EVALUATION OF THE IMMOBILIZED SOIL BIOREACTOR FOR NAPHTHENIC ACIDS DEGRADATION IN OIL SANDS PROCESS WATERS

by

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Abstract

Extraction of bitumen from Alberta oil sands produces 2 to 4 barrels of aqueous tailings per barrel of crude oil. Oil sands process water (OSPW) contains naphthenic acids (NAs), a complex mixture of carboxylic acids of the form $C_nH_{2n+Z}O_x$ that are persistent and toxic to aquatic organisms. Previous studies have demonstrated that aerobic biodegradation reduces NA concentrations and OSPW toxicity; however, treatment times are long.

The objective of this study was to evaluate the feasibility of an immobilized soil bioreactor (ISBR) for treatment of NAs in OSPW and to determine the role of ammonium and ammonium oxidizing bacteria (AOB) in NA removal. ISBRs have been used to successfully remediate water contaminated with pollutants such as pentachlorophenol and petroleum hydrocarbons. A system of two ISBRs was operated continuously for over 2 years with OSPW as the sole source of carbon. Removal levels of 30-40% were consistently achieved at a residence time of 7 days, a significant improvement compared to half-lives of 44 to 240 days reported in the literature. However, similar to biodegradation experiments in the literature, a significant portion (~60%) of the NAs was not degraded.

The role of AOB in NA removal was investigated by decreasing ammonium concentration and inhibiting AOB activity with allylthiourea, neither of which significantly affected removal, indicating that AOB did not enhance NA removal. Furthermore, high AOB populations actually inhibited the removal of a simple NA surrogate. Therefore, a moderate ammonium concentration of 0.3 g/L is recommended. NA degradation occurred with nitrate as the sole nitrogen source, however, removal levels were lower than those achieved with ammonium.

Exploratory studies involving ozonation or biostimulation were conducted with the aim of increasing NA removal. Ozonation decreased NA concentration by 94% and total organic carbon (TOC) by 6%. Subsequent ISBR treatment removed ~30% of the remaining TOC.

Addition of a NA surrogate increased heterotrophic NA-degrading populations due to the increase in available carbon, resulting in a significant increase in NA removal levels. However, use of a surrogate may result in a population that is only adapted to degradation of the NA surrogate.

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Chapter 1

Introduction

The Athabasca oil sands hold the world's second largest reserve of bitumen, with approximately 168 billion recoverable barrels (ERCB, 2013). Bitumen can be extracted from oil sands ore with alkaline hot water, producing roughly 2-4 barrels of oil sands process water (OSPW) per barrel of crude oil (Alberta Energy, 2013). This water contains naphthenic acids (NAs), a complex, toxic mixture of organic acids that has demonstrated toxicity to numerous organisms. Consequently, OSPW is not discharged, but stored in tailings ponds that exceed 720 million m^3 (ERCB, 2011) and represent a significant stakeholder concern, as this land will eventually have to be reclaimed.

A number of treatment methods are being investigated including bioremediation, ozonation, adsorption, and advanced oxidation processes. Ozonation and UV treatment can greatly reduce NA concentrations, but are prohibitively expensive. Bioremediation in reclamation ponds and laboratory studies has been shown to reduce NA concentration and toxicity, however, treatment times are long and removal is incomplete.

A possible solution for improving biological treatment of OSPW is the Immobilized Soil Bioreactor (ISBR). The ISBR is a continuous reactor with a semi-permeable membrane that serves to immobilize the microorganisms in contaminated soil particles used for inoculation and to support the subsequent biofilm that develops. It has been used to treat environmental pollutants such as pentachlorophenol (Karamanev et al., 1997), p-xylene, and naphthalene (Kermanshi et al., 2005; Jajuee et al., 2006) and has improved volumetric degradation rates (Karamanev and

Samson, 1998). On the basis of these ISBR studies, application of an ISBR to treatment of OSPW was hypothesized to improve upon the slow NA removal rates observed in the literature.

The primary objective of this work was to evaluate the potential of the ISBR for removal of NAs from OSPW (Chapter 3). Secondary objectives included determining the role of ammonium and ammonium oxidizing bacteria in NA removal (Chapter 4) and investigating ozonation and biostimulation as methods of enhancing NA removal levels (Chapter 5). Material in Chapters 3 and 4 will be submitted for publication as two journal articles.

Chapter 2

Literature review

2.1Origin and fate of OSPW

2.1.1 Athabasca bitumen reserves

The Athabasca oil sands deposit in Alberta, covering over 75,000 km^2, is the world's second largest reserve of bitumen (Zhou et al., 2008). It contains ~1.8 trillion barrels of bitumen, of which 168 billion barrels are recoverable with current technology (ERCB, 2013). Of these 168 billion barrels, ~19% can be recovered through open pit mining, while the remaining 80% of reserves are buried too deep to mine and must be recovered in-situ using steam injection. In 2012, Alberta's production of bitumen exceeded 1.9 million barrels per day (bbl/d) with surface mining and in-situ extraction accounting for 46% and 54% of production, respectively (ERCB, 2013). Production levels are expected to be at 3.8 million bbl/d by 2022 (ERCB, 2013).

2.1.2 Bitumen recovery

Bitumen from surface-mined oil sands is recovered by the Clark Process, a caustic hot water extraction procedure that achieves over 90% bitumen recovery at ~85^oC and pH 8.5 (Chalaturnyk et al., 2002). A flow diagram for a typical bitumen hot water extraction process is shown in Figure 2-1. Briefly, oil sands ore is slurried with caustic water in rotating drums, causing the ore structure to disintegrate and bitumen micelles to separate from the sand particles. Addition of NaOH protonates and solubilizes asphaltic acids, which then act as surfactants, reducing surface and interfacial tensions, causing disintegration of the oil sands ore structure (Chalaturnyk et al., 2002). In a cone-bottomed primary separation vessel, bitumen droplets

coalesce, attach to air bubbles trapped in the oil sands and float to the top of the vessel, where the bitumen froth is collected. The froth is then de-aerated and mixed with solvent to facilitate removal of residual solids and water in inclined plate settlers, cyclones and/or centrifuges, then sent for upgrade to synthetic sweet crude oil (Masliyah et al., 2004).

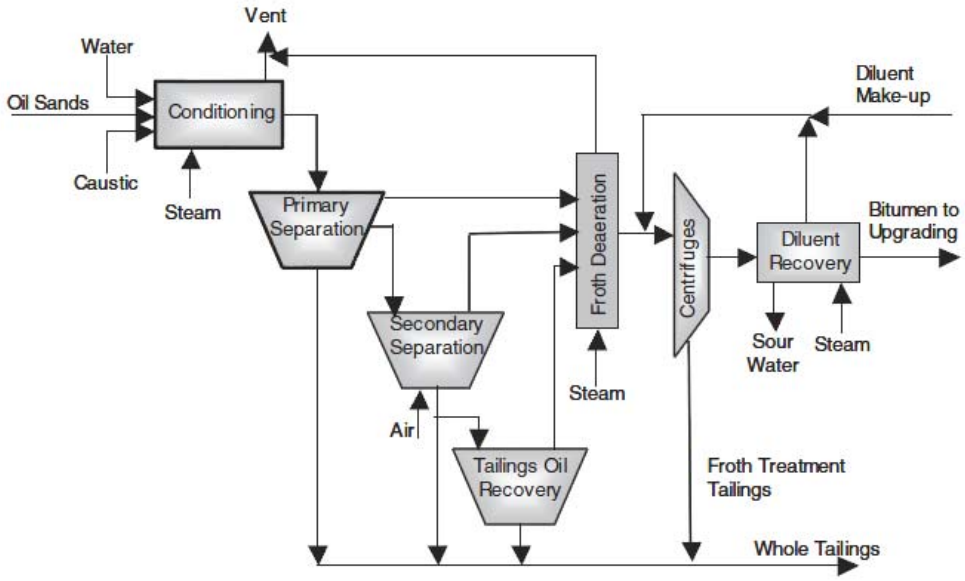

Figure 2-1: Process flow diagram of hot water process for bitumen extraction from oil sand ore, reprinted with permission from Chalaturnyk et al. (2002). Copyright 2013 Taylor & Francis.

The tailings stream from the separation vessels is sent to the tailings pond for settling. The slurry contains about 55 wt% solids composed of 82 wt% sand, 17 wt% fines, and 1 wt% residual bitumen (Chalaturnyk et al., 2002). Coarse sand particles settle quickly, leaving fine tailings with 6-10 wt% solids that settle quickly to 20 wt% and after several years to 30 wt%, at which point they are referred to as mature fine tailings (MFT) (Kasperski et al., 1992). Clarified pond water is recycled back to the extraction process. Each barrel of oil produced requires 8 to 10 barrels of water; however, approximately 70% of water used in the extraction process is recycled

from the settling ponds, resulting in a net water usage of 2-4 barrels of water per barrel of oil (Alberta Energy, 2013). Fresh water is taken from the Athabasca River, with oil sands industry usage accounting for 0.74% of the long-term average annual flow in 2010 (Alberta Energy, 2013).

Tailings ponds also serve to store the process water, as the Alberta *Environmental Protection and Enhancement Act* prohibits release of potentially toxic OSPW to ground or surface waters under a 'zero discharge policy'. Seepage and run-off are collected by sumps and interceptor ditches and pumped back to the tailings pond (Syncrude Canada Ltd., 2009). Current tailings pond volume is 720 million m^3, covering an area of ~130 km^2 (ERCB, 2011).

These ponds represent a major concern for oil sands companies as they will eventually have to be reclaimed. Under the *Environmental Protection and Enhancement Act*, oil sands operators are responsible for the reclamation of disturbed lands, with the objective of achieving land capable of supporting a range of uses similar to that of predisturbance (Province of Alberta, 2010). The Act requires posting of security bonds equivalent to the full cost of reclamation, as of March 2012, the province held over $875 million in bonds from the oil sands industry (Alberta Energy, 2013). In addition, Directive 074 was issued by the ERCB in 2009 specifying performance criteria for reclamation, with the objective of reducing fluid tailings volumes and creating trafficable deposits. Thus, there is mounting regulatory and public pressure to address the issue of OSPW and an urgent need for viable treatment options.

2.2 Characterization and composition of OSPW

2.2.1 OSPW physical and chemical properties

OSPW contains sand, clay fines, silts, dissolved ions, heavy metals and inorganic and organic compounds. Physical and chemical properties of OSPW are summarized in Table 2-1. The pH is slightly alkaline, ranging from 7.5 to 8.3. The concentration of most trace elements and nutrients are similar to those found in the Athabasca River (MacKinnon and Boerger, 1986). However, major ions such as Na^+, K^+, Cl^-, SO_4^{2-} and HCO_3^- are present at elevated levels. The dissolved organic fraction is primarily composed of naphthenic acids (NAs), which range in concentration from 40-120 mg/L (Holowenko et al., 2002). Bitumen residue and naphtha, used as a diluent in the bitumen recovery process, are also found in the organic fraction (Chalaturnyk et al., 2002). Salt and dissolved organic levels increase with time due to leaching from the ore, addition of process chemicals, and recycling of process water (Quagraine et al., 2005).

Naphthenic acids are generally considered to be a mixture of aliphatic and alicyclic carboxylic acids and will be discussed in greater detail in Section 2.2.2. NAs have a pKa between 5 and 6, exhibiting pH dependent solubilities which vary with structure; in the slightly alkaline tailings ponds they exist as water soluble naphthenate salts (Headley et al., 2002A). They also exhibit surfactant properties due to the presence of the carboxylic acid group (Quagraine et al., 2005). NAs extracted from OSPW have been shown to sorb to organic-rich soils, with a K_d value of 17.8 mL/g in saline ground water (Janfada et al., 2006). Thus, NAs may sorb to suspended particles and sink into MFT sediments (Han et al., 2009). Partitioning from OSPW to the atmosphere appears unlikely as the Henry's Law constant for NAs is approximately 8.56×10^{-6} atm m^3 mol^{-1} (Rogers et al., 2002).

Table 2-1: Physical and chemical properties of various OSPW samples, reprinted with permission from Fedorak et al. (2002). Copyright 2013 National Resource Council.

	MFT			CT			
Parameter	Syncrude	Suncor	Shell	Syncrude	Suncor	Shell (A)	Shell (B)
pH	8.0	7.5	8.3	7.8	7.8	7.9	7.9
Solids (%)	28.7	30.0	58.4	77.9	72.9	73.7	69.1
Bitumen (%)	2.7	9.0	2.2	0.1	0.3	3.5	3.5
Naphtha (%)	0.21	0.6	0.07	ND	NA	0.09	0.05
Na^+ (mg/L)*	870	410	510	1000	650	610	700
Mg^{2+} (mg/L)*	10	23	23	14	23	60	71
Ca^{2+} (mg/L)*	14	45	23	61	78	160	190
Fe (mg/L)*	ND	0.9	ND	1.7	0.7	ND	ND
Cl^- (mg/L)*	560	53	480	670	69	550	560
HCO_3^- (mg/L)*	1530	1800	530	1200	1050	1040	980
SO_4^{2-} (mg/L)*	19	34	170	1290	960	920	870
NO_3^- (mg/L)*	6†	ND	ND	ND	ND	9	5
NH_4^+ (mg/L)*	10	64	0.5	1.4	4	ND	ND
Kjehdahl N (mg/L)*	12	96	2.7	5	6	6	5
Total P (mg/L)*	0.2	0.4	4	ND	ND	ND	ND
DOC (mg/L)*	70	68	50	100	110	85	91
Naphthenic acids (mg/L)*	58	48	35	96	62	66	99

Note: MFT, mature fine tailings; CT, consolidated or composite tailings; DOC, dissolved organic carbon; ND, not detectable; NA, not analyzed.
*Pore waters.
†Syncrude results are typically <1 mg/L.

2.2.2 OSPW microbial populations

While other taxa cannot survive in untreated OSPW, microorganisms are present in appreciable populations. Foght, Fedorak, and Westlake (1983) counted populations of aerobic and anaerobic heterotrophs in the Mildred Lake Settling Basin at 10^6 cells/mL and 10^3 cells/mL, respectively. Anaerobic microorganisms that have been observed include methanogens and sulphate reducing bacteria (Fedorak et al., 2002). A variety of species of phytoplankton have also been observed (Quagraine et al., 2005). Using 16S rRNA gene pyrotag sequencing of mixed species biofilms *in vitro* the most abundant genera in oil sands sludge were *Brachymonas* (17.2%), *Acidovorax* (6.2%), *Variovora* (5.7%), *Rhodoferax* (3.7%), and *Thioalkalispira* (3.7%) (Golby et al., 2012).

2.2.3 Naphthenic acids

The term “naphthenic acids” is typically used to describe the acid-extractable organics in OSPW. They are classically defined as a complex mixture of alicyclic and aliphatic carboxylic acids, of the form, $C_nH_{2n+Z}O_2$, where Z is 0 or a negative, even integer representing the number of rings (Z= -2, represents a compound with 1-ring; Z= -4, 2-rings, etc.). Typical structures are shown in Figure 2-2. Toxicity of OSPW was first attributed to “naphthenic acids” by MacKinnon and Boerger (1986), who found that by removing the acid extractable component of OSPW, toxicity was mitigated. The Fourier transform infrared spectroscopy (FTIR) spectrum of this toxic extract was compared to that of a commercial mixture of NAs and was found to be very similar. Thus, numerous subsequent studies have focused solely on the fraction of NAs fitting the formula $C_nH_{2n+Z}O_2$, however, the acid extractable organics in OSPW have recently been found to be much more complex. Characterization of this toxic component is obviously critical for the eventual remediation and release of OSPW.

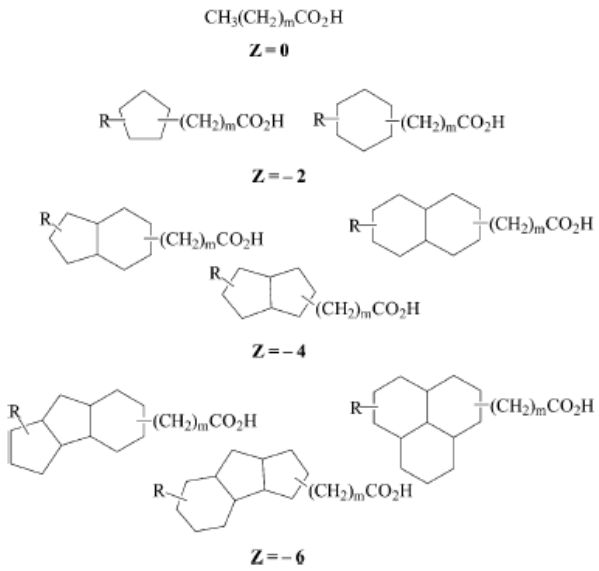

Figure 2-2: Proposed NA structures for various Z-series, reprinted with permission from Clemente et al. (2004) Environmental Science and Technology 38(4): 1009-1016. Copyright 2013 American Chemical Society.

2.2.4 Characterization of OSPW

2.2.4.1 FTIR

The industry's standard method for quantification of NAs involves a dichloromethane extraction of acidified OSPW, followed by concentration of the extract and measurement of the absorbance characteristic of carboxylic acids with FTIR (Scott et al., 2008A). The monomeric and dimeric forms of the carboxylic groups are measured at 1743 and 1706 cm^{-1}, respectively, and the absorbances summed (Clemente and Fedorak, 2004). However, this technique also detects naturally occurring carboxylic acids that are not naphthenic acids, resulting in over-estimation of the NA content (Merlin et al., 2007). While this technique can quantify NAs when

compared to a calibration curve created from commercial NAs, it does not provide information about the structural composition of the OSPW NAs.

2.2.4.2 Mass spectrometric methods for OSPW analysis

The complexity of OSPW organic content prevents the identification of individual NAs by conventional mass spectrometry (MS). Separation techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) are unable to resolve the complex mixture of compounds present in OSPW. Rather than eluting as discrete peaks, NAs elute as an unresolved hump (Holowenko et al., 2002; Lo et al., 2003). Thus, most methods organize acids into isomeric groups of the form $C_nH_{2n-Z}O_2$, based on the molecular ions detected. As the m/z value is used to assign the ion to a particular empirical formula, soft ionization techniques where the molecular ion dominates, with little further fragmentation, are required.

Soft ionization techniques that have been used include fluoride ion chemical ionization (CI) (Dzidic et al., 1988; Hsu et al., 2002), fast atom bombardment (FAB) (Fan, 1991), atmospheric pressure chemical ionization (APCI) (Hsu et al., 2000; Lo et al., 2006) and electrospray ionization (ESI) (Hsu et al., 2000; Headley et al., 2002A; Lo et al., 2003). Hsu et al. (2000) evaluated ESI, APCI, CI and FAB and found that APCI in negative mode, with acetonitrile solvent gave the cleanest spectra. In a comparison of APCI and ESI ionization for OSPW analysis, Lo et al. (2006) found that while APCI had the wider range of detection, ESI had a greater detection limit. Furthermore, APCI-MS has higher operating costs and requires frequent instrument maintenance. ESI-MS has the advantage of allowing direct analysis of OSPW, without extraction and derivatization of the organic components; however, the presence of salts can cause matrix effects, which hamper sensitivity (Bataineh et al., 2006). Use of HPLC in conjunction with

ESI-MS has been found to increase sensitivity 40 to 350 fold versus direct infusion techniques (Bataineh et al., 2006).

2.2.4.3 Analysis by gas-chromatography mass spectroscopy

Ionization by electron impact (EI), while substantially simpler and more widely available than many soft ionization techniques, results in extensive fragmentation making molecular mass information difficult to obtain. St. John et al. (1998) developed a GC-EI-MS technique for analysis of commercial preparations of NAs, which has been used widely to study NAs in OSPW (Clemente et al., 2004; Scott et al., 2005; del Rio et al., 2006).

By derivatizing NAs with N-methyl-N-(t-butyldimethylsilyl) trifluoroacteamide (MTBSTFA) to their t-butyldimethylsilyl (t-BDMS) esters, EI preferentially cleaves the tert-butyl group, producing [naphthenate + dimethylsilyl]$^+$ peaks, corresponding to [M+57]$^+$ ions, where M is the mass of the NA. Fragmentation of a derivatized NA is illustrated in Figure 2-3. The [M+57]$^+$ ions are used to calculate the molecular mass for each acid and organize the NAs into the general form $C_nH_{2n+Z}O_2$.

This technique can be used to quantify the relative amount of each isomer, but requires assuming equal ionization efficiencies. However, Clemente and Fedorak (2004) have shown that equimolar surrogate NA standards measured with GC-EI-MS yield different ion abundances, with overestimation of the relative proportions of low molecular mass acids.

Holowenko et al. (2002) plotted the relative abundances of the NA isomers as three dimensional bar graphs with n on the x-axis, Z on the y-axis, and relative abundance on the z-axis, to more easily visualize changes in NA composition. A t-test has also been developed to compare the GC-MS results used to generate the 3-D plots (Clemente et al., 2003).

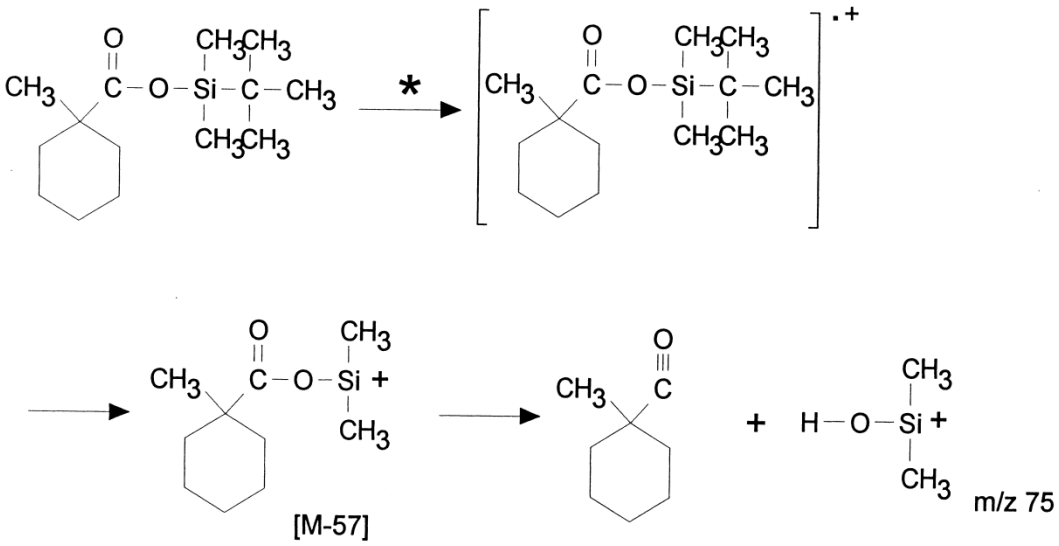

Figure 2-3: Fragmentation of the t-butyldimethylsilyl derivative of a model NA, reprinted with permission from St. John et al. (1998). Copyright 2013 Elsevier.

Although widely used, this GC-MS method results in substantial misclassification of OSPW NAs, when used in conjunction with a low resolution MS, such as a triple-quadrupole instrument. Using high resolution GC-MS, Bataineh et al. (2006) found that NA isomer classes with $n \geq 22$ result from ions containing two tert-butyldimethylsilyl derivatives, whose true identities are likely hydroxylated naphthenic acids. Additionally, fragmentation of [naphthenate + dimethylsilyl]$^+$ ions can produce fragments that may be miss assigned as low molecular weight NAs (Clemente and Fedorak, 2004).

2.2.4.4 High resolution mass spectrometric (HR-MS) techniques

Soft-ionization techniques employing unit resolution MS methods that do not use a derivatization step, such as ESI-MS, also result in substantial false positive detection of naphthenic acids (Martin et al., 2008; Headley et al., 2009A). Martin et al. (2008) compared high and low resolution ESI-MS for analysis of NAs in OSPW. The NA profile obtained from direct

injection unit resolution ESI-MS was very similar to the profile generated by Bataineh et al. (2006) using unit-resolution GC-MS, with the exception of the C22+ cluster resulting from double-derivatization. When compared to the profile generated with HPLC-HRMS, the higher m/z selectivity revealed significant false positive detection of NAs using low resolution (LR) techniques. In LR ESI-MS, isomers with n>20 contributed to 4% of the total response, while no n>20 compounds were detected with HPLC-HRMS and Z=0 compounds made up 15% of the total response, but were almost completely absent with HR-HPLC MS. Differences in the NA acid fingerprint using high and low resolution ESI-MS are illustrated in Figure 2-4. The misclassified compounds were not identified, but the authors hypothesized that they were oxidized NAs and organic N and S compounds. Although many of the ions detected do not fit the classical definition of naphthenic acids, they are still of interest as they may contribute to the toxicity of OSPW.

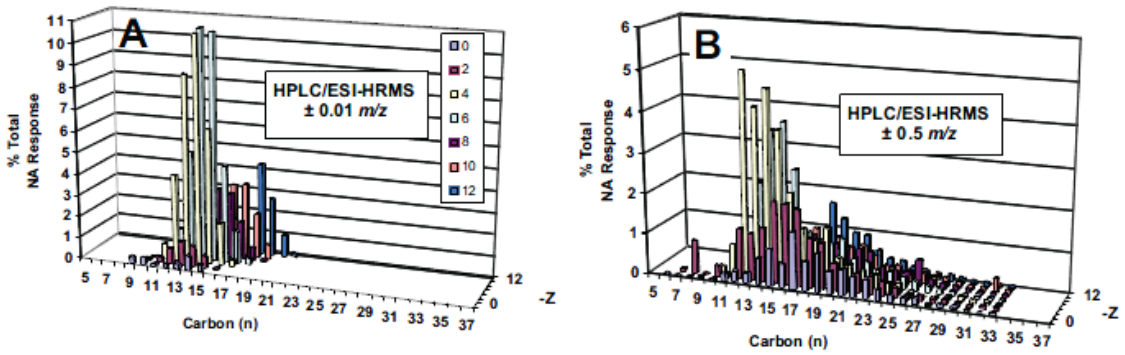

Figure 2-4: Comparison of NA profiles generated by high (A) versus low (B) resolution ESI-MS, reprinted with permission from Martin et al., (2008). Copyright 2013 Wiley.

