Orbitrap, has enabled better separation of these organics. The Orbitrap-MS has high resolution (high resolving power) meaning it can differentiate between species beyond the 3rd decimal place (i.e. 240 000 at m/z 400). In this way, subtle changes in the profile of organic species can be detected and more accurately monitored over time (Ross et al., 2012; Headley et al., 2013; Pereira et al., 2013). Select samples of live treatments which demonstrated the largest change (Mer-L2 and NAFC-L4) and controls (data not shown) were sent for Orbitrap-MS analysis. As toxicity has been attributed to classical NAs (O_2^- species), this was the focus of Orbitrap analyses.

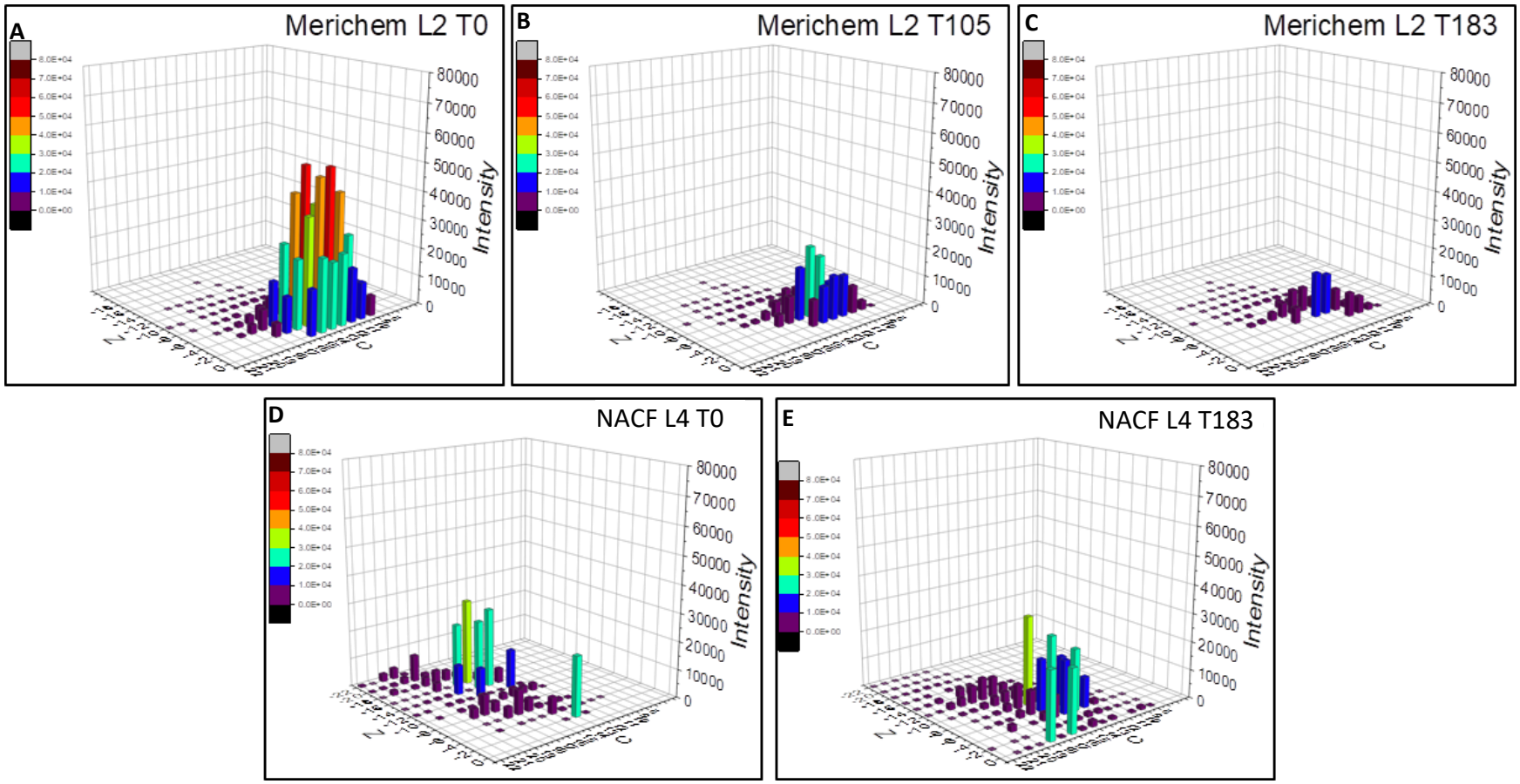

Figure 3-5: Three dimensional plots showing changes in O2- distribution of Merichem NAs and NAFCs in T. harzianum treated samples. Plots denote ion intensity vs carbon number (C) and Z-series as measured per Orbitrap-MS across time. (A), (B) and (C) are commercial Merichem NAs through Day 0, 105 and 183 respectively. (D), and (E) are NAFCs through Day 0 and 183 respectively. Data represents time series of a single live treatment of *T. harzianum*, control data in Appendix.

The output of Orbitrap analysis is summarized in three-dimensional profile plots displaying carbon number (C), hydrogen-deficiency (Z) and intensity (related to concentration). The number and distribution of Merichem NAs show major changes between days 0, 105 and 183 of the experiment (Figure 3.5). Merichem NAs decreased by 88% in 183 days; however, 80% of removal was completed before 105 days of treatment with *T. harzianum* (Figures 3-5A, B). GC-FID data indicated that most of the degradation occurred relatively quickly (by day 66) and then tapered off (Figure 3-1). By Day 105 there was a drastic 100%, 68% and 51% decrease in the Z = 0, Z = -2 and Z= -4 series, respectively. These results suggest *T. harzianum* preferentially degraded organics with simple structures (i.e. those with fewer than 4 bonds and/or rings). By Day 183, 83% and 67% organics in the Z = 0, Z = -2 and Z= -4 series were removed (Figure 3-5C). As toxicity has been attributed to classical NAs containing ≥17 carbons, remediation of these heavier organics is critical. Over 183 days there was a 77% reduction in classical NAs containing 16 or less carbons; however, more importantly there was an 85% reduction in organics containing 17 or more carbons (Figure 3-5C). This reduction of larger compounds containing ≥17 carbons could be the primary contributor to the 59% reduction of toxicity as per Microtox (Morandi et al., 2016; Hughes et al., 2017). Overall, *T. harzianum* was able to degrade a wide range of Merichem NAs that have varying carbon number and complexity.

Extrapolation of the Merichem NAs degradation results to the natural environment is limited as it is known that commercial NAs are more easily biodegradable than those in OSPW (Scott et al., 2005a). To establish a more comprehensive picture of mycoremediation, samples from microcosms containing NAFCs and *T. harzianum* were also analysed by Orbitrap-MS.

Figure 3-5D shows that the initial profile of O_2^- species in NAFCs is different than Merichem NAs (Figure 3-5A), with more NAs with Z ≤ -4 and carbons above 17. Furthermore, the initial intensities appear to be considerably different between Merichem NAs and NAFCs on Day 0. This is possibly due to the fact Orbitrap-MS data shown is entirely O_2^- species, where as OSPW sourced NAFCs contain more organic compounds than just O_2^- species. Although microcosms were given the same concentration of organics (Merichem or NAFCs), a much smaller portion of organics present in NAFCs were strictly O_2^- species (Morandi et al., 2015). Overall, NAFCs showed a 30% net increase in O_2^- intensities, the opposite to what was observed for Merichem NAs. On Day 0, NAFCs contained no species in the Z=0 series, however after 183 days there was a large intensity increase in this family of organics (Z=0). The production of simpler, less complex organics was not observed in the Merichem microcosms. As only the O_2^- species were being analyzed by Orbitrap and NAFCs contain numerous other organic species, it is possible that the non- O_2^- species were partially degraded and produced detectable O_2^- species as intermediates. In addition, as discussed above, the COD increases in live treatments also suggest that a metabolite such as EPS or an intermediate potentially could be produced. EPS produced by *T. harzianum* could be detected by both Orbitrap-MS and COD measurements. However, no production of species in the Z=0 series was noted in Merichem NA treatments, which also had a large increase in COD, indicating it is likely an intermediate product and not EPS metabolites. Regardless, since *T. harzianum* has demonstrated the ability to degrade Z=0 species in the Merichem NA microcosms, given enough time removal of these species would likely occur. Several mixed community degradation studies have also demonstrated that species in the Z=0 series are readily biodegraded by bacteria (Han et al., 2008; Toor et al., 2013b;

Mahdavi et al., 2015). Therefore, the addition of a secondary isolate could use these and other intermediates as a primary carbon source.

Despite the net increase in O_2^- intensities in NAFC microcosms, there was a 13% decrease in species containing ≥17 carbons (apart from C=18, which saw a 294% increase in 183 days). This decrease may have contributed to the 52% decrease in toxicity of this sample (as per Microtox). However, the removal of the more complex O_2^- species would not be the only factor for toxicity reduction as it is likely chemical classes other than O_2^- were degraded. A shift in species not quantified by Orbitrap-MS analysis here may also have contributed to toxicity reduction (Morandi et al., 2017). Review of all organics by Orbitrap-MS could identify species being degraded in other chemical classes which may have contributed in the decrease in toxicity.

3.3.2 Model NA Compounds

Two model NA compounds were selected to showcase the range of classical NA structures found in OSPW, using an analog of the simple single ring (CHCA) compared to a significantly more complex diamondoid ring structure (ADA) (Rowland et al., 2011a). CHCA is commonly used as an organic substrate that is a simpler carbon source and has demonstrated to be readily degradable by microorganisms native to OSPW (Herman et al., 1994; Del Rio et al., 2006; Demeter et al., 2014; Yu et al., 2019). Conversely, ADA has frequently demonstrated to be recalcitrant to biodegradation (Demeter et al., 2015; Frankel et al., 2016). With the surge of interest in utilizing bioremediation for reduction of OSPW toxicity, greater emphasis needs to

be placed on finding microorganisms capable of degrading the more complex organics in OSPW, such as ADA.

For model NA experiments, CO_2 production was used as an approximation of both microbial growth and mineralization of model NA compounds and was linked to substrate degradation measured by HPLC (Figure 3-6). Killed controls remained consistent throughout the time series, indicating the production of CO_2 and removal of substrate was due to microbial activity, not abiotic processes for both model NAs.

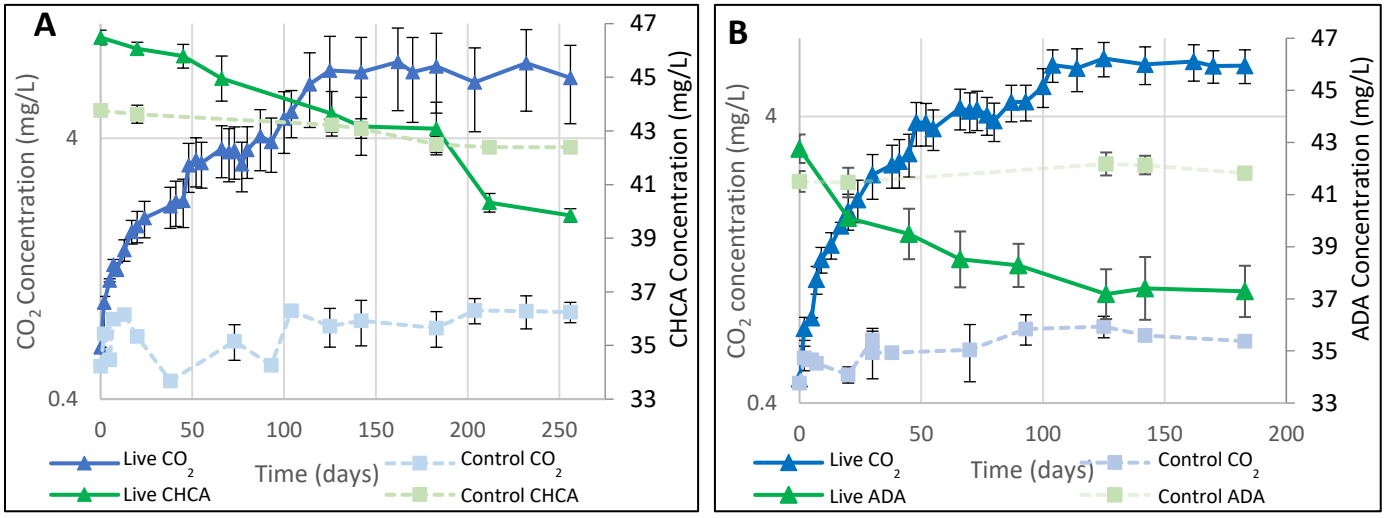

Figure 3-6: Degradation of Model NAs and subsequent CO_2 production by *T. harzianum*. (A) cyclohexane carboxylic acid (CHCA) degradation (green) with CO_2 headspace production (blue) *by T. harzianu*m. (B) 1-adamantane (ADA) degradation (green) with CO_2 headspace production (blue) *by T. harzianu*m. Results are presented as average ± standard error of replicates (5 live treatments, 4 killed controls).

Over 256 days, 14±0.5% of CHCA was removed and 6.8±2mg/L of CO_2 produced in the headspace of the microcosm, indicating a portion of the CHCA was completely mineralized by *T. harzianum* (Figure 3-6A). Similar to the Merichem NA and NAFCs results, degradation of CHCA exhibited more rapid initial degradation in 100 days, followed by a plateau at which point no

further significant degradation occurred, potentially due to the accumulation of intermediates (as demonstrated in COD analysis of Merichem/NAFCs microcosms). The first order reaction rate constant for CHCA was found to be $6x10^{-4}$ d^{-1} (r^2=0.94) (Figure 3-2). Reaction rates for CHCA degradation by specialized microbial communities consisting primarily of bacteria are often as high as 0.41 d^{-1} (Kannel and Gan, 2012), which is considerably higher than the observed rate for this experiment. Overall, these results indicate that *T. harzianum* cannot efficiently degrade CHCA in contrast to most CHCA degradation studies that utilize bacteria and suggest CHCA is readily biodegradable.

While CHCA is a simple single ring, ADA is a triple ring diamondoid structure, more representative of the complex ring structures found in OSPW. Over 183 days, 13±2% of ADA was removed and 6.1±0.5mg/L of CO_2 was produced in the headspace of the microcosm (Figure 3-6B), indicating complete mineralization of a portion of the ADA by *T. harzianum(Demeter et al., 2015)*. ADA followed the trend of a more rapid degradation phase followed by a plateau; however, the most substantial degradation occurred within the first 50 days, compared to the first 100 days for CHCA. Similarly, ADA exhibited a higher first order reaction rate constant of $7x10^{-4}$ d^{-1} (r^2=0.97) compared to $6x10^{-4}$ d^{-1} for CHCA (Figure 3-2). This is notable as CHCA is has been shown to be more readily biodegradable than ADA.

Recently, Paulssen et al. (2019) evaluated the ability of a native photosynthetic microbial community found in OSPW to degrade ADA. They found microbial communities that were dominated by algae of the order *Chlorella/Chlorellales* and genus *Actuodesmus (Scenedesmus)* (~90% relative abundance) removed approximately 80% of ADA in 90 days,

demonstrating that biodegradation of diamondoid NAFCs is possible by photosynthetic communities native to OSPW (Paulssen and Gieg, 2019). A study by Folwell et al. (2019) also found that communities sourced from OSPW could degrade ADA and 3-ethyl adamantane carboxylic acid. While Paulssen et al. (2019) and Folwell et al. (2019) evaluated the capacity for communities of microorganisms to degrade diamondoid structure carboxylic acids, our study demonstrates that a single microorganism can degrade ADA, although much less efficiently. The advantage of microbial community dynamics is in the synergistic relationships amongst microorganisms whereas an isolate must either be provided with or create all co-factors needed for efficient degradation of substrates (Demeter et al., 2015). *T. harzianum* exhibited greater Merichem NA removal (23 – 47% removal) at much faster rates ($K=2.0x10^{-3}$ - $5.3x10^{-3}$ d^{-1}) compared to both CHCA (14% removal; $K=6x10^{-4}$ d^{-1}) and ADA (13% removal; $K=7x10^{-4}$ d^{-1}). It is possible that mixtures of compounds are better removed by single microorganisms as they can preferentially degrade the compounds that are most suited to them.

While *T. harzianum* initially used the supplied organics as a carbon source, a metabolite or intermediate may have built up within the bottles that inhibited further degradation. Folwell et al. (2019) identified metabolites produced by ADA degradation, however within their microbial community this inhibitory metabolite could be consumed by another species and not accumulate. This synergistic metabolic process within a microbial community would allow *T. harzianum* to continue to degrade the provided organics. Regardless, the fact that a single microorganism, *T. harzianum,* consumed even a potion of ADA is novel and makes it a desirable option to add to other specialized microbial consortia for degrading various NAFCs. In

particular, its slight preference for ADA over CHCA is notable and implies this fungus prefers different, and sometimes more complex, compounds than bacteria.

3.4 Conclusion

This study determined that *T. harzianum* isolated from OSPW could utilize CHCA, ADA, Merichem NAs and NAFCs as a sole source of carbon, as seen by the 14%, 13% and 23-47% reduction of CHCA, ADA and Merichem NAs respectively. Additionally, Orbitrap-MS revealed shifts in the chemical profiles of Merichem NAs and NAFCs that led to a 59% and 52% decrease in toxicity over the course of the experiment, respectively. In addition, results suggested a build up of unidentified nontoxic metabolites or intermediates during the degradation process. A significant outcome of this study was that a single microorganism, the fungus *T. harzianum,* degraded a portion of ADA, which was previously thought to be recalcitrant, at a rate faster than for the more labile CHCA.

Overall, this indicates that *T. harzianum* may be an ideal addition to a microbial community, with synergistic relationships where metabolites are consumed, leading to more comprehensive NAFC removal. However, treatment technologies and applications of *T. harzianum* requires further research, such as elucidating the degradation pathways for model compounds CHCA and ADA, to improve understanding of how fungi can support the remediation of OSPW.

With the continuing development of the oil sands region, an effective remediation strategy will be important for stored OSPW. Biological treatment of this water fraction has become an attractive alternative to costly chemical options. This proof of concept experiment

showed that a fungal species associated with oil sands tailings ponds can degrade complex organics found within OSPW including multi-ring diamondoid structures, which has never been demonstrated before.

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Chapter 4: A dual-step approach: Bioremediation of oil sands process affected water sourced naphthenic acid fraction compounds with pre-treatment of ultraviolet photocatalytic oxidation with TiO_2

4.1 Introduction

The rapid and expanding development of the Alberta oil sands has made remediation of oil sands process affected water (OSPW) critical to reclamation efforts. An organic class within OSPW, naphthenic acid fraction compounds (NAFCs) (Dzidic et al., 1988; St John et al., 1998) are known to be the primary contributors to toxicity of OSPW (Defined in Section 2.2.1) (MacKinnon and Boerger, 1986; Kavanagh et al., 2013; Li et al., 2017). Remediation of these complex organics is therefore required. With the ample presence of organic compounds in OSPW such as unrecovered bitumen, diluent (naphtha), microbial communities are highly active in tailings ponds. This activity degrading more labile organics in OSPW indicates the potential for bioremediation of recalcitrant NAFCs may be possible (Siddique et al., 2007; Siddique et al., 2019). Previous studies have shown that NAFCs can be eliminated from OSPW through microbial degradation under aerobic conditions (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). Although bioremediation is cost effective, it is slow, and several organic compounds are proving to be recalcitrant to the process. In contrast, advanced oxidation processes (AOPs) indiscriminately oxidize all organic compounds thus proving to be very effective in OSPW remediation, but costs can become prohibitive due to the vast inventory of OSPW requiring treatment (Allen, 2008b).

UV photocatalytic oxidation with TiO_2, is an effective and powerful advanced oxidation treatment technique that has emerged for treatment of NAFCs in OSPW. It is an economical option, as compared to other AOPs, with TiO_2 being a relatively low cost, chemically stable, and

biologically inert material that can be recovered and reused (Leshuk and Gu, 2014; Leshuk et al., 2016). Photocatalysis treatment with TiO_2 has been shown to completely mineralize target NAFC organics within a 14h treatment (25 Mj/m^2, 0.5g/L TiO2 loading), and eliminate toxicity as measured through Microtox® bioassay. With approximately 80% of the NAFC organics being mineralized within the first 6h, and the remaining 20% requiring an additional 8h, the efficiency of this treatment could be improved (Leshuk et al., 2016). A secondary treatment step after a photocatalytic oxidation pretreatment could more efficiently degrade the remaining fraction. Livera et al. (2018) found that photocatalytic oxidation was significantly more efficient in oxidizing NAFC compounds with greater structural complexity, which are often recalcitrant to biodegradation. Increased carbon number, aromaticity, and the degree of cyclicity in NAFCs are properties that often decrease biodegradability but increase the chemical oxidation potential. Using microbial degradation as a secondary treatment is currently being investigated; the first step in this process would oxidize and break up the complex recalcitrant organic fraction making it more bioamenable to the microbial community. Using this multistep treatment plan could provide a more economic and energetically conservative treatment process than remediation by photocatalytic oxidation alone as the remaining 20% of organics required eight hours of treatment (Leshuk et al., 2016). Microbial treatment of the remaining fraction by the native community, would best simulate a real-life scenario of treatment and release into wetlands for natural attenuation.