Recent use of high resolution time of flight (TOF) and ultrahigh resolution Fourier transform ion cyclotron resonance (FT-ICR) has helped to expand understanding of the

components present in OSPW acid extractable organics. Traditional NAs of the form $C_nH_{2n+Z}O_2$, seem to be dominated by 2 and 3 ring compounds (Z=-2 and Z=-4), with a mean carbon number of 13.9 to 14.7 (Han et al., 2009; Grewer et al., 2010; Bataineh et al., 2006). However, using HPLC-ESI-FT-ICR MS, NAs of this form have been found to make up only 19 to 35.6% of the total ion abundance (Grewer et al., 2010). Both TOF and FT-ICR techniques have detected NAs of the form $C_nH_{2n+Z}O_x$, where x=2 to 5, termed "oxy-naphthenic acids" (Han et al., 2008; Barrow et al., 2010; Headley et al., 2009A), however, even this expansion of the NA definition only accounts for 36.1 to 47.4% of the total ion abundance detected in OSPW extracts (Grewer et al., 2010). For the characterization of oxy-naphthenic acids, high resolution TOF-MS seems to be sufficient; Headley et al. (2009A) found that the same general trends for oxy-naphthenic acids were observed using TOF and FT-ICR MS.

Elemental analysis of OSPW extracts indicates that heteroatomic species may account for a significant part of the unidentified ions present. West In-Pit (WIP) tailings extracts were found to contain 3.38% S and 0.51% N by mass (Grewer et al., 2010). For characterization of these heteroatomic species, FT-ICR MS, capable of measuring exact masses to m/z ± 0.001, is required to resolve species such as $C_{17}H_{25}O_5$ (mw=309.1772) and $C_{14}H_{25}SO_5$ (mw=309.17411) (Grewer et al., 2010).

HPLC-ESI-FT-ICR MS has detected the presence of O_6, O_7, SO_x (x=2-6) and NO_4 species in OSPW extracts (Headley et al., 2009A; Barrow et al., 2010; Grewer et al., 2010). Negative-mode ESI is well suited for acidic species such as oxy-NAs, however, Barrow et al. (2010) pointed out that this ionization method is not suitable for less acidic or basic species, such as S-containing heteroatomics. Species of the form C_nH_{2n}, $C_nH_{2n+Z}N$, $C_nH_{2n+Z}S$, $C_nH_{2n+Z}NO_x$ were identified, with APPI generally providing accessibility to a greater range of compounds (Barrow

et al., 2010). Additionally, the solvent used for ESI-FT-ICR-MS has been shown to significantly affect the spectra obtained (Headley et al., 2007).

2.2.4.5 Identification of individual naphthenic acids

While ultra high resolution MS techniques provide more insight into the isomers present in OSPW, without sufficient separation, they cannot provide structural information about the compounds detected. With two dimensional comprehensive gas chromatography (GCXGC) coupled with a time of flight-MS, Rowland et al. (2011A; 2011B) identified a number of peaks that were sufficiently resolved to glean EI spectra containing molecular and fragment ions. Interpretation of these spectra and comparison of reference acid spectra and GCxGC retention times allowed positive identification of a number diamondoid tricyclic acids, including adamantine-1-carboxylic acid and 3-ethyl adamantine-1-carboxylic acid (Rowland et al., 2011 A) in addition to pentacyclic acids diamantine-1-carboxylic acid and diamantine-3-carboxylic acid (Rowland et al., 2011B) (Figure 2-5). Tetracyclic diamantines were tentatively identified as ring-opened biotranformation products of penta-cyclic diamantines (Rowland et al., 2011B). The authors proposed that these acids are biotransformation products of alkyladamantane and alkyldiamantane hydrocarbons.

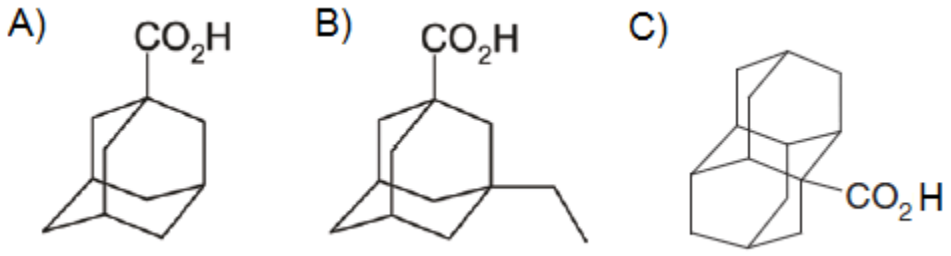

Figure 2-5: Diamondoid carboxylic acids identified by 2D-GC MS; A) adamantine-1-carboxylic acid B) 3-ethyl adamantine-1-carboxylic acid, reprinted with permission from Rowland et al., (2011A) Environmental Science & Technology 45(7): 3154-3159. Copyright 2013 American Chemical Society C) Diamantine-1-carboxylic acid, reprinted with permission from Rowland et al. (2011B). Copyright 2013 Wiley.

Two-dimensional chromatography has also confirmed the presence of aromatic species. 3 C-ring monoaromatic hydroxyl steroid carboxylic acids have been tentatively identified that have similar structures to known estrogens (Figure 2-6). However, authentic standards were not available for confirmation (Rowland et al., 2011C). The authors hypothesized that these compounds are degradation products of C_{20}-C_{21} steroidal hydrocarbons. Previous work using mass spectrometry coupled with ^{13}C-NMR (Hsu et al., 2000) and synchronous fluorescence spectroscopy has indicated that aromatic acids are present in OSPW (Kavanaugh et al., 2009). However, Jones et al. (2012) used GCxGC-MS coupled with argentation chromatography to separate aromatic acidic compounds from alicyclic acidic compounds and found that acid extractable organics contain a higher fraction of aromatics than previously thought.

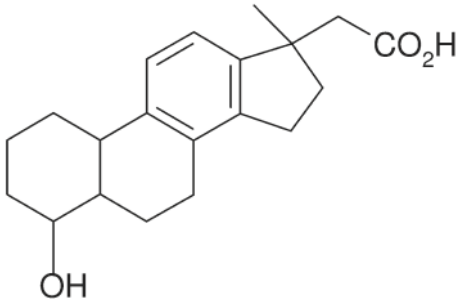

Figure 2-6: Tentatively identified 3 C-ring monoaromatic hydroxyl steroid carboxylic acid found in OSPW, reprinted with permission from Rowland et al. (2011C) Environmental Science & Technology 45(22): 9806-9815. Copyright 2013 American Chemical Society.

2.3 OSPW toxicity

2.3.1 Toxicity to various species

Oil sands process water is toxic to a variety of organisms. Native process water has an effective concentration at which 50% of the maximal toxic effect is observed (EC_{50}) when the process water has been diluted less than 30% v/v, as measured by Microtox (MacKinnon and Boerger, 1986). The Microtox test is an acute toxicity assay that determines EC_{50} based on the concentration that causes a 50% reduction in light output by the luminescent bacteria *Vibrio fischeri*. Fresh tailings water is acutely toxic to aquatic organisms, with rainbow trout and water fleas exhibiting lethal concentrations for 50% of the test organisms (LC_{50}) less than10% v/v (MacKinnon and Boerger, 1986). At a concentration of 6.8 mg/L of OSPW NAs, 100% mortality was observed in yellow perch (Nero et al., 2006). Non-lethal effects observed in fish species are tainting (an unnatural flavor or aroma) (Koning and Hrudey, 1992), degeneration and inflammation of liver cells, and proliferation of mucous cells and epithelial cell necrosis in gill tissue (Nero et al., 2006). NAs were detected in the flesh of rainbow trout exposed to OSPWs with a NA concentration of 15 mg/L after 96 hours (Young et al., 2007). A toxicity study with

Wistar rats exposed to OSPW contaminated drinking water found that under worst-case exposure conditions, acute toxicity was unlikely for small, wild mammals, however, chronic exposure to low concentrations of NAs may have adverse effects such as suppressed growth and liver effects including increased glycogen storage and suppressed plasma cholesterol concentrations (Rogers et al., 2002). NAs have varying effects in plants. Commercial sodium naphthenates decreased hydraulic conductivity, net photosynthesis and leaf growth in aspen (Kamaluddin and Zwaizek, 2002). However, only unionized OSPW NAs showed significant toxic effects in wetland plants at pH<6 (Armstrong et al., 2009). In wetlands receiving oil sands effluents, photosynthetic rates in cattails were actually increased, possibly due to higher levels of available phosphate and ammonium (Bendell-Young et al., 2000).

2.3.2 Mode of toxicity

Naphthenic acids are considered to be the primary toxic component of OSPW (MacKinnon and Boerger, 1986). Since NAs have surfactant properties, the probable mode of action for acute toxicity is narcosis (Frank et al., 2008). Narcosis is a non-specific mode of toxicity, where the cell cytoplasmic membrane is disrupted by a hydrophobic compound, which alters membrane properties, affecting function and ultimately causing cell death (Frank et al., 2008).

Components of OSPW also appear to have the potential to disrupt normal endocrine function. Exposure to OSPW was found to significantly reduce testosterone and 17β-estradiol levels in the plasma of goldfish (Lister et al., 2008) and yellow perch (van den Heuvel et al., 1999). Estrogenic and anti-androgenic effects have been observed – exposure to OSPW caused cells from the human H295R adrenocarcinoma cell line to produce less testosterone and more 17β

-estradiol (He et al., 2010). He et al. (2011) found that OSPW exposure resulted in estrogen receptor agonist and androgen receptor antagonist properties.

2.3.3 Determining OSPW fraction responsible for toxicity

Although NAs are thought to be responsible for OSPW toxicity, the contribution of specific isomers or NA fractions to this toxicity is unknown. In field studies, the acute toxicity of OSPW has been shown to decrease with time (MacKinnon and Boerger, 1986). This decrease was correlated to an increasing abundance of naphthenic acids with $n \geq 22$, suggesting that lower molecular weight compounds (n=13-16) are more toxic than larger molecules (Holowenko et al., 2002). NA fractionation via Kugelrohr distillation and subsequent toxicity testing of the fractions supported these results, with the lowest molecular weight fraction having the highest potency (Frank et al., 2008). However, both these studies utilized unit resolution MS techniques, which have been shown to result in substantial misclassification (Bataineh et al., 2006). Additionally, an increase in molecular weight does not support narcosis as the probable mode of acute toxicity, as a larger molecule would be more hydrophobic and cause greater disturbance to the structure of the cell membrane (Frank et al., 2008).

Frank et al. (2009) found that the higher molecular weight NAs had greater carboxylic acid content, which would decrease hydrophobicity and explain the decreased toxicity observed for the higher molecular weight NA fraction. Additionally, Han et al. (2009) determined that the ratio of mono and dioxidized NAs to parent NAs increased with increasing age of the Syncrude OSPW, which supports this conclusion, as aged OSPW displays reduced toxicity compared to fresh OSPW (MacKinnon and Boerger, 1986). OSPW fractions separated on the basis of solubility did not differ significantly in Microtox toxicity (Lo et al., 2006).

2.4 OSPW treatment options

2.4.1 Industry OSPW reclamation efforts

The two primary reclamation techniques being considered by the oil sands industry are the "dry" and "wet" landscape approaches. In the dry landscape approach, fine tailings will be dewatered, mixed with sand, buried and capped with soil (Allen, 2008), followed by watershed and topography reconstruction and revegetation (BGC Engineering Inc., 2010). Typically, the fines fraction is mixed with coarse sand and a coagulant aid such as gypsum to produce a non-segregating deposit (MacKinnon et al., 2001). Under self-weight consolidation, water is released, leaving the fines trapped within the coarse solids matrix. However, the released water exhibits toxicity due to the presence of NAs and high salt concentrations (MacKinnon et al., 2001). Pore water released from MFT mixed with gypsum was found to have a NA concentration of 43 mg/L, measured by FTIR (Han et al., 2009). Additionally, there are concerns about the NA contaminated process water that might drain or leach from the reclamation area (Quagraine et al., 2005A).

The "wet landscape" reclamation approach entails pumping of mature fine tailings into a mined pit area and capping with a layer of process and river water, the ultimate goal being a reclaimed pond with environmentally acceptable water quality that is capable of supporting wildlife and requires no long-term maintenance (Nix and Martin, 1992). This approach is attractive as it is a cost-effective method to reclaim soft tailings and eliminates the need for heavy equipment and reclamation material required for the dry landscape approach (BGC Engineering Inc., 2010). Natural attenuation has been shown to decrease NA concentration and acute toxicity with time and Han et al. (2009) estimated a half-life of 12.8 to 13.6 years for NAs in-situ. Mature fine tailings capped with Mildred Lake Settling Basin water and aged 11 years showed a decrease

in NA concentration from 49 mg/L to 24 mg/L (Holowenko et al., 2002). The EC_{50} measured by Microtox increased from 32% and 100%, and the EC_{20} increased from 7% to 100%. A potential problem associated with this approach is methane evolution, which has been observed in the Mildred Lake Settling Basin since the early 1990s. Methane released to overlying waters could potentially increase the dissolved oxygen demand due to aerobic methane consumption, leading to an anoxic environment that could prevent establishment of a functioning lake ecosystem (BGC Engineering, Inc., 2010). Additionally, bubbles of released methane may re-suspend fines, destabilizing the fine-tailings interface (Fedeorak et al., 2002).

Constructed wetlands have also been built to incorporate the principles of a fixed film bioreactor and were designed to remove NAs via settling, sediment/plant sorption and microbial mineralization (Quagraine et al., 2005A). Samples collected from the discharge of these wetlands have shown a decrease in NA concentration from 68 mg/L to 42 mg/L. This reduction was mainly attributed to dilution, but changes in the NA fingerprint show some reduction in the lower molecular weight NAs (Holowenko et al., 2002). However, preliminary results indicate that this approach requires long hydraulic retention times and would need large areas of land to treat the large volume of process water (Quagraine et al., 2005A).

Although 6-12 months of aging eliminates the acute toxic response of OSPW (MacKinnon and Boerger, 1986), aged process water still displays chronic toxic effects. Exposure to process water aged >15 years completely inhibited spawning of fathead minnows and reduced secondary male sexual characteristics (Kavanagh et al., 2011). A model benthic invertebrate was found to exhibit significantly less pupation and emergence in reclamation ponds that had been aged over 18 years (Anderson et al., 2012). Additionally, wetland treated OSPW effluent inhibited germination and reduced the fresh weight of seedlings in plant species such as rye,

wheat, pea, canary grass, and clover (Crowe et al., 2002). It seems that based on the persistence of chronic effects in species exposed to aged process water that passive wet reclamation schemes do not sufficiently remediate OSPW and more directed approaches are required.

2.4.2 Bioremediation of OSPW

2.4.2.1 Biological treatment of surrogate naphthenic acids

Due to limitations in the analytical methods used to measure NAs, many studies have focused on biodegradation of NA model compounds or commercial NA preparations. Commercial preparations of NAs are readily biodegradable by microbial consortia indigenous to oil sands tailings water, to lake biofilms (Headley et al., 2010A), and to rhizosphere microorganisms (Biryukova et al., 2007) with no previous exposure to NAs. *Pseudomonas putida* and *Pseudomonas fluorescens* have been shown to degrade >95% of commercial mixtures (del Rio et al., 2006). In shake flask experiments, Merichem and Kodak commercial NA preparations can be reduced by 90% in approximately 10 days (Clemente et al., 2004; Scott et al., 2005) and to 1.7% in 28 days (Han et al., 2008). Approximately 50-60% of the NAs are released as CO_2 (Clemente et al. 2004; Herman et al. 1994). NA degradation has been shown to result in an increase of oleic, linoleic, palmitic, and stearic acids, fatty acids found in the membranes of prokaryotic cells (Clemente et al., 2004; Biryukova et al., 2007).

Studies with surrogate and commercial NAs have been useful in determining relationships between NA structure and biodegradation rate. Structural differences result in varying half-lives for each isomer class, ranging from 1 to 8 days (Han et al., 2008). For commercial mixtures, low molecular weight acids (n=7-13) are degraded more readily than high molecular weight ones (n=14-21) (Clemente et al., 2004; Biryukova et al., 2007). However, the degree of cyclicity has a much stronger influence on persistence, with the half life increasing with

ring number, while the n number for each Z-series has a limited effect on half-life (Han et al., 2008). Using surrogate NAs, Herman et al. (1993) found that methyl and aliphatic substitutions on a carboxylated cycloalkane ring are more recalcitrant than carboxylated side chains. It is hypothesized that the least branched commercial NAs are preferentially degraded (Han et al., 2008; Bataineh et al., 2006). As side-chain alkyl branching increased in butylcyclohexylbutanoic acids, biotransformation by sediment enrichment cultures decreased (Smith et al., 2008). This trend was also observed with butylphenylbutanoic acid isomers with varying degrees of alkyl side chain branching (Johnson et al., 2011). For alicyclic model NAs, the cis-isomer was more recalcitrant than the trans-isomer, most likely due to intramolecular H-bonding that occurs only in the cis-isomers (Headley et al., 2002B).

2.4.2.2 Biological treatment of native process water

While commercial NA preparations are readily biodegradable, attempts to degrade OSPW NAs have met with limited success. Cultures capable of 50% to 95% reduction in commercial NAs have been unable to significantly decrease NA content in OSPW (Herman et al. 1994; del Rio et al., 2006). Scott et al. (2005) found that a commercial mixture could be biodegraded completely within 14 days, while with the same consortium only 25% of OSPW NAs were degraded after 40-49 days. Han et al. (2008) reported half-lives ranging from 44 to 240 days for OSPW NAs in a shake flask study. In a laboratory study using simulated wetlands, a hydraulic residence time of 400 days resulted in a 64-74% reduction in NA concentrations; however, treatment did not significantly affect Microtox toxicity (Toor et al., 2012).

The degradation rate of commercial NA mixtures is not affected by the presence of OSPW (Scott et al., 2005). Thus, it is hypothesized that the difference in biodegradability is due

to the relatively high alkyl branching of OSPW NAs, compared to commercial preparations, rather than dissolved organics such as cresols, mercaptans, and thiophenols found in OSPW.

Cyclization appears to be a factor in persistence, with biodegradation rate decreasing with decreasing Z number (i.e. Z= -2 < Z= -4 < Z= -6 < Z= -8) (Han et al., 2008). However, Han et al. (2009) found there was no enrichment of highly cyclic naphthenic acids in aged tailings reclamation ponds compared to active settling basins. Freshly extracted ore samples found a significant response from Z= 0 and Z= -2 compounds that is absent in active settling pond extracts, indicating that there is a labile aliphatic and monocyclic fraction that undergoes rapid degradation upon discharge (Han et al., 2009). Similar to commercial NAs, Han et al. (2008) found that the n number had a limited impact on biodegradability, with only NAs in the Z= -6 series having a statistically significant correlation between n number and half-life.

2.4.2.3 Factors affecting degradation

Non-structure related factors have also been found to affect NA degradation. Aerobic degradation may be nutrient limited, since the addition of N and P stimulated CO_2 production and O_2 uptake in degradation studies with commercial and OSPW NAs (Herman et al., 1994). In shake flask experiments with surrogate naphthenic acids, Lai et al. (1996) found that phosphate addition increased degradation rates two fold. NA degradation is generally thought to be an aerobic process, as a decrease in oxygen concentration resulted in a decrease in NA degradation (del Rio et al., 2006; Herman et al., 1993, 1994; Lai et al. 1996). Decreasing temperature has also been found to decrease the rate of degradation of model NA compounds (Lai et al. 1996; Headley et al., 2002B). In a study with surrogate NAs, Headley et al. (2002B) found that degradation rates at pH 6 compared to pH 8.7 were not significantly different. However, due to the effect of the pH on NA solubility, it is expected that as the pH decreases and NAs partition into the solid phase,

becoming less bioavailable, the rate of OSPW NA degradation would decrease (Quagraine et al., 2005A).

The source of the microorganisms used in degradation studies has a significant effect on the degradation of OSPW NAs, with previous exposure being critical to successful degradation. Comparison of microbial communities found in sediments with exposure to varying concentrations of NAs found that NA content had a major influence on the composition of the community, even at low concentrations (Hadwin et al., 2006). Del Rio et al. (2006) found that while CHCA was readily degraded by both river and process water-affected sediments, mineralization of the bicyclic NA surrogate proceeded significantly faster in process affected sediments. Similarly, while lake biofilms with no previous adaptation to OSPW were able to degrade commercial naphthenic acids, no dissipation was evident for OSPW NAs (Headley et al., 2010A).

2.4.2.4 Proposed degradation pathways for naphthenic acids

The preferred pathway for the aerobic biodegradation of aliphatic and alicyclic carboxylic acids is the β-oxidation pathway (Quagraine et al., 2005B); other proposed pathways include combined α and β oxidation (Rontani and Bonin, 1992) and aromatization (Taylor and Trudgill, 1978; Blakley, 1974). The first step of degradation for all pathways produces a hydroxylated intermediate (Rontani and Conin, 1992). β-oxidation proceeds via a sequence of oxidation processes at the β-carbon, producing a new carboxylic acid with two carbons fewer than the parent compound (Quagraine et al., 2005B). In degradation studies with butylcyclohexylbutanoic acid and butylphenylbutanoic acid isomers, degradation was initiated by removal of two carbons from the carboxyl side-chain, indicative of β-oxidation (Smith et al., 2008; Johnson et al., 2011). Cleavage of an alicyclic ring with a side chain containing an odd

number of carbons proceeds effectively, however, compounds with an even number of carbons in the side chain (e.g. cyclohexylacetic acid) are generally more recalcitrant as oxidation is blocked by the relative positions of the carboxyl group and cyclohexyl ring (Quagraine et al., 2005B). Rontani and Bonin (1992) found that *Alcaligenes* sp. PHY 12 overcomes this problem by degrading cyclohexylacetic acid via a combination of α and β oxidation. Blakley (1974) found that an *Arthrobacter* strain degraded cyclohexylcarboxylic acids via aromatization. Degradation begins with hydroxylation at the para-position, followed by dehydrogenation of the hydroxyl group to form a ketone and electron withdrawal to form para-hydroxybenzoic acid. It should be noted that the proposed degradation pathways are based on studies using monocyclic naphthenic acids, which are not particularly abundant in OSPW (Martin et al., 2008), thus their relevance is questionable.

The proportion of oxidized NAs has been found to be greater in aged process water compared to OSPW from active settling basins (Han et al., 2009). This seems to support production of a hydroxylated intermediate as the first step in the degradation pathway of NAs. However, in laboratory degradation studies the concentration of hydroxylated NAs did not change over 98 days and it is unclear if hydroxylated NAs are more persistent than their parent NAs, or if they are being formed and degraded at the same rate (Han et al., 2009).

2.4.3 Ozonation

The resistance of highly branched NAs to biodegradation has prompted research into chemically oxidizing NAs with ozone. Scott et al. (2008B) found that sparging OSPW samples with ozone (dissolved ozone concentration ~35 mg/L) for 130 min reduced typical NA concentrations by 95%. Reductions in total organic carbon (TOC) and chemical oxygen demand (COD) were low, indicating that NAs are partially oxidized to small organic molecules, rather

than completely mineralized (Scott et al., 2008B; El-Din et al., 2011). However, the biodegradability of the organic fraction appears to increase with ozonation based on increases in the BOD/COD ratio (Scott et al., 2008B; El-Din et al., 2011). The effect of ozonation on OSPW toxicity is still unclear. In studies with greater than 90% NA removal, Microtox toxicity was completely eliminated (Scott et al., 2008B; El-Din et al., 2011), however, Martin et al. (2010) found that a reduction of NAs by 74% did not affect Microtox toxicity. It was hypothesized that oxidation of OSPW might produce hydroxylated products with steroidogenic activity; however, ozonation attenuated steroidogenic effects in a human cell line (He et al., 2010).

Although clearly superior to bioremediation for removal of NAs and toxicity from OSPW, implementation of ozonation is impeded by the large volumes of OSPW in the tailings ponds and the high cost of ozone generation. To address this issue, Martin et al. (2010) investigated mild ozonation as a pre-treatment step in hopes of accelerating subsequent NA biodegradation. Ozonating for only 90 s at a flow rate of 2 mL/min and ozone concentration of 106 g/m^3 reduced the NA concentration by 73%. This pre-treatment significantly decreased the half-life of all residual NA classes, and decreased the incubation time required to observe a reduction in acute toxicity. The authors hypothesized that the acceleration of NA and toxicity removal was due to removal of the more recalcitrant NA fraction. This hypothesis seems to be supported by a structure-reactivity study that found ozonation more rapidly depleted NAs with more rings and higher carbon numbers (Perez-Estrada et al., 2011).

2.4.4 Other proposed remediation options

2.4.4.1 Phytoremediation

Phytoremediation is the treatment of environmental contaminants with plants via biotransformation by rhizosphere microorganisms, reducing contaminant transport via adsorption

or uptake by the plant followed by metabolism, volatilization or accumulation (Quagraine et al., 2005B). Phytoremediation of NAs has had limited success. A study with an OSPW NA extract and emergent macrophytes, using unit resolution ESI-MS, found that NA concentration was not significantly reduced over a 30 day period, leading to the conclusion that NAs are not sequestered by wetland plants (Armstrong et al., 2008). In a study using the common reed for treatment of MFT, no significant difference was found in NA concentration between treated and untreated samples (Armstrong et al., 2010). However, a study using ultra-high resolution MS found that exposure to cattails changed the NA finger-print with preferential dissipation of low and high carbon number O_2 species, while highly oxygenated and heteroatomic species were recalcitrant (Headley et al., 2009A). It was unclear whether the dissipation mechanism was adsorption, absorption, or biotransformation by rhizosphere microorganisms. The large-scale application of this technology has been limited due to concerns of NAs and heavy metals leaching into ground water and the long-term activity of the plant (Knight et al., 1999).

2.4.4.2 Advanced oxidation processes

Advanced oxidation processes are also being investigated for NA removal. Photolysis of NAs by natural and artificial UV light is limited, as partial photolysis was limited to UV irradiation at 254 nm, and UV in the visible range had no effect on NA concentrations (McMartin et al., 2004). UV illuminated H_2O_2 and UV illuminated persulfate have been found to reduce NA concentration to below 5 mg/L at an estimated cost of 415 USD/kg TOC and 483 USD/kg TOC, respectively (Liang et al., 2011). However, UV/$S_2O_8^{2-}$ treatment produces treated water with high residual sulphate content. Headley et al. (2010B) found that UV treatment in the presence of a TiO_2 catalyst and a combination of UV and microwave radiation are effective in removing O_2 NA species, however, microwave irradiation alone did not significantly decrease concentrations. O_3,

O_5 and O_6 classes were only found in the UV and microwave treated samples, suggesting oxidation of the parent acids. This treatment was effective in eliminating acute toxicity in the treated water, measured by Microtox assay (Mishra et al., 2010). Photodegradation with TiO_2 irradiated under natural conditions reduced NA concentrations by 75%, and analysis of the NA composition suggested that a high degree of cyclicity may affect efficacy due to steric hindrance (Headley et al., 2009B).