Survivability of the native microbial community through the chemical treatment step in multi-step process is not well understood or researched. Studies using ozone oxidation of NAFCs and microbial degradation have indicated microbial community survivability is plausible. A study

by Brown et al. (2013) combined ozonation of NAFCs in OSPW, and microbial degradation by the native microbial community. The native community survived the ozonation and was able to re-establish itself to the same microbial density as before treatment (based on *rpoB* gene copy measurements), while maintaining the same NAFC removal (11-13mg/L in 84d) potential as non-ozonated microbial communities (5mg/L in 84d). As ozonation and UV photocatalysis with TiO_2 both produce highly reactive hydroxyl radicals, microbial stress experienced with TiO_2 treatment would be analogous to ozonation (Scott et al., 2008; Martin et al., 2010). However, survivability of microbial communities has not been established specifically with UV photocatalysis with TiO_2 in treatments of NAFCs.

Several studies have researched the use of ozonation in the treatment of OSPW sourced NAFCs, coupled with bioremediation however, no studies have been conducted on UV photocatalysis oxidation of NAFCs coupled with bioremediation (Brown et al., 2013; Hwang et al., 2013b; Wang et al., 2013b). It is hypothesized that the native community will survive oxidation and continue on to biodegrade NAFCs produced by the UV photocatalytic oxidation step. The primary focus of this work is to determine 1) if the native microbial community could survive varying doses of UV photocatalysis with TiO_2; 2) if varying doses of UV photocatalysis with TiO_2 of OSPW would improve NAFC biodegradability to the native community; and 3) if addition of a known NAFC degrader would improve removal.

4.2 Materials and Methods

Unless otherwise stated, all materials were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA)

4.2.1 Microcosm Set up

OSPW was sent to Dr. Gu's lab at the University of Waterloo where they treated it via UV photocatalysis. Details on their methodology can be found in Leshuk et al. (2016). Three treatment doses were targeted: 1) light treated OSPW (approximately 25% reduction of NAFCs), 2) heavy treated OSPW (approximately 75% reduction of NAFCs) and 3) untreated OSPW (no UV oxidation treatment). Treated OSPW was then shipped back to UofA and the experiment was set-up as shown in Figure 4-1. 1 L Fisherbrand™ glass media bottles with cap (modified with a 20 mm blue butyl septa for periodic gas and liquid sampling) were filled aseptically with 470 mL of light or heavy treated OSPW, or untreated OSPW; and 30 mL Bushnell Haas (BH) media (Bushnell and Haas, 1941). BH media was chosen as it was specifically developed for hydrocarbon degradation studies by bacteria, and would give the native community the best nutrient conditions for optimal degradation (Bushnell and Haas, 1941).. Treatments included: (1) live test bottles with the native microbial community in OSPW exposed to UV photocatalytic treatment (light and heavy) or not (untreated) (in triplicate); same set-up as the live test bottles previously mentioned, but with an inoculation of a known NAFCs degrader *Pseudomonas sp.* (Miles et al., 2019) to a target optical density at 600nm (OD_{600}) of 0.005 (in triplicate); and abiotic killed controls set-up identically to each live experimental bottle condition, and then autoclaved (every day for a three day period) and 0.04% (w/v) sodium azide was added to ensure the microorganisms were killed (in triplicate). All bottles were incubated at room temperature (20±2°C) with aluminum foil covers for light restriction constantly shaking horizontally at 150rpm. Dissolved oxygen was monitored periodically to ensure aerobic conditions were maintained, and it was found that the 50 liquid: 50 headspace set up provided

sufficient oxygen throughout the experiment (data not shown). Liquid samples (5mL-25mL) were taken periodically, filtered with 0.22µm nylon filters and stored at 4˚C until analysis.

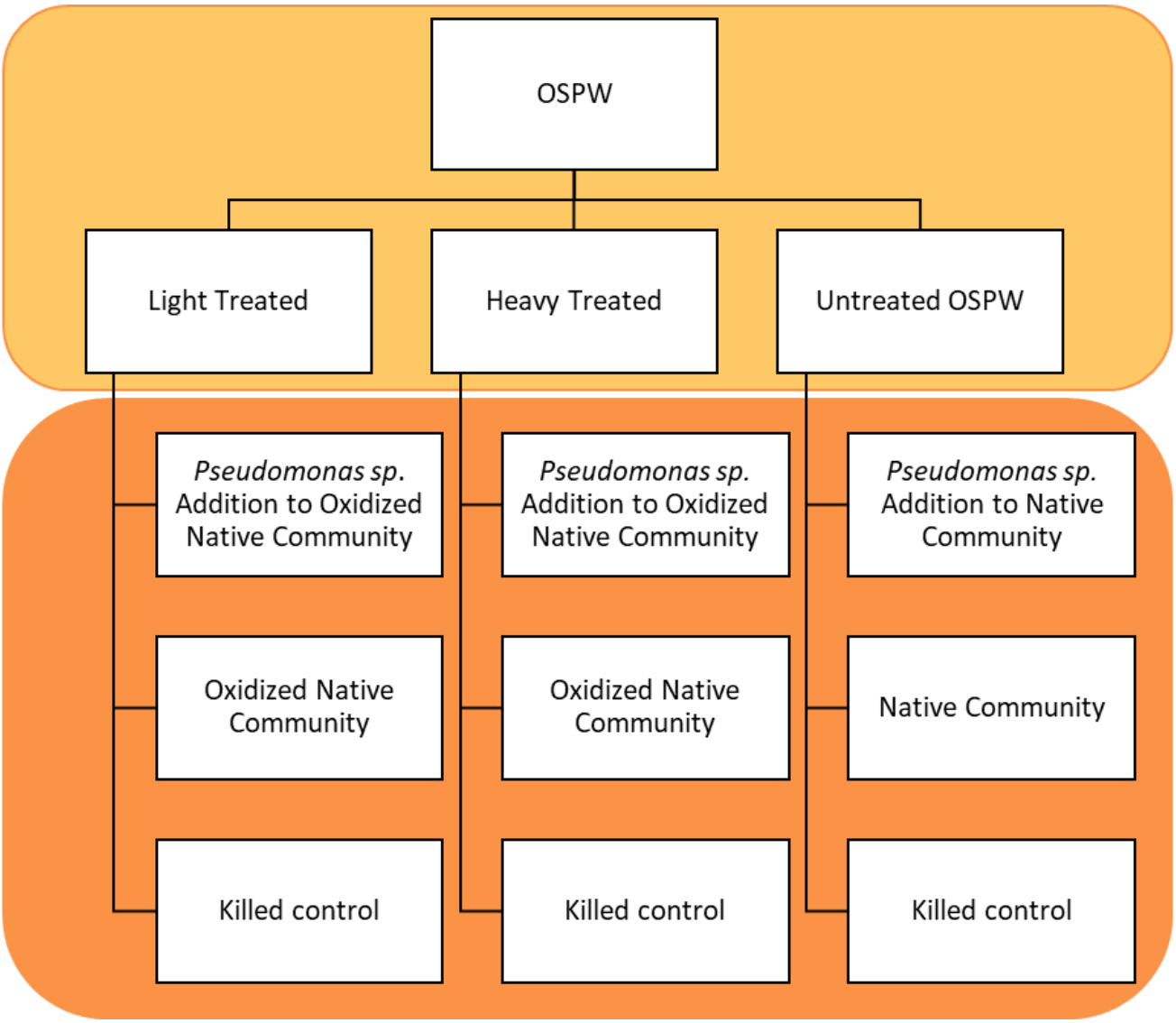

Figure 4-1: Flowchart of microcosm experiment treatment plan. Each subheading was constructed in triplicate as per microcosm set up methods section.

4.2.2 Headspace CO_2 Analysis

Headspace $CO_{2(g)}$ was measured to track CO_2 production as a metabolic byproduct of biodegradation with an Agilent 7890A gas chromatograph equipped with a thermal conductivity detector (GC-TCD) (Agilent HP-PLOT/Q column: 30 m x 320 µm x 0.2 µm). The oven temperature gradient was as follows: 50 °C for 2 min, then increased at a rate of 30 °C/min to 150 °C which was maintained for 2 min. Helium was used as carrier gas with the following flow program: 8.83 mL/min for 2 min, decreasing to 5.67 mL/min until the end of the separation. Total run time was 7.33 min. The detector was maintained at 200 °C, and the injection port at 300 °C. The makeup gas (helium) was set to 5 mL/min. The injector split ratio was set to 5:1 (no gas saver), with a column flow of 8.89 mL/min, split vent flow of 44.4 mL/min, and a septum purge flow of 58.3 mL/min under a pressure of 30 psi. A typical injection volume was 100 µL. Gas standards were created using various concentrations of CO_2 and N_2 gas mixes.

4.2.3 NAFCs Analysis

NAFCs were extracted under methods previously described (Grewer et al., 2010) using 5mL of liquid sample, derivatized by 50µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (Agilent J&W 122-5512 column: 15 m x 250 µm x 0.25 µm). The temperature gradient was as follows: 50 °C for 2 min, then increasing at a rate of 30 °C/min to 280 °C which was maintained for 8 min. Helium was used as carrier gas with the following flow program: 0.15 mL/min for 2 min, then decreased to 0.063 mL/min which was maintained until the end of the separation. Total run time was 17.7 min. The detector was

maintained at 300 °C. Column flow plus makeup gas (helium) was set to 32.1 mL/min, air flow 450 mL/min and H_2 flow 40 mL/min. A typical liquid injection volume was 1 µL. Merichem NAs (Merichem Chemicals and Refinery Services LLC) were used as standards for quantification (Calibration curves given in Appendix A2), and fluorene-9-carboxylic acid (97% FCA) was used as an internal standard to determine extraction efficiency.

4.2.4 Dissolved Organic Carbon Analysis

Dissolved organic carbon (DOC) was measured with a Shimadzu Model TOC-L_{CPH} ASI-L Shimadzu auto sampler. Non-Purgeable Organic Carbon (NPOC) method was used for DOC analysis which measures the non-volatile dissolved carbon content during the purging process (Brown et al., 2013). Samples were filtered with a 0.22 µm nylon filter and diluted to a suitable range before analysis. Concentrations were determined by external calibration using potassium hydrogen phthalate.

4.2.5 Statistics

Significance was determined using Microsoft Excel (Microsoft 365) software, using a two tailed t-test analysis. Variance between sample pools were tested first using F-test, to determine if variance is equal, or unequal. Differences were deemed significant if the t-test resulted in a p-value that was less than 0.05 (higher significance also noted).

4.3 Results and Discussion

4.3.1 UV photocatalytic oxidation treatment

OSPW was treated at one of two doses of oxidation, heavy dose (target 75% removal of NAFCs) and light dose (target 25% removal of NAFCs), with untreated OSPW as a control. Initial OSPW NAFC concentrations were measured at 32.8±0.5mg/L, final concentrations after UV photocatalysis oxidation were 26.8±0.7mg/L and 14.4±0.2mg/L for light and heavy treatment, respectively. This results in approximately an 18% and 56% reduction in NAFCs for light and heavy treatments, respectively. Through the entire multi step process, no treatment yielded complete removal of NAFCs; heavy treated OSPW removed 59% of total NAFCs, lightly treated OSPW removed 35% of total NAFCs and untreated removed 16% of total NAFCs. Interestingly, the light treated OSPW and untreated OSPW can account for the same amount of total removal of NAFCs due to biodegradation with approximately 5±1mg/L of NAFCs being biodegraded, indicating that the light treated oxidation step did not necessarily provide more biodegradable NAFCs. Further high-resolution characterization would be required to determine if the 5±1mg/L of NAFCs removed from both treatments is the same organic fractions as all species and groups are combined with GC-FID method.

Table 4-1: Naphthenic acid fraction compound concentrations. Analyses at day 0 (T0) and day 20 (T20) was done by GC-FID. Live treatment denotes both *Pseudomonas* sp. added, and native community treatments averaged together. Average given of 6 live treatment replicates and 3 killed control replicates ± one standard error. **p<0.01, T-test to determine significance of change within a treatment by comparing T0 to T20 within a treatment, *n*=6. (p values given in Appendix). ***p<0.001, T-test to determine significance rate difference between live treatment and controls over first 20d of experiment.

UV Photocatalytic treatment	Biological treatment	T0 NAFC Concentration (mg/L)	T20 NAFC Concentration (mg/L)	NAFC Removal after Oxidation %	Degradation Rate (mg/L/day)
Light Treated	Live Treatment [a]	26.8±0.7	21.5±1.3**	19.9	1.80±0.02***
	Killed Control	27.9±0.2	28.4±0.8	-2.0	-0.12±0.01
Heavy Treated	Live Treatment [a]	14.4±0.2	13.6±0.0**	5.8	1.74±0.02***
	Killed Control	14.5±0.2	14.6±0.0	-0.8	0.00±0.00
Untreated	Live Treatment [a]	32.8±0.5	27.5±0.4**	16.0	1.69±0.06***
	Killed Control	32.8±0.3	33.0±0.6	-1.8	0.08±0.01

[a] Both live treatments (oxidized native community, and *Pseudomonas sp.* addition to oxidized native community) were averaged as they showed no (p>0.05) significant difference in NAFC degradation in any treatments tested (Figures 4-2, 4-3, and 4-4).

Organics in OSPW that are not categorized as NAFCs are measured in DOC analysis. Similar initial values of DOC for all treatments (Figure 4-2, 4-3, and 4-4) 61±1mg/L, 69±1mg/L, and 72±3mg/L for heavy treated, light treated and untreated, respectively indicates that DOC measurements characterize approximately 2x more organic compounds that are not NAFCs. The oxidation process often produces by-products that are no longer characterized as NAFCs, which would not be measured by GC-FID, but measured in the larger more encompassing DOC measurements. Although DOC analysis does not differentiate between specific organic compounds, it is a good analog for NAFCs in OSPW, with a DOC decrease corresponding with decreasing NAFC concentrations. Both NAFC and DOC measurements indicate that the microbial community was capable of degrading organics present in the OSPW to a point, where

it then plateaus leaving a portion of NAFCs that is not capable of being biodegraded at its current state. No lag period was observed in any analyses, indicating the microbial community did not require time to recover due to the oxidative stress. The heavy, light, and untreated OSPW all took approximately 20d to plateau, rates calculated during this degradation phase (Table 4-1) show that the light treated OSPW had the faster rate of degradation, followed by heavy treated then untreated. This slightly faster rate indicates that the organics produced by the light oxidation treatment were consumed faster than the organics produced by the heavy oxidation treatment.

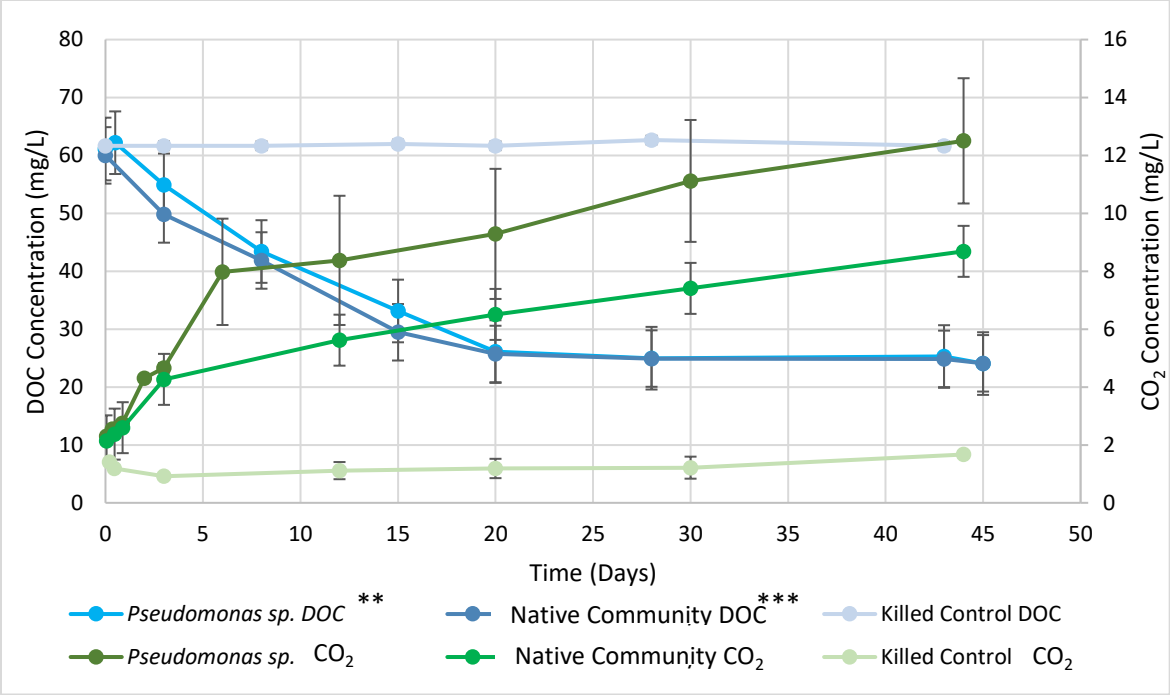

Figure 4-2: Heavy UV photocatalytic oxidation treated OSPW. Microcosms of heavy treated OSPW with either native community or added *Pseudomonas* sp. inoculation. Dissolved organic carbon concentrations (mg/L) are used to track organic degradation, CO_2 concentrations are monitored to track growth of the microbial communities. All treatments are in triplicate, error bars denote one standard error. ***p<0.01, ***p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T45 within a treatment, *n*=3. (p values given in Appendix)

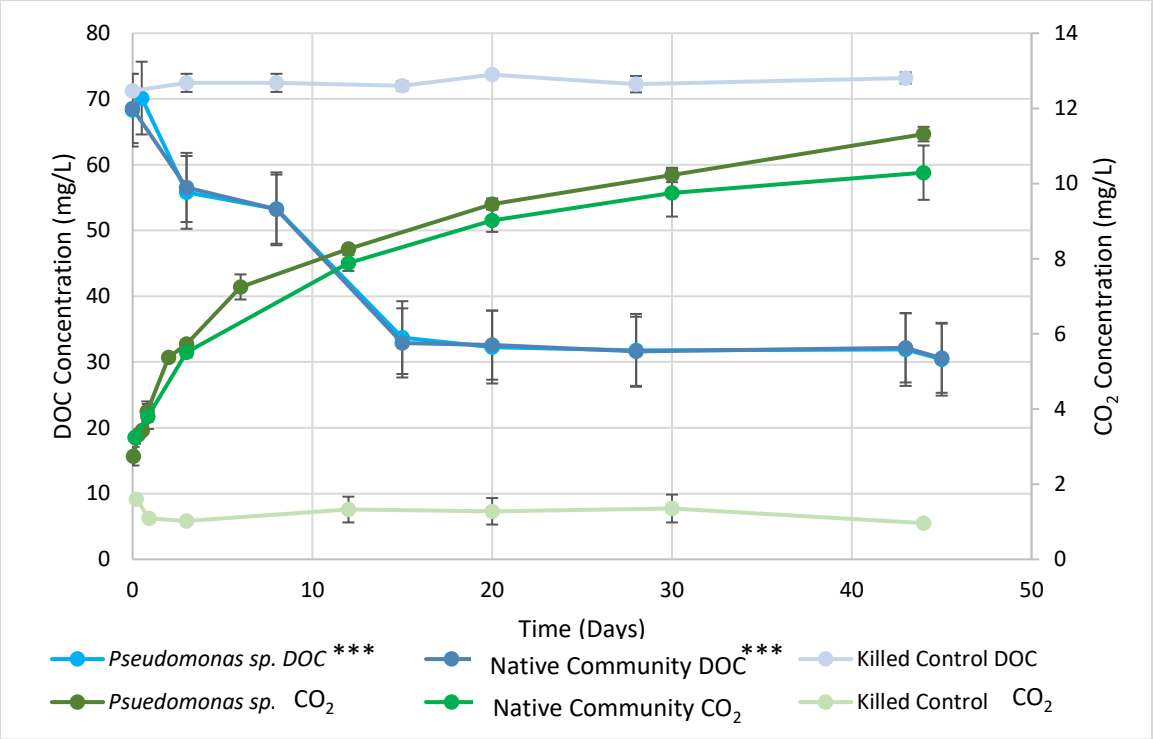

Figure 4-3: Light UV photocatalytic oxidation treated OSPW. Microcosms of light treated OSPW with either native community or added *Pseudomonas* sp. inoculation. Dissolved organic carbon concentrations (mg/L) are used to track organic degradation, CO_2 concentrations are monitored to track growth of the microbial communities. All treatments are in triplicate, error bars denote standard error. ****p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T45 within a treatment, $_n$=3. (p values given in Appendix)