2.4.4.3 Adsorption

Petroleum coke adsorption has also been investigated for NA removal. El-Din et al. (2011) found that addition of 20 wt% petroleum coke reduced the NA concentration by 85%, and eliminated toxicity to *V. fischeri*. Adsorption of NAs has also been studied on commercially available weak and strong anion-exchange resins (Gaiker and Maiti, 1996). Problems associated with adsorption include potential fouling from bitumen and the need for acidification as alkalinity is needed for bitumen extraction in recycled water (Allen, 2008).

2.4.4.4 Membranes

Membrane systems have also been investigated for OSPW treatment. Nanofiltration was found to reduce NAs and water hardness by over 95% (Peng et al., 2004). Reverse osmosis using a poly-amide membrane, achieved 95% removal of TOC and 98% removal of total dissolved solids (Mehrotra and Banerjee, 1986). Problems associated with membrane treatment include fouling from bitumen and possibly biofouling, membrane replacement costs, and brine disposal (Allen, 2008).

2.5 Immobilized soil bioreactors for treatment of recalcitrant environmental pollutants

An emerging technology for the degradation of relatively recalcitrant pollutants is the Immobilized Soil Bioreactor (ISBR). An ISBR immobilizes contaminated soil, to harness the intrinsic biodegradation activity of the soil's microorganisms arising from natural selection. The ISBR was first proposed by Karamanev et al. (1997) for the biodegradation of pentachlorophenol (PCP) and it has since been used for bench-scale (Kermanshahi et al., 2005; Kermanshi et al. 2006; Jajuee et al., 2006) and pilot-scale ISBR (Jajuee et al., 2007) degradation studies of p-xylene and naphthalene.

2.5.1 Principles of operation

The ISBR is a modification of the airlift reactor, a pneumatically agitated reactor that is divided vertically into an aerated and non-aerated zone (Jajuee et al., 2006). The aerated zone, or riser, is sparged with air, decreasing the bulk density of the water compared to the non-aerated zone and thus inducing fluid circulation, upwards in the riser and downwards in the downcomer (Jajuee et al., 2006). The ISBR is cylindrical with a rectangular, non-woven polyurethane barrier separating the aerated from the non-aerated zone. The geotextile barrier is semi-permeable and allows liquid flow, but is impenetrable to gas bubbles. The geotextile has a large pore size distribution, typically 1-1000 μm (Karamanev et al., 1997). Because the hydrostatic head on the non-aerated side of the reactor is less than the head at the same reactor height on the aerated side, the pressure difference induces horizontal fluid flow in addition to the vertical fluid flow seen in airlift fermenters (Figure 2-7).

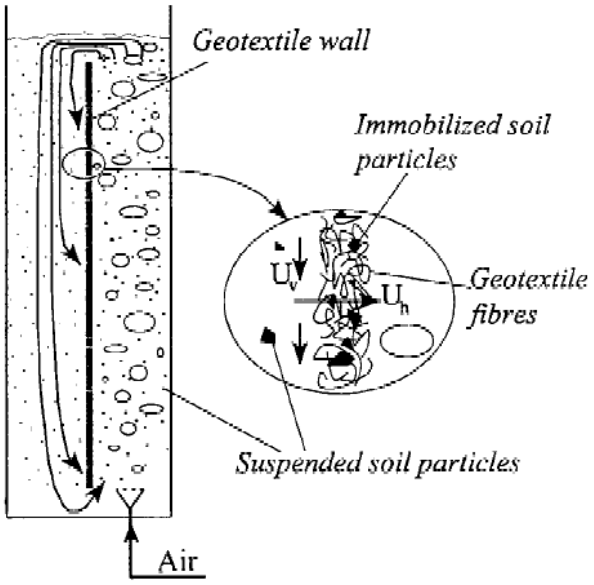

Figure 2-7: Fluid flow in an ISBR, reprinted with permission from Karamanev et al., (1998). Copyright 2013 Wiley.

In an ISBR, the source of the inoculum is contaminated soil. The semi-permeable membrane serves to immobilize these soil particles and support the biofilm that forms. For recalcitrant compounds, it can be difficult and time-consuming to isolate and cultivate a pure strain capable of efficient degradation (Otte et al., 1994). Using contaminated soil as the source for the target pollutant-degrading microorganisms capitalizes on the intrinsic biodegradation activity of the soil due to natural selection. While in-situ microbial activity is often low due to the lack of nutrients and oxygen under natural conditions, using favourable conditions, the microbial population can be activated and used to efficiently degrade the xenobiotic compound. The fluid flow characteristics in the ISBR prevent soil from leaving the reactor and minimize friction between soil particles, allowing a biofilm to form (Karamanev and Samson, 1998). For a soil particle size distribution of 150 to 250 μm, Karamanev et al. (1997) found that 90% of the soil was immobilized within 12 min.

The horizontal liquid flow also serves to transport substrate into the depths of the geotextile, making the substrate more accessible to microbes within the biofilm and promoting uniform biofilm formation – an important scale-up issue (Kermanshi et al., 2005). These fluid flow characteristics also decrease mixing time and increase k_La, compared to the classic airlift reactor (Karamanev et al., 1997).

2.5.2 ISBR applications

Immobilization of microbial cells provides a number of potential advantages (Karel et al., 1985).

1. Easy separation and reuse of cells; in particular, cells with a low specific growth rate can be easily retained
2. Attainment of high cell concentrations, resulting in high volumetric productivities
3. Elimination of cell washout condition allows operation at high dilution rates
4. Protection of fragile cells from shear forces
5. May provide cells with favourable microenvironment conditions

The ISBR combines the advantages associated with immobilized cell reactors with good substrate and oxygen transfer (due to the fluid flow patterns) and a novel method for development of contaminant-degrading microbial consortia. Furthermore, it is simple in design, and requires little operational attention.

In the case of environmental pollutants (PCP, p-xylene, and naphthalene), where substrate concentration is limited by solubility, the ISBR has achieved high volumetric cell concentrations. In the degradation of PCP, a cell concentration of 0.4 g/L was achieved in the ISBR, compared to concentrations of 0.0002 to 0.06 g/L reported in suspension culture

(Karamanev and Samson, 1998). Increased cell concentration resulted in a volumetric degradation rate of 920 mg/L-h, 4-3000 times higher than previously reported rates (Karamanev and Samson, 1998).

Despite higher volumetric cell concentrations, the ISBR maintains good mass transfer. The planktonic cell concentration in most ISBR fermentations is negligible, thus there is very little accumulation of biopolymers in the bulk medium and oxygen transfer rate is not depressed due to changes in viscosity (Jajuee et al., 2007). A substrate penetration study, with an ISBR degrading p-xylene, found a substrate concentration gradient consistent with a shallow biofilm (Kermanshi et al., 2005). Furthermore, for a range of substrate concentrations, the effectiveness factor was greater than 0.9, leading the authors to conclude that the reaction was kinetically controlled.

A limitation of the ISBR is the potential for the pores of the geotextile membrane to become clogged, decreasing performance due to decreased horizontal fluid flow (Karamanev and Samson, 1998). Consequently, sustained operation would require replacement or flushing of the semi-permeable membrane. Additionally, biofilm samples are difficult to obtain without disrupting operation.

2.5.3 ISBRs for OSPW treatment

An ISBR is a promising remediation technique for the treatment of OSPW. Biodegradation of OSPW NAs is slow in suspension culture, with NA half-lives ranging from 44 to 240 days (Han et al., 2008). As OSPW volumes are rapidly accumulating, faster treatment options are required, however, bioremediation studies with OSPW in the literature have focused almost exclusively on degradation in suspension culture, carried out in shake flasks (MacKinnon

et al., 1986; Herman et al., 1994; Lai et al., 1996; Scott et al., 2005; del Rio et al., 2006; Bataineh et al., 2006; Han et al., 2008).

An immobilized system is well suited to the low NA concentrations in OSPW, ranging from 40 to 120 mg/L (Holowenko et al., 2002). This concentration range is similar to that used for p-xylene and naphthalene in ISBR studies of 0-200 mg/L and 0-25 mg/L, respectively (Kermanshi et al., 2005). Paslawski et al. (2009) found that the use of an immobilized cell system increased the biodegradation rate of a model NA, trans-4-methyl-1-cyclohexane carboxylic acid, by almost two orders of magnitude, compared to a continuous stirred reactor. Additionally, as successful degradation requires previous exposure to OSPW (Herman et al., 1994; del Rio et al., 2006), the ISBR offers an easy method for developing and maintaining a NA-degrading microcosm. In shake flask studies, centrifuged OSPW is used as an inoculum and maintained by repeated transfer to fresh medium (Scott et al., 2005).

Although the ISBR has only been studied at the bench and pilot scale, it shows great promise for treatment of contaminated water on a larger scale. In a pilot scale study, the maximum volumetric degradation rates achieved for p-xylene and naphthalene improved by 35% and 60%, respectively, compared to bench scale studies (Jajuee et al., 2007). This improvement was attributed to more uniform bubbling, resulting in an increased oxygen transfer rate and better mixing. There is also the potential for treatment of OSPW in-situ. Kermanshi et al. (2005) proposed adaptation of the principles of the ISBR to a biobarrier. The microcosm would be activated and cultivated in an ISBR, then subsequently used to inoculate the biobarrier, sparging on one side of the barrier to induce circular and horizontal fluid flow.

2.6 Conclusions

The Athabasca oil sands is a valuable energy resource; however, the massive volume of OSPW held in tailings ponds is a major concern for the oil sands industry, as this land will ultimately have to be reclaimed. Remediation is especially challenging due to the complex nature of naphthenic acids, the composition and toxicity of which are still not fully understood.

While both laboratory and field studies have shown that bioremediation will reduce the concentration of NAs over time, degradation is slow and incomplete. Abiotic methods such as ozonation and UV treatment are prohibitively expensive. Thus, there is currently a great need for a techno-economically viable treatment option.

Bioremediation studies have primarily been limited to suspension cultures. Studies examining degradation in a continuous, immobilized cell reactor are required to truly assess the potential of bioremediation as a treatment option. A possible candidate is the ISBR, which has been successfully used for the treatment of petroleum hydrocarbons and TCE.

Chapter 3

Feasibility of the ISBR for removal of NAs in OSPW

3.1 Introduction

Previous OSPW NA biodegradation studies have demonstrated the ability of microorganisms to reduce NA concentrations and toxicity in shake flask (Herman et al., 1994; Scott et al., 2005; Han et al., 2009) and simulated wetlands (Toor et al., 2012). However, required treatment times are long. A model NA has been treated in a CSTR and in an immobilized cell reactor (Paslawski et al., 2009), but to date, native process water has not been successfully treated in a continuous flow bioreactor. Therefore, existing studies are not adequate in assessing the potential of bioremediation as a treatment option.

As discussed in Section 2.5.3, the ISBR is a promising technology for OSPW treatment. Use of immobilized cells has been shown to increase treatment rates compared to suspension culture (Paslawski et al., 2009). Furthermore, the ISBR has successfully treated contaminants such as PCP and petroleum hydrocarbons at concentrations similar to that of NAs in OSPW. However, the ISBR has only been evaluated for treatment of synthetic contaminated water (Jajuee et al., 2007; Karamanev and Samson, 1998; Kermanshi et al., 2005). A complex wastewater may result in operating and monitoring challenges.

The present work evaluates the potential for two ISBRs connected in series to remove NAs from OSPW. Typical NA removal levels are established and the change in NA profile with ISBR treatment determined. Operating and monitoring concerns associated with OSPW treatment are identified and discussed.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 OSPW

OSPW was obtained from the West-In-Pit (WIP), an active settling basin on the Syncrude Canada site in Fort McMurray. Water was stored in sealed 20 L carboys at room temperature, with periodic measurement to ensure that NA concentrations were constant.

OSPW composition is summarized in Table A1 in Appendix A. NA concentration determination is described in Section 3.2.3. OSPW was analyzed by the Queen's Analytical Services Unit for metals, anions and TOC. Acid-digested OSPW samples were analyzed for metals with a 7700x inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Mississagua, ON). Anions (chloride, sulphate, nitrate, and nitrite) were determined with a Dionex DX300 ion chromatographic system (Sunnyvale, CA) equipped with a Dionex IonPac AS11 column. TOC was measured with a Shimadzu TOC 505A non-purgeable organic carbon analyzer (Columbia, MD) as the average of triplicate injections of 100 μL of sample. Prior to analysis, 5 mL samples were acidified to pH 2 with 1 M HCl and sparged with high purity nitrogen for 1 min immediately prior to analysis to remove inorganic carbon.

3.2.1.2 Source of inoculum

A mixture of soils from a natural wetland, a consolidated tailings wetland, and a high sulphate wetland was used as the inoculum for the ISBRs. The soils were mixed with water to form a slurry that was poured onto the geotextile used as the biofilm support. The OSPW fed to the ISBRs was not autoclaved and can also be considered a source of NA-degrading organisms.

3.2.1.3 Mineral salts medium (MSM)

The MSM composition was varied based on the objective of each experiment; however, the recipe under normal operation is reported in Table 3-1 and is the feed to Reactor 1. Micronutrients were not provided as it was assumed that the levels in the OSPW would be sufficient. The MSM was not sterilized prior to use.

Table 3-1: Components of the mineral salts medium in distilled water

Component	Mass (per litre)
$(NH_4)_2SO_4$	1.10 g
Na_2HPO_4	1.33 g
KH_2PO_4	0.13 g
$MgSO_4 \cdot 7H_2O$	1.77 mg
$CaCl_2 \cdot 2H_2O$	1.33 mg

3.2.2 Reactor set-up and operation

Reactor 1 (R1) set-up is shown in Figure 3-1. Each ISBR is a cylindrical glass vessel with an internal diameter of 16.3 cm, a height of 30.5 cm, and a working volume of ~4.8 L. In the centre of the cylinder is a 3 cm thick rectangular geotextile (polyester fibre furniture stuffing, Wal-Mart), 16.3 cm by 23 cm, matrix secured between two stainless steel screens. Reactors were operated in a continuous flow regime with OSPW and mineral salts medium (MSM) fed to R1 separately via peristaltic pumps through Masterflex Tygon tubing (Cole-Parmer Canada Inc., Montreal, QC) at flow rates of 48 mL/h and 12 mL/h, respectively, resulting in an 80 hour residence time in each reactor. R1 effluent was fed directly to Reactor 2 (R2). Reactors were operated at room temperature, 22 ± 2 ˚C.

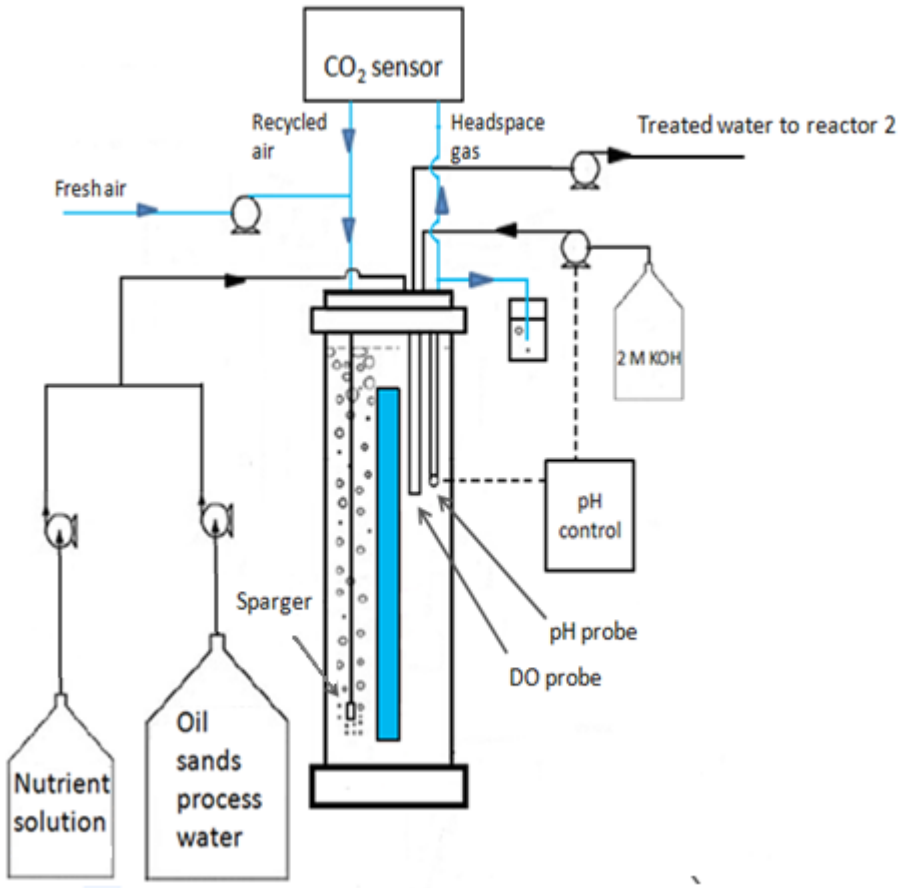

Figure 3-1: Reactor 1 set-up under normal operation

The pH in each reactor was measured by a Cole Parmer (Montreal, QC) high-pH, double junction pH electrode, attached to an αlpha (Eutech Instruments, Vernon Hills, IL) pH800 pH/ORP controller. The low pH set point was 8.00 with a hysteresis band of 0.1 and 2 M KOH was used for pH adjustment.

Fresh air was added to each reactor from the building`s compressed air line at a rate of 20 mL/min, controlled by an Omega (Stamford, CT) FMA-2600A series mass flow controller and distributed with a fish tank sparger. The headspace gas was recirculated at a flow rate of ~150 ml/min by a diaphragm pumps in the CO_2 sensor. Moisture was removed from the air with a

Drierite (Xenia, OH) drying column prior to measuring the CO_2 concentration in the headspace with a Topac Inc. (Cohasset, MA) Guardian CO_2 monitor. O_2 in the headspace gas was monitored during experimentation with a differential oxygen analyzer (DOX) (S104 DOX system, Qubit Systems, Kingston, ON). The DOX was calibrated prior to each use and headspace oxygen monitored for a minimum of 3 hours, with measurements recorded every 90 s. Dissolved oxygen (DO) was measured with a Sensorex (Garden Grove, CA) 12 mm DO probe.

pH, DO, and CO_2 were constantly monitored. Instrumentation output signals were collected by an ADR 2000 board (Ontrak Control Systems Inc., Sudbury, ON) and recorded with Labview 6 (National Instruments, Vaudreuil-Dorion, QC). Measurements were recorded every 90 seconds.

3.2.3 Sample collection and analysis

Reactor samples were collected via a sampling port drawing from both the aerated and non-aerated sides of the reactor, at a depth of ~5 cm below the liquid level. Untreated OSPW and reactor samples were centrifuged at 14,475 xg for 20 min to remove suspended sediment and microorganisms. The supernatant was collected and stored at -20˚C in 100 mL glass vials until analysis.

Planktonic cell samples were obtained by centrifuging reactor samples at 14,475 xg for 20 min. After aspirating the supernatant, the pellet was re-suspended in 1 mL of distilled water containing 15% glycerol in a 1.5 mL plastic microcentrifuge tube and stored at -80˚C. Biofilm samples were collected by opening the ISBR and cutting off a piece of the geotextile membrane. The sample was placed in a plastic tube with glass beads and 40 mL of phosphate buffered saline and vortexed to separate the cells from the support. The liquid was centrifuged at 8,000 xg for 10

min and the supernatant discarded. 0.7 mL of suspended cells were mixed with 0.3 mL of 50% glycerol in distilled water in a 1.5 microcentrifuge tube and stored at -80˚C.

3.2.3.1 Ammonium concentration determination

Ammonium concentration of reactor samples was determined by a colorimetric phenol-hypochlorite reaction developed by Weatherburn (1976). In 13 mm test tubes, 2.5 mL of reagent A containing 10 g/L phenol and 0.05 g/L sodium nitroprusside was added to 40 μL of sample, followed by addition of reagent B containing 5 g/L sodium hydroxide and 0.42 g/L sodium hypochlorite. Tubes were vortexed and incubated at room temperature for 30 min, at which point the reaction was stopped by immersing the tubes in an ice bath for 5 min. Absorbance was measured at 630 nm within 30 min and compared to a calibration curve of ammonium standards from 0 to 0.1 g/L (Figure A1, Appendix A). All samples were tested in triplicate, with the number of samples reported (n) representing the average ammonium concentration of n distinct reactor samples.

3.2.3.2 Phosphate concentration determination

Phosphate concentration of reactor samples was measured via reduction of phosphomolybdate to molybdene blue (Lanzetta et al., 1979). In a test tube, 0.1 mL of standard/sample was mixed with 1 mL of reagent 1 (solution of 19.8 g/L sodium borate and 18.1 g/L sodium metabisulfite), 0.2 mL of reagent 2 (24.2 g/L sodium molybdate in 1.288 M sulfuric acid) and 0.2 mL of reagent 3 (10 g/L 4-(methylamine) phenol sulfate and 30.4 g/L sodium metabisulfite). Reactant mixture was incubated for 15 min at room temperature, followed by addition of 2 mL of reagent 4 (42.4 g/L sodium carbonate and 6.9 g/L sodium sulfite), mixing and incubation for a subsequent 15 min. Absorbance was measured at 750 nm and compared to a

calibration curve generated from phosphate standards ranging in concentration from 0 to 0.5 g/L (Figure A2, Appendix A). Samples were tested in triplicate, with n representing the average concentration of n distinct reactor samples.

3.2.3.3 Extraction and derivatization of NAs

20 mL reactor or OSPW sample aliquots were placed in a 250 mL separatory funnel with 100 mL of distilled water and spiked with 100 µL of a stock solution of 0.1 g/L 9-fluorene carboxylic acid (9-fca) in dichloromethane (DCM). The solution was acidified to ~pH 2 with 7 M H_2SO_4 and extracted a total of three times, 1X 10 mL and 2X 5 mL, with dichloromethane (DCM). The solvent extract was concentrated under a gentle stream of high purity N_2 then transferred to a 2 mL GC vial, where it was evaporated until dry. After the addition of 50 µL of N-methyl-N-(t-butyldimethylsilyl) trifluoroacteamide (MTBSTFA) derivatizing agent, the mixture was incubated at 60 ˚C for 20 min, then the MTBSTFA was driven off with N_2 and the derivatized extract was re-dissolved in 0.5 mL DCM.

3.2.3.4 GC-MS analysis of NA extracts

Samples (2 µL) were analyzed by a Varian 3800 gas chromatograph (Agilent Technologies, Mississagua, ON) equipped with a Varian FactorFour capillary 5 MS column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness) connected to a Varian 1200 single quadrupole MS. The carrier gas was helium at a flow rate of 0.7 mL/min. The injector, source, and transfer line temperatures were 290 ˚C, 250 ˚C, and 290 ˚C, respectively. The column oven temperature was initially held at 80 ˚C for 6 min, then increased to a final temperature of 280 ˚C at a rate of 7 ˚C/min and held for 8 min. The MS was operated in EI ionization mode with a source voltage of 70 eV. A 6 min collection delay was used to prevent saturation of the detector while the solvent eluted. Ions were detected over a range of 60 to 550 m/z, with additional

single ion monitoring (SIM) at m/z=267, with dwell times of 0.250 and 0.100 s, respectively. The major ion for 9-fca is 267, thus SIM at this mass allows accurate measurement of the internal standard, without interfering ions that are detected in the total ion chromatogram. With a sample volume of 20 mL, the GC-MS can detect NA concentrations ≥ 0.5 mg/L (Merlin et al, 2007).

3.2.3.5 NA quantification

For NA quantification, the area under the total ion chromatogram (A_{hump}) was integrated from 10-35 min (beyond 35 min, NAs could not be distinguished from column bleed) and compared to the area under the 9-fca peak (A_{std}). An example chromatogram can be found in Appendix A, Figure A3. The A_{hump}/A_{std} ratio was compared with a calibration curve, for determination of the mg/L concentration of NAs. This calibration curve (Figure A4, Appendix A) was generated by adding varying masses of Merichem naphthenic acids to 2 mL GC vials with 10 µg of 9-FCA, derivatizing and analyzing by GC-MS as previously described. Plotting the A_{hump}/A_{std} ratio versus mass of Merichem added to the GC-vial and dividing by the extracted OSPW volume gives the NA concentration. Quantification of NAs in this way considers all acid-extractable organics (AEOs) rather than just NAs fitting the $C_nH_{2n+Z}O_x$ formula. However, this concentration is only an estimate as OSPW NAs and commercial NAs have been found to have different NA profiles, and thus different ionization efficiencies.

Additionally, a calibration curve (Figure A5, Appendix A) was created by extracting various volumes of OSPW and plotting the resulting A_{hump}/A_{std} ratios versus volume extracted as a percentage of the 20 mL volume used for sampling, with adjustment for dilution from addition of mineral salts medium (MSM) during reactor operations. In this case, the area under the 267 ion hump was used for quantification and integrated between 15 and 30 min. The 267 mass

corresponds to the most abundant ion in the process water and has been used in literature for quantification (Scott et al., 2008A). The advantage of this method is that it eliminates variation from the baseline that sometimes affects results when considering the total ion chromatogram. The concentration or removal levels reported and the corresponding standard deviations were calculated from n extracted and analyzed samples.

3.2.3.6 Determination of NA composition

The peak ion intensities eluting in the NAs hump (retention time 10-35 min) were averaged and divided by the area under the internal standard peak and corrected for volume extracted, so that spectra could be directly compared. 57 mass units were subtracted from each mass to account for the mass arising from derivatization. A Mathematica (Wolfram, Research, Champaign, IL) program was then used to match the ions to an isomeric group of the form $C_nH_{2n+Z}O_2$, the program code can be found in Appendix A. For a match to be accepted, the difference between the actual and theoretical mass was required to be less than 1000 ppm.