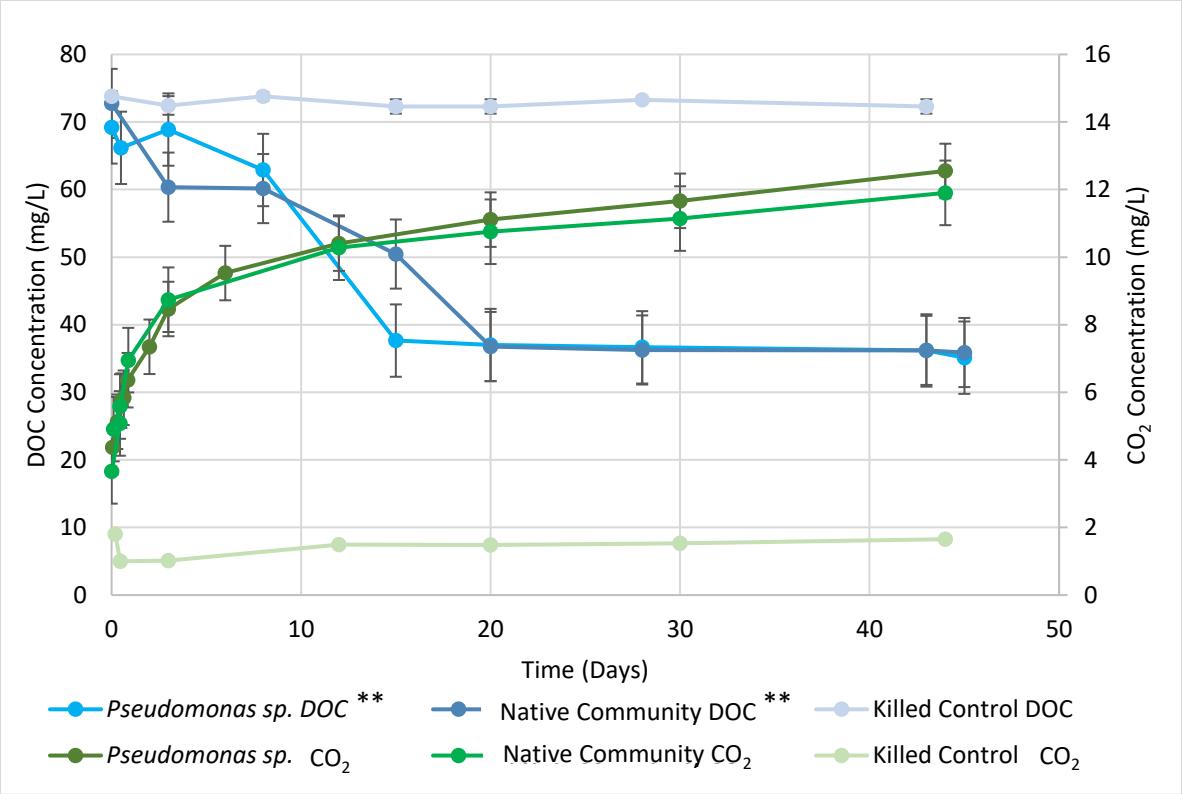

Figure 4-4: Untreated OSPW. Microcosms of untreated OSPW with either native community or added *Pseudomonas* sp. inoculation. Dissolved organic carbon concentrations (mg/L) are used to track organic degradation, CO_2 concentrations are monitored to track growth of the microbial communities. All treatments are in triplicate, error bars denote standard error. ***p<0.01,* T-test to determine significance of change within a treatment by comparing T0 to T45 within a treatment, *n*=3. (p values given in Appendix)

4.3.2 Microbial Community

With a demonstrated capacity for the native microbial community to degrade NAFC compounds in OSPW, understanding this community under stress is key (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). With the aim of an advanced oxidation step to transform complex recalcitrant organics into a more biodegradable form for the microorganisms, the microbial community must be able to survive and tolerate the harsh oxidative stress. Several studies have demonstrated that the native microbial community found within OSPW can endure oxidative

stress of ozonation and continue to degrade NAFCs (Martin et al., 2010; Zhang et al., 2019). A study by Brown et al. (2013) specifically looked at survivability of the native microbial community in OSPW to oxidative stress of ozonation (50mg/L ozone dose). The native community was able to withstand the oxidative stress of the ozonation treatment as indicated by *rpoB* gene copy measurements, and retain the capacity to degrade NAFCs and produced organics by the treatment (11-13mg/L degraded, opposed to 5mg/L in untreated) (Brown et al., 2013).

The study by Brown et al. (2013) only tested one dosage of ozonation, however this study tested two dosages of UV photocatalytic oxidation with TiO_2. A high dosage (target 75% removal of NAFCs) and low dosage (target 25% removal of NAFCs), were chosen to replicate the organic removal noted by Leshuk et al. (2016) where 80% of NAFCs were removed in the first 6h of treatment. The lower treatment dosage was chosen as it was hypothesized the microbial community would not be able to withstand the high dose oxidation treatment. However, the microbial community for both high and low oxidation doses survived as evident by the significant production of CO_2 in the live treated bottles over 56d in comparison to the abiotic controls (Figure 4-2, and 4-3) and significant degradation of NAFCs in live bottles in comparison to the controls (Table 4-1). In comparison to the killed control, the live treatment microcosms thrived and removed organics in both oxidation treatment doses with no lag time. These results are similar to Brown et al. (2013), which noted no delay in recovery from oxidative stress. The light oxidized OSPW treatment produced on average 4mg/L more CO_2 in live bottles than the heavy oxidized OSPW treatment however no significant difference was found in amount of CO_2 produced in all three OSPW treatments ($p>0.05$).

After initial period of removal (approximately 18-20d) removal stagnated in all treatments, leaving a recalcitrant fraction of organics that microorganisms could not degrade in that time period. To ensure this stagnation was due to the recalcitrant nature of NAFCs remaining, bottles were dosed with 20mL of BH media and re-inoculated with *Pseudomonas* sp. and 5mL of untreated OSPW for the unstressed microbial community, which provided no additional removal of DOCs (data not shown). Although the UV photocatalysis oxidation degraded recalcitrant NAFCs to biodegradable forms, it is apparent a remaining fraction is still inaccessible to microbial degradation.

Untreated OSPW was used as a control to demonstrate how the NAFCs present in OSPW are recalcitrant, as the microbial community has had ample time to degrade the fraction of NAFCs available. However, these microcosms once given BH also showed a drop in NAFCs over the 18-20d following the same trend as the treated OSPW bottles with a plateau of removal at 20d (Figure 4-4). Given the OSPW was not pre-treated with oxidation to produce more biodegradable organics, it is evident there are NAFCs present in OSPW amenable to remediation but are not being degraded due to lack of nutrients. With the addition of mineral media (BH) and constant aeration through shaking, possibly the microbial community is starved of nutrients required to degrade all the biodegradable NAFCs present in OSPW in an efficient way. Without the benefit of oxidation pre-treatment, the native community with the boost of mineral media was able to remove 16% of NAFCs present (Table 4-1). These findings support that OSPW is nutrient depleted, and organics presenting in OSPW are not necessarily solely a recalcitrant fraction.

4.3.3 Microbial Isolate

Various microbial species have been isolated and identified that show an affinity to NAFC degradation and enrichment over time in a diverse OSPW community. A study by Yu et al. (2019) tested model NAs degradation of both the native OSPW community and microbial isolate *C. kessleri* alone and in co-culture. Degradation of model NA cyclohexanebutyric acid and cyclohexane carboxylic acid was improved with co-culture of *C. kessleri* with the OSPW microbial community versus the algae isolate alone, or OSPW culture alone. The microbial diversity in the OSPW native community was vastly improved with the addition of *C. kessleri*, enriching known hydrocarbon degraders like *Brevundimonas* sp. (Rochman et al., 2017), *Hydrogenophaga, Parvibaculum , Pseudofulvimonas* and *Hyphomonas* (Song et al., 2018). With the improved biodiversity, removal was improved supporting that microbial communities can provide a synergistic environment for microbial isolate addition and optimizing NAFC degradation.

The addition of an isolate known for NAFC degradation was thought to similarly improve degradation potential of the native microbial community in OSPW pre-treated with UV photocatalysis with TiO_2. A *Pseudomonas sp*. isolate from OSPW previously enriched on NAFCs with a demonstrated resistance to NAFCs was added to treatment bottles in addition to the native microbial community (Miles et al., 2019). However, no significant difference was measured (NAFC or DOC) between live treatments with or without the addition of the *Pseudomonas sp.* in all three OSPW treatments studied (Figure 4-2, 4-3, and 4-4). Comparing the native microbial community alone, and the community with the isolate added, no benefit to

degradation was found. There was no increase to rate of removal or increase in removal potential. Although *Pseudomonas sp.* have been shown to have the potential to be strong candidates for NAFC bioremediation (Del Rio et al., 2006; Johnson et al., 2013; Demeter et al., 2014), there was no increase in NAFC removal with the addition of *Pseudomonas sp*. in this case.

4.4 Conclusions

This study aimed to characterize the coupling of UV photocatalytic oxidation with TiO_2 pre-treatment, with a bioremediation secondary step, as this has never been examined. As this oxidation process mineralizes 80% of organics within the first 6h of a 14h treatment, and requires 8h for the remaining 20%, a secondary treatment step is ideal to efficiently remove target NAFCs (Leshuk et al., 2016). With UV photocatalytic oxidation treatment degrading larger more complex organics at a faster rate than simpler compounds, this oxidation pre-treatment should make recalcitrant NAFCs biodegradable for the native community to fully degrade (Livera et al., 2018). Critical for this coupling to be effective is the survival of the microbial community, as it is required to degrade organics produced by the oxidation pre-treatment. This experiment showed significant removal of NAFCs (5.8%, 19.9% and 16% for heavy, light and untreated OSPW) during the biological step and produced significantly more CO_2 compared to abiotic controls ($p<0.001$). Therefore, the microbial community survived the harsh oxidative stress of both high and low doses. With no measured lag period, it is possible the resilient microorganisms present could withstand a much larger dose of oxidative stress. Future experiments may include higher dosages of oxidation, the microbial community present in

OSPW can degrade produced organics even after experiencing oxidative stress. However, the addition of an unstressed, known NAFC degrader *Pseudomonas* sp. did not improve outcomes.

With a fraction of organics remaining in all treatments, a larger amount of oxidation may prove beneficial in making this fraction biodegradable for the microbial community. Untreated OSPW surprisingly had NAFCs amenable to degradation with the provided boost of the mineral media, which indicates a portion of NAFCs in OSPW are not being degraded by microorganisms in tailings ponds due to potential nutrient limitations. If there is a fraction in OSPW that is biodegradable as noted in this experiment (16% NAFC degradation in untreated OSPW in 20d), then a first treatment step of nutrient addition to stimulate the biological activity would reduce all biodegradable organics present, leaving a truly recalcitrant fraction for secondary treatment with oxidation. Based on this study results, a multi step approach for treatment with a biological step first, may prove most beneficial for an efficient effective remediation strategy of OSPW sourced NAFCs.

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Chapter 5: A multi-step approach: Coupling of biodegradation and UV photocatalytic oxidation with TiO_2 for remediation of naphthenic acid fraction compounds in oilsands process affected water

5.1 Introduction

Production of one of the worlds largest proven reserves of bitumen in the world, Albertan oil sands, requires substantial amounts of water for extraction. This post extraction water is termed oil sands process affected water and is stored in ponds due to enrichment of compounds such as salts, minerals, heavy metals, residual bitumen and organic compounds such as naphthenic acid fraction compounds (NAFCs) (Allen, 2008a; Giesy et al., 2010). The organic fraction (NAFCs) solubilized into the water during extraction are the principle group of compounds in OSPW that contribute extensively to toxicity (MacKinnon and Boerger, 1986; Holowenko et al., 2002; Clemente et al., 2004; Kavanagh et al., 2013; Marentette et al., 2015a).

NAFCs are naturally present in bitumen and become solubilized and concentrated in OSPW during the extraction process (Frank et al., 2008; Yang et al., 2019). The definition of NAFCs has expanded over time to include not only classically defined naphthenic acids, but also a complex mixture of oxidized organics that contain three or more oxygen atoms, sulphur and nitrogen heteroatoms (Grewer et al., 2010; Rowland et al., 2011a; Rowland et al., 2011b). Further specificity on what NAFCs are can be found in Section 2.2. Toxicity associated with NAFCs in OSPW have been attributed to the O_2^- species, and compounds containing ≥17 carbons (Hughes et al., 2017). Morandi et al. (2017) found that while other non-acidic species (O^+, O_2^+, SO^+, NO^+) contributed to the toxicity for *Escherichia coli*, it was much less compared to that of the O_2^- chemical class. Clearly, the unique components in NAFC plays a role in toxicity, and therefore remediation methods must aim to address the complete mixture of organic compounds within OSPW.

Previous studies have indicated active microbial communities within OSPW can eliminate NAFCs present under aerobic conditions (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). Although bioremediation is cost effective, time required for treatment is often long, whereas chemical oxidation is an effective fast treatment option (Allen, 2008b). NAFCs have been shown to be successfully oxidized using advanced oxidation processes (Liang et al., 2011; Drzewicz et al., 2012; Afzal et al., 2015; Abdalrhman and El-Din, 2020). UV photocatalytic oxidation using TiO_2 has been investigated as potential cost effective, efficient treatment of NAFCs in OSPW (Leshuk et al., 2016). Photocatalysis treatment with TiO_2 has been shown to completely mineralize target NAFC organics within a 14h treatment (25 Mj/m^2, 0.5g/L TiO2 loading), and eliminate toxicity as measured through Microtox® bioassay. Livera et al. (2018) found that photocatalytic oxidation was significantly more efficient in oxidizing NAFC compounds with greater structural complexity, which are often recalcitrant to biodegradation. Increased carbon number, aromaticity, and the degree of cyclicity in NAFCs are properties that often decrease biodegradability but increase the chemical oxidation potential. With this finding, coupling with biodegradation would be ideal as oxidation would provide more biodegradable NAFCs for the microbial community.

Several studies have demonstrated that microbial communities found in OSPW can withstand the oxidative stress of NAFC ozonation, and continue to biodegrade organics (Brown et al., 2013; Hwang et al., 2013b; Wang et al., 2013b). However, no studies have been conducted to determine if the advanced oxidation process UV photocatalytic oxidation with TiO_2 can be coupled in a multi step process with biodegradation enhanced by nutrient addition. It is

hypothesized that a multistep treatment of OSPW will provide optimal degradation and toxicity reduction in OSPW. Addition of an UV photocatalytic oxidation step will provide more biodegradable NAFCs for the native microbial community. As OSPW is nutrient limiting, the addition of mineral media can increase the community's ability to degrade NAFCs. It is hypothesized that nutrient addition increases the microbial community's ability to degrade NAFCs within the untreated OSPW, therefore otherwise thought recalcitrant NAFCs will be degraded over time. With a multistep approach, it's hypothesized that the native microbial community can survive the oxidation treatment, and continue on to degrade the remaining organics within the OSPW in step three. The primary focus of this work is to determine: 1) if the native microbial community in OSPW can be stimulated with nutrients to biodegrade otherwise considered recalcitrant NAFCs and which nutrients are key for this process; 2) if an oxidative step can make recalcitrant NAFCs biodegradable; 3) if the microbial community present can withstand the oxidative stress of UV photocatalytic oxidation and recover to degrade remaining NAFCs (larger dose than Chapter 4 experiments). Overall reduction in relative toxicity is the target of this multi-step treatment of NAFCs in OSPW.

5.2 Materials and Methods

5.2.1 Microcosm Set Up

Step one microcosm set up- 1L Fisherbrand™ reusable glass media bottles with cap (modified with a 20 mm blue butyl septa for periodic gas and liquid sampling) were filled aseptically with 470 mL of OSPW and 30 mL Bushnell Haas media (compound list can be found in the Supplemental Materials). All bottles were incubated at room temperature (20±2°C) with light restriction constantly shaking horizontally at 150rpm. Unless otherwise stated, all materials

were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). These biotic bottles are herein referred to as “Live” bottles (denoted with an L) and were conducted in triplicate. Abiotic controls were set-up (in triplicate) identically to each live test bottle condition with the addition of 0.04% (w/v) sodium azide to kill the native community and are referred to as “Killed” bottles (denoted with a K). Dissolved oxygen was monitored periodically to ensure aerobic conditions were maintained, and it was found that the 50/50 liquid/headspace set up provided sufficient oxygen throughout the experiment (data not shown). Liquid samples (5mL-25mL) were taken periodically, filtered with 0.22µm nylon filters and stored at 4˚C until analysis.

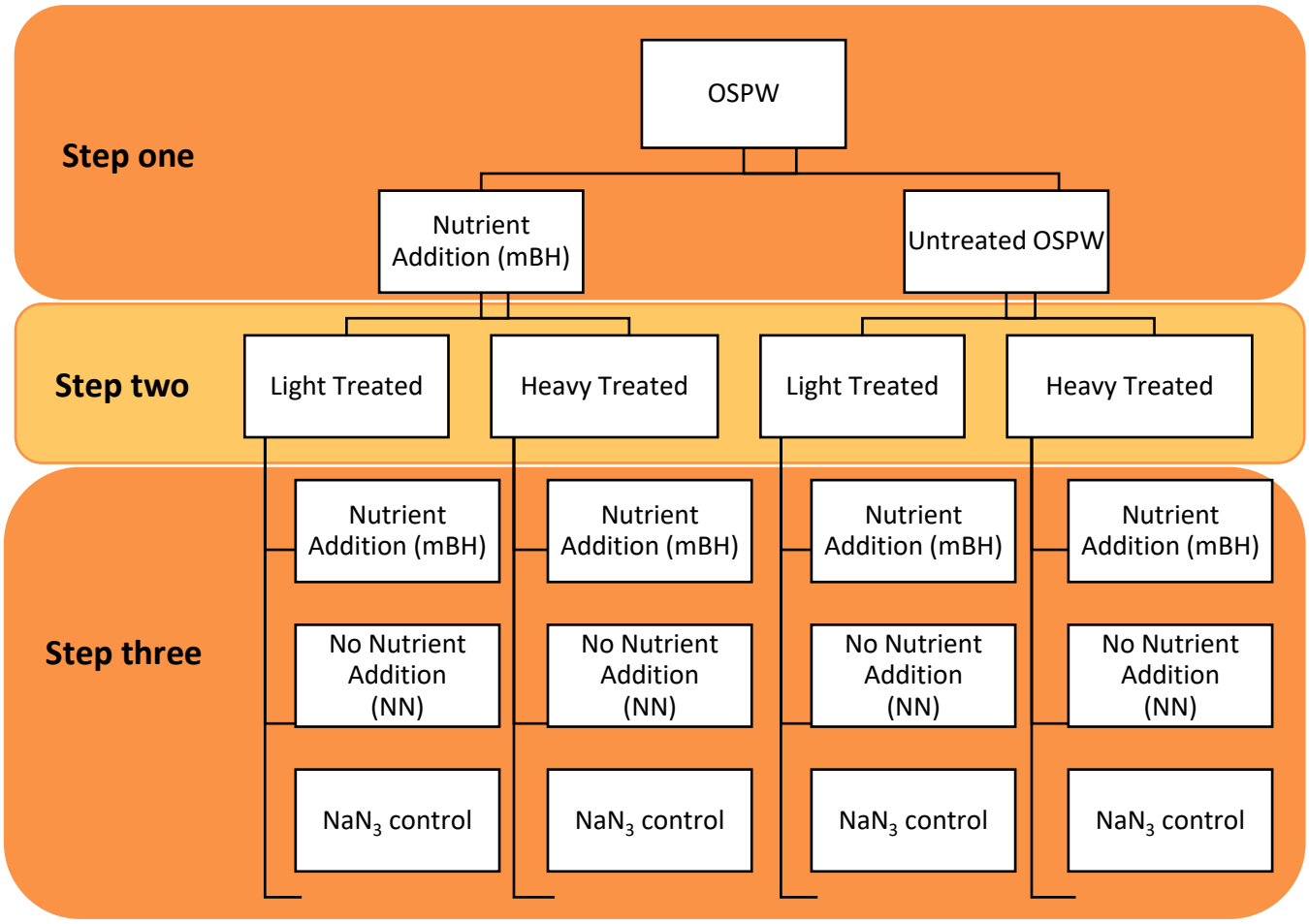

Figure 5-1: Flowchart of experimental set up for multi step treatment of OSPW. Step one, conducted at University of Alberta, a biological stimulation treatment of mineral nutrients to reduce all organics present to recalcitrant compounds. Step two, conducted at University of Waterloo, a UV photocatalytic oxidation with TiO_2 step of two doses to oxidize recalcitrant NAFCs and provide amenable organics for the native microbial community. Step three, conducted at the University of Alberta, a biological stimulation treatment with additional nutrients to stimulate the microbial community to degrade the remaining NAFCs and oxidized organics present.

Step one large scale set up- 20L Thermo Scientific™ Nalgene™ polypropylene carboys were filled with 12L of OSPW, or 700mL mBH and 11.3L of OSPW, in replicate. Caps were modified with 20 mm blue butyl septa for periodic gas and liquid sampling. Carboys were incubated at

room temperature (20±2°C) and shaken continuously at 150rpm to maintain oxygenation. Liquid samples (5mL-25mL) were taken periodically, filtered with 0.22µm nylon filters and stored at 4˚C until analysis.

Step two oxidation treatment- 24L of step one untreated OSPW, and 24L of step one mBH treated OSPW was sent to Dr. Gu's lab at the University of Waterloo where is was treated via UV photocatalysis. Details on their methodology can be found in Leshuk et al. (2016). Two treatment doses were targeted: 1) light treated OSPW (approximately 25% reduction of NAFCs), 2) heavy treated OSPW (approximately 75% reduction of NAFCs). Due to algae present limiting light penetration, OSPW was prefiltered using a 1.5µm glass microfibre filter Whatman 934-AH. Doses of UV (kJ/L) were approximately 50.9kJ/L and 50.5kJ/L for light treated untreated OSPW, and mBH treated OSPW respectively. Heavy UV doses of 170.3kJ/L and 159.9kJ/L were used for untreated OSPW and mBH treated OSPW, respectively. It should be noted the heavy dose oxidation had issues with continuous stirring for the first 50-100kJ/L of UV dose, thus exceeded the 100kJ/L target. Treated OSPW was then shipped back to UofA and the experiment was set-up as shown in Figure 5-1.

Step three microcosm set up- 500mL Fisherbrand™ reusable glass media bottles with cap (modified with a 20 mm blue butyl septa for periodic gas and liquid sampling) were filled aseptically with 250mL of treated OSPW(-BH), OR 235 mL of OSPW and 15 mL modified Bushnell Haas media (+BH) (compound list can be found in the Supplemental Materials)(both treatments in quadruplicate). Abiotic controls were set-up (in duplicate due to limited volume) identically to live +BH test bottle conditions with the addition of 0.04% (w/v) sodium azide to kill the native community and are referred to as "killed "control bottles. All bottles were

incubated at room temperature (20±2°C) with light restriction constantly shaking horizontally at 150rpm. Dissolved oxygen was monitored periodically to ensure aerobic conditions were maintained, and it was found that the 50/50 liquid/headspace set up provided sufficient oxygen throughout the experiment (data not shown). Liquid samples (5mL-25mL) were taken periodically, filtered with 0.22µm nylon filters and stored at 4˚C until analysis.

5.2.2 NAFC Analysis using GC-FID

Merichem was extracted under methods previously described (Grewer et al., 2010) using 5mL of sample, derivatized by 50µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (Agilent J&W 122-5512 column: 15 m x 250 µm x 0.25 µm). The temperature gradient was as follows: 50 °C for 2 min, then increasing at a rate of 30 °C/min to 280 °C which was maintained for 8 min. Helium was used as carrier gas with the following flow program: 0.15 mL/min for 2 min, then decreased to 0.063 mL/min which was maintained until the end of the separation. Total run time was 17.7 min. The detector was maintained at 300 °C. Column flow plus makeup gas (helium) was set to 32.1 mL/min, air flow 450 mL/min and H_2 flow 40 mL/min. A typical liquid injection volume was 1 µL. Merichem NAs were used as standards for quantification (Calibration curves given in Appendix A2)., and fluorene-9-carboxylic acid (97% FCA) was used as an internal standard to determine extraction efficiency.

5.2.3 NAFC Analysis using Orbitrap-MS

Samples were analyzed as per Headley et al. (2002). In brief, sample extracts (5 µL) were introduced into the mass spectrometer by loop injection (flow injection analysis) using a Surveyor MS pump (Thermo Fisher Scientific Inc.) and a mobile phase of 50:50 acetonitrile/water containing 0.1% NH4OH. Mass spectrometry analysis was carried out using a dual pressure linear ion trap−orbitrap mass spectrometer (LTQ Orbitrap Elite, Thermo Fisher Scientific, Bremen, Germany) equipped with an ESI interface operated in negative ion mode. Data was acquired in full scan mode from m/z 100 to 600 at a setting of 240,000 resolution (average mass resolving power (m/Δm50%) was 242,000 at m/z 400). Mass accuracy error of less than 1 ppm were obtained using a lock mass compound (n-butyl benzenesulfonamide) for scan-to-scan mass calibration correction.

A previously characterized Athabasca Oils Sands OSPW large volume extract was used as standard for the quantification of NA in the AEO samples. Linear equations, forced to zero, were derived from the data and used to calculate sample concentrations. There is currently no standard method for the quantification of NAs, therefore the results here are considered semi-quantitative.

5.2.4 Microbial Community Analysis

DNA Extraction- 100mL samples of single microcosms were filtered on .22µm nylon filters and stored at -20˚C until extraction. DNA was isolated from filters using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the DNA isolation protocol suggested by the manufacturer.

DNA Sequencing- The sequencing was performed at RTL Genomics (Lubbock, Texas). Samples were amplified for sequencing in a two-step process. The forward primer was constructed with

(5’-3’) the Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and the V4 region 515F universal primer. The reverse primer was constructed with (5’-3’) the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the V4 region 806R universal primer. Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1µl of each 5µM primer, and 1µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95°C for 5 min, then 35 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold.

Products from the first stage amplification were added to a second PCR based on qualitatively determine concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using SPRIselect Reagent (BeckmanCoulter, Indianapolis, Indiana) in a 0.75 ratio for both rounds. Size selected pools were then quantified using the Qubit 4 Fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2x300 flow cell at 10pM.

The raw sequences obtained were processed and analyzed using the QIIME2 pipeline version 2019.10 (Bolyen et al., 2019). After trimming the primer region with the cutadapt plug-in,

sequences were clustered into amplicon sequence variants (ASVs) using the DADA2 plug-in with the following settings: p-trunc-len-f = 240, p-trunc-len-r = 180 or 220 bp, p-max-ee = 3. Taxonomy classification was performed using the Silva-132-99-nb classifier (silva-132-99-515-806-nb-classifier.qza) trained to match the 515f and 806r primer amplicon and provided at the QIIME2 web site. Then the sequence data set was subsampled to an equal depth of 17,000 reads per sample prior to analysis to minimize the bias caused by different read-depth. The amplicon data was further analysed using the Phyloseq package V1.26 (McMurdie and Holmes, 2013) in R. Data shown is of single microcosm bottles.

5.2.5 Headspace CO_2 Analysis

Headspace $CO_{2(g)}$ was measured to track CO_2 production as a metabolic byproduct of biodegradation with an Agilent 7890A gas chromatograph equipped with a thermal conductivity detector (GC-TCD) (Agilent HP-PLOT/Q column: 30 m x 320 µm x 0.2 µm). The oven temperature gradient was as follows: 50 °C for 2 min, then increased at a rate of 30 °C/min to 150 °C which was maintained for 2 min. Helium was used as carrier gas with the following flow program: 8.83 mL/min for 2 min, decreasing to 5.67 mL/min until the end of the separation. Total run time was 7.33 min. The detector was maintained at 200 °C, and the injection port at 300 °C. The makeup gas (helium) was set to 5 mL/min. The injector split ratio was set to 5:1 (no gas saver), with a column flow of 8.89 mL/min, split vent flow of 44.4 mL/min, and a septum purge flow of 58.3 mL/min under a pressure of 30 psi. A typical injection volume was 100 µL. Gas standards were created using various concentrations of CO_2 and N_2 gas mixes (Calibration curves given in Appendix A2).

5.2.6 DOC & Total Nitrogen Analysis

Dissolved organic carbon (DOC) was measured with a Shimadzu Model TOC-L_{CPH} ASI-L Shimadzu auto sampler. Non-Purgeable Organic Carbon (NPOC) method was used for DOC analysis which measures the non-volatile dissolved carbon content during the purging process (Brown et al., 2013). Total nitrogen was measured by Natural Resources Analytical Laboratory on a Shimadzu TOC-L_{CPH} using a chemiluminescence detector. Samples were filtered with a 0.22 µm nylon filter and diluted to a suitable range before analysis. Concentrations were determined by external calibration using potassium hydrogen phthalate for NPOC and ammonium or nitrate salts for total nitrogen.

5.2.7 Cation Analysis

Standard array of cations were analysed by Natural Resources Analytical Laboratory on Thermo iCAP6300 Duo (N. America) inductively coupled plasma-optical emission spectrometer (ICP-OES). Multi-element certified standard solutions purchased from SCP Scientific are used for calibration, and separate certified multi-element solutions are used as external reference standards.

5.2.8 Microtox™ assay

Microtox™ is routinely used to assess the toxicity of OSPW and NAFCs (Herman et al., 1994; Lo et al., 2006; Frank et al., 2008; Toor et al., 2013a). Toxicity of samples at the beginning and end of the experiment was determined using the Microtox™ toxicity assay (Osprey Scientific, Edmonton, Alberta, Canada). Day 0 samples were taken within 2 hours of set up, filtered at .22µm to sterilize and stored at 4°C until analysis. The 81.9% Basic Test protocol for 5 min and 15 min acute exposure toxicity assay were performed on a Microbics model M500

analyzer (AZUR Environmental Corporation, Fairfax, California, USA) according to the manufacturer's recommendations. Both 5 and 15 minute acute toxicity was measured, however there was no significant difference in toxicity measured between tests and therefore only the 5 min data is described. The IC_{50} values were recorded representing percentage of the sample resulting in a 50% decrease in bioluminescence of the target microorganism *Vibrio fischeri*. Toxicity units (TU) were then derived from IC_{50} ($TU=100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

5.2.9 Statistics

Significance was determined using Microsoft Excel (Microsoft 365) software, using a two tailed t-test analysis. Variance between sample pools were tested first using F-test, to determine if variance is equal, or unequal. Differences were deemed significant if the t-test resulted in a p-value that was less than 0.05 (higher significance also noted).

5.3 Results and Discussion

5.3.1 Step one- Microcosm study of nutrient enhanced NAFC biodegradation

The results of Chapter 4 demonstrate that a multistep approach to remediation of NAFCs in OSPW is a potential strategy that should be investigated. Based on previous results in Chapter 4 shows OSPW may be nutrient limited as well as analysis (Figure 5-3) indicates OSPW is limited in phosphorus, a nutrient addition step before oxidation would be beneficial to allow the native microbial community to degrade all possible organics present. The oxidation step would then only target remaining organics that are recalcitrant to biodegradation. A microcosm

study was conducted in triplicate to determine the extent OSPW is nutrient limited, and how much this limitation impacts biodegradation of NAFCs present in OSPW. Two media mixes were tested to determine if i) added micronutrients (trace metals, like iron, nickel, zinc) (mBH) could provide a boost to microbial activity and degradation in comparison of traditional BH media and ii) added macronutrients (nitrogen, phosphorus) improve biodegradation in comparison to OSPW alone. Bushnell-Haas media mix was chosen as the base as it was developed specifically for microbial degradation studies of hydrocarbons (Bushnell and Haas, 1941; Clemente et al., 2004; Videla et al., 2009). CO_2 production is used as a monitor of microbial growth, live treatments produced CO_2 in their metabolic processes and the killed control bottles produced negligible amounts (0.1 ± 0.0 mg/L CO2 produced, 0±2mg/L of NAFC removed in 50d) indicating NAFC degradation is due to biological activity and not abiotic processes (Figure 5-1). Between the two media mixes tested, the mBH bottles produced 11.7± 1.5 mg/L of CO_2 and degraded 26 ± 2% of NAFCs over 50d and the BH bottles produced 14.8 ± 3.8 mg/L of CO_2 and degraded 25 ± 3 % of NAFCs over 50d. There are no significant differences ($p>0.05$) of NAFCs degraded (at T50) between BH and mBH treated microcosms, indicating the additional nutrients in mBH had no significant benefit over the simpler BH nutrient media. Lai et al. (1996) determined that phosphate addition significantly improved degradation of model NA palmitic acid (Z=0) and decahydro-2-naphthoic acid (Z=-4) by microbial communities present in OSPW. Addition of nutrients such as phosphate will be critical to improve and boost native microbial degradation of NAFCs. Although there was no significant difference indicated by this microcosm study between mBH and BH, going forward in the large-scale step one set up of mBH will be used to ensure micronutrients present in OSPW do not become limited.

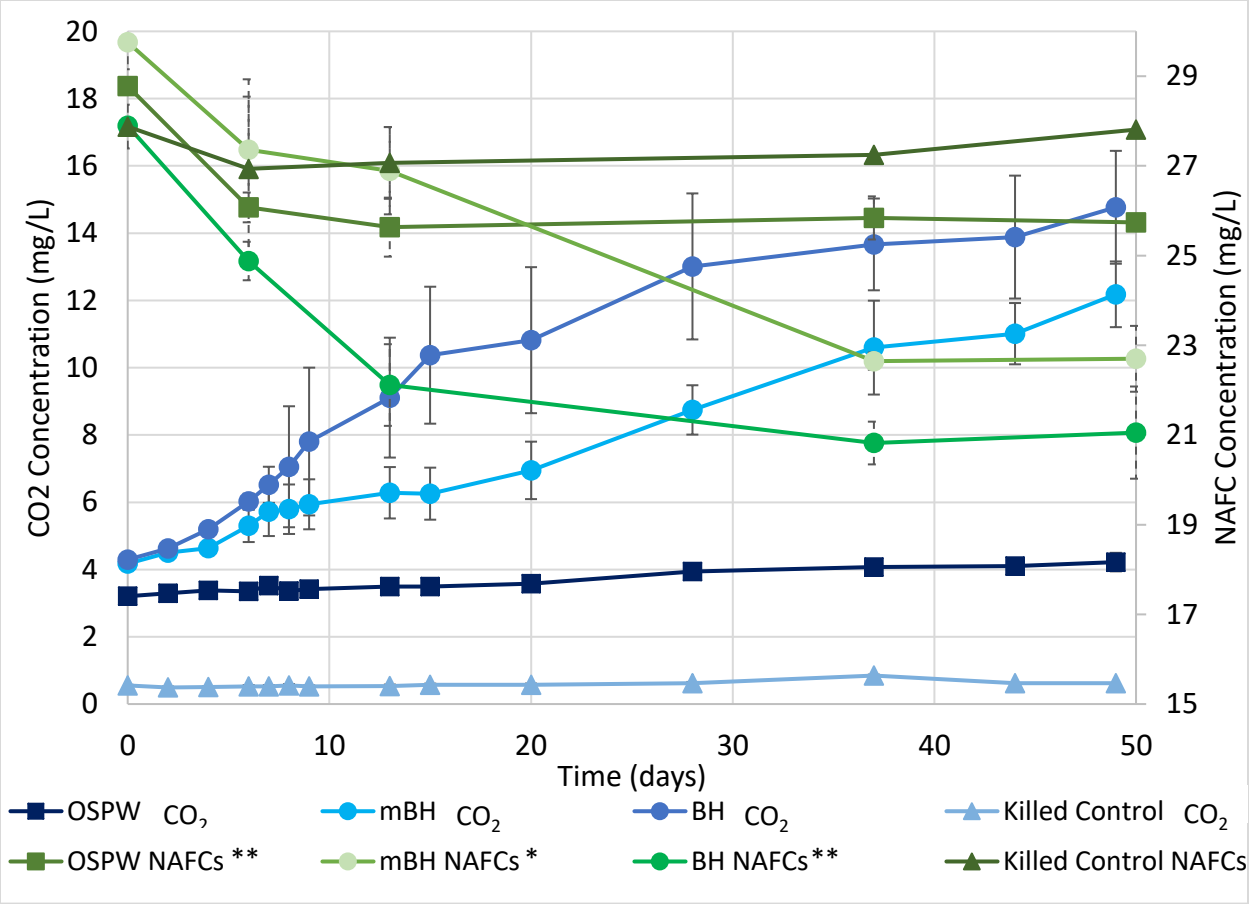

Figure 5- 2: CO_2 production and NAFC concentration for small scale step one experiment. "mBH" denotes OSPW supplemented with modified BH media, "BH" denotes OSPW supplemented with standard BH media, "OSPW" denotes untreated OSPW, and "killed control" denotes NaN_3 killed mBH amended microcosms. Averages are of triplicate 1L bottles ± standard error. **p<0.05, **p<0.01* T-test to determine significance of change within a treatment by comparing T0 to T89 within a treatment, *n*=3. (p values given in Appendix)

Lai et al. (1996) found that oxygen consumption can particularly limit the capacity for a microbial community to actively degrade NAFCs. All microcosms were provided with half headspace of air, and O_2 monitored periodically to ensure no oxygen limitations. Although no nutrients were added to Untreated OSPW microcosms, 1.4 ± 0.8 mg/L of CO_2 was produced and 11 ± 3% of NAFCs were degraded in 50d (Figure 5-1). With constant aeration through shaking, the microbial community was able to degrade 3.0 ± 0.5mg/L of NAFCs present without the benefit of additional nutrients. Substantially less CO_2 was produced in comparison to the mBH

added microcosms, indicating only a small fraction of NAFCs were completely mineralized. Misti et al. (2013) found that although significant degradation of NAFCs was possible by the commercial NA enriched microbial community from OSPW, CO_2 mineralization only occurred in a small fraction. Only 28.5% of commercial NAs (NA sodium salts, 125mg/L initial liquid concentration) were detected as CO_2, with 44% of NAs being used for biomass growth. Although only a small amount of CO_2 was produced by the Untreated OSPW microcosms (100µmol), NAFC removal was significant ($p<.05$).

This microcosm study showed that the microbial community in OSPW benefited from the addition of BH media, and no significant difference of effectiveness between BH and mBH media was found ($p>0.05$). Nutrient addition significantly increased degradation with 26 ± 2% of NAFCs being degraded in 50d, indicating OSPW is limited in nutrients such as nitrogen and phosphorus present in BH media. Nutrients will be tracked in the large scale set up of step one due to sample availability in small scale microcosm study (Figure 5-3). Untreated OSPW just shaken aerobically resulted in significant 11 ± 3% degradation of NAFCs present in OSPW from T0 to T50 ($p<0.01$), indicating oxygen availability may be limited in OSPW. Based on these findings, both mBH treated OSPW and Untreated OSPW will be replicated in large scale 30L carboy containers to provide adequate volume for step two oxidation and step three additional microbial degradation.

5.3.2 Step One-Large volume treated with nutrient enhanced NAFC biodegradation

To provide sufficient volume (25L per treatment) for step two and three, large-scale treatment of OSPW was conducted (Section 5.2.1 in methods). Additionally, this provided liquid volume for additional step one measurements that were limited in the microcosm study. NAFC

degradation for mBH treated and Untreated OSPW were on par with small scale microcosm study, with 25% and 11% decrease in NAFCs for mBH treated and untreated OSPW, respectively. Monitoring of CO_2 produced followed the same trend as the microcosm study, however at 33d a notable green colour of the water indicated a potential algae bloom (light restriction was not done in error). This bloom consumed most of the CO_2 produced, which was not an issue in the microcosm study since it did not develop an algal bloom. However, it should be noted that NAFC degradation (as per GC-FID) was not affected by the apparent algae bloom, indicating the algae present did not have a significant role in NAFC degradation in this study.

Since our microcosm study indicated that our OSPW samples were nutrient limited, cations were tracked for the large-scale treatment. It was found that a key macronutrient, phosphorus was not detectable in OSPW (detection limit of 0.011mg/L). Phosphorus is a critical nutrient required for microbial life, required for synthesis of nucleic acids and phospholipids (Madigan, 2006). With no available phosphorus in the water, this limitation would negatively impact the microbial community's ability to degrade NAFCs. OSPW treated with mBH had 22.6mg/L of phosphorus, and used 7.3mg/L in 89d, a significant decrease in concentration. With no limitation of phosphorus in the mBH treatment, the plateau in NAFC degradation would be attributed to the recalcitrant nature of the NAFCs left, not a nutrient limitation.