As discussed in Section 2.2.3, GC coupled with a quadrupole MS lacks sufficient resolution to distinguish O_2 species from those that are more highly oxidized. Thus, it was assumed that only O_2 species are present and detected ions were assigned to isomer groups that fit the empirical formula $C_nH_{2n+Z}O_2$, as outlined by Holowenko et al. (2002). However, Bataineh et al. (2006) determined that ions eluting with $n \geq 22$ are artifacts caused by double derivatization of hydroxylated NAs. Thus, the presence of hydroxylated NAs and general increases/decreases in this group can be detected.

3.2.4 Denaturing gradient gel electrophoresis analysis of reactor cell samples

Total genomic DNA was extracted from biofilm and planktonic samples using an E.Z.N.A Standard Bacterial DNA Isolation Kit (Omega Bio-Tek, Norcross, GA) with an additional bead beating step.

Partial sequences of the 16S rRNA gene including the variable V3 region were amplified using two universal bacterial primers, tP2 (5′-ATTACCGCGGCTGCT-3′) and tP3 (5′-CCTACGGGAGGCAGCAG-3′) (Muyzer et al., 1993). The PCR program was run on an Eppendorf Mastercycler (Model 6331, Mississagua, ON) as follows, temperature was held at 94˚C for 5 min; for the first cycle, 30 s at 94˚C, 30 s at 65˚C (0.5˚C decrease after each cycle), 40 s at 72˚ x 20 cycles; for the second cycle, 30 s at 94˚C, 30 s at 56˚C, and 40 s at 72˚ x 10 cycles; followed by a 7 min hold at 72˚C. 200 base pair DNA fragments were confirmed on a 1.5% agarose gel. The PCR reconditioning program was as follows, hold at 95˚C for 10 min, followed by 5 cycles of 1 min at 94˚C, 1 min at 56˚C, and 1 min at 72˚C, finishing with a 7 min hold at 72˚C.

A 6% polyacrylamide gel was used with a deionized formamide and urea denaturing gradient of 30 to 60%. The gel was run in 1x TAE buffer (40mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) at 60˚C and 80 V for 16 hours. The gel was stained with SYBR Gold and visualized with a transilluminator with a SYBR Gold filter.

3.2.5 Sorption test

A batch sorption test in 20 mL glass vials was conducted to determine if NAs would sorb to the geotextile used as the biofilm support. A 75 mg/L solution of Merichem NAs was prepared in distilled water using the MSM described in Table 3-1, and adjusted to pH 8, to simulate reactor contents. 20 mL aliquots of this solution were placed in scintillation vials with geotextile in

masses varying from 0 to 1.2 g/L. The vials were sealed and placed on a shaker at 200 rpm and room temperature for 7 days. 3 mL samples were removed from each vial and the NA concentration determined as described in Section 3.2.4.3.

3.2.6 Effect of inorganic carbon on reactor operation study

The effect of inorganic carbon (IC) on ISBR operation was investigated by feeding IC-free OSPW to R1. Inorganic carbon (IC) was removed from a 20-L bucket of OSPW by decreasing the pH to ~2 with 7 M H_2SO_4 and sparging with N_2 for 30 min under mixing. pH was readjusted to pH 8 with 2M KOH prior to use. IC-free OSPW was fed for 11 days, and with a residence time of 3.33 days in each reactor, this allowed for ~1.5 volume changes of the ISBR system contents.

3.2.7 Effect of pH on reactor operation study

To determine the effect of pH on reactor activity, pH control was removed from R2. The pH was allowed to reach a new steady-state value. After 4 weeks at a reduced pH, the pH was adjusted to 8 with 2 M KOH and pH control via base addition was resumed.

3.3 Results and discussion

3.3.1 Sorption test

To ensure all decreases in NA concentration are due to biological degradation, the geotextile membrane used as the biofilm support was investigated as a potential source of abiotic NA removal. OSPW NAs are known to sorb to soils, exhibiting a K_d of 17 mL/g (Janfada et al., 2006) and the geotextile membrane has been found to sorb p-xylene and naphthalene in previous ISBR studies (Jajuee et al., 2007).

To assess the potential for sorption of NAs to the ISBRs' membranes, a sorption experiment was carried out using Merichem NAs and the geotextile used as the biofilm support. The percent reduction in equilibrium NA concentration with mass of sorbent after 1 week is shown in Figure 3-2. One week approximates the residence time in the ISBR system and should provide ample time for equilibrium to be established; in a study of NA sorption on soils, Janfada et al. (2006) observed that equilibrium was achieved after 3 hours. The sorption isotherm does not appear to be linear, the K_d calculated decreased with increasing mass of sorbent, ranging from 5 to 14.5 mL/g, indicating that more sorption will occur at higher NA concentrations. The concentration of NAs in the vials with 0 g of sorbent was 57.0 ± 0.7 mg/L, compared to initial concentration of 75 mg/L added to the scintillation vials. This discrepancy could be due to sorption to the glass of the scintillation vials and/or a result of inefficient extraction of the NAs. With a Henry's Law constant of 8.56×10^{-6} (Rogers et al., 2002), NAs are not volatile, and evaporative losses are not expected.

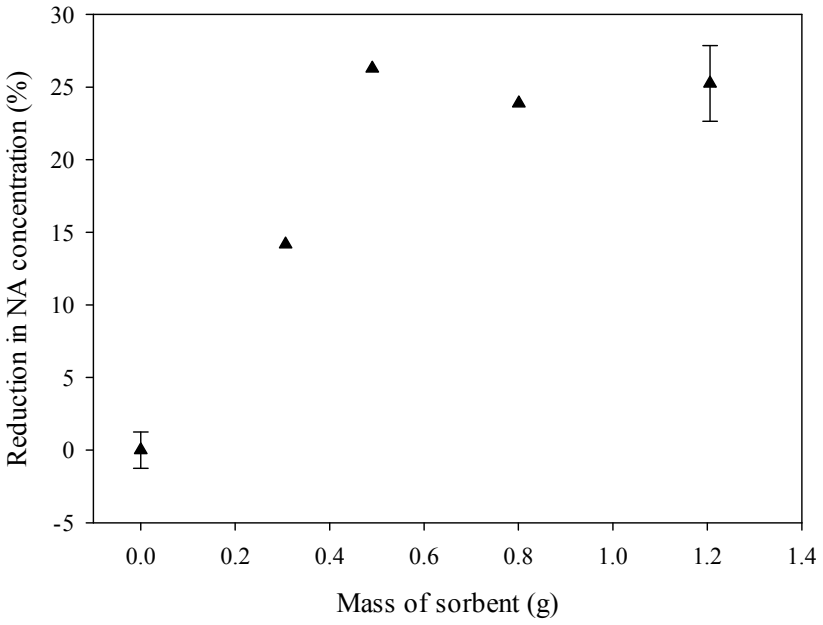

Figure 3-2: Reduction in NA concentration on varying masses of biofilm support after 1 week of equilibration; error bars represent the standard deviation of replicate samples (n=2)

These results indicate that some sorption of NAs to the biofilm support should be expected, especially in start-up experiments. However, in the case of p-xylene and naphthalene sorption on the ISBR biofilm support, the system was quickly saturated (Jajuee et al., 2006). With a residence time of 3 hours, 44% of the p-xylene and 37% of the naphthalene were sorbed and subsequent introduction of feed streams with varying p-xylene and naphthalene compositions showed no reduction in concentration. Experimental results reported in this work all occurred after more than 10 months of continuous operation, therefore abiotic loss of NAs due to sorption should not occur.

3.3.2 NA removal in the ISBR

Typical NA removal in the ISBR is summarized in Table 3-2. These results were obtained after 16 months of continuous operation. At this time point, ISBR treated OSPW shows a decrease in NA concentration of ~40%, with the bulk of degradation occurring in R1. This removal level compares favorably to those reported in the literature for shake flasks. Han et al. (2008) reported 52% removal after 98 days of treatment; however, only ion peaks matching the form $C_nH_{2n+Z}O_2$ were considered in quantification. Considering all compounds with a carboxylic acid group, Clemente et al. (2004) reported 20% removal in 40-49 days. Furthermore, with a residence time of 80 hours in each reactor, the total treatment time is ~7 days, which is substantially shorter than any previously reported (Table 3-3). It should be noted that the removal levels reported in this study do not necessarily indicate mineralization. Transformed NAs may have lower ionization efficiencies or elute outside of the 10-35 min range used for quantification.

Table 3-2: Typical NA degradation in the ISBRs achieved with an 80 hour residence time per reactor

Sample	NA concentration[a] (mg/L)		Total NA removal[b] (%)	
OSPW *(n=4)*	49.0	(2.1)[c]		
Reactor 1 *(n=3)*[d]	34.6	(5.8)	31	(9)
Reactor 2 *(n=3)*[d]	30.2	(1.4)	40	(12)

a) calculated using Figure A4 calibration curve
b) calculated using Figure A5 calibration curve
c) standard deviations reported in parentheses
d) based on 3 consecutive days of operation

Table 3-3: Comparison of ISBR treatment with bioremediation of OSPW in the literature

Treatment description	NA Removal	Treatment time	NA quantification	Author
ISBR treatment with Syncrude WIP OSPW + MSM	*40%*	*7 days*	*GC-MS analysis of MTBSTFA derivatized acid extractable organics*	*This study*
Shake flask study; 190 mL Syncrude WIP OSPW + 10 mL Bushnell Haas (BH) medium; microorganisms indigenous to the OSPW were used as the inoculum	52%	98 days	HPLC-HRMS monitoring of relative change in ions with exact masses fitting the formula $C_nH_{2n+Z}O_2$	Han et al. (2008)
Shake flask study; 190 mL Syncrude WIP OSPW + 10 mL BH medium; microorganisms indigenous to the OSPW were used as the inoculum	20%	40 days	HPLC coupled with an UV-visible diode array detector for detection of nitrophenylhydrazide derivatized carboxylic acids (Yen et al., 2004)	Scott et al. (2005)
Flow through simulated wetlands (glass, aerated aquarium with 9:1 ratio of water to non-OSPW impacted wetland sediment); OSPW was from Syncrude's MLSB[a]	40% 74%	40 days 400 days	FTIR analysis of NA extracts	Toor et al. (2013)
Shake flask study; Syncrude WIP OSPW + BH medium; microorganisms indigenous to the OSPW were used as the inoculum	Total NA half-life of 83 days	98 days	UPLC[b]-HRMS monitoring of relative change in ions with exact masses fitting the formula $C_nH_{2n+Z}O_2$	Martin et al. (2010)

a) Mildred Lake Settling Basin

b) Ultra pressure liquid chromatography

The NA profile generated for untreated OSPW (Figure 3-3A) is similar to other profiles in the literature by this GC-MS method, Figure 3-3B (Holowenko et al., 2002; Bataineh et al., 2006). However, the NA profile will vary with the OSPW source. For the Syncrude WIP tailing

samples used in this study, two and three-ring compounds (Z= -4 and Z= -6) dominate, and ions are present in the C22+cluster, indicating the presence of hydroxylated NAs. There is a significant amount of aliphatic compounds present in this OSPW. However, a number of sources have reported that the presence of Z=0 compounds is quite limited and that unit resolution MS misclassifies ions with similar molecular weights as aliphatic NAs (Bataineh et al., 2006; Martin et al., 2008). Analysis using HPLC–ESI–HRMS confirmed the presence of these isomers for n=9 to 18 in our OSPW; see Figure A4 in Appendix A. Our results are consistent with those of Grewer et al. (2010) who found that Z=0 compounds account for 18% of total $C_nH_{2n+Z}O_2$ species in WIP OSPW analyzed with ESI-FT-ICR-MS.

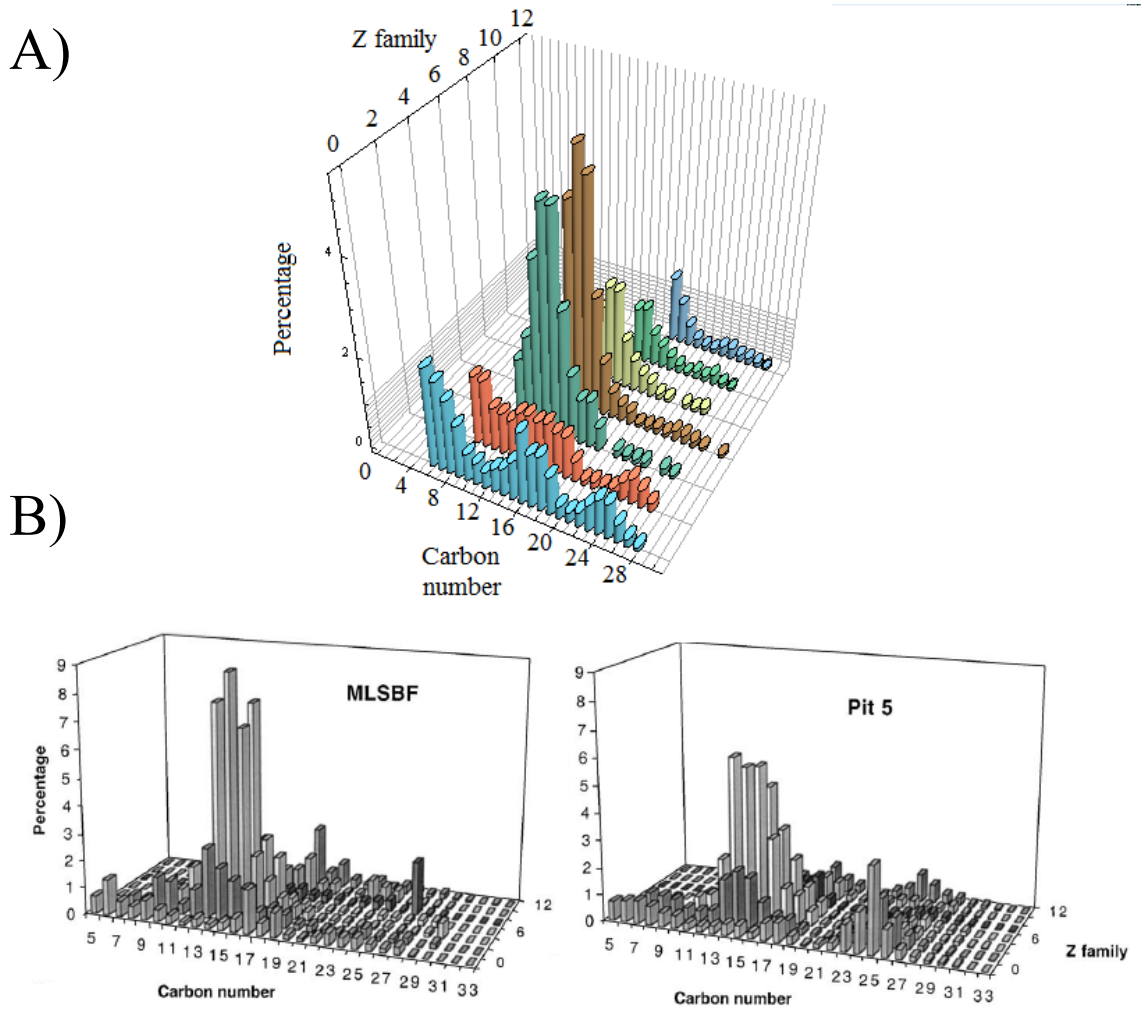

Figure 3-3: GC-MS generated NA profiles of A) WIP OSPW used and analyzed in this study and B) Mildred Lake Settling Basin and Pit 5 OSPW, reprinted with permission from Holowenko et al. (2002). Copyright 2013 Elsevier.

The change in NA profile with ISBR treatment (Figure 3-4A) is compared to a biodegradation study by Han et al. (2005) where HPLC-HR MS was used for NA analysis (Figure 3-4B). Visual examination of the plots shows significant degradation of two of the most abundant Z-series, Z= -4 and Z= -6 in the ISBR treated samples. However, aliphatic NAs appear to be recalcitrant. In order to determine if there were statistically significant changes to the NA profile with ISBR treatment, the distributions of four untreated OSPW samples were averaged and

compared to the averaged distributions of four ISBR samples generated under the same treatment conditions.

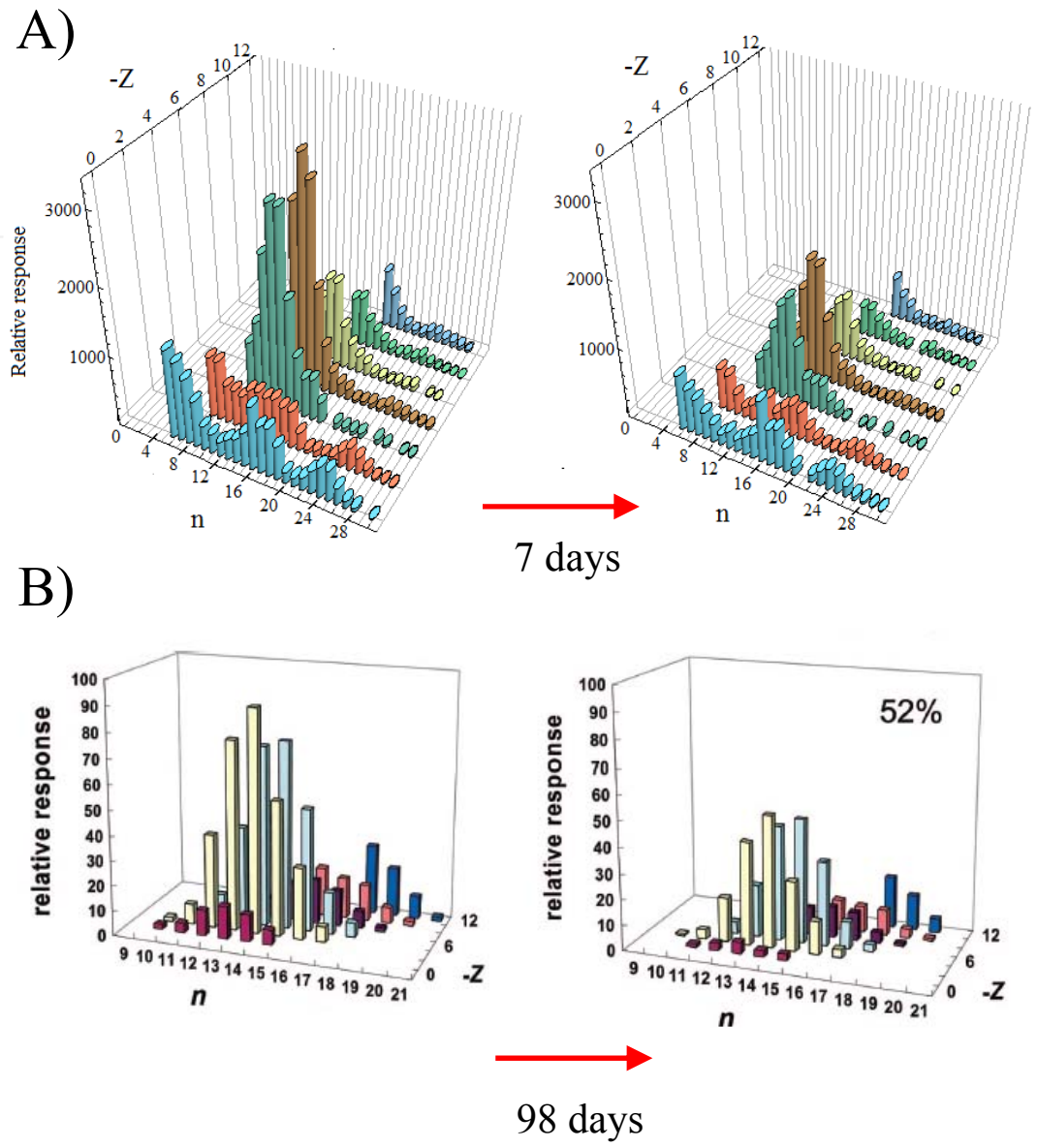

Figure 3-4: A) Change in NA profile with A) ISBR treatment B) batch degradation in shake flask, reprinted with permission from Han et al. (2008) Environmental Science & Technology 42(4): 1290-1295. Copyright 2013 American Chemical Society.

The changes in average n-number and Z-series distribution with ISBR treatment are reported in Tables A2 and A3 in Appendix A. Figures 3-5A and 3-5B, showing the percentage decrease or increase, respectively, in each n and Z combination, more clearly delineate the change to the NA profile. The Z= 0 series shows a statistically significant decrease in percent of total response, while the Z= -12 and Z= -4 series show statistically significant decreases at the 95% confidence level. Welch's t-test was used to determine significance, calculations can be found in Appendix A. The Z= -4 series is the most abundant and exhibits the greatest percentage decrease with treatment. The distribution of the remaining classes remains relatively constant, with a general trend of 1-, 2-, and 3- ring compounds decreasing in proportion, while 4-, 5- and 6-ring isomers increase in proportion. This trend is consistent with the findings of Han et al. (2008) that biodegradation rate in OSPW NAs decreases with increasing cyclicity. However, there is no enrichment of highly cyclic NAs in aged tailing pond waters, compared to active settling basins (Han et al., 2009). Thus, it may be that while the degradation *rate* of less cyclic NAs is greater, the higher Z-series NAs are not recalcitrant.

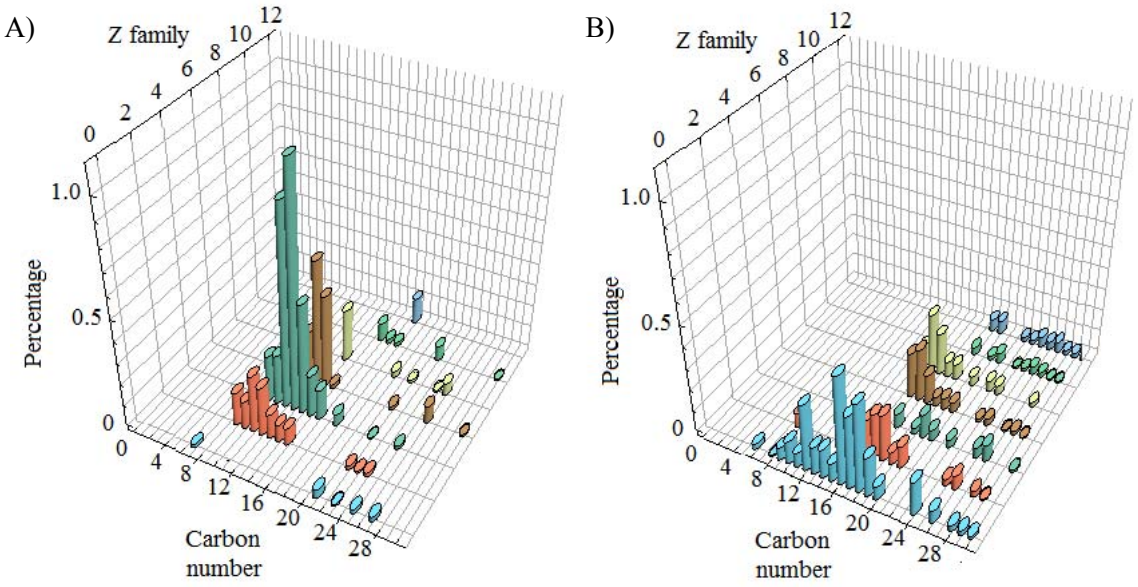

Figure 3-5: A) Isomers decreasing and B) increasing in percentage of total NAs with ISBR treatment; calculated as $[(\frac{isomer\ count}{total\ NA\ count})_{OSPW} - (\frac{isomer\ count}{total\ NA\ count})_{ISBR\ treated}] \cdot 100$

Closer examination of the Z=0 series shows that the ions corresponding to Z=0 isomers expressed as a percentage of total NAs increases regardless of n number. However, the greatest increases occur for n=12 and n=16 to 18. For these isomers, not only does the proportion compared to total NAs increase, the actual ion count increases from OSPW to ISBR treated samples, indicating that they are not just refractory, but are being created within the ISBR. These compounds may be branched degradation products. Using surrogate NAs, biotransformation has been found to decrease with increased branching (Smith et al., 2008; Johnson et al., 2011). Alternatively, they may be heteroatomic compounds which have been mis-classified as oxy-NAs. In WIP OSPW, N and S account for 3.89% and 0.5%, respectively, of the mass of the acid extractable organics (Grewer et al., 2010). Degradation of commercial NAs has been found to produce palmitic ($C_{16}H_{32}O_2$) and stearic ($C_{18}H_{36}O_2$) acids (Clemente et al., 2004). The authors

hypothesized that these fatty acids, found in the membranes of Gram-negative bacteria, were the result of microbial growth, prompting us to investigate whether these acids were responsible for the increase in $C_{16}H_{32}O_2$ and $C_{18}H_{36}O_2$ concentration. The elution peak time and fragmentation pattern of palmitic and stearic acid standards were used to confirm the presence of these acids in both treated and untreated OSPW samples. However, there was no significant increase in concentration with treatment, likely because ISBR cells are immobilized and consequently, their lipids are not extracted in reactor samples.

There is a slight increase in average n for all Z-series with treatment, however, this increase is only statistically significant at the 95% confidence level for Z= -2, Z= -4, and Z= -6 (Appendix A). For each Z-series, there is no direct linear correlation between carbon number and change in relative proportion, with the exception of Z= -6. However, in general, for Z= -2 to -12, isomers with n<15 decrease in proportion with treatment, see Figure 3-6. These results are consistent with the work of Han et al. (2008), who found that isomer half-lives were not significantly associated with carbon number.