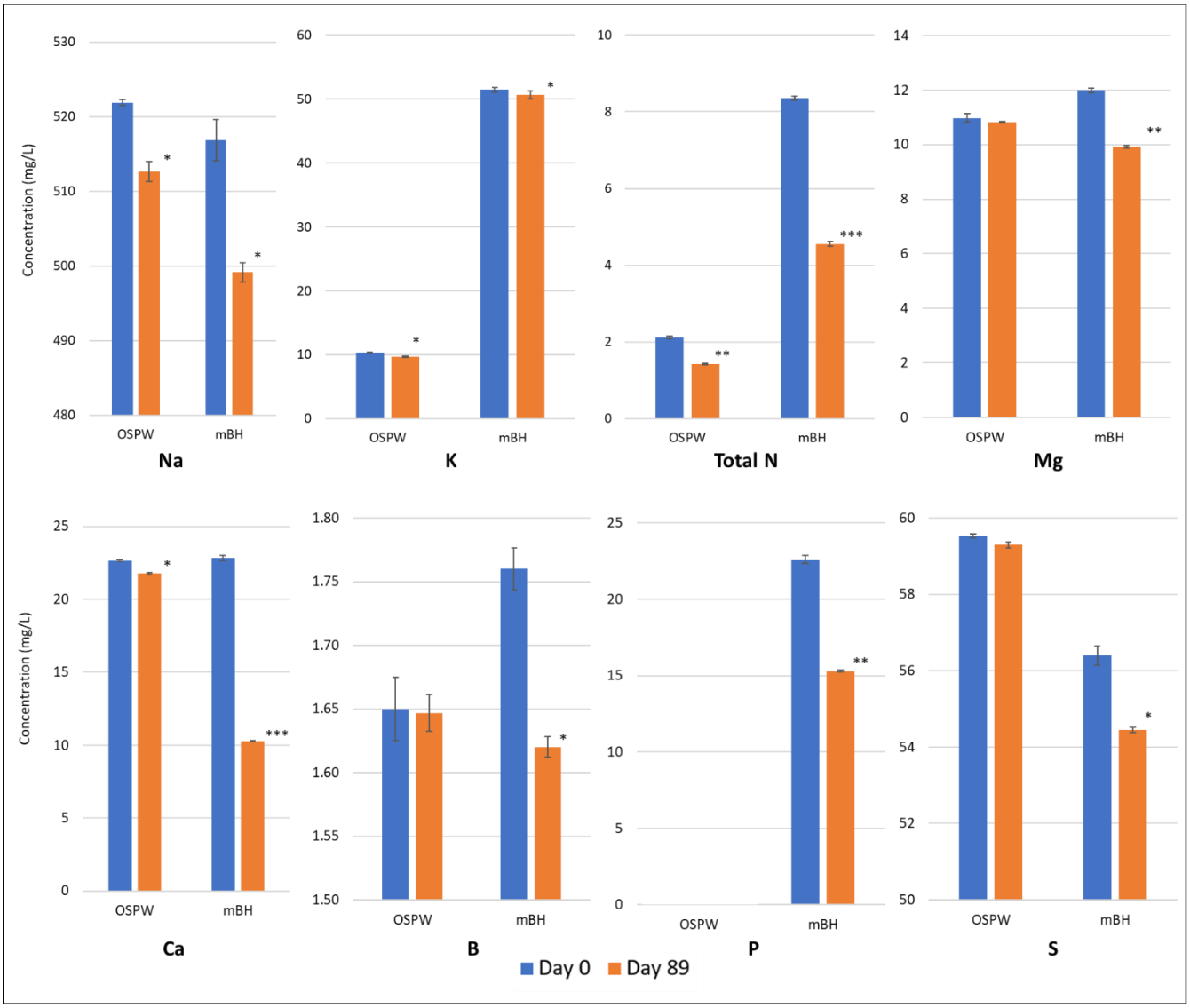

Figure 5-3: Cation analyses for large scale step one treatment of OSPW. Cations were analysed from large scale set up. Mn, Fe, Cu, and Zn were below detection limit (<DL) for both treatments. Li showed no change in concentration. **p<0.05, **p<0.01, ***p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T89 within a treatment, *n*=3. (p values given in Appendix)

Several other cations showed significant decreases over the 89d experiment, such as nitrogen (Figure 5-3, p values given). Nitrogen again is a macronutrient required for cellular life, it is a major element in proteins, nucleic acids, and many cell constituents. By dry weight, nitrogen comprises 12% of total cell mass, a critical component for microbial life. Untreated OSPW contained 2.1mg/L of total nitrogen, and used 0.7mg/L by 89d. OSPW treated with mBH

began with 8.4mg/L of total nitrogen (given as ammonium) and ended with 4.6mg/L, which is a 3.8mg/L decrease in 89d. mBH treated OSPW used more total nitrogen in 89d than what OSPW contained originally, indicating the amount of biomass produced in mBH treatment may not be achievable in OSPW without added nutrients to support cell growth. Videla et al. (2009) examined the nitrogen use by native microbial communities found in OSPW via stable isotope analysis. By adding nitrogen to microcosms, biomass became enriched in ^{15}N, supporting that additional nitrogen is beneficial for microbial communities to degrade NAFCs in OSPW. A study by Herman et al. (1993) tested the biodegradability of four model NAs with a microbial community native to OSPW. It was determined that by adding nitrogen and phosphorus three out of four model NAs were degraded in 40d. Cyclopentane carboxylic acid and cyclohexane carboxylic acid were degraded within 10d of nitrogen and phosphorus addition, with 2-methyl-1-cyclohexane carboxylic acid being the slowest at 40d. However 1-methyl-1-cyclohexane carboxylic acid did not benefit from addition, with no degradation at all (Herman et al., 1993). As in this study, nitrogen and phosphorus have been identified as key macronutrients that effect the biodegradation of NAFCs by native microbial communities in OSPW.

To get more in-depth characterization of NAFC profiles, samples collected in the large-scale set up was analyzed using an Orbitrap-MS (Figure 5-3). Characterization of NAFCs has been evolving for the last 20 years, with the advent of powerful mass spectrometers like the Orbitrap, a greater separation of organics is possible. The Orbitrap-MS has high resolving power making it possible to differentiate between species beyond the 3rd decimal place (i.e. 240 000 at m/z 400) (Ross et al., 2012; Headley et al., 2013; Pereira et al., 2013). The output of the Orbitrap analysis is summarized in three-dimensional profile plots displaying carbon number

(C), double bond equivalents (DBE), and intensity (related to concentration). As toxicity has been attributed to the O_2^- species, this was the focus of Orbitrap analyses (Morandi et al., 2017). The initial intensities for day 0 for mBH and untreated OSPW (Figure 5-4A, C) appear to be considerably different. This is likely due to variability in OSPW used within this experiment. As large volumes were required, several buckets were used throughout set up. Variation in OSPW sampled at the source is common, homogenization was not possible due to such large volumes and time between multi-step set ups. The focus is on comparing changes over time in the same treatment as opposed to comparisons between treatments.

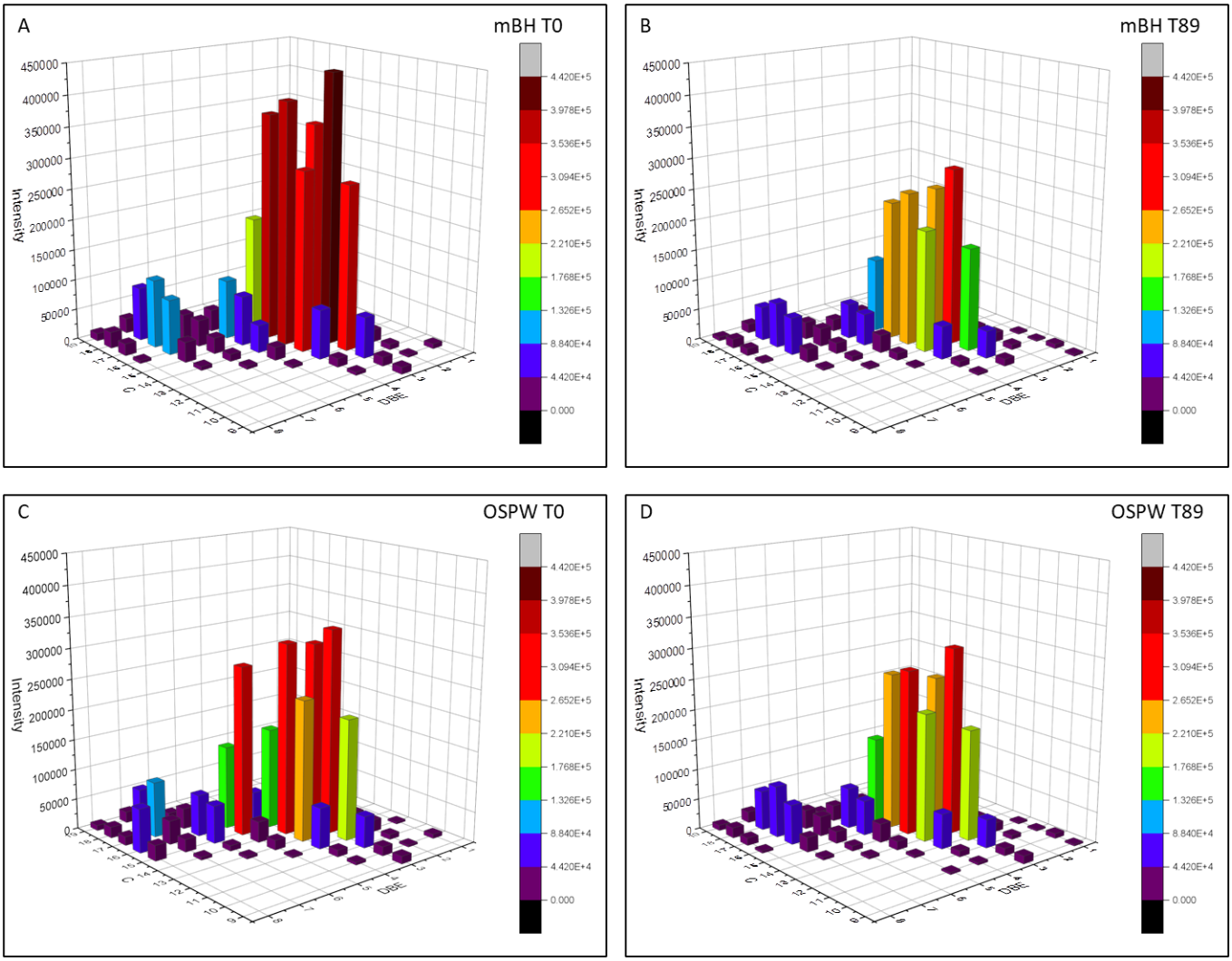

Figure 5-4: Three dimensional plots showing changes in O_2^- distribution of mBH and untreated OSPW. Plots denote ion intensity vs carbon number (C) and double bond equivalents (DBE) as measured per Orbitrap-MS across time. (A), and (B) are mBH treated OSPW through day zero and day 89, respectively. (C) and (D) are untreated OSPW through day zero and day 89, respectively. Data represents time series of the large-scale treatment of step one of the multistep remediation experiment.

The number and distribution of NAFCs in mBH treatment show major changes between days 0 and 89 (Figure 5-4A, B). By day 89 there was a drastic 42% reduction in the DBE 8, as DBE is a measure of degree of unsaturation, or the number of rings and double bonds, reduction in the higher DBE groups is critical for reduction in associated toxicity. In comparison only 6% of

DBE 8 was degraded in untreated OSPW. On average, 34% of DBE 2 to DBE 7 was degraded by 89d, indicating there was no preferential degradation of one group over the other in mBH treated OSPW. Untreated OSPW had a slightly different trend with only 8% degradation on average for DBE 3 through DBE 8, showing no difference in the DBE 8 degradation unlike the 42% reduction in mBH treated. However, DBE 2 showed a 27% decrease in both mBH treated OSPW, and untreated OSPW, indicating this group of compounds was amenable to biodegradation even without the boost given by the nutrients to the microbial community. In both treatments, DBE 1 increased substantially with a 113% and 65% increase in mBH and untreated OSPW, respectively. This increase in DBE 1 suggests that smaller less complex compounds are being produced through microbial degradation, the microbial community is breaking down the higher DBE NAFCs into the simpler DBE 1 compounds, or producing metabolites. Similarly, compounds containing 9 carbons increased by 27% in untreated OSPW, which supports that simpler, less heavy NAFCs are being produced. These by-products of biodegradation may be consumed given enough time as simpler compounds (DBE 1, Z=0) are often depleted in aged OSPW and have been demonstrated to be readily biodegraded by bacteria (Han et al., 2008; Toor et al., 2013b; Mahdavi et al., 2015).

As toxicity has been attributed to O_2^- species containing ≥17 carbons, remediation of these heavier organics is critical (Hughes et al., 2017). NAFCs containing 10 to 16 carbons on average were degraded 38%, with 9 carbon compounds being reduced 69% in the mBH treated OSPW. More importantly, 19 carbon NAFCs were reduced 44%, which is critical for toxicity reduction (Figure 5-3A, B). Initial toxicity assessment by Microtox™ bioassay indicates a significant decrease in toxicity for both untreated OSPW, and mBH treated OSPW (Figure 5-5).

mBH treated OSPW had a 33% reduction in toxicity in 89d. This large decrease in toxicity can be attributed to the 39% reduction of NAFCs containing ≥17 carbons (Hughes et al., 2017). However, untreated OSPW did not have this large decrease in compounds containing ≥17 carbons with only a 2% average reduction in this group. Overall untreated OSPW did have a significant decrease in toxicity, with a 11% reduction of total toxicity (Figure 5-4). Although O_2^- species has been largely attributed to the acute toxicity of OSPW, other species still contribute to toxicity to a lesser extent (Morandi et al., 2017). A shift in species not quantified by the O_2^- Orbitrap-MS analysis may have contributed to the reduction in toxicity as well. While Bartlett et al. (2017) has indicated that Microtox™ toxicity bioassay may not accurately assess toxicity of NAFCs or OSPW to native species, it is an effective screening tool to compare relative toxicity changes within an experiment such as this.

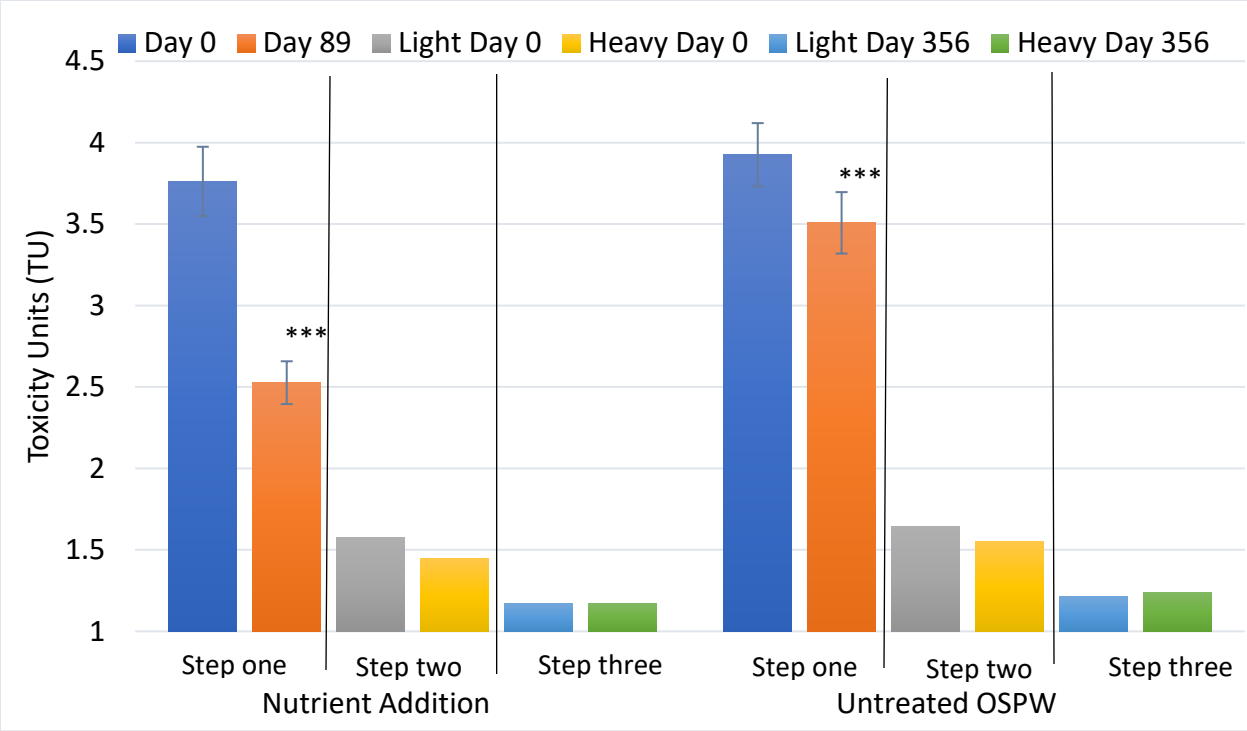

Figure 5-5: 5-minute acute toxicity Microtox™ analysis multistep remediation of OSPW. All samples under Nutrient Added were treated in large scale step one with the addition of mBH media. All samples under OSPW were unamended in large scale step one with no addition of nutrients. Day 0 and day 89 are the beginning and end of the step one time series. Bars represent triplicate ± one standard error, the remaining bars are single analyses due to limited sampling volume. Heavy/Light refers samples after oxidation in step two of treatment. Day 0 and day 356 are the beginning and end of the step three time series of biological treatment. These samples were the microcosms amended with additional nutrients in step three. Toxicity units were derived from IC_{50} ($TU=100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control (data not shown). ****p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T89 within a treatment, n=3. (p values given in Appendix)

As NAFCs contribute to the toxicity of OSPW, studies of NAFC degradation are the focus of much research. Numerous studies focus on enrichment of the native microbial species through model NAs or NAFC mixtures (Herman et al., 1994; Clemente et al., 2004), or by isolating pure cultures (Del Rio et al., 2006; Demeter et al., 2014). However, with the development of high-throughput sequencing direct analysis of the microbial community is possible. Samples were taken at various time points throughout this multistep treatment and

analysed using 16S rRNA to determine dominant bacteria in those treatments. Large variability is possible within OSPW (Richardson et al., 2019), and was also seen in our work as demonstrated by the slight difference in microbial community abundance (Figure 5-5). Time zero samples for mBH treated and untreated OSPW show slight differences in microbial community composition, where the mBH treatment was dominated by Proteobacteria, specifically *Xanthomonadales, Rhizobiales, Sphingobacteriales* and *Burkholderiaceae.* Whereas untreated OSPW has more abundance of Actinobacteria *Sporichthyaceae,* Proteobacteria *Rhodocyclaceae Sulfuritalea* and *Burkholderiaceae* and with Actinobacteria *Microbacteriaceae*. Although variations in abundance exist, Proteobacteria, and Actinobacteria have been consistently found in OSPW (Penner and Foght, 2010; Johnson et al., 2011; Ramos-Padron et al., 2011; Siddique et al., 2011; Foght, 2015; Foght et al., 2017; Zhang et al., 2018; Siddique et al., 2019).

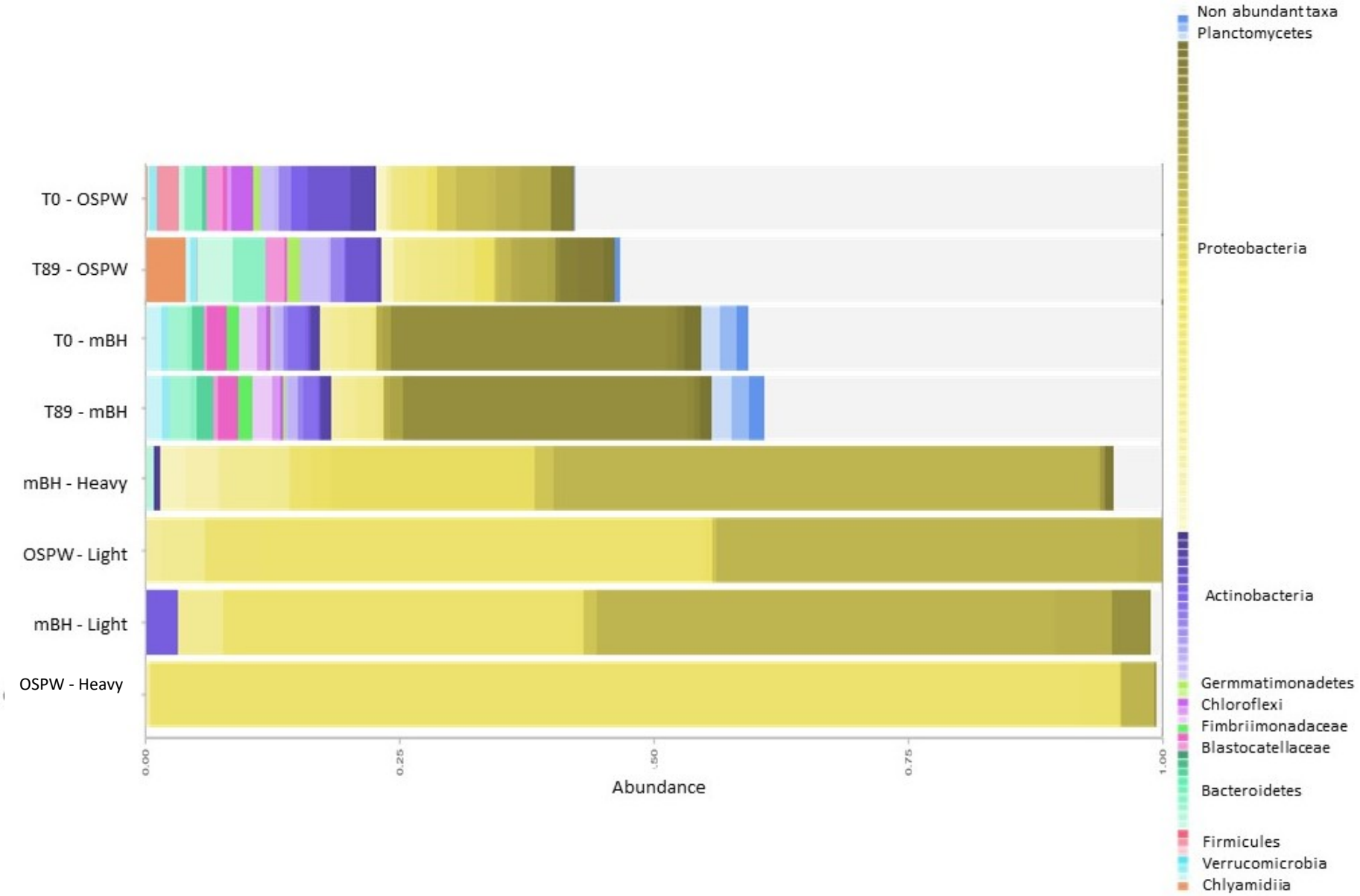

Figure 5-6: Microbial community abundance analysis for large scale multi-step treatment, step one and two. All samples treated in large scale step one with either OSPW unamended or with the addition of mBH media. Day 0 and Day 89 are the beginning and end of the step one time series. Heavy or light refers samples after oxidation in step two of treatment. Due to sample volume restrictions, two microcosms were combined of the treatment quadruplicates for analysis.

Relative abundance changes over the course of step one remained relatively unchanged with only minor shifts over the 89d for mBH treated OSPW. With Proteobacteria *Acidibacter* enriching the most by 105 OTU copies, bacteria belonging to *Planctomycetes* and *Chloroflexi* (26 and 81 copies, respectively) also increasing slightly over 89d. These bacteria have been noted as constituents of effective NAFC degrading biofilm reactors treating OSPW sourced NAFCs (Zhang et al., 2020a). Untreated OSPW increased in abundance of known degraders *Burkholderiaceae* (528 increase in copies) and *Rhizobiales* (253 increase in copies) (Johnson et al., 2011; Golby et al., 2012). These variations in abundance indicates that nutrient limitations such as total nitrogen, and phosphorus can affect the microbial community, with these bacteria thriving in a more nutrient rich environment. Cation analysis revealed that OSPW is depleted in phosphorus and low on total nitrogen. Both phosphorus and total nitrogen were significantly decreased in mBH treated OSPW over the 89d indicating these nutrients are required and used by the microbial community to actively degrade NAFCs in OSPW. With a 25% reduction of NAFCs, and 33% reduction in toxicity, nutrient addition treatment significantly increased degradation of NAFCs in OSPW.

5.3.3 Step Two – UV photocatalyitic oxidation with TiO_2

Large volumes of both mBH treated OSPW and untreated OSPW were sent for oxidation treatement to Dr. Frank Gu's lab at University of Waterloo. As per chapter 4 results, the oxidation doses were targeted with a light and heavy dose of oxidation. It has been demonstrated that NAFCs that are more complex in structure were preferentially degraded by advanced oxidation processes such as UV/H_2O_2 and ozonation treatements (Perez-Estrada et

al., 2011; Afzal et al., 2012). Livera et al. (2018) determined that rate of photocatalytic oxidation treatement significantly increased with NAFC structural complexity, specifically due to carbon number, aromaticity and degree of cyclicity. As these characteristics are what often lead to NAFCs being recalcitrant to biodegradation, treatment with UV photocatalyic oxidation with TiO_2 could produce organics that are more biodegradable. For step two, oxidation of both mBH and untreated OSPW treatments, the targeted heavy dose was three times stronger than the light dose. Heavy dose degraded approximately 80% of NAFCs, and light dose degraded approximately 60% of NAFCs. Associated toxicity of treated water in step two was reduced 53%, 56%, 38% and 42% for untreated OSPW light and heavy, mBH treated light and heavy respectively (Figure 5-5).

Understanding the oxidative stress experienced by the microbial community during oxidation of NAFCs is critical for coupling of advanced oxidation with microbial degradation. NAFCs have been shown to be amenable to ozonation treatment with innoculation with unstressed OSPW sourced microbial communities (Martin et al., 2010). However, coupling ozonation with biodegradation of the oxidant stressed community has demonstrated that the microbial community can survive oxidation and recover to continue on to degrade the new improved biodegradable NAFCs produced (Brown et al., 2013; Zhang et al., 2019). Chapter 4 has demonstrated that OSPW sourced microbial communities can withstand the oxidative stress of UV photocatalyic oxidation with TiO_2, however community make up was not characterized. Samples sent for 16S rRNA sequencing display which bacteria in OSPW are present after oxidation, however it cannot determine which cells are alive, or dead. Figure 5-5 shows the relative microbial abundance of organisms in the microbial community after heavy and light UV

photocatalytic oxidation treatement. Compared to the diversity of microbial community analysis of step one mBH treatement, and untreated OSPW, the microbial community post oxidation (regardless oxidation dose) has shifted to predominantly Proteobacteria and Actinobacteria, losing much of the diversity seen in pre-oxidation analysis. Specifically the genus *Rhodococcus, Pseuodomonas, Sphingomonas and Brevundimonas* are dominant in these samples. Although these genera are strongly represented in abundance in these samples post oxidation, this does not indicate that they will be the microbial genera that go on to degrade NAFCs and organics produced by the advanced oxidation step.

5.3.4 Step Three- Microcosm study of nutrient enhanced NAFC biodegradation post oxidation

The results of chapter 4 show that a multistep approach to NAFC degradation with UV photocatalytic oxidation with TiO_2 and biodegradation should be investigated as an effective remediation strategy. Based on step one results, OSPW is nutrient limited specifically in total nitrogen and phosphorus. Therefore, a third biodegradation step with nutrient addition would be beneficial to provide the native microbial community the resources required to recover from the oxidative stress experienced and biodegrade oxidized organics. To determine if the microbial community can rebound from the oxidative stress, go on to biodegrade organics present, and decrease associated toxicity, a microcosm study was conducted. Set up is described in methods section (5.2.1) and demonstrated in Figure 5-1.

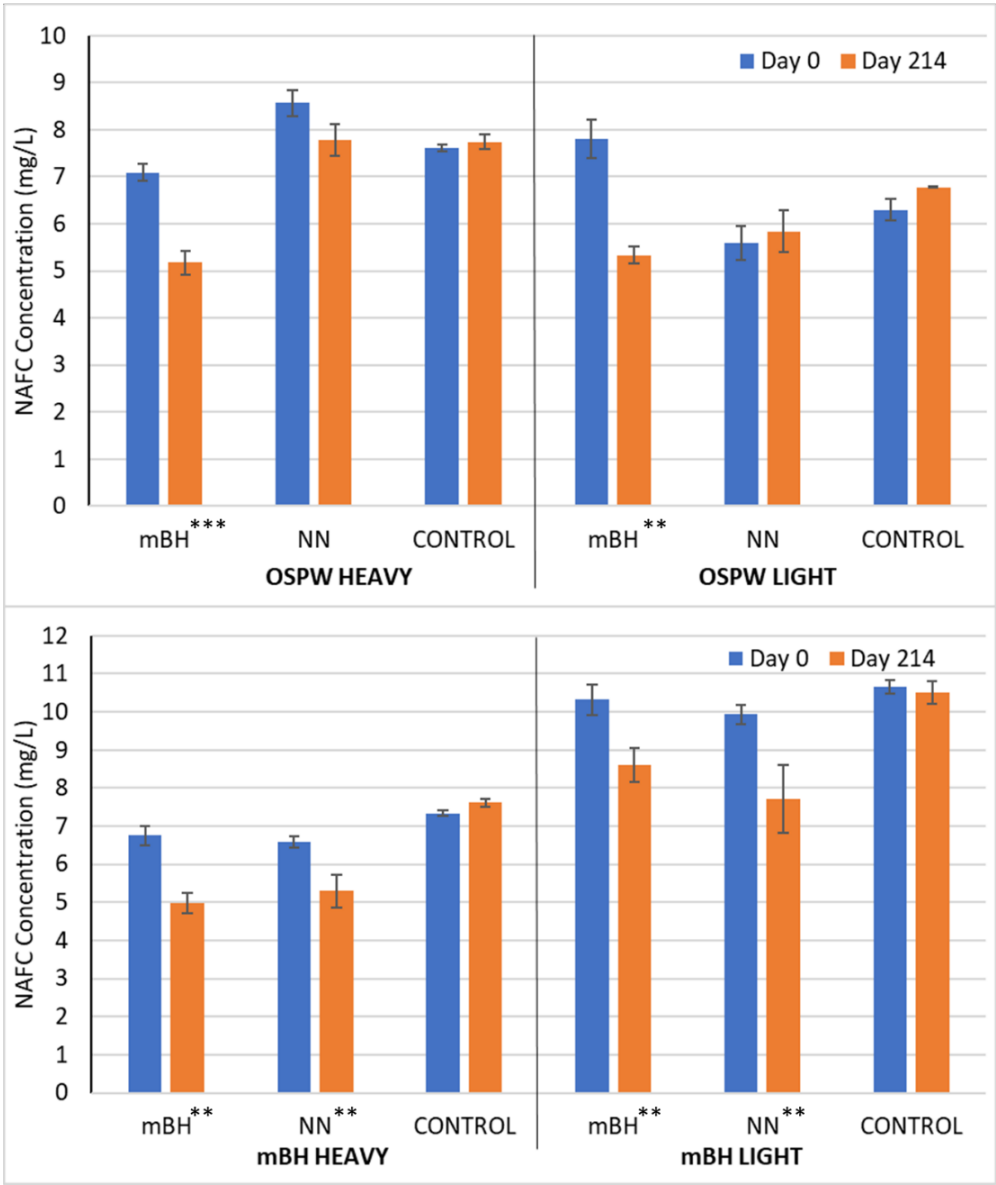

Figure 5- 7: NAFC concentration analysis for step three of the multistep treatment plan. NAFC concentration is average of quadruplicates for live treatments, and average of duplicates for controls ± one standard error. Top graph is untreated OSPW in step one, with either heavy or light oxidation treatment in step two. Bottom graph is mBH addition OSPW in step one, with either heavy or light oxidation treatment in step two. Horizontal axes labels, mBH, NN, and control indicate the step three treatment given (mBH nutrient addition; NN no nutrient addition; control is sodium azide killed control). ***p<0.01, ***p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T214 within a treatment, n=4 for live treatments, n=2 for controls. (p values given in Appendix)

Due to limited liquid volumes, only two time points were take for NAFC concentration analysis for GC-FID, and end point characterization for Orbitrap-MS. Killed controls were used to compare to live treatments to indicate whether changes are do to live microbial activity or abiotic processes. All controls NAFC concentration remained constant throughout the experiment, with no significant change (Figure 5-7). Therefore, any changes observed in live treatments can be attributed to microbial degradation by the native community that survived the oxidative stress. In all live treatments but two (OSPW-light-NN; OSPW-heavy-NN), significant biodegradation occurred. Of the NAFCs remaining after untreated OSPW heavy oxidation, 27% and 8% of NAFCs were degraded for mBH added and NN, respectively. Of the NAFCs remaining after untreated OSPW light oxidation, 31% of NAFCs were degraded for mBH added, and NN showed no degradation. Of the NAFCs remaining after mBH treatment then heavy oxidation, 26% and 20% of NAFCs were degraded for mBH added and NN, respectively. Of the NAFCs remaining after mBH treatment then light oxidation, 16% and 23% of NAFCs were degraded for mBH added and NN, respectively (Figure 5-7).

Treatments that show no significant NAFC degradation ($p>0.05$) in step three, OSPW-light-NN, and OSPW-heavy-NN, were the only treatments which received no nutrient addition at all throughout the multistep process. Phosphorus analysis in step one (Figure 5-3) shows that it is limited within raw OSPW, with no mBH addition throughout step three also shows no phosphorus (Figure 5-8). As these are the only treatments that saw no NAFC degradation in step three, phosphorus limitation may be a large factor in degradation of these organics. However, total nitrogen decreased significantly over time in these treatments ($p<0.01$) with controls holding constant over the 356d. Decrease of nitrogen in live treatments and not in

controls indicates that microbial activity is still occurring (supported by microbial diversity increase in Figure 5-9). Perhaps the limited phosphorus treatments would still degrade NAFCs, but in a longer timeframe of this experiment.

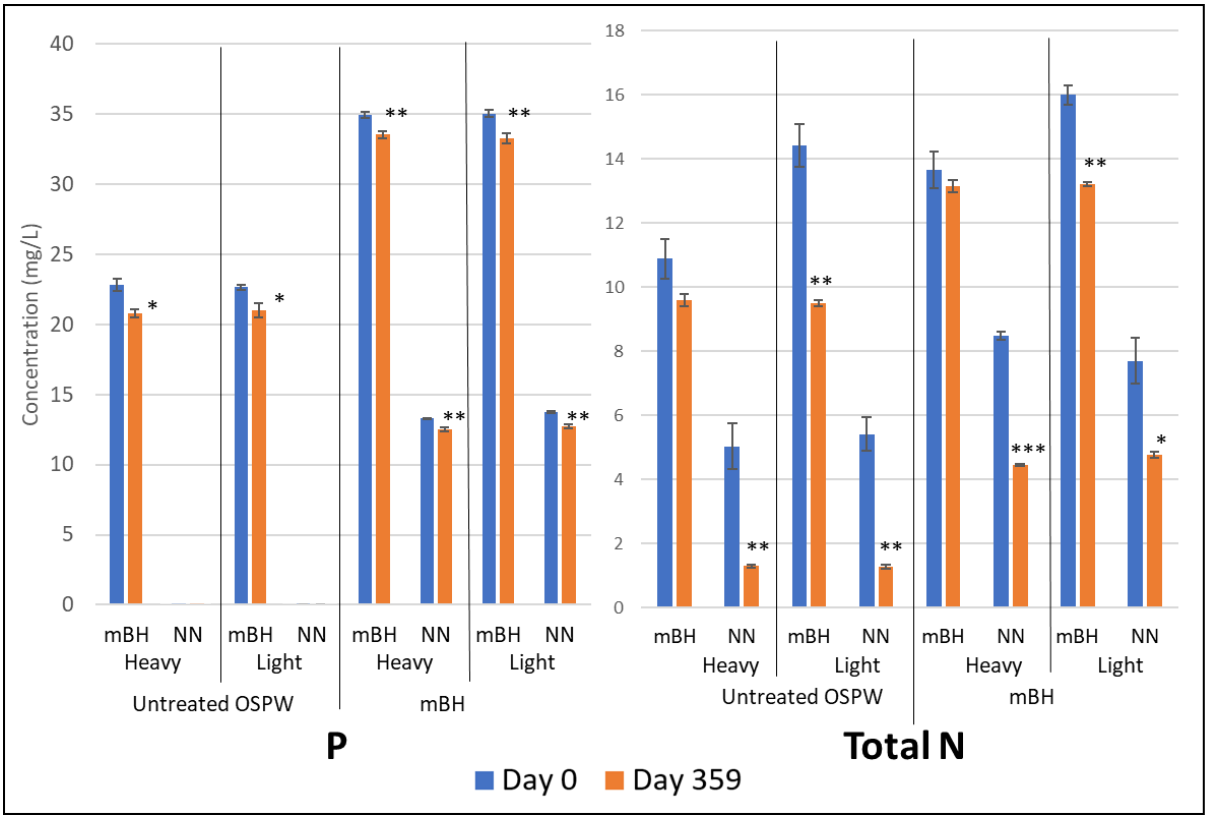

Figure 5-8: Phosphorus and total nitrogen analysis for step three of multistep treatment of OSPW. **p<0.05, **p<0.01, ***p<0.001* T-test to determine significance of change within a treatment by comparing T0 to T359 within a treatment, n=4 (p values and controls data given in Appendix).

Other treatments all saw significant (p<0.01) degradation of NAFCs (Figure 5-7) over the 214d measured and saw significant decrease of phosphorus and total nitrogen over the 359 (Figure 5-8). These findings support findings in step one that indicate OSPW is nutrient limited and impacting the degradation of NAFCs. The addition of key nutrients phosphorus and nitrogen significantly improve the microbial community's ability to degrade NAFCs in all treatments regardless the oxidative dose received.

Although none of the multi-step treatments brought the NAFC concentration to zero during the experimental time frame, toxicity measurement via Microtox™ bioassay indicate a drastic decrease in relative toxicity throughout (Figure 5-5). Due to restrictions in sample volume, only single samples were available for end point toxicity testing so statistical significance can not be determined. Additionally, mBH treated samples in step three were given priority due to their NAFC degradation profiles (Figure5-7). These samples show an additional decrease of toxicity in step three, with 25% decrease for mBH-heavy-mBH, 29% decrease for mBH-light-mBH, 14% decrease for untreated OSPW-heavy-mBH, and 23% decrease for untreated-light-mBH. All treatments analysed have an end toxicity measured at 1.1-1.2TU, which brings the relative toxicity to approximately the toxicity of water (1TU).

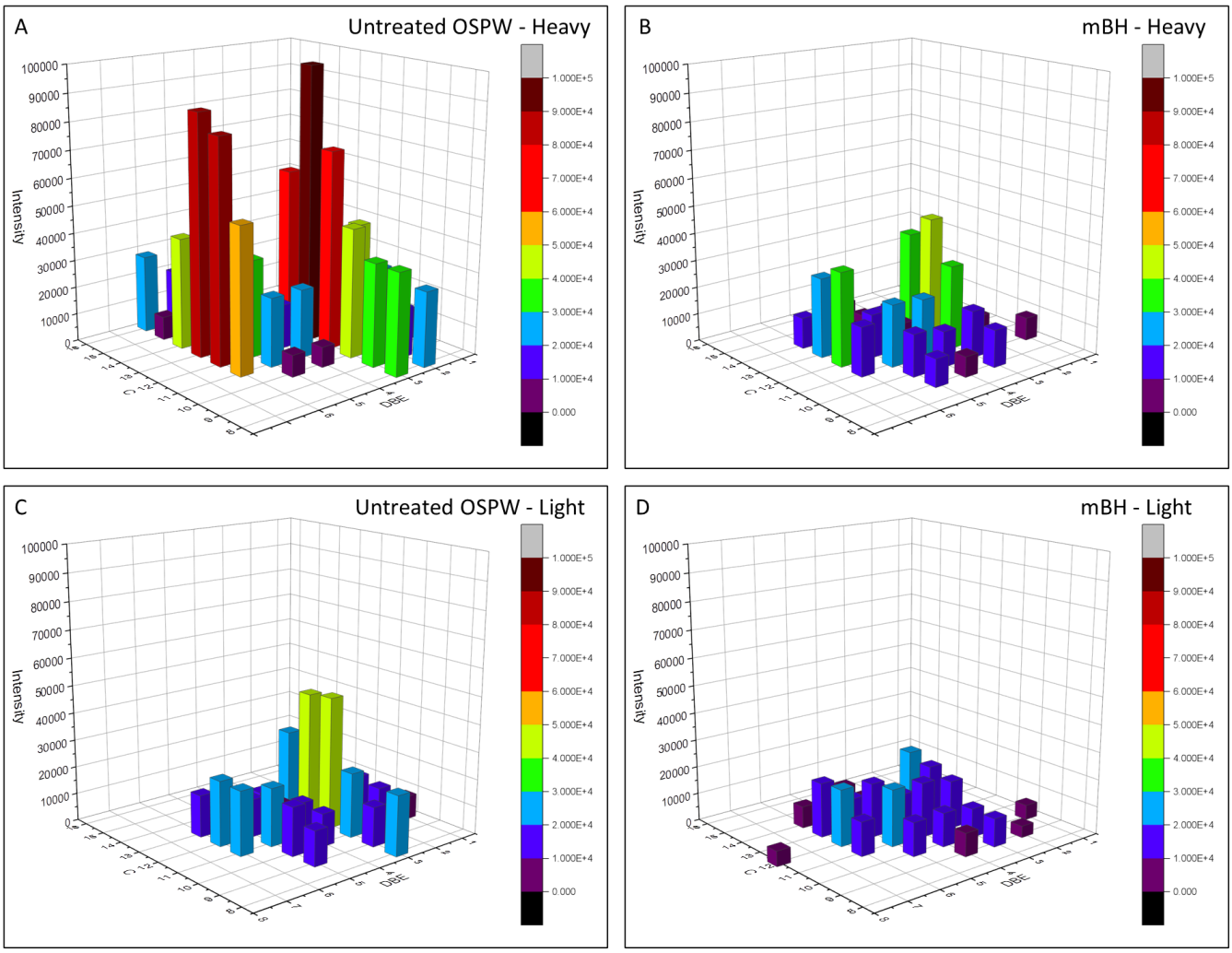

Figure 5-9 Three dimensional plots showing changes in O2- distribution of step three mBH treated microcosms. Plots denote ion intensity vs carbon number (C) and double bond equivalents (DBE) as measured per Orbitrap-MS across time. All plots are endpoint day 356 of mBH treated samples**.** (A) Step one: Untreated OSPW, step two: heavy oxidation. (B) Step one: mBH treated, step two: heavy oxidation. (C) Step one: Untreated OSPW, step two: light oxidation. (D) Step one: mBH, step two: light oxidation.

As toxicity has been attributed to O_2^- species, with ≥17 carbons, further characterization by Orbitrap-MS was completed for step three mBH treated microcosms (Figure 5-9) (Morandi et al., 2016; Hughes et al., 2017). All remaining O_2^- characterized by Orbitrap-MS do not contain any NAFCs with ≥17 carbons. OSPW-light treated with mBH completely biodegraded all NAFCs ≥14 carbons, and DBE 7 and 8. Overall, the relative intensity measured decreased more than

75% for NAFCs from beginning of step one, to the end of step three mBH addition. Although NAFCs were not entirely eliminated in any of the treatments, the significant reduction of relative toxicity is a strong indicator that a multistep treatment of OSPW can reduce toxicity. The remaining fractions of NAFC as characterized by Orbitrap-MS (step three mBH treated) indicate they are compounds containing less than 14 carbons, and DBE under 6. Lighter less complex NAFCs are often preferentially degraded by microorganisms, given enough time this remaining fraction may be completely reduced (Scott et al., 2005a; Biryukova et al., 2007; Han et al., 2008; Misiti et al., 2014). Understanding which microorganisms are present with the potential for remediation is key to determining if this remaining fraction will eventually be degraded, given enough time.