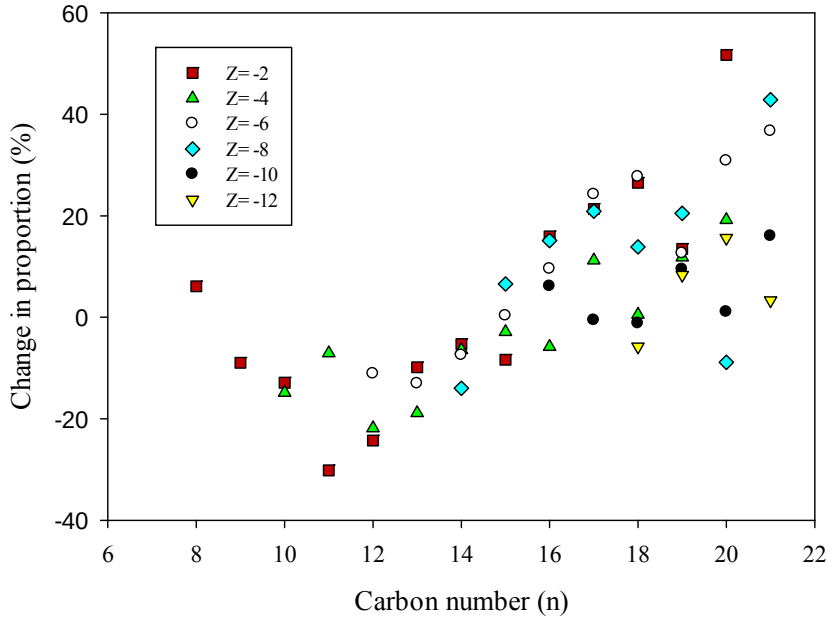

Figure 3-6: Change in proportion of total NAs with carbon number (n) for Z= -2 to -12 with ISBR treatment

Comparing various fresh and aged OSPW, Holowenko et al. (2002) found that aged process water had a higher proportion of NA isomers with n≥22, and reduced Microtox toxicity. Isomers with n≥22 in derivatized samples have been found to be hydroxylated naphthenics that have been doubly-derivatized. Consequently, Holowenko's et al. (2002) results likely support Han's et al. (2009) finding that the proportion of oxidized NAs is greater in aged process water than in active settling basins and an increase in the C22+ cluster can be interpreted as an increase in hydroxylated NAs. With ISBR treatment, the proportion of C22+ NAs increased modestly from 9.6 ± 0.4% to 10.6 ± 0.4%. When the actual ion count is compared there is little difference, indicating that the concentration of these compounds remains the same. This is consistent with laboratory studies where the concentration of hydroxylated NAs has been found to decrease

(Martin et al., 2010) or remain constant (Han et al., 2008). It appears that enrichment of oxidized NAs is an in-situ phenomenon, but is not observed in laboratory degradation studies.

3.3.3 Microbial population

Biofilm and planktonic cell populations from R1 and R2 were analyzed using denaturing gradient gel electrophoresis (DGGE). A clone library was developed by Siqing Yue from the original contaminated sediment used to inoculate the ISBRs. DNA sequencing and comparison to a database allowed identification of a number of genera present in the inoculum. DNA extraction of these sequenced plasmids allowed construction of a marker that was used for comparison and tentative identification of some of the genera present in reactor samples, Figure 3-7.

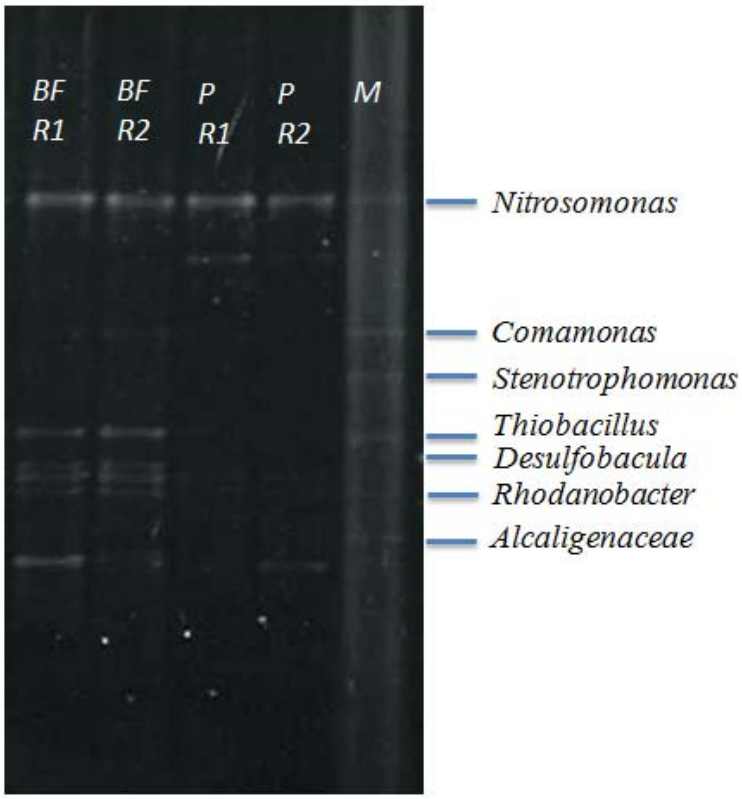

Figure 3-7: DGGE gel for reactor cell samples; BF-biofilm, P-planktonic, M-marker

The conditions at which these bacterial samples were collected were slightly different than those described in Section 3.3.2. Ammonium (only N-source) concentration fed to R1 was 0.9 g/L, with 0.51 g/L ammonium measured in the feed to R2. NA removal was 31±5% in R1 and 0±8% in R2.

The genera present in the reactors were tentatively identified to include *Comamonas*, *Stenotrophomonas*, *Thiobacillus* and *Rhodanobacter*. *Nitrosomonas*, an ammonium oxidizing bacterium, appears to be present in significant amounts, based on the density of the bands in Figure 3-7, as planktonic cells and in the biofilm of R1 and R2 and will be discussed in greater detail in Section 3.3.4.2.

3.3.4 ISBR operating and monitoring considerations

ISBR operation at the time point discussed in Section 3.3.2 is summarized in Figure 3-8. This data is representative of typical operation. The effluent of R1 flows to R2. N is provided as ammonium in R1, but in limited amounts, and as nitrate in R2, in excess. Phosphate is provided to both reactors in excess. The nutrients for R2 are provided in the effluent of R1, with the exception of nitrate, which is supplied in a separate feed. The pH is maintained at 7.95, with base addition. O_2 transfer within the ISBRs is good; DO under typical operation is steady at 40%.

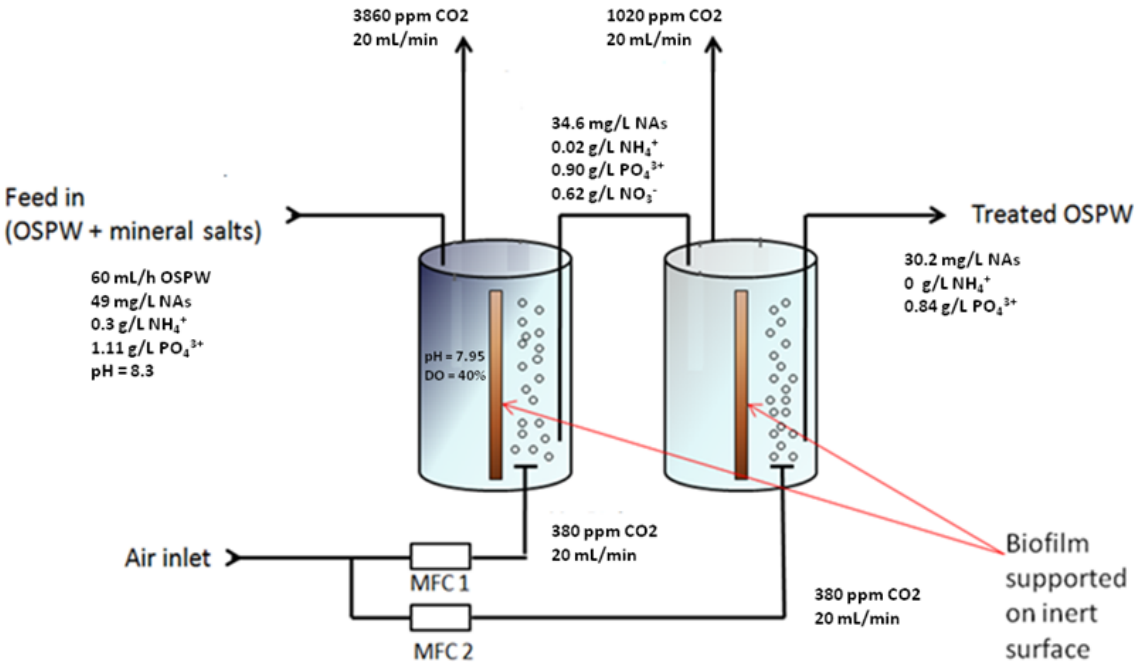

Figure 3-8: Typical ISBR operation

3.3.4.1 Effect of inorganic carbon on ISBR monitoring

The carbon balance of the ISBR system at this time point is reported in Table 3-4. Theoretical C consumption due to NA degradation accounts for only 14% and 24% of the C released as CO_2 in R1 and R2, respectively, indicating that NA degradation is not the only source of CO_2 production (CPR). The carbon consumption (X_{cons}) in mg C/h was estimated using the NA removal in mg/L, using equation 3-2. The most abundant NA isomer, $C_{13}H_{22}O_2$ was used to approximate the mass of C/mass of NAs.

$$X_{cons} = (C_o - C) \cdot F \cdot \frac{156\ mg\ C}{210\ mg\ NAs} \cdot Y \qquad (3\text{-}2)$$

C_o is NA concentration entering the reactor (mg/L), C is the NA concentration in the reactor (mg/L), F is the flow rate of the liquid stream (L/min), and Y is the percent conversion of NA carbon content to CO_2. A conversion value of 50% was used based on conversion levels of

20% for OSPW NAs and 50% for commercial NAs, of organic carbon to CO_2 via microbial activity reported in the literature (Herman et al., 1994).

Table 3-4: Carbon balance for the ISBRs

Stream	Reactor 1 (mg C/h)			Reactor 2 (mg C/h)		
	Feed	*Effluent*	*Difference*	*Feed*	*Effluent*	*Difference*
Liquid	1.09	0.77	-0.32	0.77	0.67	-0.10
Air	0.24	2.48	2.24	0.24	0.66	0.41
Total	1.33	3.25	1.92	1.01	1.33	0.31

It was hypothesized that the remaining CPR is due to the conversion of inorganic carbon (IC) to carbon dioxide. IC concentrations are high in OSPW. Pore water from Syncrude's MFT had a concentration of 1530 mg/L HCO_3^- (Fedorak et al., 2002) and total inorganic carbon (TIC) in the WIP OSPW used in experimentation was estimated at 161 mg/L (Table A1, Appendix A). Inorganic carbon exists in solution in four different forms: aqueous carbon dioxide, bicarbonate (HCO_3^-), carbonate ion (CO_3^{2-}) and carbonic acid (H_2CO_3), which are related by the equilibria in equation 3-1 (Dickson, 1981).

$$CO_2\,(aq) + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H+ \rightleftharpoons CO_3^{2-} + 2H^+ \qquad (3\text{-}1)$$

The reduction in pH from 8.3 in untreated OSPW to 7.95 in the ISBR shifts the equilibrium to the left, producing aqueous CO_2 which is converted to gaseous CO_2 by sparging of air in the reactors.

To confirm that IC is contributing to CPR in the ISBRs, OSPW that had been purged of IC was fed to the reactor. The CPR in R1 and R2 decreased rapidly following introduction of the OSPW containing no IC, while the oxygen uptake rate (OUR) remained constant, Figure 3-9. CPR was regained following introduction of OSPW containing IC. The residence time in each reactor is 3.33 days, but steady state took approximately 5 days to achieve. NA degradation was

constant, confirming that the majority of CO_2 production can be attributed to the conversion of IC to CO_2. CPR decreased by ~80% in R1 and ~90% in R2. Comparing the carbon consumed by NA degradation and produced by CPR in Table 3-5, IC removal resolves the carbon balance in R2 (within error); however, excess carbon is unaccounted for in R1, possibly due to incomplete removal of IC during the purge procedure.

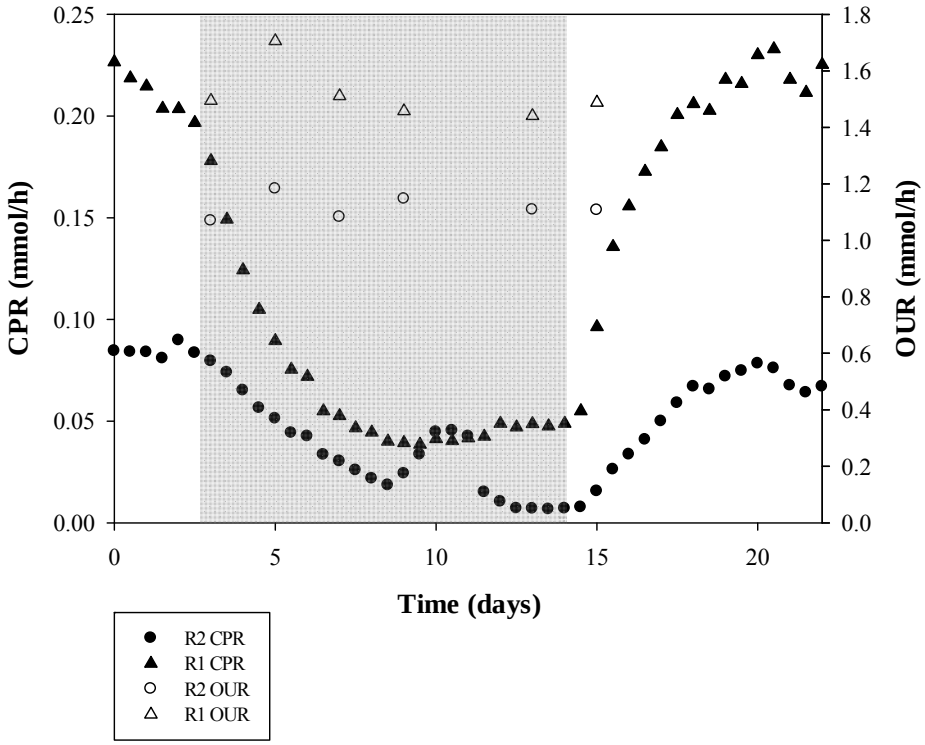

Figure 3-9: Effect of IC on CPR and OUR in the ISBRs, IC free OSPW was fed beginning at day 3, and is indicated by the gray shading; OSPW feed recommenced at day 14

Table 3-5: Effect of IC on the ISBR carbon balance

Reactor	IC	NA consumption (mg/L)[a]	Carbon balance			RQ
			NA consumption (mg C/h)	*CPR (mg C/h)*	*Difference*	
1	+	6.8 (1.6) n=5	0.15 (0.03)	2.56 (0.12)[b]	2.41	0.134
1	-		0.15 (0.03)	0.58 (0.04)[c]	0.43	0.034
2	+	1.4 (2.2) n=5	0.03 (0.05)	1.01 (0.04)[b]	0.98	0.08
2	-		0.03 (0.05)	0.11 (0.04)[c]	0.08	0.01

a) NA consumptions in R1 and R2 were constant over days 0 to 15; therefore the average over this time period was used to calculate a single NA consumption in mg C/h
b) Average of days 0 to 2
c) Average of days 11.5 to 14.5

As IC in OSPW had a stronger effect on the CPR than NA degradation, it is not a useful indicator of heterotrophic microbial activity. Even small fluctuations in pH have a significant effect on CPR. Herman et al. (1994) addressed this issue in an OSPW degradation study conducted in shake flasks by extracting OSPW NAs and re-suspending in dissolved water. Treated samples were acidified to < pH 2 to convert all IC to CO_2, and CO_2 in the headspace of sample flasks compared to inoculated controls with no carbon source. This is clearly not a feasible solution for a bioreactor, and consideration should be given to this issue for future applications of bioreactors to OSPW treatment.

3.3.4.2 Effect of nitrifying bacteria on reactor conditions and monitoring

The large population of *Nitrosomonas* detected in the ISBRs via DGGE appears to have a significant effect on the ISBR system. Ammonium provides N for cell growth in addition to providing an energy source for *Nitrosomonas*, thus consumption in the ISBRs is high. To meet this demand, nutrients are provided in excess for heterotrophic activity, with N and P feed concentrations of 0.233 g/L and 0.377 g/L, respectively, although only 49 mg NA/L are available

as a carbon source. These levels are substantially higher than the recommended C:N:P ratio for in-situ biodegradation of 100:13:3 for petroleum hydrocarbons (Young and Cerniglia, 1995).

The nitrification process is described by equations 3-2 and 3-3 (Volk and Brown, 1997). Ammonium oxidation produces hydrogen ions and is hypothesized to be responsible for the downward trend of pH in the ISBRs, requiring KOH addition to maintain a pH of 7.95.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H^+ + 2H_2O \quad (3\text{-}2)$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \quad (3\text{-}3)$$

High ammonium consumption (0.993 mmol/h) accounts for the high OUR in R1, 1.501 mmol/h compared to ~0.020 mmol CO_2/h produced by NA degradation, Table 3-6. The proportion of OUR contributed by heterotrophic microbial activity cannot be estimated from ammonium consumption as it is used for cell growth in addition to oxidation. Consequently, OUR is not a good indicator of NA degradation under normal operation. Under wetland conditions, it has been hypothesized that microbial nitrification of ammonium inhibits NA degradation due to competition for phosphate and/or oxygen (Allen et al., 2008). However, under typical ISBR conditions, DO is 40% and there is an excess of phosphate, thus nitrification does not appear to have a deleterious effect on the heterotrophic bacteria. The role of AOB in the ISBRs and NA degradation are explored further in Chapter 4.

Table 3-6: Summary of OUR, CPR, and NH_4^+ consumption in the ISBRs[a]

Reactor	OUR (mmol/h)		CPR (mmol/h)		NH_4^+ consumption rate (mmol/h)		DO (%)	NA removal (%)
1	1.501	(0.048)	0.186	(0.008)	0.993	(0.001)	40	31 (9)
2	0.273	(0.001)	0.034	(0.002)	0.007	(0.001)	40	8 (4)

a) Average and standard deviations were calculated from 3 consecutive days of operation, for NA removal and ammonium consumption n=3

3.3.4.3 pH effects

NAs are known to have pH dependent solubilities, and are most soluble at higher pH in their ionized form (Headley et al., 2002A). To ensure all NAs are in solution, reactor pH is maintained between pH 7.9 and 8.0. However, few bacteria do well above pH 8 (Volk and Brown, 1997). To determine if this pH is inhibitory to growth, R2 was operated without pH control. Without KOH addition, the pH of R2 reached a steady state at 6.95; reactor operation at pH 6.95 versus 8.00 is summarized in Table 3-7.

The effect of pH on NA degradation could not be determined due to the limited degradation occurring in R2 at this time point. However, the NA concentration was constant; indicating that alkaline pH is not inhibiting biodegradation in R2 and that NA solubility at this concentration is not affected by a more neutral pH. Additionally, the pH change did not affect reactor microbial activity, as evidenced by the constant OUR. This is in agreement with Headley et al. (2002B), who found that the half-life of NA surrogates was only slightly increased at pH 6 compared to pH 8.7.

Table 3-7: R2 operation at low and high pH

pH	NA concentration (mg/L)	OUR (mmol/h)	CPR (mmol/h)
6.95	22.6 (2.5)[a]	1.11 (0.11)[a]	0.15 (0.01)[a]
8	23.6 (0.4)[b]	1.13 (0.05)[b]	0.08 (0.01)[b]

a) n=3, for OUR and CPR this represents the average over 3 days of operation
b) n=4

CPR increased from 0.08 to 0.15 mmol/h when the pH was decreased. This was expected as lower pH drives the production of gaseous carbon dioxide from bicarbonate, as previously

discussed in Section 3.3.4.1, and further demonstrates the strong effect of inorganic carbon on CPR in the reactors.

3.4 Conclusions

The efficacy of the ISBR for treatment of OSPW NAs has been demonstrated. Removal levels comparable to those in the literature were achieved in significantly less time. However, challenges were identified for treatment of OSPW in a continuous reactor with a mixed microbial population. Namely, OUR and CPR are not good indicators of NA degradation, although OUR is a good indicator of microbial activity. Consequently, NA removal can only be monitored by extracting and analyzing a reactor sample.

Chapter 4

Role of ammonium and ammonium oxidizing bacteria in NA removal

4.1 Introduction

The ISBRs` biofilms are comprised of a mixed microbial population, thus aerobic, heterotrophic degradation is likely not the only process occurring. DGGE analysis of the biofilms previously showed the presence of *Nitrosomonas* (Section 3.3.3), with examination of OUR and ammonium consumption confirming that *Nitrosomonas* is a dominant group (Section 3.3.4.2).

Ammonium oxidizing bacteria (AOB) obtain energy from the oxidation of ammonium to nitrate, and can obtain carbon through carbon dioxide fixation. However, AOB have also been found to enhance the mineralization rate of 17α-ethinylestradiol (Khunjar et al., 2011) and transform other natural and synthetic estrogens (Shi et al., 2004) having similar structures to NAs. The nitrifier, *N. europaea,* has been found to oxidize a number of hydrocarbons including alkanes, alkenes, and aromatic aliphatic compounds (Hyman et al., 1998; Keener and Arp, 1994). Thus, AOB may enhance NA degradation in the ISBRs. However, it has been suggested that nitrification of ammonium inhibits NA degradation in wetlands by competing for phosphate and/or oxygen (Quagraine et al., 2005A).

If AOB are not enhancing degradation, then their population in the reactor should be limited to increase the amount of oxygen available for NA degradation, decrease their consumption of nutrients (eg N and P sources), and to obtain OUR data that may be directly related to NA degradation. Thus, the objective of the work in this chapter was to determine the role of AOB and ammonium in NA degradation within the ISBRs. This was investigated by

comparing NA degradation under the following conditions 1) low and high ammonium concentration 2) with ammonium oxidation inhibition and 3) with nitrate as the sole N-source.

4.2 Materials and Methods

4.2.1 The effect of ammonium concentration

To examine the effect of ammonium on NA degradation, the strength of the MSM fed to R1 was gradually reduced by a factor of 3, from medium A to medium B in Table 4-1. This reduced the ammonium concentration entering R1 from 0.9 to 0.3 g/L NH_4^+.

Table 4-1: Components of the mineral salts mediums in distilled water fed to R1 for the effect of ammonium concentration experiment

Nutrient	Concentration (g/L)	
	Medium A	Medium B
$NaNO_3$	-	-
$(NH_4)_2SO_4$	16.52	5.51
Na_2HPO_4	20.00	6.67
KH_2PO_4	2.00	0.67
$MgSO_4 \cdot 7H_2O$	0.0265	0.0088
$CaCl_2 \cdot 2H_2O$	0.0200	0.0067

The two ISBRs were operated in series. Consequently, resulting nutrient concentrations in R2 were lower than those in R1. A nitrate feed of 1.03 g NO_3^-/L to R2 provided N in an amount equivalent to 0.3 g/L of ammonium, to ensure that the microorganisms had sufficient nitrogen. The nutrient feeding program is shown in Figure 4-1.

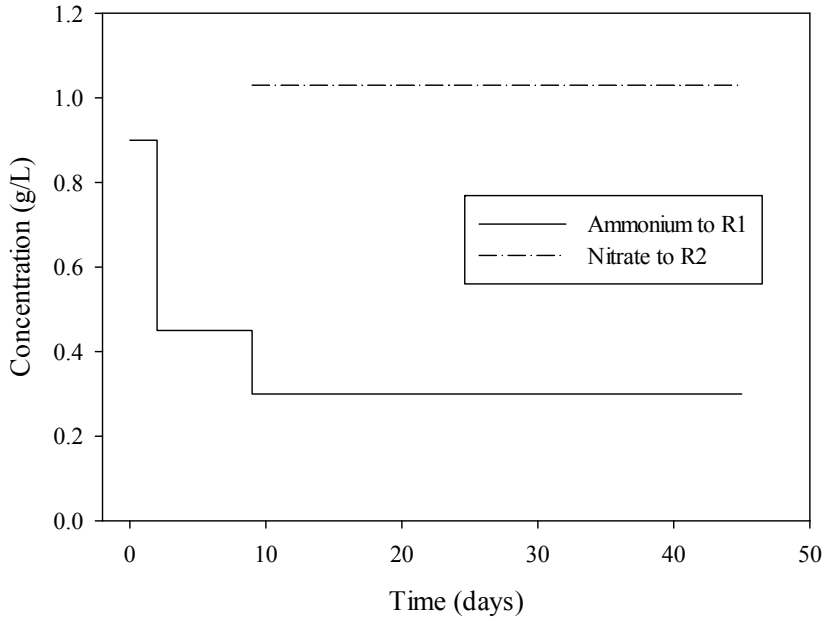

Figure 4-1: Feeding program for the effect of ammonium concentration experiment

4.2.2 The effect of nitrate as the N-source

To investigate the effect of ammonium and ammonium oxidizers on NA removal, ammonium was also replaced by nitrate as the N-source in R2. R1 and R2 were decoupled, with R2 receiving fresh process water and an independent nutrient feed, rather than R1 effluent. The MSM fed to R2 is shown in Table 4-2. The $(NH_4)_2SO_4$ in Medium B was replaced with $NaNO_3$, such that the molar amount of N fed to the reactor in Medium C was equal to molar amount in Medium B.

Table 4-2: Components of the MSM in distilled water fed to R2

Nutrient	Concentration (g/L) Medium C
$NaNO_3$	7.09
Na_2HPO_4	6.67
KH_2PO_4	0.67
$MgSO_4 \cdot 7H_2O$	0.0088
$CaCl_2 \cdot 2H_2O$	0.0067

4.2.3 Ammonium oxidation inhibition study

The role of ammonium oxidizers in NA degradation was investigated by inhibiting ammonium oxidation with allylthiourea (ATU) in a decoupled R1. Ten milliliters of ATU (4.8 g/L) was added to R1 to achieve a reactor concentration of 10 mg/L, which was maintained by adding ATU to the R1 nutrient feed at a concentration of 50 mg/L (nutrients were fed at a 1:4 ratio with OSPW, resulting in a feed concentration of 10 mg ATU/L). In the literature, 10 mg/L ATU was sufficient to completely inhibit ammonium oxidation with nitrifying activated sludge in shake flask (Shi et al., 2004) and chemostat (Khunjar et al., 2011) culture. MSM was medium A in Table 4-1.

4.2.4 Degradation of surrogate naphthenic acids

As small changes in NA concentration can often be difficult to detect with certainty, a NA surrogate, cyclohexane carboxylic acid (CHCA), was used to compare degradation under the following conditions:

a) High ammonium concentration

b) High ammonium concentration with ATU inhibition of AOB

c) Nitrate as the sole N-source

Ammonium CHCA experiments were carried out in R1. In the case of ATU addition, ATU was added 3 days prior to CHCA addition. The nitrate CHCA experiment was performed in R2 after ~5 months of operation with nitrate as the sole N-source. For all three conditions, CHCA was added as a slug injection of 100 mL of 24 g/L CHCA solution, to achieve a final reactor concentration of 0.5 g/L. CHCA has been used in the literature as a NA surrogate (Herman et al, 1994) and as a single-ring carboxylic acid with no branched side chains, it represents an easily degradable NA. The concentration of 0.5 g/L CHCA was chosen based on results of CHCA degradation studies by Tobiah Newton and Sarah Partanen with OSPW isolates that show CHCA can be inhibitory at concentrations greater than or equal to 1.0 g/L.