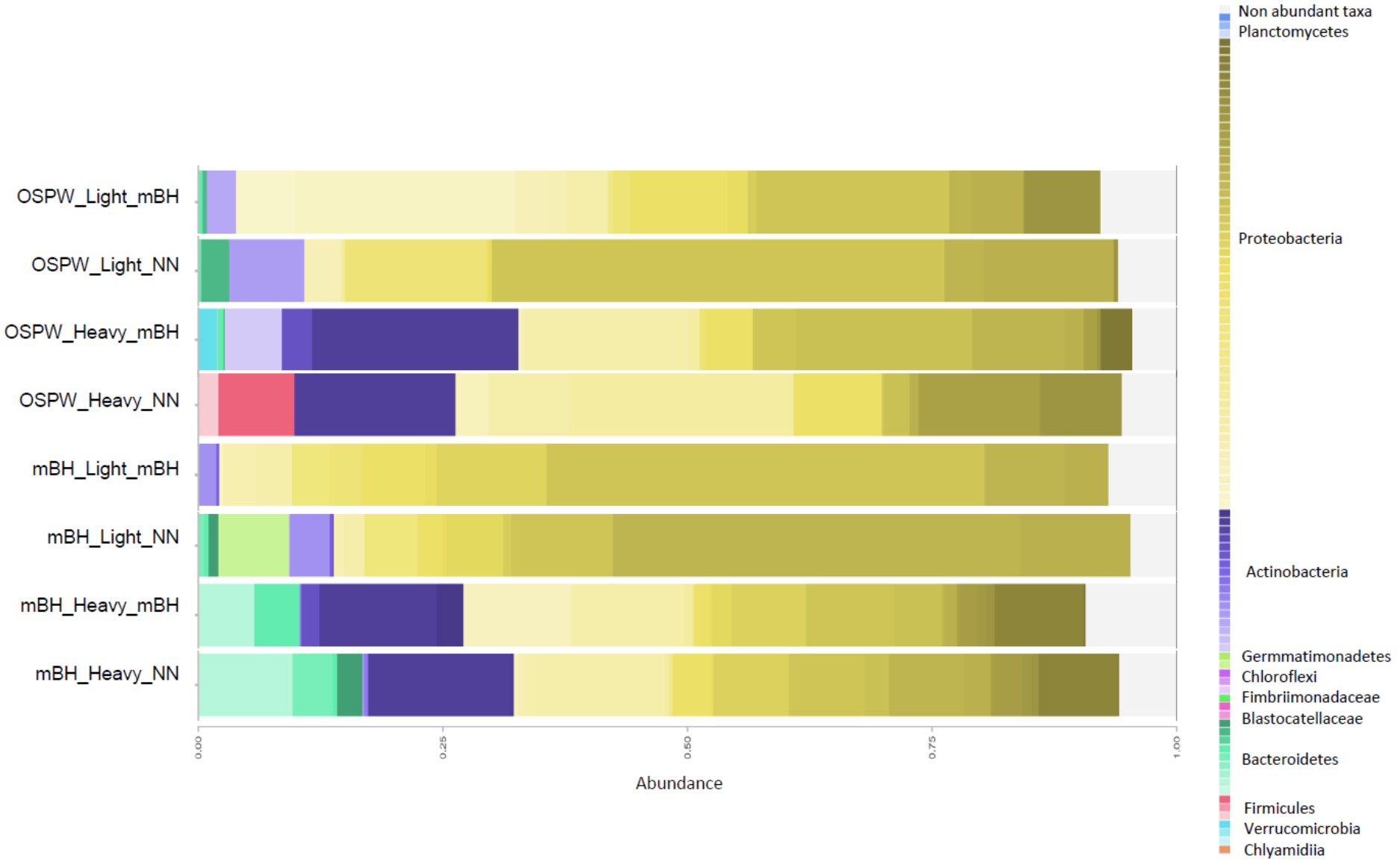

Figure 5-10: Microbial community abundance analysis for large scale multi-step treatment, step three. All samples treated in large scale step one with either OSPW unamended (OSPW) or with the addition of mBH media (mBH). Heavy or light refers to oxidation strength in step two of treatment. The third acronym refers to treatment received in step three of the multistep process, either more nutrient addition (mBH) or no nutrient addition (NN). Control refers to abiotic killed control. Due to sample volume restrictions, two microcosms were combined of the treatment quadruplicates for analysis.

The microbial community was sampled and analysed using 16S rRNA gene at 356d. Figure 5-10 shows microbial community abundance analysis for step three of the multistep treatment. Visually its evident that the microbial community diversity has recovered from the oxidative stress experienced in step two (Figure 5-6). Although Proteobacteria still holds a large portion of the genetic abundance, several other microbial groups have recovered, such as Actinobacteria, and Bacteroidetes. Interestingly, in all heavy treated OSPW, *Corynebacteriales* recovered with over 3000 copies increasing since oxidative step two, but not in light treated OSPW. In addition to the Actinobacteria, a *Pseudomonas sp*. also increased in these bottles, with 1500 to 7800 copies increasing in 356d. *Pseudomonas sp*. has been commonly noted as a resilient significant species within OSPW that can degrade hydrocarbons and NAFCs (Del Rio et al., 2006; Johnson et al., 2013; Demeter et al., 2014).

Most notable is the recovery of the microbial community post oxidation, genetic diversity pre oxidation was 283 OTUs and 365 OTUs for mBH treated and untreated OSPW, respectively. After step two oxidation, all treatments were reduced to 20-25 OTUs, a drastic decrease in diversity. These findings are comparable to Islam et al. (2014) which found ozonation decreased biodiversity of planktonic microbial communities in comparison to biofilm communities. Over the course of step three, microbial diversity recovered substantially, however in the two treatments that did not receive mBH at any step only doubled in diversity to 40-50 OTUs. All other treatments which saw significant degradation of NAFCs has a 4x increase in diversity of OTUs in step three. Increased diversity of microbial community is important for synergistic relationships between microorganisms, as one species may be able to

degrade metabolites more effectively or use substrates more efficiently (Demeter et al., 2015; Folwell et al., 2019; Yu et al., 2019).

5.4 Conclusions

This study aimed to characterize the multistep coupling of biological degradation by native microbial communities found in OSPW, and UV photocatalytic oxidation with TiO_2. A first step treatment of OSPW with nutrient addition showed that OSPW is limited in phosphorus, and the addition of phosphorus improves degradation of otherwise thought recalcitrant NAFCs present in OSPW. Untreated OSPW, with no nutrient addition still saw an 11% decrease in NAFCs and 11% decrease in toxicity in 89d just with constant shaking increasing oxygen penetration. mBH treated OSPW saw a 25% decrease in NAFCs in 89d and 33% reduction of toxicity as per Microtox™ toxicity bioassay. 16S rRNA gene sequencing showed a shift in the mBH treated OSPW in 89d with *Acidibacter* enriching the most by 105 OTU copies and bacteria belonging to *Planctomycetes* and *Chloroflexi* (26 and 81 copies, respectively) also increasing slightly. Untreated OSPW saw an increase of different microbial species, specifically an increase of abundance of known degraders *Burkholderiaceae* (528 increase in copies) and *Rhizobiales* (253 copies). Step two oxidative treatment massively decreased the biodiversity found in the OSPW microbial community going from 250-353 OTUs to 20-25OTUs post oxidation. However, throughout step three of biodegradation biodiversity recovered from the oxidative stress, increasing 4x OTUs in 356d. This recovery of diversity is key to demonstrate that the microbial community can withstand the harsh oxidative stress of UV photocatalytic oxidation and continue to degrade NAFCs present.

Overall reduction of toxicity brought the Microtox™ toxicity analysis of treated OSPW to nearly that of water (1TU), which toxicity reduction is the goal of NAFC degradation in OSPW treatment. This multi step treatment of OSPW addresses key factors, 1) the nutrient limitation on biodegradation of otherwise thought to be recalcitrant NAFCs present, 2) oxidation of complex recalcitrant NAFCs present to smaller lighter compounds, 3) recovery of the microbial community to continue on to degrade organics provided by the oxidative step. A multi step coupling of biodegradation and UV photocatalytic oxidation such as this could prove to be an important strategy for NAFC remediation in OSPW.

5.5 References

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Chapter 6: Conclusions and Recommendations for Future Work

6.1 State of knowledge

Increasing focus is being placed upon the management and reclamation of vast stores of oil sands process affected water (OSPW) in northern Alberta, Canada. Due to toxicity of naphthenic acid fraction compounds (NAFCs) within OSPW, remediation efforts have heavily focused on this fraction (Scott et al., 2005a; Grewer et al., 2010; Quesnel et al., 2011a; Hughes et al., 2017). Several different remediation strategies have emerged to treat NAFCs in OSPW and its associated toxicity. These treatment technologies have traditionally utilized an advanced oxidation process such as ozonation (Wang et al., 2013b), UV photocatalysis (Leshuk et al., 2016), and potassium ferrate (VI) (Wang et al., 2016), or physical processes through the use of adsorbents such as activated carbon (Niasar et al., 2019). While these treatments are fast acting and reliable, they may create unintended unwanted by-products and be cost prohibitive for large scale treatment of OSPW. Alternatively, with the abundance of organic compounds found in tailings ponds, the native microbial community are very active, diverse and well adapted to the environment (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012). Given this activity, bioremediation of NAFCs in OSPW is a plausible method for treating vast quantities of OSPW. Studies of microbial communities native to OSPW have traditionally focused on bacteria and algae, (Herman et al., 1994; Del Rio et al., 2006; Quesnel et al., 2011b; Demeter et al., 2014; Mahdavi et al., 2015; Paulssen and Gieg, 2019), studies have indicated fungal species are often present in OSPW (Zafra et al., 2014; Lee et al., 2015; Andreolli et al., 2016; Marchand et al., 2017). Miles et al. (2019) isolated a fungi *Trichoderma harzianum,* and demonstrated this isolate from OSPW was capable of growing on a pure drop of Merichem NAs as a sole source of

carbon on agar. These results indicated that *T. harzianum* could be a prime candidate for mycoremediation of the complex and toxic organic fraction found in OSPW and thus the focus of Chapter 3.

As advanced oxidation processes have a proven capability for NAFCs remediation, and it has been demonstrated that microbial communities native to OSPW have the potential for biodegradation, coupling of these two processes would be an effective remediation strategy. Study by Leshuk et al. (2016) has shown that the use of solar photocatalytic oxidation to degrade OSPW NAFCs is a effective, however inefficient in complete degradation. With 80% of NAFCs being degraded in the first 6h of treatment, and the remaining 20% requiring 8h of insolation; coupling with a secondary treatment for the remaining 20% would be a more efficient remediation strategy. No studies have focusing on the coupling of solar photocatalytic oxidation with TiO_2 catalyst, with biodegradation and thus the focus of Chapters 4 and 5.

6.2 Summary of Results and Recommendations of Future work and Application

6.2.1 Oil sands process affected water sourced *Trichoderma harzianum* demonstrates capacity for mycoremediation of naphthenic acid fraction compounds

A comprehensive study done within the microbial community found in OSPW has revealed substantial eukaryotic genetic diversity present (Richardson et al., 2019). Analysis with 18S rRNA revealed that the two most abundant OTU of the data set were fungi, indicating that fungi posses the capacity to resist the harsh OSPW environment. Repas et al. (2017) isolated *T. harzianum* from plant roots growing in coarse tailings, and found it had the capacity to remediate complex organic compounds. Based on these findings, there is an opportunity to

understand OSPW sourced fungi species for their potential for mycoremediation of OSPW sourced NAFCs. Therefore, the aim of this study was to establish if the OSPW sourced isolate T. *harzianum* possess the capacity to degrade complex OSPW sourced NAFCs and determine how chemical structures of two model NAs effect degradation. It was determined that that *T. harzianum* could utilize cyclohexane carboxylic acid (CHCA), 1-adamantance carboxylic acid (ADA), Merichem NAs and OSPW sourced NAFCs as a sole source of carbon, as seen by the 14%, 13% and 23-47% reduction of CHCA, ADA and Merichem NAs respectively. An important finding of this study was that a single fungi isolate was able to degrade a portion of ADA, which was previously thought to be recalcitrant to biodegradation, at a faster rate than the more labile CHCA. Orbitrap-MS revealed shifts in the chemical profiles of Merichem NAs and NAFCs especially compounds containing ≥17 carbons, that led to a 59% and 52% decrease in toxicity over the course of the experiment as per Microtox™ bioassay, respectively.

Overall, this study indicates that *T. harzianum* has the capacity to biodegrade NAFCs sourced from OSPW, however metabolites accumulated within the microcosms. Although it did not affect toxicity, this build up may have had a detrimental effect on growth. *T. harzianum* may be an ideal addition to a microbial community, with a synergistic relationship for the metabolites to be consumed, leading to an overall more substantial NAFC degradation. Functions of a fungi isolate in remediation efforts at the oil sands tailings could have a few applications. As this study confirmed the capacity for OSPW sourced NAFC degradation, with the more complex ADA degradation, *T. harzianum* could be used in a bioreactor treatment scenario in association with a mixed community. A biofiltration treatment of OSPW sourced NAFCs by Zhang et al. (2020b) found that although fungi OTUs were significantly lower than the

bacteria, fungal phylum were observed throughout the biofilter. Although *Cryptomycota* was determined to be the dominant fungal phylum in OSPW, growth of an unclassified fungi *Incertae sedis* was observed. With a proven established ability to degrade NAFCs, *T. harzianum* could be used as an additional isolate to biofiltration treatment of OSPW.

With the addition of work by Repas et al. (2017), that found *T. harzianum* allowed tomatoes to germinate and the seedlings to thrive on course tailings, increasing germination speed and biomass accumulated, without the addition of fertilizer. This demonstrated the ability of *T. harzianum* to grow on course tailings and facilitate growth of plants in a harsh environment. These findings indicate that NAFC remediation in tailings could potentially be optimized through the addition of *T. harzianum*, promoting growth of important plants in a reclamation strategy. Further work to investigate the prospective use of *T. harzianum* in a biofiltration system or tailings pond reclamation scenario. Fully characterizing the complete potential for *T. harzianum* to degrade complex structures of NAFCs will give a comprehensive strategy for applying this OSPW sourced fungal isolate for future remediation treatments.

6.2.2 A multi-step approach: Coupling of biodegradation and UV photocatalytic oxidation with TiO_2 for remediation of naphthenic acid fraction compounds in oilsands process affected water

With ample presence of organic compounds in OSPW such as unrecovered bitumen and diluent, microbial communities are highly active in tailings ponds possible (Siddique et al., 2007; Siddique et al., 2019). Although bioremediation is cost effective, it is slow and several organic compounds are proving to be recalcitrant to biodegradation, therefore advanced oxidation

processes have more commonly been used for NAFC treatment in OSPW (Allen, 2008b). UV photocatalytic oxidation with TiO_2 has been proposed as an effective, powerful advanced oxidation treatment technique of NAFCs in OSPW (Leshuk et al., 2016; Leshuk et al., 2018a; Leshuk et al., 2018b; Livera et al., 2018). Livera et al. (2018) found that photocatalytic oxidation was significantly more efficient in oxidizing NAFC compounds with greater structural complexity, which are often recalcitrant to biodegradation. Increased carbon number, aromaticity, and the degree of cyclicity in NAFCs are properties that often decrease biodegradability but increase the chemical oxidation potential. Based on this preferential degradation of complex NAFCs with UV photocatalytic oxidation, coupling with biodegradation would be ideal to biodegrade the byproducts. Survivability of the native microbial community through the chemical treatment step in multi-step process is not well understood or researched. Studies using ozone oxidation of NAFCs and microbial degradation have indicated microbial community survivability is plausible (Scott et al., 2008; Martin et al., 2010; Brown et al., 2013). However, survivability of microbial communities has not been established specifically with UV photocatalysis with TiO_2 in treatments of NAFCs.

Microcosm studies were conducted using a heavy and light dose of UV photocatalytic oxidation, and untreated OSPW (Chapter 4). This study showed significant removal of NAFCs (5.8%, 19.9% and 16% for heavy, light and untreated OSPW) during the biological step and produced significantly more CO_2 compared to abiotic controls ($p<0.001$). These results indicate that the native microbial community found in OSPW survived the harsh oxidative stress of both high and low doses of UV photocatalytic oxidation. No lag period was observed in this experiment (biodegradation occurring from T0 to T20), with degradation rates varying slightly

between treatments, 1.80±0.02mg/L/day, 1.74±0.02 mg/L/day, and 1.69±0.06 mg/L/day for light, heavy and untreated OSPW, respectively (degradation rates significant in comparison to abiotic controls). Unexpectedly, untreated OSPW degraded significant portion of NAFCs with BH addition, indicating that OSPW contains biodegradable NAFCs but is nutrient limited.

Based on all the results of Chapter 4, a multi-step approach for NAFCs is a strong candidate for remediation strategies, with a first step of nutrient addition to allow the native microbial community to degrade all possible NAFCs it can. This multi-step approach was the basis of Chapter 5, with a three-step treatment of OSPW. Step one being nutrient addition to stimulate the native microbial community, step two of UV photocatalytic oxidation in heavy and light doses, and a third step of additional nutrient addition to allow the surviving native microbial community the best conditions for biodegradation of organics produced in step two.

The addition of nutrients in the first step allowed for the native community to degrade 25% of NAFCs and a 33% reduction of toxicity as per Microtox™ toxicity bioassay in 89d. Untreated OSPW, without the addition of nutrients was able to degrade 11% of NAFCs, and a 11% reduction in toxicity as per Microtox™ toxicity bioassay in 89d. Analysis showed that OPSW is limited in phosphorus, and the addition of phosphorus improves degradation of otherwise thought recalcitrant NAFCs present in OSPW. Two treatments throughout the multistep treatment received no mBH addition at all, these bottles showed no significant ($p>0.05$) NAFC degradation post oxidative step. Indicating nutrient addition, specifically phosphorus and nitrogen are critical in NAFC degradation. Step two oxidative treatment massively decreased the biodiversity found in the OSPW microbial community going from 250-353 OTUs to 20-

25OTUs post oxidation. However, throughout step three of biodegradation biodiversity recovered from the oxidative stress, increasing 4x OTUs in 356d. This recovery of diversity is key to demonstrate that the microbial community can withstand the harsh oxidative stress of UV photocatalytic oxidation and continue to degrade NAFCs present. The oxidation step targeted larger more complex NAFCs leaving compounds with <14 carbons, and DBE <6. The remaining NAFCs are the fraction often found to be more biodegradable (Han et al., 2008; Smith et al., 2008; Misiti et al., 2014).

Although successful in reducing overall toxicity to that approximately of water (1TU as per Microtox), further characterization of toxicity would be required to fully understand how this multistep process remediates OSPW. Further work would be required to fully characterize the microbial community present that's active in NAFC degradation. Understanding what nutrients are required, in specific concentrations would be key as large doses of phosphorus and nitrogen can have detrimental effects on microbial growth. Application of this multistep process is a practical solution to the vast quantities of OSPW stored in tailings ponds. Nutrient addition to stimulate the native microbial community is possible. Although not investigated in this study, algae has been shown to degrade NAFCs in OSPW which benefits greatly from nitrogen addition possibility increasing NAFC reduction (Mahdavi et al., 2015; Yu et al., 2019). Large scale treatment of OSPW with UV photocatalytic oxidation is possible, as the TiO2 catalyst has been shown to be recoverable and recyclable for multiple uses (Leshuk and Gu, 2014). In situ treatment of OSPW by UV photocatalytic oxidation has been developed by using floating photocatalysts that float on the air water interface that allows for passive solar photocatalysis. The natural sunlight with the gentle mixing of tailings pond water would allow for constant

passive oxidation of NAFCs in the OSPW (Leshuk et al., 2018a). This passive oxidation of NAFCs would allow for the native community to sustain their capacity for degradation of the more biodegradable compounds provided through this process (Livera et al., 2018). Therefore, with this technology in addition to the results of this study, economical and effective *in situ* treatment of NAFCs in OSPW with this multi step approach is a strong potential for remediation of immense quantities water in the oil sands region.

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Appendix A1- Nutrient media recipe

Table A1: Modified Bushnell Haas media composition

	Concentration (mg/L)
KH_2PO_4	419.2
K_2HPO_4	857
H_3BO_3	0.6
ZnCl	0.2
$(NH_4)_6Mo_7O_{24}\bullet 7H_2O$	1.46
$NiCl_2\bullet 6H_2O$	1.5
$MnCl_2\bullet 4H_2O$	2
$CuCl_2\bullet 2H_2O$	0.2
$CoCl_2\bullet 6H_2O$	3
NA_2SeO_3	0.04
$AL_2(SO_4)_3\bullet 18H_2O$	0.2
NH_4Cl	535
$CaCL_2\bullet 6H_2O$	20
$MgSO_4$	200
$(NH_4)_2SO_4$	500
$MnSO_4$	1.8
$FeCl_3$	2

Appendix A2: Calibration Curves

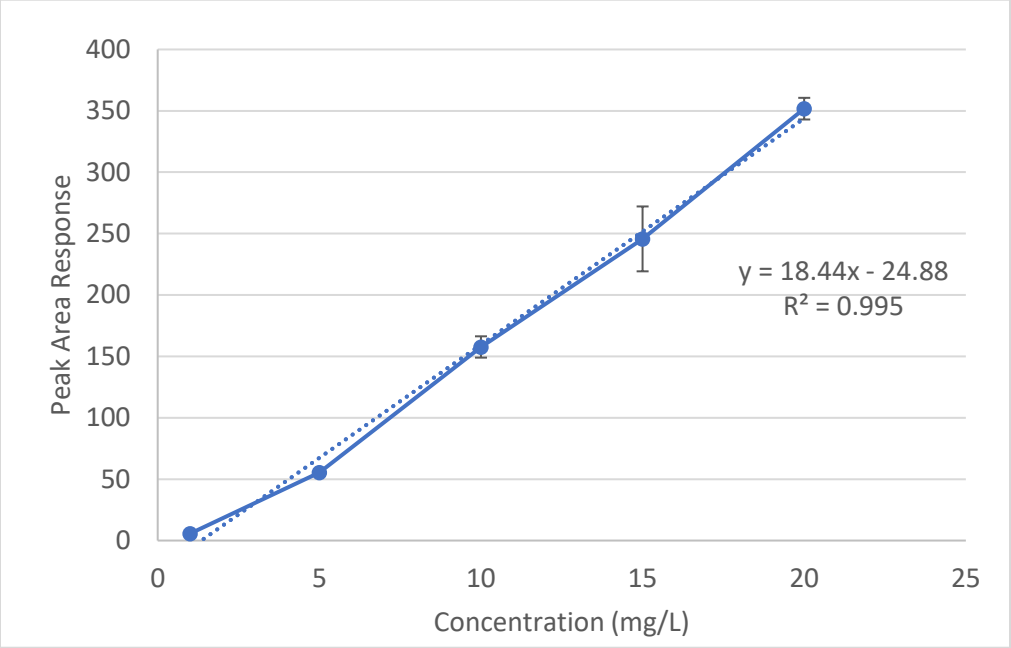

Figure A1 – Standard calibration curve for cyclohexane carboxylic acid on HPLC (Chapter 3). Error bars represent ± one standard error, *n*=5.