4.2.5 Analyses

4.2.5.1 Ammonium analysis

Ammonium concentration of reactor samples was determined by colorimetric phenol-hypochlorite reaction as described in Section 3.2.3.1. Briefly, 40 µL of sample was reacted with 2.5 mL of reagent A containing 10 g/L phenol and 0.05 g/L sodium nitroprusside and 2.5 mL of reagent B containing 5 g/L sodium hydroxide and 0.42 g/L sodium hypochlorite. Tubes were vortexed and incubated at room temperature for 30 min. Absorbance was measured at 630 nm and compared to a calibration curve of ammonium standards.

4.2.5.2 NA analysis

NA concentration and composition in sample extracts were determined via GC-MS as described in Section 3.2.3. Briefly, 20 mL samples were centrifuged, spiked with an internal standard, extracted in DCM, and derivatized with MTBSTFA. Samples were analyzed with a

Varian 3800 gas chromatograph connected to a Varian 1200 single quadrupole MS detecting ions over a range of 60 to 550 m/z (Agilent Technologies, Mississagua, ON). The area under the naphthenic acids hump was then compared to a calibration curve generated from OSPW NAs to determine the NA removal. The NA composition was determined by averaging the peak intensities eluting in the NA hump and matching the ions to an isomeric group of the form $C_nH_{2n+Z}O_2$.

4.2.5.3 CHCA analysis

An internal standard (100 µL of 10 g/L of 2-methy-1-cyclohexane carboxylic acid in DCM) was added to 4 mL of centrifuged reactor supernatant in screw cap test tubes and the pH adjusted to < 2 with 7 M H_2SO_4. Samples were extracted twice, each time by adding 1 mL of DCM, vortexing for 30 seconds, then allowing phase separation for 30 min. The solvent layer was recovered with a glass pipette and placed in 2 mL GC vials.

Underivatized samples were analyzed by GC-MS, as described in Section 3.2.3.4 with slight modification to the method. The injector, source and transfer line temperatures were 190 ˚C, 250 ˚C, and 190 ˚C, respectively. The column oven temperature was initially held at 60 ˚C for 3 min, then increased to a final temperature of 180 ˚C at a rate of 8˚C/min and held for 4 min. A 5 min collection delay was used to prevent saturation of the detector while the solvent eluted. Ions were detected over a range of 50 to 300 m/z, with a dwell time of 0.250 s. CHCA concentration was determined by comparing the ratio of the area under the CHCA peak to the area under the IS standard to a calibration curve generated from extraction of 4 mL standard solutions ranging from 0 to 1.0 g CHCA/L, Figure S4-1 in the appendix.

4.3 Results and Discussion

4.3.1 Effect of ammonium concentration on NA removal

The role of ammonium and AOB was investigated by reducing the ammonium feed concentration to R1 from 0.9 to 0.3 g NH_4^+/L. Since the effluent of R1 flows to R2, the ammonium concentration entering R2 was reduced from 0.51 to 0.006 g NH_4^+/L after a two week period (Figure 4-2A). R2 nutrients were supplemented with $NaNO_3$, to ensure that sufficient nitrogen was available for cell growth and maintenance. After ~2 weeks, the effluent concentration of NH_4^+ in R2 was 0, confirming that the ammonium concentration fed was limiting. Phosphate concentration was also reduced during the experiment, however, concentrations were monitored throughout the study and were not found to be limiting. The changes observed with decreasing inlet ammonium concentration are reported in Table 4-3.

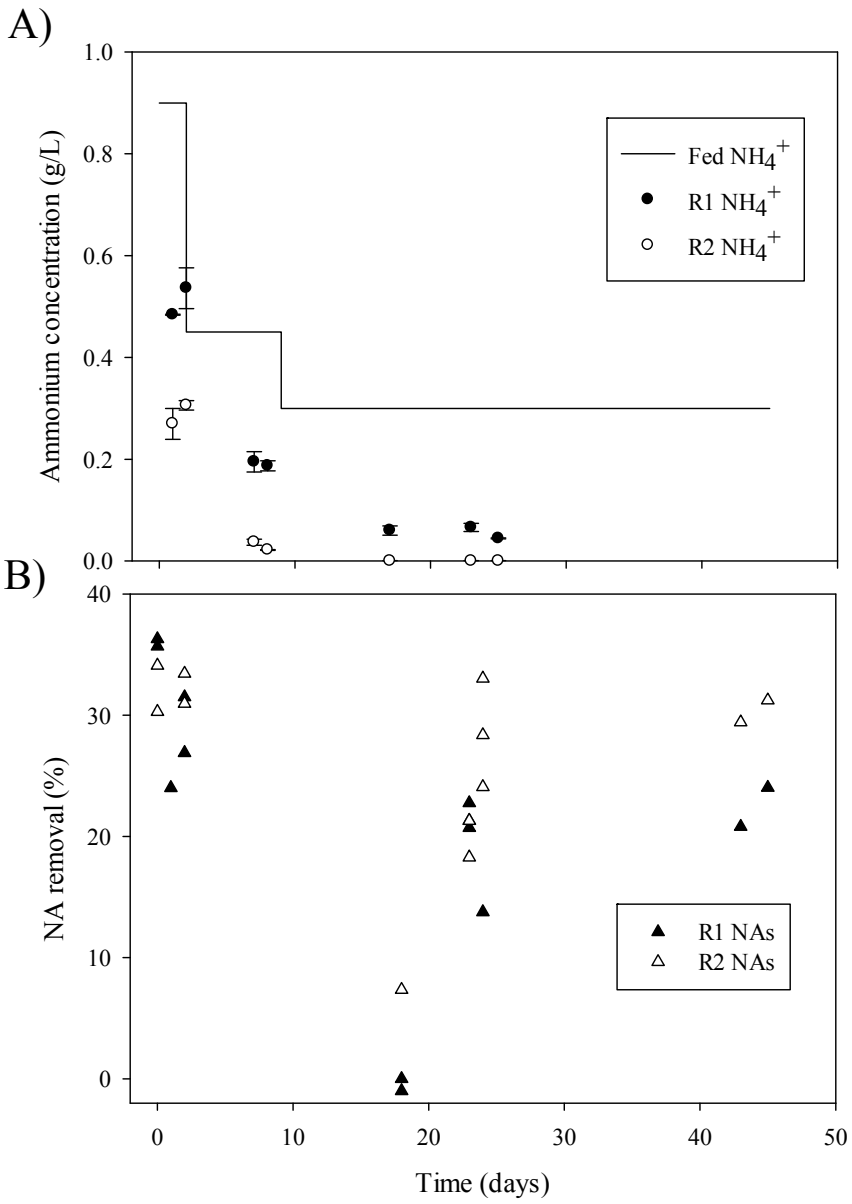

Figure 4-2: Effect of reduction in inlet ammonium concentration in R1 and R2 on A) ammonium concentration and B) NA removal; R2 removal represents total removal compared to OSPW

Table 4-3: Summary of ISBR performance at high and low ammonium concentration

	Inlet N concentration (mmol/h)		OUR (mmol/h)		NH_4^+ consumption (mmol/h)		NA removal (%)	
	NH_4^+	NO_3^-						
Reactor 1								
0.9 g/L NH_4^+[a]	3	0	1.62 (0.09)	*n=3*	1.3 (0.12)	*n=2*	31 (5)	n=5
0.3 g/L NH_4^+								
+3-5 weeks[b]	1	0	1.11 (0.01)	*n=3*	0.81 (0.04)	*n=3*	22 (2)	n=2
+16 weeks[c]	1	0	1.55 (0.06)	*n=3*	0.997 (0.001)	*n=3*	31 (9)	n=3
Reactor 2								
0.9 g/L NH_4^+	1.70 (0.13) n=2	0	1.09 (0.04)	*n=3*	0.74 (0.21)	*n=2*	1 (8)[d]	n=4
0.3 g/L NH_4^+								
+3-5 weeks	0.19 (0.04) n=3	1	0.6 (0.02)	*n=3*	0.19 (0.04)	*n=3*	8 (4)[d]	n=2
+16 weeks	0.003 (0.001) n=3	1	0.24 (0.04)	*n=3*	0.003 (0.001)	*n=3*	8 (12)[d]	n=3
Total ISBR performance								
0.9 g/L NH_4^+	3	0	2.71 (0.13)[e]		2.04 (0.33)[e]		32 (3)[f]	n=4
0.3 g/L NH_4^+								
+ 3-5 weeks	1	1	1.71 (0.03)[e]		1 (0.08)[e]		30 (2)[f]	n=2
+16 weeks	1	1	1.79 (0.10)[e]		1 (0.002)[e]		39 (3)[f]	n=3

a) Days 0 to 2 b) Days 37 to 42 c) Days 114 to 116

d) Calculated as R2 removal (average) - R1 removal (average), stdev is the sum of R1 and R2 stdevs

e) Sum of R1 and R2 values at this time point, standard deviations were summed

f) Based on NA removal in R2

4.3.1.1 Effect on NA degradation

Initially, reducing the ammonium feed concentration had a detrimental effect on NA degradation in R1 (Figure 4-2B). NA removal in R1 at high ammonium concentration was 31 ± 5 %. However, all NA removal initially stopped when the inlet ammonium was decreased to 0.3 g/L. After 5 weeks, 22 ± 2% removal was regained and after 16 weeks, the original NA removal level of 31 ± 9% was re-established.

In R2, NA concentrations were initially not significantly different from the R1 effluent, but following ammonium limitation, the removal level increased to 8 ± 4%. This increase was initially attributed to the higher NA concentration in the feed to R2, resulting from decreased NA removal in R1. However, R2 NA removal of 8 ± 12% was sustained even after the initial R1 removal level of 30% was regained. It appears that NA degradation decreased in R1 due to competition for ammonium between AOB and heterotrophs, but in R2 this effect was not observed due to the nitrate feed providing an alternate N-source.

Overall, decreasing the ammonium feed had a positive effect on NA degradation, with NA removal in the ISBR system increasing from 32 to 39%, albeit after 16 weeks of operation.

4.3.1.2 Effect on OUR and ACR

In both reactors, OUR decreased as the ammonium feed concentration decreased (Figure 4-3). Initially, the OUR in both R1 and R2 decreased by approximately 0.50 mmol O_2/h, with corresponding reductions in ammonium consumption rate (ACR) of 0.49 and 0.55 mmol NH_4^+/h, respectively. Stoichiometrically, oxidation of 1 mol of ammonium to nitrite requires 1.5 mol of O_2 (Equation 3-2), while 0.5 mol are required to oxidize 1 mol of nitrite to nitrate (Equation 3-3). Therefore, if ammonium oxidation is assumed to require between 1.5 and 2 mol of O_2, the 1:1 ratio of change in OUR to change in ACR indicates that a significant portion of the decrease in ACR is due to decreased ammonium use for cell-growth.

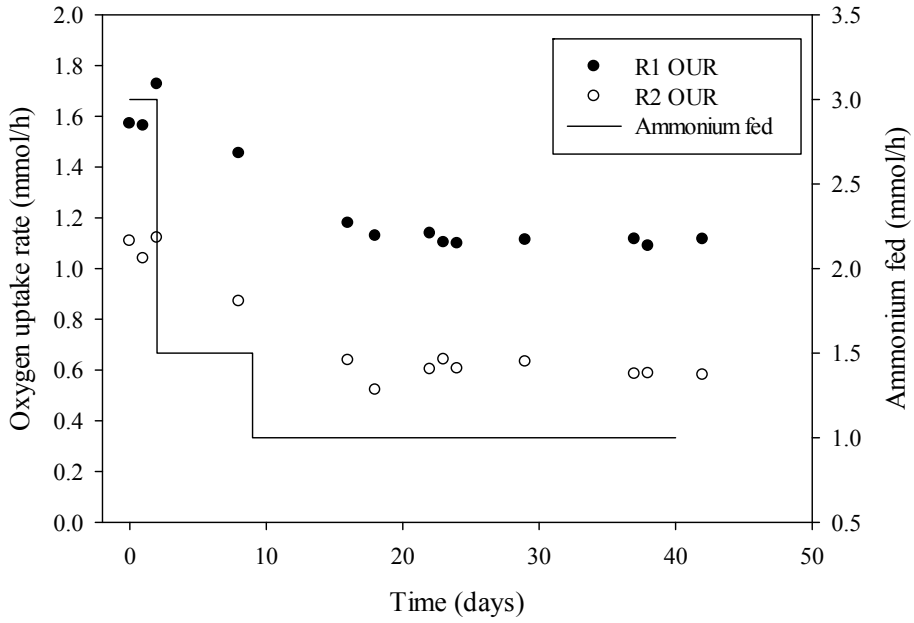

Figure 4-3: Change in OUR with ammonium feed concentration in the ISBRs

After 16 weeks at the reduced ammonium concentration, the OUR in R1 increased to 1.55 mmol/h, just slightly lower than the OUR observed at 0.9 g/L NH_4^+. The ratio of OUR/AUR at high ammonium concentration was 1.25, but after 16 weeks at the lower concentration, it increased to 1.55. The increase in this ratio seems to indicate that the proportion of ammonium being used for ammonium oxidation increased relative to ammonium use for nutrition in other bacterial species. The increase in ammonium use occurring in R1 between week 6 and 16 appears to be responsible for the decrease in ACR and OUR in R2. A decrease in ACR of 0.19 mmol/h was observed, corresponding to a 0.36 mmol/h decrease in OUR. Thus, it seems that only nitrification activity was affected, due to the auxiliary nitrate feed.

4.3.2 Effect of ammonium oxidation inhibition on NA degradation

To decouple the roles of AOB and ammonium in NA degradation, ammonium oxidation was inhibited in R1 with ATU in the presence of a high ammonium concentration. An additional aim of this experiment was to determine the proportion of the OUR that was related to ammonium oxidation rather than heterotrophic NA degradation.

ATU is thought to inhibit oxidation by chelating copper at the active site of the ammonia monooxygenase (AMO) enzyme catalyzing reaction 4-1 (Ginestet, et al. 1998). Results are summarized in Table 4-4.

$$NH_3 + O_2 + AH_2 \rightarrow NH_2OH + H_2O + A \qquad (4\text{-}1)$$

Table 4-4: Effect of inhibition of AOB with ATU on R1 performance

Condition	NA removal (%)		OUR (mmol/h)	NH_4^+ consumption (mmol/h)	
3 mmol/h NH_4^+ fed +ATU inhibition *(days 9 to 28)*	41.8 (9.9)	n=7	0.09 (0.01)	0.24 (0.14)	n=3
3 mmol/h NH_4^+ fed *(days 0, 1, 57-70)*	36.2 (4.2)	n=5	1.59 (0.04)	1.1 (0.10)	n=3

The change in OUR with ATU addition to the reactor is shown in Figure 4-4. The abiotic decrease in ATU concentration (S in mg/L) was estimated based on equation 4-2.

$$S = S_o e^{\frac{-F}{V}t} \qquad (4\text{-}2)$$

S_o is the ATU concentration prior to cessation of the ATU feed in mg/L, F is the reactor flow rate in L/day, V is the reactor volume in L, and t is the time since the ATU feed was stopped in days.

Upon addition of 10 mg/L of ATU, the OUR began to decrease within 10 min to a steady state value of 0.09 ± 0.01 mmol/h, a substantial reduction compared to the OUR of 1.59 ± .04 mmol/h without inhibition. Nitrite concentration was not monitored; therefore 100% inhibition of ammonium oxidation cannot be stated with certainty. However, OUR in R1 did not begin to increase until the ATU concentration was below 2 mg/L, indicating that 10 mg/L ATU was inhibitory.

The >95% decrease in OUR with ATU addition confirms the hypothesis discussed in Section 3.3.4.2 that ammonium oxidation is responsible for the bulk of oxygen consumption in R1. Consequently, at high ammonium feed concentrations, OUR is not a good indicator of NA degradation. The 41.8 ± 9.9 % NA removal observed with AOB inhibition required an estimated OUR of 0.076 mmol/h, assuming NAs are completely mineralized and 1 mol of C requires 1 mol of O_2 for metabolism (calculation can be found in Appendix B). With a corresponding OUR of 0.09 mmol/h at this time point, OUR becomes a good measure of NA degradation when ammonium oxidation is inhibited.

Inhibition of ammonium oxidation did not have a detrimental effect on NA degradation (Figure 4-5). With ATU addition, NA removal in R1 was 41.8 ± 9.9%, compared to 36.2 ± 4.2% without AOB inhibition. Comparison of the average n number for each Z-series and Z-series distribution of ISBR treated samples with and without AOB inhibition found no significant differences in average values based on student t-tests at the 95% confidence level. Therefore, it can be concluded that the enzyme catalyzing ammonium oxidation, ammonium monooxygenase (AMO), does not play a significant role in NA degradation in R1. Our results are consistent with those of Misiti et al. (2013), who found that commercial NAs were not degraded under strictly

nitrifying conditions, however, the nitrifying culture used in their study had no previous exposure to NAs.

AMO has been found to be directly involved in the biotransformation of estrogens. ATU addition resulted in inhibition of 17β-estradiol (Shi et al., 2004) and 17α-ethinylestradiol (Yi and Harper, 2007; Khunjar et al., 2011) degradation by AOB. Oxidation of hydrocarbons by *N. europaea* is catalyzed by AMO (Hyman et al., 1998; Keener and Arp, 1994). However, nitrifying activated sludge was able to degrade estrone, 17β-estradiol, estriol, and 17α-ethynylestradiol even in the presence of ATU (Shi et al., 2004). Therefore, during inhibition with ATU, there may have been nitrifying organisms present capable of NA degradation via a different enzymatic pathway.

Interestingly, as ATU reactor concentration decreased and the OUR began to increase, NA removal appeared to briefly stop, before returning to typical levels. This trend is similar to the temporary decrease in NA removal observed in R1 when the ammonium feed concentration was decreased.

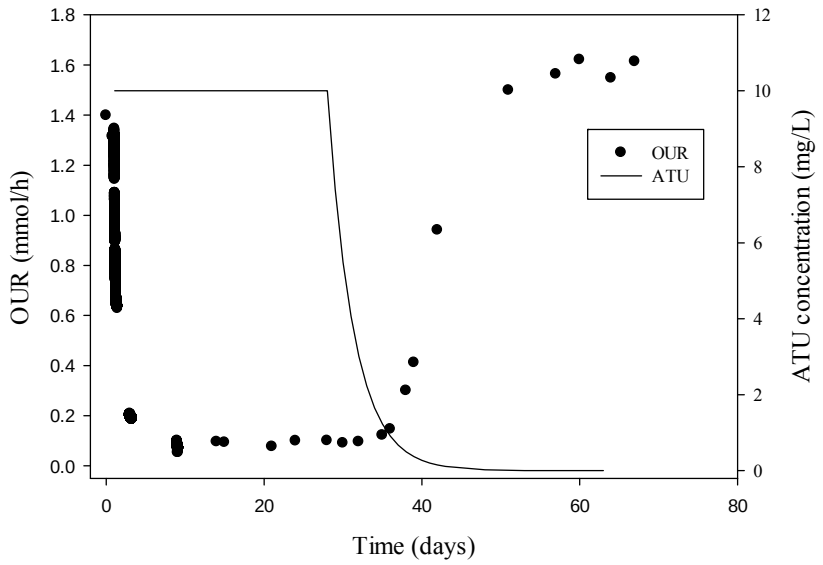

Figure 4-4: Effect of ammonium oxidation inhibition by ATU on OUR in R1

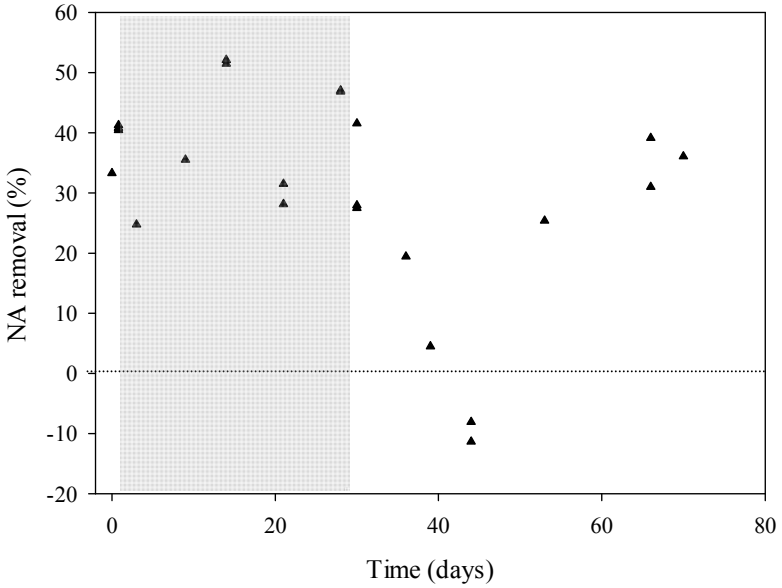

Figure 4-5: Effect of ammonium oxidation inhibition by ATU on NA removal[a]. The shaded area represents the time period during which ATU was present in R1 at 10 mg/L

a) *Negative removals are a result of variation in GC-MS quantification and/or upsets in the nutrient to OSPW feed ratio*

4.3.3 Effect of nitrate as sole N-source on NA degradation

To determine if ammonium and AOB were necessary for NA degradation, nitrate was provided as the sole N-source to R2. The R1 effluent feed to R2 was replaced with fresh OSPW and Medium C, containing no ammonium. Following replacement of the R1 effluent feed with fresh OSPW, the OUR in R2 decreased from 0.24 ± 0.04 (days 0 to 4, n=4) to 0.055 ± 0.003 mmol/h (days 7 to 22, n=4) (Figure 4-6). Prior to decoupling R2 from R1, only 0.006 mmol/h of NH_4^+ were being consumed, thus the remaining difference in OUR is likely due to nitrite oxidation. The OUR appeared to increase slightly with time –after 8 months the OUR averaged 0.068 ± 0.001 mmol/h (days 230 to 261, n=6).

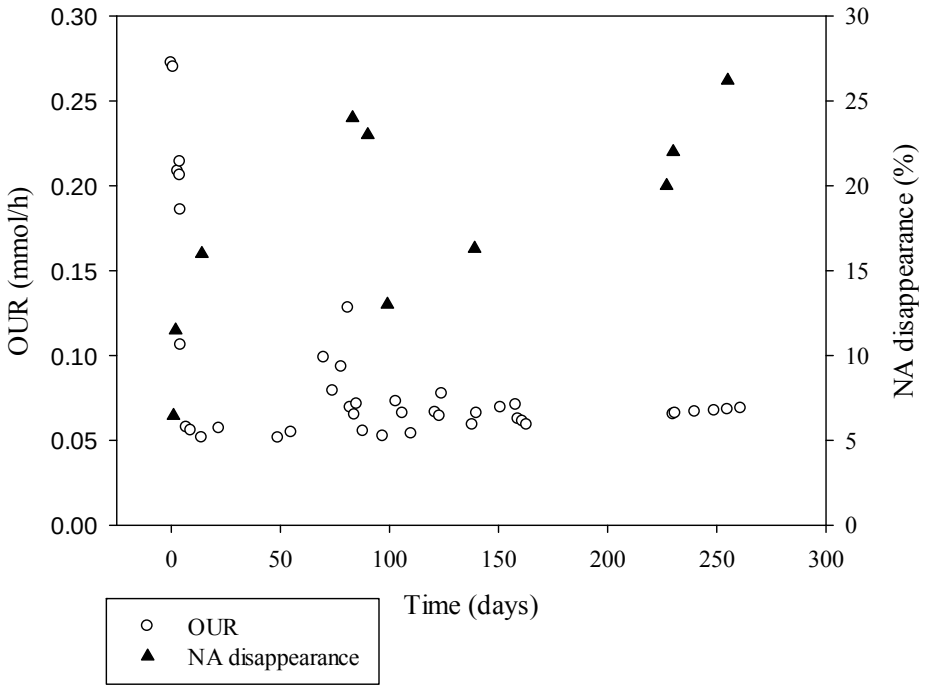

Figure 4-6: OUR and NA removal in R2 with nitrate as the sole N-source. Beginning at day 4, R1 effluent was replaced with fresh OSPW and ammonium-free MSM as the R2 influent

Without ammonium, the pH in R2 no longer decreased and base addition was no longer required, confirming the hypothesis discussed in Section 3.3.4.2 that ammonium oxidation was responsible for alkalinity consumption in the ISBRs. Acid control was added, using 0.1 N HCl to maintain a pH between 7.9 and 8.0.

Prior to reactor decoupling, NA removal was 8 ± 5% (days 0 to 2, n=2). NA removal increased to 23± 3% (days 230 to 261, n=3) after 8 months of operation with nitrate as the sole N-source. Part of this increase can be attributed to a fresh OSPW feed being supplied to R2, rather than the R1 effluent, containing the more recalcitrant NAs. The increase in OUR supports the increase in NA removal observed and indicates that there may be potential for increased NA removal as the R2 biofilm develops.

These results indicate that NA degradation can occur without ammonium or ammonium oxidizing bacteria. However, the removal level achieved is less than the 41.8 ± 9.9 % achieved in R1 in the presence of ammonium.

Nitrate assimilation reduces nitrate to ammonium in the cytoplasm of the cell, according to Equation 4-3 (Moreno-Vivian et al., 1999).

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+ \qquad (4\text{-}3)$$

This conversion may slow remediation. Jackson and Pardue (1999) found that almost 5x the required nitrate compared to ammonium (mg-N/L) was needed to achieve the same increase in the biodegradation rate of crude oil. Nitrate measurement during experimentation confirmed that the concentration provided was not limiting. However, the NA degradation in R2 did increase with time, therefore additional work is required to determine if the discrepancy in NA removal could be resolved by allowing additional time for the R2 biofilm to develop.

4.3.4 Effect of ammonium and AOB on degradation of a NA surrogate

As changes in NA degradation can be subtle and difficult to detect with certainty due to the large standard deviations associated with quantification, CHCA, a simple NA surrogate, was used to assess the role of ammonium and AOB in NA degradation. The three conditions investigated were high ammonium, active AOB; high ammonium, ATU-inhibited AOB; and no fed ammonium. These conditions are summarized in Table 4-5.

Table 4-5: Summary of reactor conditions used in CHCA degradation experiments

Experiment		Details
Ammonium	*AOB*	
+	+	R1; 0.9 g/L NH_4^+ fed as N-source
+	-	R1; 0.9 g/L NH_4^+ fed as N-source; ATU inhibition of ammonium oxidation
-	-	R2; 1.03 g/L NO_3^- fed as N-source

The change in CHCA concentrations with time is shown in Figure 4-7A, with the corresponding change in OUR in Figure 4-7B. CHCA was rapidly degraded in the absence of AOB. At high ammonium concentration and ATU inhibition of AOB, the CHCA concentration reached zero within 56.5 hours. With nitrate as the sole source of nitrogen, CHCA was removed in 53.25 hours. In both cases there was a lag time of ~36 hours for the CHCA degrading population to increase and/or adapt before the OUR began to increase. Degradation in both these cases was accompanied by a large spike in OUR. However, with an active AOB population at high ammonium concentration, complete CHCA removal took 162 hours, despite a high OUR throughout the experiment. The concentration profile is quite similar to the abiotic loss of CHCA due to dilution, calculated with Equation 4-1. However, there is a slight spike in OUR at ~150 hours, corresponding to complete removal of CHCA, thus it is likely that some degradation eventually occurred.

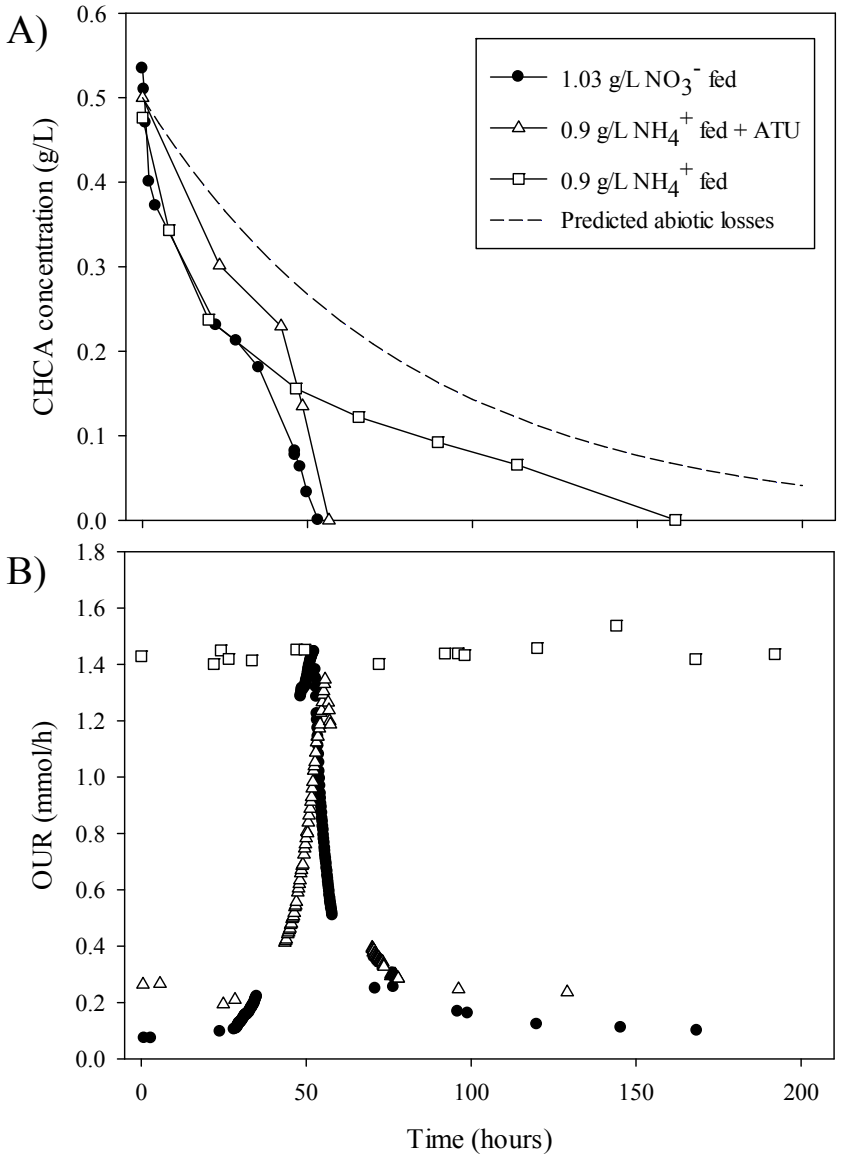

Figure 4-7: A) CHCA removal and B) OUR following injection of 0.5 g/L CHCA at t=0

The results of these experiments are consistent with results obtained with OSPW NAs. Namely, that ammonium and AOB activity are not required for NA degradation. The time required for CHCA degradation was the same with and without ammonium, which seems to support the hypothesis that reduced NA removal with nitrate as the sole N-source was due to

insufficient time for biofilm development. However, CHCA represents a very simple and easy to degrade NA, and degraders of more complex NAs may require ammonium. AOB were unable to degrade CHCA and appear to have inhibited the growth of CHCA degraders. The effect of CHCA addition on NA removal is discussed in Chapter 5.

4.4 Conclusions

Ammonium and ammonium oxidizers were not required for NA degradation, with removal occurring in the absence of ammonium, at low and high concentrations of ammonium, and with and without an active AOB population.

While NA removal and ammonium oxidation occur concurrently, there were a number of instances where AOB activity appeared to inhibit NA degradation.

- Competition for ammonium between AOB and NA-degrading heterotrophs in R1, due to the reduced feed concentration, initially resulted in a decrease in NA removal.
- Return of AOB activity in R1 after ATU inhibition suppressed degradation.
- CHCA degradation took more than 3 times as long with an active AOB population, compared to one that was inhibited with ATU.
- In R2, NA degradation only became significant when the ammonium concentration was drastically decreased with nitrate as an additional source of N.

Therefore, it appears that AOB will not inhibit NA degradation in established populations of NA degraders under regular operation; but a large population of AOB can prevent growth in the size of the NA-degrading population and consequently prevent higher levels of NA removal. However, although NA degradation could occur in the absence of ammonium, the removal levels

achieved are lower with nitrate as the sole N-source. Thus, there is a need to balance the ammonium concentration provided, insuring sufficient N for cell growth of NA-degrading heterotrophs, but preventing the growth of large populations of AOB, Figure 4-8. A moderate NH_4^+ feed concentration of 0.3 g/L is recommended for optimal NA removal.

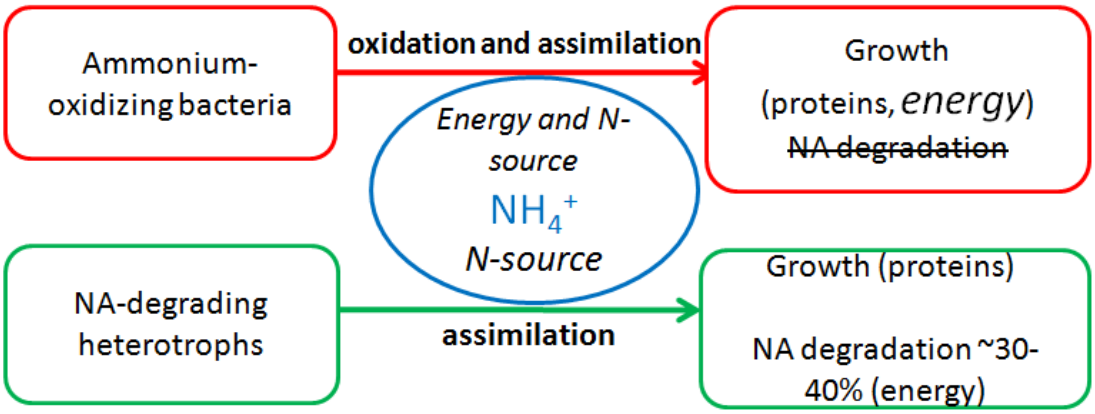

Figure 4-8: Competition for ammonium between AOB and NA-degrading heterotrophs within the ISBR

Chapter 5

Exploratory techniques for improving NA removal in the ISBRs

5.1 Introduction

Biological treatment has been shown to reduce the toxicity and NAs concentration in OSPW (Scott et al., 2005; Han et al., 2009; Martin et al., 2010), however, treatment times are long and removal is incomplete. ISBR treatment has increased the rate of NA removal from a half life of ~83 days in shake flask (Martin et al., 2010) to 30-40% removal in 1 week, but has not been able to completely remove NAs. Therefore, it is likely that biologically treated OSPW would still have some toxicity as OSPW aged in reclamation ponds for >15 years was found to display a number of chronic effects (Kavanagh et al., 2011; Anderson et al., 2012). Consequently, a pre- or post-treatment is needed to enhance the NA removal via ISBR.

Ozonation was shown to be very effective for NA removal (Scott et al., 2008B; El-Din, 2011; He et al., 2010; He et al., 2011). However, it is expensive and may not remove all toxic effects (He et al., 2011; Martin et al., 2010). Martin et al. (2010) investigated ozonation as a pre-treatment to biological treatment. Mild ozonation was found to decrease the half-life of the residual NAs and shorten the biological treatment time required to reduce toxicity. Another approach to enhance NA removal is to add an exogenous carbon source, as NA concentrations are very low. This technique has been found to increase the rate and level of petroleum hydrocarbon removal in C-poor soils (Sarkar et al., 2004).

This chapter evaluates these two techniques for enhancing NA removal in the ISBRs. Ozonation was investigated as a pre-treatment step to biological treatment. Changes in NA concentration, NA profile and TOC were monitored. Additionally, carbon supplementation with a

simple surrogate NA, cyclohexane carboxylic acid (CHCA), was investigated as a technique to increase the population of NA-degraders and consequently NA removal.

5.2 Materials and methods

5.2.1 Ozonation of OSPW

Uncentrifuged OSPW at a pH of 8.1 was ozonated with an Ozotec Type S ozonator (Hankin Ozone, Mississagua, ON) in 1.25 L batches in a 2 L Erlenmeyer flask. OSPW was mixed with a magnetic stir bar while sparging with air containing 2% ozone at a flow rate of 2 L/min regulated by a Matheson flow meter (Model 8170, Newark, CA) for 30 minutes. Batches were combined in a 20 L carboy (totaling 12 L of ozonated water) and sparged with N_2 for 10 min under mixing to remove any residual ozone. Treated OSPW was stored for one week at 4 ˚C prior to experimentation.

The treatment time of 30 minutes was chosen to partially degrade the NAs to a concentration of ~20 mg/L, based on a curve of NA concentration with ozonation time constructed prior to experimentation (Figure C1, Appendix C). However, the final NA concentration in the ozone treated OSPW was 3 ± 1 mg/L, possibly due to residual ozone reactions occurring in the carboy prior to sparging with N_2.

5.2.2 Biological treatment of ozone-treated OSPW

Ozonated water was biologically treated in R2 in two batches. Batch 1 was treated for 4 days, Batch 2 for 6. Prior to experimentation, R2 had been operating in continuous mode, treating unozonated process water supplemented with MSM (7.09 g/L $NaNO_3$; 6.67 g/L Na_2HPO_4; 0.67 g/L KH_2PO_4; 8.8 mg/L $MgSO_4 \cdot 7H_2O$; 6.7 mg/L $CaCl_2 \cdot 2H_2O$) in a 4:1 ratio. To transition from continuous treatment of OSPW to batch-treatment of ozonated water, reactor contents were

drained and refilled with 4 L of ozone-treated OSPW mixed with 1 L of MSM. Following treatment of Batch 1, R2 was operated in continuous mode with unozonated OSPW for 4 days prior to treatment of Batch 2, to ensure that treatment conditions were consistent.

5.2.3 Biostimulation experiments

In order to increase the population of NA degraders in the reactors, an easily degradable NA, cyclohexane carboxylic acid (CHCA) was added. The reactors were operated independently, under two different conditions. In R2, the MSM provided nitrate as the N-source (7.09 g/L $NaNO_3$; 6.67 g/L Na_2HPO_4; 0.67 g/L KH_2PO_4; 8.8 mg/L $MgSO_4 \cdot 7H_2O$; 6.7 mg/L $CaCl_2 \cdot 2H_2O$). In R1, the MSM provided ammonium as the N-source (16.52 g/L $(NH_4)_2SO_4$, 20 g/L Na_2HPO_4, 2 g/L KH_2PO_4, 26.5 mg/L $MgSO_4 \cdot 7H_2O$, 20 mg/L $CaCl_2 \cdot 2H_2O$) with ATU inhibition of AOB. Both reactors were operated continuously with OSPW and MSM fed in a 5:1 ratio. In R1, an aged OSPW was used, with a NA concentration of 37 ± 2 mg/L compared to 49 ± 2 mg/L in OSPW fed to R2 and used under normal operation. Figures C1 and C2 in Appendix C compare the TIC and 267 ion chromatogram of aged OSPW and OSPW used under normal operation

A volume of 100 mL of 24 g/L CHCA solution was injected into each reactor to achieve an initial concentration of 0.5 g CHCA/L. CHCA concentration, NA removal and OUR were monitored with time. However, NA concentration was only monitored at low CHCA concentrations to avoid saturation of the MS detector.

5.2.4 Analyses

5.2.4.1 Total organic carbon (TOC) analysis

TOC in centrifuged samples was measured with a Shimadzu (Columbia, MD) TOC 505 a non-purgeable organic carbon analyzer. Five mL samples were acidified to pH 2 with 1 drop of 1 M HCl and sparged with high purity N_2 for 1 min immediately prior to analysis to remove inorganic carbon. Results are the average of triplicate injections of 100 µL of sample.

5.2.4.2 NA analysis

NA concentration and composition in sample extracts from the ozonation and biostimulation experiments were determined via GC-MS as described in Section 3.2.3. Briefly, 20 mL samples were centrifuged, spiked with an internal standard, extracted in DCM, and derivatized with MTBSTFA. Samples were analyzed with a Varian 3800 gas chromatograph connected to a Varian 1200 single quadrupole MS detecting ions over a range of 60 to 550 m/z (Agilent Technologies, Mississagua, ON). The area under the NA hump was then compared to a calibration curve generated from Merichem NAs to determine the NA concentration. The detection limit for a 20 mL NA extract is 0.5 mg/L using this method. The NA composition was determined by averaging the peak intensities eluting in the NA hump and matching the ions to an isomeric group of the form $C_nH_{2n+Z}O_2$.

5.3 Results and discussion

5.3.1 Effect of ozonation and biological treatment on OSPW

Ozonation of OSPW reduced the NA concentration by 94%, from 49.0 ± 2.0 to 3.1 ± 0.7 mg/L. TOC was only slightly reduced from 54 ± 1 to 50.6 ± 0.8 mg/L (Table 5-1). These results are consistent with ozonation of NAs in the literature, which report drastic decreases in NA

concentration, but only modest decreases in TOC (Scott et al., 2008B; El-Din et al., 2011). El-Din et al. (2011) hypothesized that the NAs were degraded into smaller organic acids that were not detected by the GC-MS, but contributed to the TOC. These acids may have had a m/z ratio below the range of detection of the GC-MS (60-550 m/z), or alternatively, they may have eluted with the solvent front before the MS detector had been turned on.

Table 5-1: Change in TOC and NA concentration with ozone and biological treatment

OSPW Treatment	TOC (mg/L)			NAs (mg/L)		
Untreated	54.1[a]	±	1	49	±	2[b]
Biologically treated only	47.6	±	0.6	36.5	±	1.3[b]
O_3 treated only	50.6	±	0.8	3.1	±	0.7[b]
O_3 + biological treatment						
Batch 1, t=0 days	51.1	±	0.7	6.3		
t=4 days	36.9	±	1.3	7.3		
Batch 2, t=0 days	46.7	±	1.8	3.2		
t=6 days	32.5	±	0.8	3.5		

a) TOC results are the average of three analyses ± standard deviation
b) n=2

Ozonation also significantly changed the distribution of isomers in the remaining NAs, Figure 5-1. All isomer classes were reduced; however, cyclic NAs were particularly susceptible to ozonation. Three, 4-, 5-, and 6-ring compounds decreased in proportion, see Table C1 in Appendix C. These results are consistent with preferential removal of cyclic NAs in the literature (El-Din et al., 2011; Perez-Estrada et al., 2011). In the case of 4- to 6-ring compounds, the NAs with a lower n number showed the largest decrease in proportion. The n-number for these Z-classes significantly increased with ozonation. This seems to indicate that within these Z-groups lower molecular weight compounds were preferentially ozonated. However, Perez-Estrada et al. (2011) found that degradation increased with increasing n number for NAs of the form

$C_nH_{2n+Z}O_2$. Therefore, it is likely that the residual isomers of n>22 are double-derivatized oxy-NAs or heteroatomic NAs.

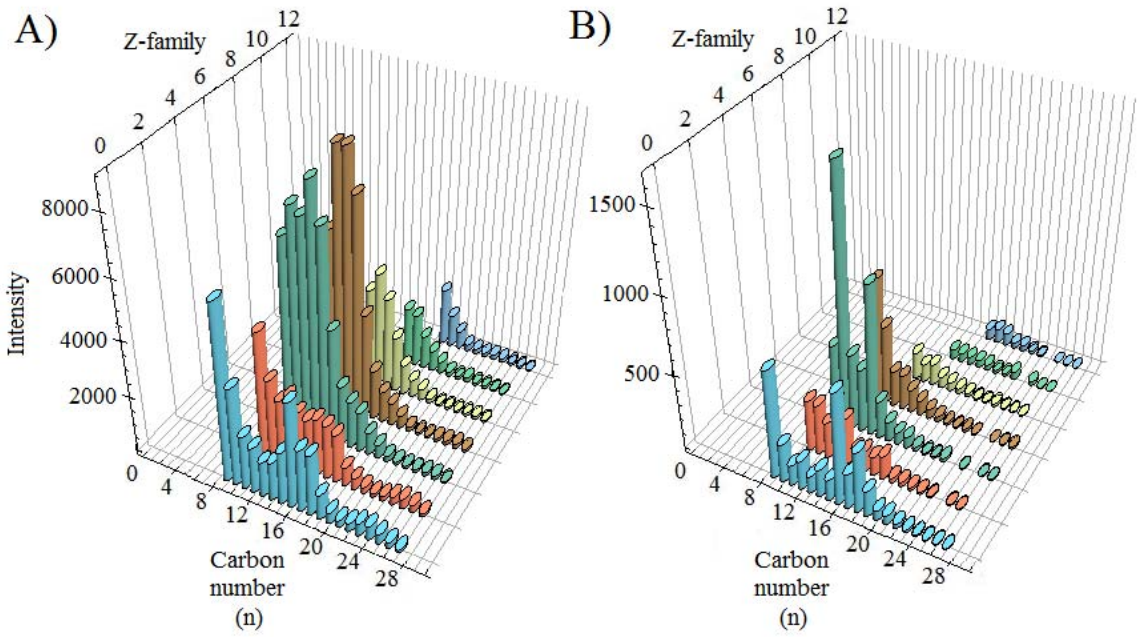

Figure 5-1: NA profiles of A) untreated OSPW and B) ozone-treated OSPW

Biological treatment of the ozonated OSPW reduced the TOC by 14.2 mg/L in both Batch 1 and Batch 2, ~30%. Similarly, Scott et al. (2008B) and El-Din et al. (2011) found that ozonation increased the biological oxygen demand (BOD) of OSPW by 13 mg/L and 17 mg/L, respectively. The majority of this removal occurred in the first day of treatment, Figure 5-2A. This large initial decrease is supported by a corresponding spike in the OUR, Figure 5-2B. Residual TOC concentrations after biological treatment for Batches 1 and 2 were 36.9 ± 1.3 and 32.5 ± 0.8 mg/L, respectively. Thus, there is a fraction of organic carbon remaining that is not detectable via GC-MS, nor easily degraded by biological treatment. However, the overall

mineralization of 30-40% of the TOC with combined ozonation and biological treatment is quite promising, as biological treatment is known to only mineralize ~20% of OSPW NAs (Herman et al., 1994).

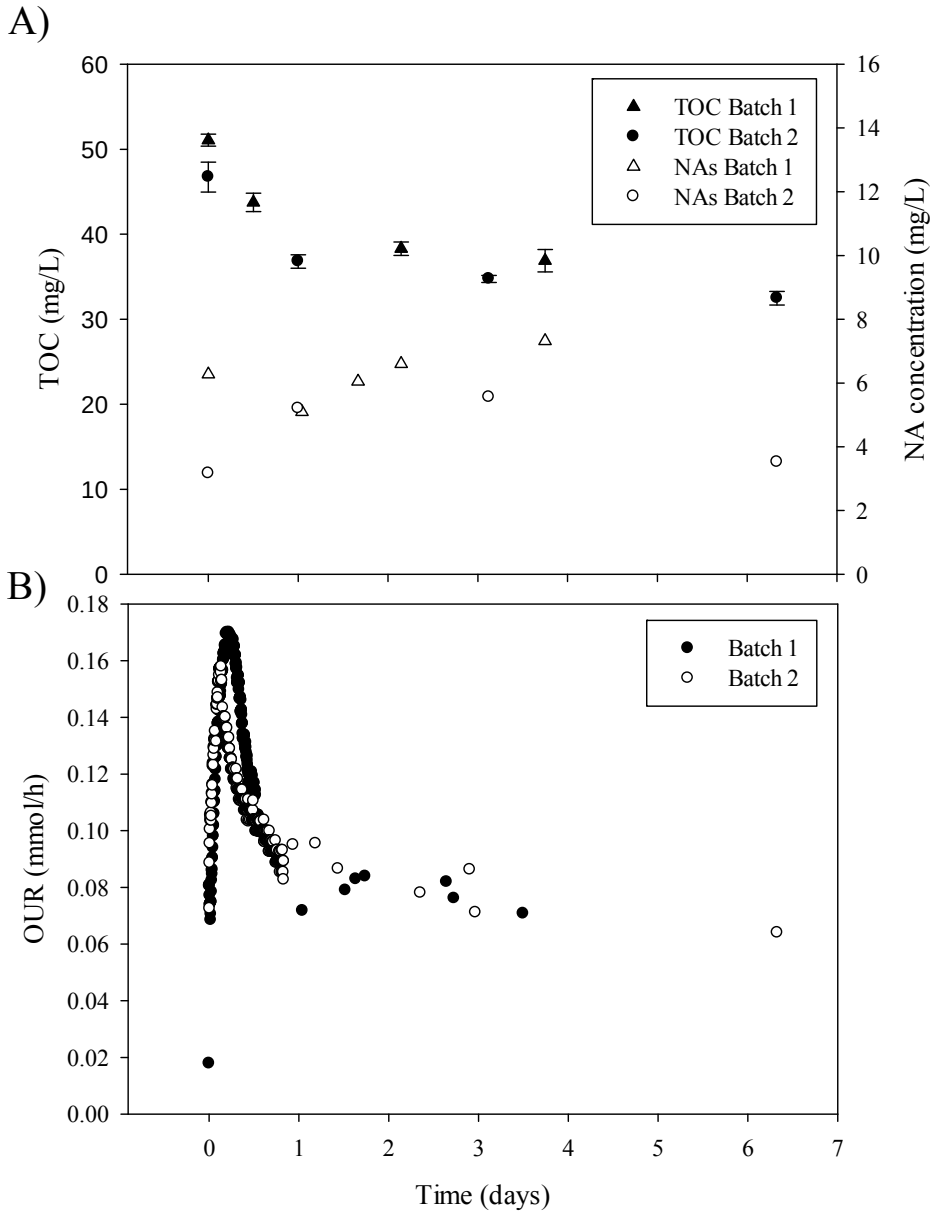

Figure 5-2: Batch biological treatment of ozonated OSPW A) TOC and NA removal B) OUR

NA concentration was unaffected by biological treatment in ozone-treated samples, increasing from 6.3 to 7.3 mg/L in Batch 1, and from 3.2 to 3.5 mg/L in Batch 2. The NA profile was also relatively unchanged with biological treatment, Figure 5-3 (with the exception of a decrease in the low carbon number Z=0 series) indicating that the residual NAs following ozonation are recalcitrant. However, Martin et al. (2010) observed that the half-lives of residual NAs were decreased with ozone pre-treatment. Perez-Estrada et al. (2011) hypothesized that post-ozonation residual NAs are the least refractory, based on their finding that the most recalcitrant NAs (those with high cyclicity and branching) are more susceptible to ozonation. Therefore, it was expected that any residual NAs would be easily degraded. However, Martin et al.'s (2010) study used only mildly ozonated water, resulting in 73% NA removal. Therefore, the residual NAs detected in this study are likely not classical NAs, but instead could be mis-classified heteroatomic NAs and/or heteroatomic fragment ions that retained a carboxylic acid moiety, as WIP OSPW extracts are known to contain 3.38% S and 0.51% N, by mass (Grewer et al., 2010). Analysis with a high-resolution MS is required to more accurately characterize the residual acid extractable organics remaining following ozonation.

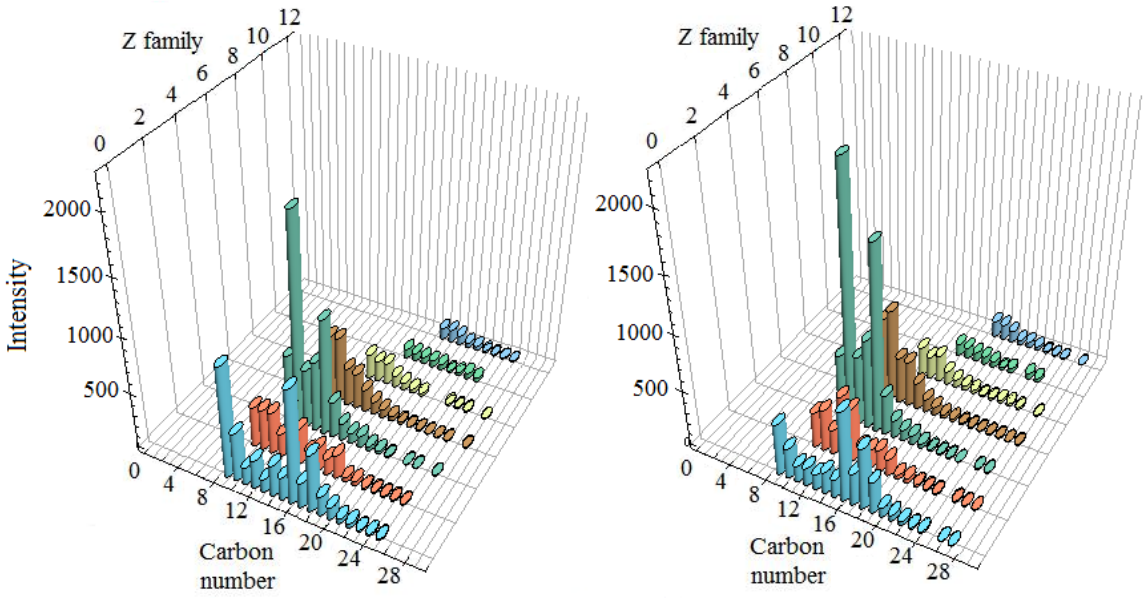

Figure 5-3: NA profile of Batch 2 ozonated OSPW A) prior to biological treatment and B) after 6 days of ISBR treatment

5.3.2 Effect of biostimulation on NA degradation

Addition of CHCA successfully boosted biological activity in the reactors. Its consumption significantly increased the microbial population in the reactor, as indicated by increases in oxygen consumption (Figures 5-4 and 5-5) and turbidity (Figure C3, Appendix C); however, the effect on NA removal was mixed. Results of biostimulation are summarized in Table 5-2.