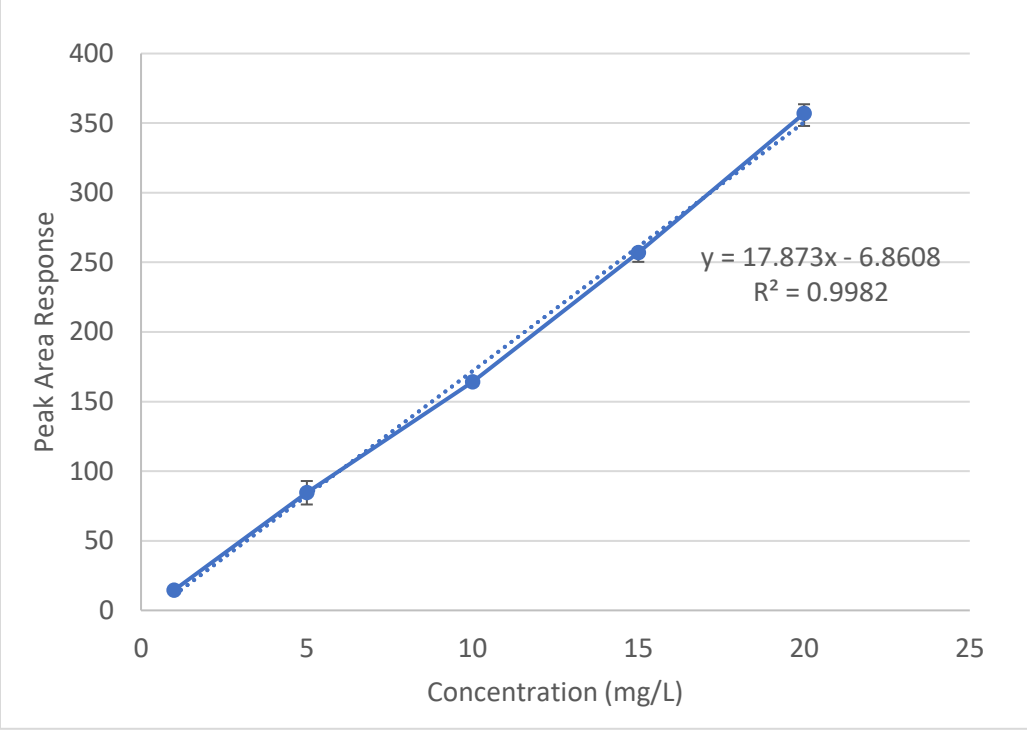

Figure A2 – Standard calibration curve for 1-adamantane carboxylic acid on HPLC (Chapter 3). Error bars represent ± one standard error, *n*=5.

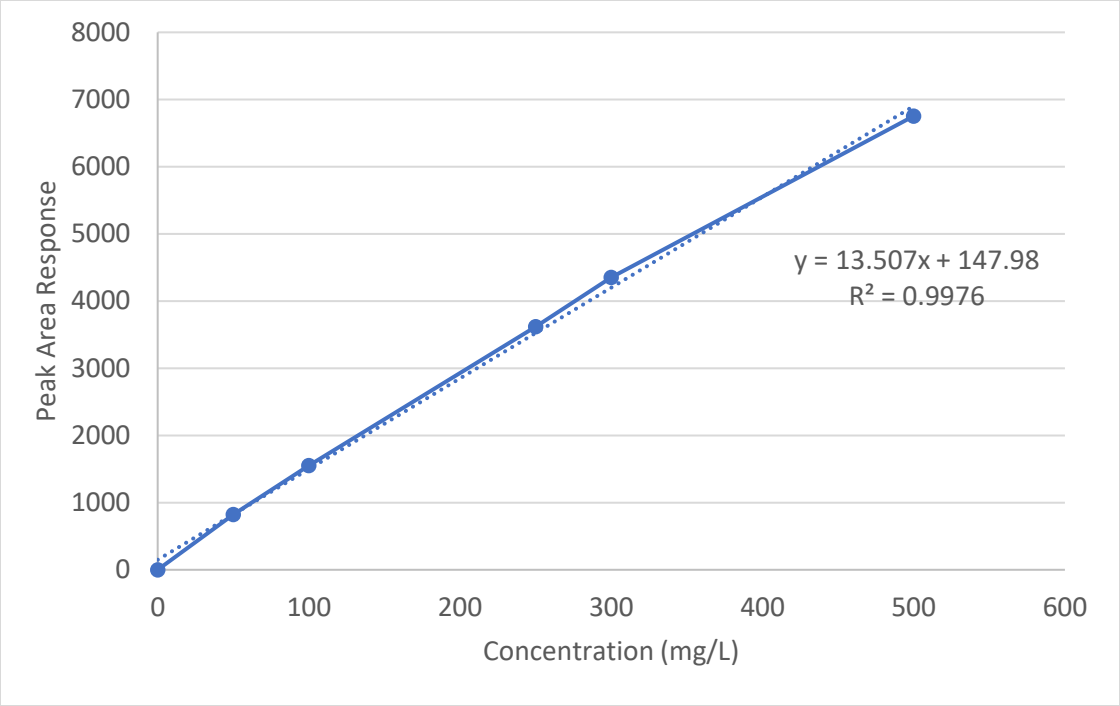

Figure A3 – Standard calibration curve for Merichem on GC-FID (Chapter 3, Chapter 4, and Chapter 5).

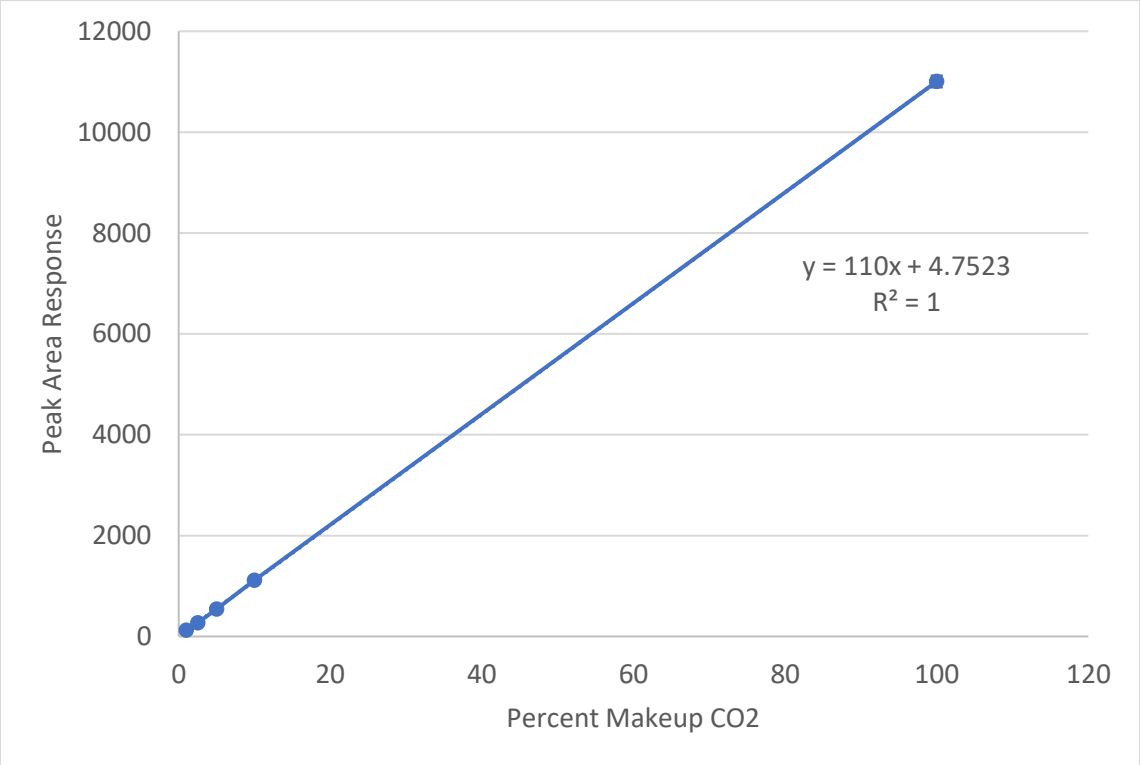

Figure A4 – Standard calibration curve for CO_2 on GC-TCD (Chapter 3, Chapter 4 and Chapter 5). Error bars represent ± one standard error, *n*=25.

Appendix A3 -Additional Control Data

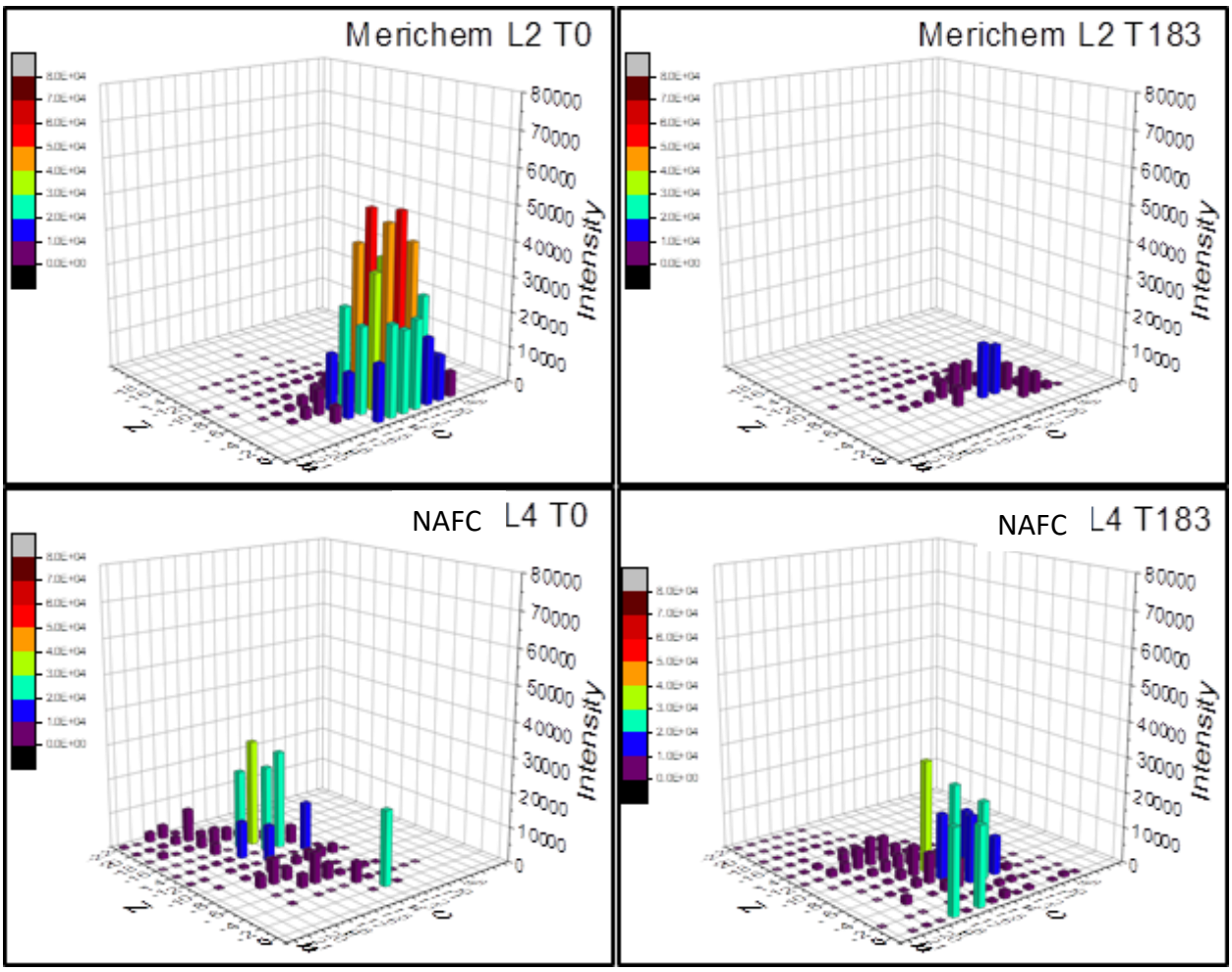

Figure A5 - Three dimensional plots showing changes in O_2^- distribution of Merichem NAs and NAFCs in *T. harzianum* treated samples. Plots denote ion intensity vs carbon number (C) and Z-series as measured per Orbitrap-MS across time.

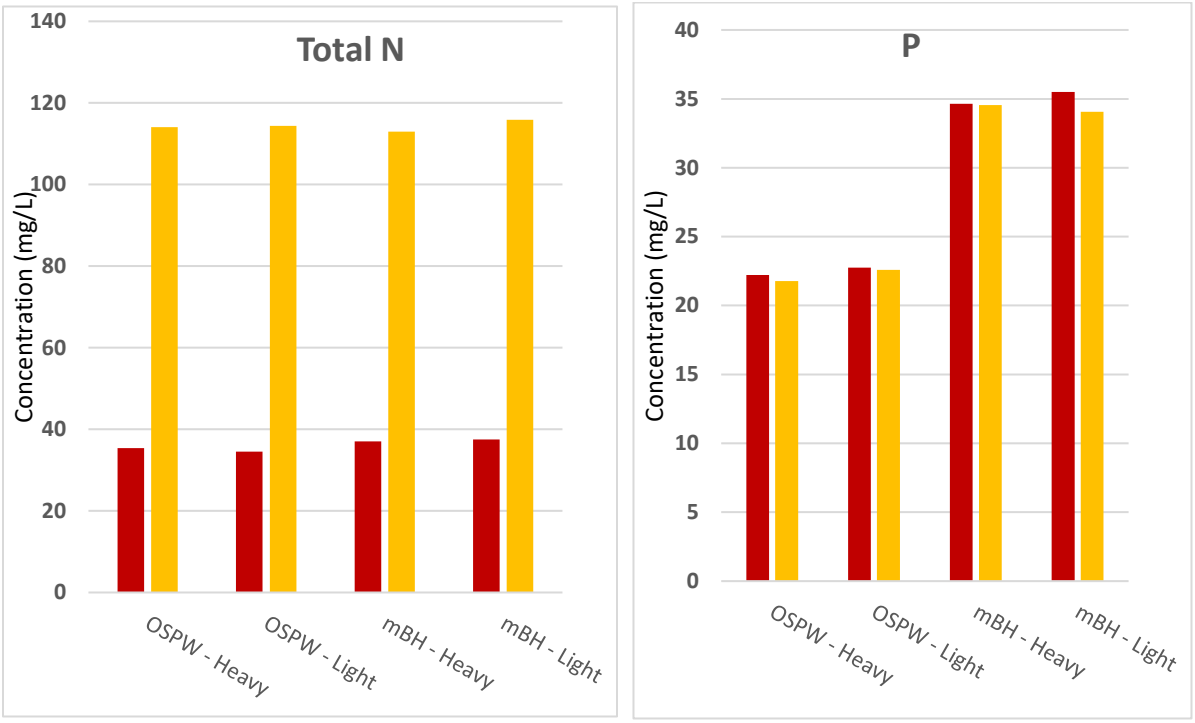

Figure A6 – Control data for total nitrogen, and phosphorus analysis of step three of the multistep treatment (Chapter 5). Total nitrogen increased due to addition of sodium azide NaN_3

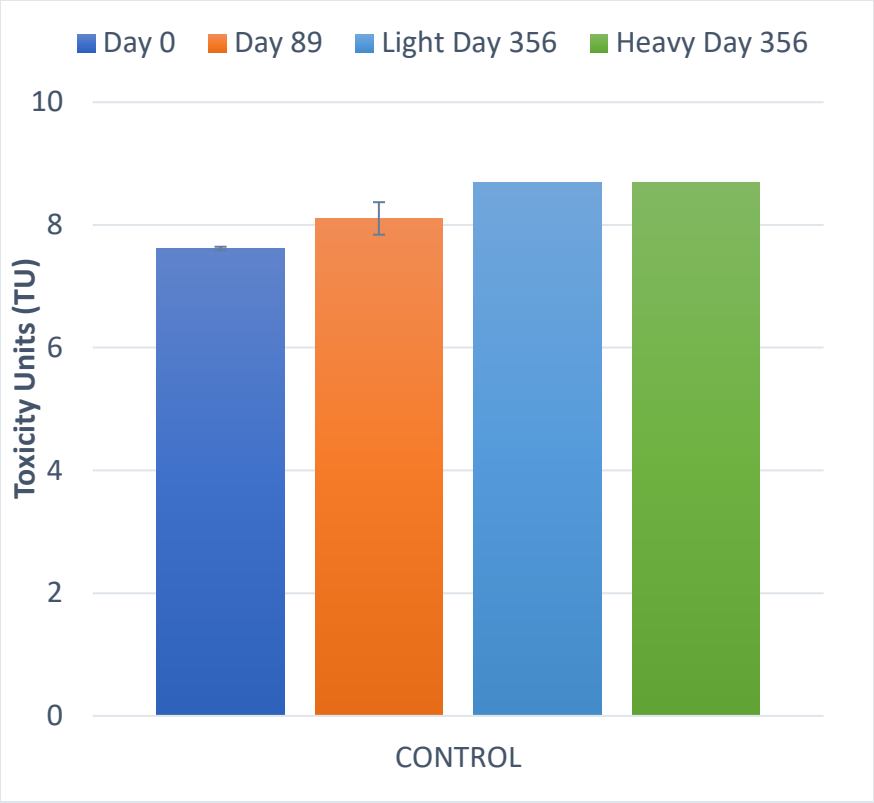

Figure A7 - 5-minute acute toxicity Microtox™ analysis multistep remediation of OSPW. Control toxicity analysis, Day 0 and Day 89 are the beginning and end of the step one time series of the small-scale microcosm set up. Bars represent triplicate ± standard error, the remaining bars are single analyses due to limited sampling volume. Heavy/Light refers samples after oxidation in step two of treatment. Day 356 is end of the step three time series of biological treatment. Toxicity units were derived from IC_{50} ($TU=100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

Appendix A4: Stats p values

Table A2: P values for Chapter 4 data. Values calculated using Excel, *n*=3 for live, *n*=2 for controls.

		DOC (T0 to T45)	NAFC (T0 to T45)	DOC (Comparison of T45 Live treatments)
Pre-treatment	**Microcosm Treatment**	**P value**	**P value**	**P value**
OSPW	Native Community	0.001		
	Pseudomonas sp. Added	0.001		
	Killed Control	0.241	0.392	
	Live pooled		0.011	0.235
Heavy	Native Community	0.000		
	Pseudomonas sp. Added	0.005		
	Killed Control	0.500	0.355	
	Live pooled		0.025	0.405
Light	Native Community	0.000	0.048	
	Pseudomonas sp. Added	0.000		
	Killed Control	0.124	0.318	
	Live pooled		0.048	0.386

Table A3: P values for Chapter 5 step one treatment data. Values calculated comparing T0 to Tend data using Excel, *n*=3.

Analysis	Set up	Treatment	P value
NAFC	small	BH	0.001
NAFC	small	OSPW	0.005
NAFC	small	mBH	0.011
NAFC	small	Control	0.477
NAFC	large	mBH	0.007
NAFC	large	OSPW	0.016
5 min Microtox™	large	mBH	0.000
5 min Microtox™	large	OSPW	0.000
5 min Microtox™	large	Control	0.036

Table A4: P values for Chapter 5 step one large scale treatment data. Values calculated comparing T0 to T89 data using Excel, *n*=3.

Cation	Na	K	Mg	Ca	B	P	S	Total N
OSPW	0.024	0.019	0.267	0.015	0.475	-	0.214	0.002
mBH	0.019	0.012	0.001	0.000	0.021	0.001	0.033	0.000

Table A5: P values for Chapter 5 step three treatment data. Values calculated comparing T0 to T356 data using Excel, n=4 for mBH/NN, *n*=2 for control. (n/a as single samples were analysed)

Treatment Set up			P	Total N	NAFC
Step one	**Step two**	**Step three**	**P value**	**P value**	**P value**
OSPW	Heavy	mBH	0.014	0.089	0.000
OSPW	Heavy	NN	0.357	0.007	0.061
OSPW	Heavy	Control	0.113	n/a	0.269
OSPW	Light	mBH	0.030	0.002	0.001
OSPW	Light	NN	0.125	0.002	0.341
OSPW	Light	Control	n/a	n/a	0.081
mBH	Heavy	mBH	0.006	0.265	0.002
mBH	Heavy	NN	0.007	0.000	0.016
mBH	Heavy	Control	n/a	n/a	0.086
mBH	Light	mBH	0.018	0.001	0.015
mBH	Light	NN	0.002	0.015	0.028
mBH	Light	Control	0.106	n/a	0.349