Table 5-2: Summary of effects of biostimulation with CHCA on NA removal

Reactor	Condition	OUR (mmol/h)	NA removal (%)	NA concentration (mg/L)
1	0.9 g/L of ammonium in feed, with AOB inhibited with ATU *(Days -1 to 0)*	0.24 ± 0.03 →	7 ± 4 (n=3) →	34 ± 1 →
	Biostimulation with CHCA (Days 2 to 5)	>1.34*	27 ± 8 (n=5)	26 ± 3
2	Nitrate provided as sole source of nitrogen *(Days -1 to 0, 8)*	0.07 ± 0.01 →	24 ± 3 (n=3) →	37 ± 2 →
	Biostimulation with CHCA (Days 2 to 6)	>1.44*	26 ± 10 (n=5)	36 ± 5

** indicates highest value reached*

In R1, addition of CHCA had a clear, positive effect on OSPW treatment, with NA removal increasing from 7 ± 4 to 27 ± 8%. The OUR, CHCA concentration, and NA removal with time are shown in Figure 5-4. NA removal increased following consumption of CHCA, reaching a maximum of 38% removal approximately 2 days later, before decreasing to the original removal level. Therefore, it seems a biostimulation strategy would require a constant feed of a supplementary carbon source, or periodic pulse injections to sustain high removal rates.

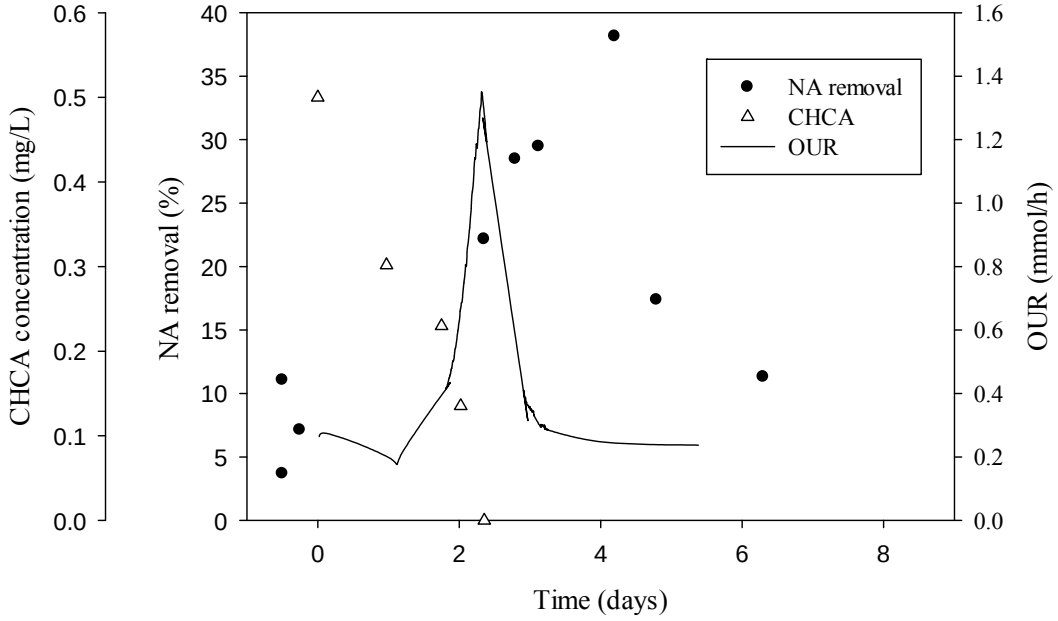

Figure 5-4: Biostimulation of NA-degradation with CHCA in R1 (ammonium N-source, with ATU inhibition of AOB)

In R2, the effect of CHCA addition on NA removal was mixed, Figure 5-5. Following CHCA addition, there was an initial boost in removal from 22.7 ± 1.3% to 29.7 ± 1.8%, before decreasing to 11%. The reason for this temporary suppression of NA removal was not clear. NA removal was observed with small concentrations of CHCA remaining, confirming that NAs can be removed concurrently. A slightly enhanced NA removal level of 33% was achieved three days following CHCA consumption. However, the increase in NA removal was not as significant as the increase in R1, and may be due to variation in reactor removal and/or GC-MS quantification. As discussed in Chapter 4, ammonium may be required to reach high levels of NA removal, which could account for the reduced enhancement observed with biostimulation with nitrate as the sole N-source.

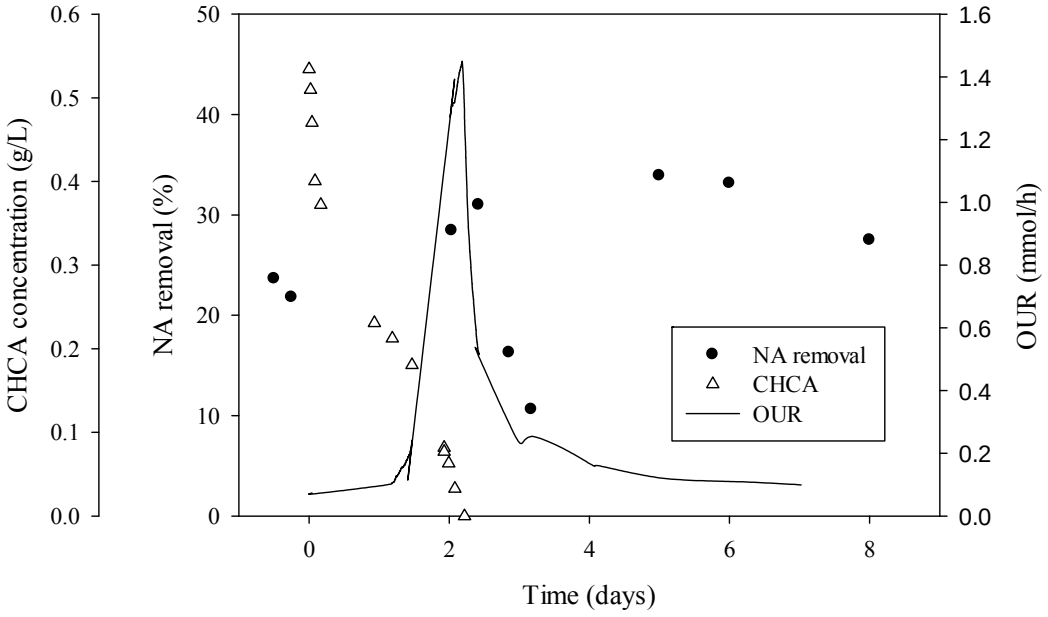

Figure 5-5: Biostimulation of NA-degradation with CHCA in R2 (nitrate N-source)

The increase in NA removal observed in R1 is promising; however, the use of a NA surrogate may result in a population that is adapted only to degradation of the NA surrogate. More information on the bacterial population and/or populations that grew on CHCA in R1 and R2 is needed. In the literature, supplementation of OSPW inoculations with Kodak NAs did not increase removal of OSPW NAs (Scott et al., 2005).

5.4 Conclusions

Ozonation combined with biological treatment is a promising method for NA removal. Ozonation drastically reduced the NA concentration, while subsequent biological treatment rapidly removed 40% of the TOC. However, the remaining TOC and acid extractable organics appeared to be recalcitrant and further work is required to determine the composition and toxicity of this fraction. Biostimulation increased the removal of NAs by 3-fold in the presence of

ammonium, but only modestly increased removal with nitrate as the sole N-source. Nonetheless, this technique shows promise and warrants further investigation.

Chapter 6

Conclusions

To date, OSPW NA biodegradation studies have largely been limited to shake flask studies and passive treatment in reclamation ponds and wetlands, resulting in long treatment times. This work has shown that biological treatment times can be significantly reduced with ISBR treatment. In the ISBR, 30-40% NA removal was consistently achieved in 7 days of treatment over ~2 years of continuous operation. However, like previous degradation studies, there was a persistent NA fraction that could not be degraded. Challenges associated with treatment of native process water in a continuous bioreactor were identified. Most significantly, inorganic carbon present in OSPW prevents meaningful measurement of CPR. Additionally, in the presence of AOB, OUR is not a good indicator of NA removal.

The effect of AOB and ammonium on NA removal was established. AOB do not appear to contribute to NA degradation –NA removal was unaffected by ATU inhibition of AOB and by a three-fold decrease in fed ammonium concentration. Furthermore, large populations of AOB can inhibit the growth of NA-degrading heterotrophs. This was most clearly demonstrated by the inhibition of CHCA degradation in the presence of AOB. NA removal did not require ammonium; however, treatment with nitrate as the sole N-source resulted in reduced NA removal. Consequently, ammonium cannot be completely eliminated. Ammonium concentrations of 0.3 g/L or lower are recommended to provide N for cell growth in heterotrophs, while limiting the size of AOB populations.

Techniques for improving NA removal with the ISBR were investigated. It was shown that ozonation combined with biological treatment is a powerful technique for NA and TOC

removal. Ozonation greatly reduced NA concentration, while subsequent ISBR treatment removed 24-28% of the residual TOC. However, a recalcitrant fraction remained that could not be well characterized. Biostimulation also shows promise for increasing NA removal, addition of an NA surrogate temporarily boosted NA removal by 3-fold.

Chapter 7

Recommendations for future work

Further work is required to determine the effect of reactor conditions investigated in this work such as high ammonium, low ammonium, and nitrate as the N-source and ATU inhibition on ISBR populations. Next generation sequencing of planktonic and biofilm samples collected at these time points will allow identification of the bacterial species present and determination of their relative amounts. For biostimulation experiments, this technique would allow identification of the CHCA degrader and/or degraders. Correlating changes in NA removal with changes in biofilm populations would be a powerful tool for enhancing NA degradation via reactor conditions and/or bioaugmentation.

The toxicity of ISBR-treated OSPW must be examined, as the ultimate goal of treatment is not complete NA removal, but rather detoxification. Comparison of toxicity at different reactor time points may reveal changes in ISBR performance that were not detectable via GC-MS. For instance, OSPW is known to exhibit estrogenic effects (He et al., 2010; He et al., 2011) and AOB have been found to enhance the removal of estrogens. Therefore, to determine if AOB enhance OSPW detoxification, comparison of the estrogenic effects exhibited by samples treated with and without AOB using a yeast estrogenic assay would be prudent. The effect of combined ozone and biological treatment on toxicity is also of interest. However, to fully characterize changes in toxicity with ISBR treatment, acute and chronic toxicity tests with representative species from a variety of trophic levels would be needed.

Use of high resolution MS is recommended for analysis of ISBR-treated and OSPW samples. There is a significant fraction of the acid extractable organics that seems to be quite

recalcitrant, even with biological and/or ozone treatment. Identification of these compounds could help to direct treatment strategies such as bioaugmentation. Advanced NA separation and identification techniques would be particularly powerful when used in conjunction with toxicity testing. If toxicity can be attributed to a certain group of compounds, these compounds could be targeted for treatment.

References

Alberta Energy (2013). “Oil sands: Facts and statistics.” http://www.energy.gov.ab.ca/OilSands/791.asp#Environment

Allen, E. W. (2008). "Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives." Journal of Environmental Engineering and Science **7**(2): 123-138.

Anderson, J., Wiseman, S. B., Moustafa, A., El-Din, M. G., Liber, K. and Giesy, J. P. (2012). "Effects of exposure to oil sands process-affected water from experimental reclamation ponds on *Chironomus dilutus*." Water Research **46**(6): 1662-1672.

Armstrong, S. A., Headley, J. V., Peru, K. M. and Germida, J.J. (2008). "Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes." Journal of Environmental Science and Health A: Toxic/Hazardous Substances and Environmental Engineering **43**(1): 36-42.

Armstrong, S. A., Headley, J. V., Peru, K. M. and Germida, J. J. (2009). "Differences in phytotoxicity and dissipation between ionized and nonionized oil sands naphthenic acids in Wetland Plants." Environmental Toxicology and Chemistry **28**(10): 2167-2174.

Armstrong, S. A., Headley, J. V., Peru, K. M., Mikula, R. J. and Germida, J. J. (2010). "Phytotoxicity and naphthenic acid dissipation from oil sands fine tailings treatments planted with the emergent macrophyte *Phragmites australis*." Journal of Environmental Science and Health A: Toxic/Hazardous Substances and Environmental Engineering **45**(8): 1008-1016.

Barrow, M. P., Witt, M., Headley, J. V. and Peru, K. M. (2010). "Athabasca oil sands process water: characterization by atmospheric pressure photoionization and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry." Analytical Chemistry **82**(9): 3727-3735.

Bataineh, M., A., Scott, A. C., Fedorak, P. M. and Martin, J. W. (2006). "Capillary HPLC/QTOF-MS for characterizing complex naphthenic acid mixtures and their microbial transformation." Analytical Chemistry **78**(24): 8354-8361.

Bendell-Young, L. I., Bennett, K. E., Crowe, A., Kennedy, J., Kermode, A. R., Moore, M. M., Plant, A. L. and Wood, A. (2000). "Ecological characteristics of wetlands receiving an industrial effluent." Ecological Applications **10**(1): 310-322.

Biryukova, O. V., Fedorak, P. M. and Quideau, S. A. (2007). "Biodegradation of naphthenic acids by rhizosphere microorganisms." Chemosphere **67**(10): 2058-2064.

Blakley, E. R. (1974). "The microbial degradation of cyclohexanecarboxylic acid: a pathway involving aromatization to form -hydroxybenzoic acid." Canadian Journal of Microbiology **20**(10): 1297-1306.

Chalaturnyk, R. J., Scott, D. J. and Ozum, B. (2002). "Management of oil sands tailings." Petroleum Science and Technology **20**(9-10): 1025-1046.

Clemente, J. S., Prasad, N. G. N., MacKinnon, M. D. and Fedorak, P. M. (2003). "A statistical comparison of naphthenic acids characterized by gas chromatography–mass spectrometry." Chemosphere **50**(10): 1265-1274.

Clemente, J. S. and Fedorak, P. M. (2004). "Evaluation of the analyses of tert-butyldimethylsilyl derivatives of naphthenic acids by gas chromatography–electron impact mass spectrometry." Journal of Chromatography A **1047**(1): 117-128.

Clemente, J. S., MacKinnon, M. D. and Fedorak, P. M. (2004). "Aerobic biodegradation of two commercial naphthenic acids preparations." Environmental Science and Technology **38**(4): 1009-1016.

Crowe, A. U., Plant, A. L. and Kermode, A. R. (2002). "Effects of an industrial effluent on plant colonization and on the germination and post-germinative growth of seeds of terrestrial and aquatic plant species." Environmental Pollution **117**(1): 179-189.

Del Rio, L. F., Hadwin, A. K. M., Pinto, L. J., MacKinnon, M. D. and Moore, M. M. (2006). "Degradation of naphthenic acids by sediment micro-organisms." Journal of Applied Microbiology **101**(5): 1049-1061.

Dickson, A. (1981). "An exact definition of total alkalinity and a procedure for the estimation of alkalinity and total inorganic carbon from titration data." Deep Sea Research Part A. Oceanographic Research Papers **28**(6): 609-623.

Dzidic, I., Somerville, A. C., Raia, J. C. and Hart, H. V. (1988). "Determination of naphthenic acids in California crudes and refinery wastewaters by fluoride-ion chemical ionization mass-spectrometry." Analytical Chemistry **60**(13): 1318-1323.

El-Din, M. G., Fu, H., Wang, N., Chelme-Ayala, P., Perez-Estrada, L., Drzewicz, P., Martin, J. W., Zubot, W. and Smith, D. W. (2011). "Naphthenic acids speciation and removal during petroleum-coke adsorption and ozonation of oil sands process-affected water." Science of the Total Environment **409**(23): 5119-5125.

Energy Resources Conservation Board (ERCB) (2011). “EnerFAQs 12: Oil Sands”. http://www.ercb.ca/enerfaqs/EnerFAQs12-OilSands.pdf

Energy Resources Conservation Board (2013). “ST98-2013 Alberta’s energy reserves 2012 and supply/demand outlook 2013-2022.” http://www.ercb.ca/sts/ST98/ST98-2013.pdf

Fan, T. P. (1991). "Characterization of naphthenic acids in petroleum by fast atom bombardment mass spectrometry." Energy & Fuels **5**(3): 371-375.

Foght, J., Fedorak, P.M., Westlake, D.W.S and Boergeret, H.J. (1985). "Microbial content and metabolic activities in the Syncrude tailings pond." AOSTRA J. Res **1**: 139-146.

Frank, R. A., Kavanagh, R., Burnison, K. B., Arsenault, G., Headley, J. V., Peru, K. M., van der Kraak, G. and Solomon, K. R. (2008). "Toxicity assessment of collected fractions from an extracted naphthenic acid mixture." Chemosphere **72**(9): 1309-1314.

Frank, R. A., Fischer, K., Kavanagh, R., Burnison, B. K., Arsenault, G., Headley, J. V., Peru, K. M., van der Kraak, G. and Solomon, K.R. (2009). "Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids." Environmental Science and Technology **43**(2): 266-271.

Gaikar, V. and Maiti, D. (1996). "Adsorptive recovery of naphthenic acids using ion-exchange resins." Reactive and Functional Polymers **31**(2): 155-164.

Ginestet, P., Audic, J.M., Urbain, V. and Block, J.C. (1998). "Estimation of nitrifying bacterial activities by measuring oxygen uptake in the presence of the metabolic inhibitors allylthiourea and azide." Applied and Environmental Microbiology **64**(6): 2266.

Golby, S., Ceri, H., Gieg, L.M., Chatterjee, I., Marques, L.L., and Turner, R.J. (2012). "Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond." FEMS Microbiology, Ecology **79**(1): 240-250.

Grewer, D. M., Young, R. F., Whittal, R. M. and Fedorak, P. M. (2010). "Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured?" Science of the Total Environment **408**(23): 5997-6010.

Hadwin, A. K. M., del Rio, L. F., Pinto, L. J., Painter, M., Routledge, R. and Moore, M. M. (2006). "Microbial communities in wetlands of the Athabasca oil sands: genetic and metabolic characterization of microbial communities in wetlands of the Athabasca oil sands." FEMS Microbiology Ecology **55**(1): 68-78.

Han, X., Scott, A. C., Fedorak, P. M., Bataineh, M. and Martin, J. W. (2008). "Influence of molecular structure on the biodegradability of naphthenic acids." Environmental Science & Technology **42**(4): 1290-1295.

Han, X., MacKinnon, M. D. and Martin, J. W. (2009). "Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS." Chemosphere **76**(1): 63-70.

He, Y., Wiseman, S. B., Zhang, X., Hecker, M., Jones, P. D., El-Din, G. M., Martin, J. W. and Giesy, J. P. (2010). "Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line." Chemosphere **80**(5): 578-584.

He, Y., Wiseman, S. B., Hecker, M., Zhang, X., Wang, N., Perez, L. A., Jones, P. D., Gamal El-Din, M., Martin, J. W. and Giesy, J. P. (2011). "Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water." Environmental Science & Technology **45**(15): 6268-6274.

Headley, J. V., Peru, J. V., McMartin, D. W. and Winkler, M. (2002). "Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry." Journal of AOAC International **85**(1): 182.

Headley, J. V., Tanapat, S., Putz, G. and Peru, K. M. (2002B). "Biodegradation kinetics of geometric isomers of model naphthenic acids in Athabasca river water." Canadian Water Resources Journal **27**(1): 25-42.

Headley, J. V., Peru, K. M., Barrow, M. P. and Derrick, P. J. (2007). "Characterization of naphthenic acids from Athabasca oil sands using electrospray ionization: The significant influence of solvents." Analytical Chemistry **79**(16): 6222-6229.

Headley, J. V., Peru, K. M., Armstrong, S. A., Han, X., Martin, J. W., Mapolelo, M. M., Smith, D. F., Rogers, R. P. and Marshall, A. G. (2009A). "Aquatic plant-derived changes in oil sands naphthenic acid signatures determined by low-, high- and ultrahigh-resolution mass spectrometry." Rapid Communications in Mass Spectrometry **23**(4): 515-522.

Headley, J. V., Du, J., Peru, K. M. and McMartin, D. W. (2009B). "Electrospray ionization mass spectrometry of the photodegradation of naphthenic acids mixtures irradiated with titanium dioxide." Journal of Environmental Science and Health . Part A: Environmental Science and Engineering and Toxicology **44**(6): 591.

Headley, Peru, J., K., Adenugba, A., Du, J. L. and McMartin, D.W. (2010A). "Dissipation of naphthenic acids mixtures by lake biofilms." Journal of Environmental Science and Health, Part A **45**(9): 1027-1036.

Headley, J. V., Peru, K. M., Mishra, S., Meda, V., Dalai, A. K., McMartin, D. W., Mapolelo, M. M., Rodgers, R. P. and Marshall, A. G. (2010B). "Characterization of oil sands naphthenic acids treated with ultraviolet and microwave radiation by negative ion electrospray Fourier transform ion cyclotron resonance mass spectrometry." Rapid Communications in Mass Spectrometry **24**(21): 3121-3126.

Herman, D. C., Fedorak, P. M. and Costerton, J. W. (1993). "Biodegradation of cycloalkane carboxylic acids in oil sand tailings." Canadian Journal of Microbiology **39**(6): 576-580.

Herman, D. C., Fedorak, P. M., MacKinnon, M. D. and Costerton, J. W. (1994). "Biodegradation of naphthenic acids by microbial populations indigenous to oil sands tailings." Canadian Journal of Microbiology **40**(6): 467-477.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P.M. (2002). "Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry." Water Research **36**(11): 2843-2855.

Hsu, C. S., Dechert, G. J., Robbins, W. K. and Fukuda, E. K. (2000). "Naphthenic Acids in Crude Oils Characterized by Mass Spectrometry." Energy Fuels **14**(1): 217-223.

Hyman, M. R., Murton, I.B. and D.J. Arp (1988). "Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes." Applied and Environmental Microbiology **54**(12): 3187-3190.

Jackson, W. A. and J. H. Pardue (1999). "Potential for enhancement of biodegradation of crude oil in Louisiana salt marshes using nutrient amendments." Water, Air, and Soil Pollution **109**(1): 343-355.

Jajuee, B., Margaritis, A., Karamanev, D. and Bergougnou, M.A. (2006). "Mass transfer characteristics of novel three-phase airlift contactor with a semipermeable membrane." Chemical Engineering Journal **125**:119–126.

Jajuee, B., Margaritis, A., Karamanev, D. and Bergougnou, M. A. (2007). "Kinetics of biodegradation of p-xylene and naphthalene and oxygen transfer in a novel airlift immobilized bioreactor." Biotechnology Bioengineering. **96**(2): 232 -242

Janfada, A., Headley, J., Peru, K., and Barbour, S. (2006). "A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils." Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering **41**(6): 985-997.

Johnson, A.J. (2011). Miller & Freund's Probability and Statistics for Engineers. 9th Edition.

Johnson, R. J., Smith, B. E., Sutton, P. A., McGenity, T. J., Rowland, S. J. and Whitby, C. (2011). "Microbial biodegradation of aromatic alkanoic naphthenic acids is affected by the degree of alkyl side chain branching." ISME Journal **5**(3): 486-496.

Jones, D., West, C. E., Scarlett, A. G., Frank, R. A. and Rowland, S. J. (2012). "Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract." Journal of Chromatography A **1247**: 171-175.

Kamaluddin, M. and Zwiazek, J. J. (2002). "Naphthenic acids inhibit root water transport, gas exchange and leaf growth in aspen (*Populus tremuloides*) seedlings." Tree Physiology **22**(17): 1265-1270.

Karamanev, D. G., Chavarie, C. and Samson R. (1997). "Hydrodynamics of soil immobilization in the immobilized soil bioreactor." American Institute of Chemical Engineers Journal **43** (5) 1163–1170.

Karamanev, D. G. and Samson, R. (1998). "High-rate biodegradation of pentachlorophenol by biofilm developed in the immobilized soil bioreactor." Environmental Science & Technology **32**(7): 994-999.

Karamanev, D. G., Chavarie, C. and Samson, R. (1998). "Soil immobilization: New concept for biotreatment of soil contaminants." Biotechnology and Bioengineering **57**(4): 471-476.

Karel, S., Libick, S. and Robertson, C. R. (1985). "The immobilization of whole cells: Engineering principles." Chemical Engineering Science **40**: 1321-1354.

Kavanagh, R. J., Burnison, B. K., Frank, R. A., Solomon, K. R. and Van Der Kraak, G. (2009). "Detecting oil sands process-affected waters in the Alberta oil sands region using synchronous fluorescence spectroscopy." Chemosphere **76**(1): 120-126.

Kavanagh, R. J., Frank, R. A., Oakes, K. D., Servos, M. R., Young, R. F., Fedorak, P. M., MacKinnon, M. D., Solomon, K. R., Dixon, D. G. and Van der Kraak, G. (2011). "Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters." Aquatic Toxicology **101**(1): 214-220.

Keener, W. K. and Arp, D.J. (1994). "Transformations of aromatic compounds by *Nitrosomonas europaea.*" Applied and Environmental Microbiology **60**(6): 1914-1920.

Kermanshahi, A., Karamanev, D. and Margaritis, A. (2005). "Biodegradation of petroleum hydrocarbons in an immobilized cell airlift bioreactor." Water Resources **39** (15): 3704-3714.

Kermanshahi, A., Karamanev, D. and Margaritis, A. (2006). "Kinetic modeling of the biodegradation of the aqueous p-xylene in the immobilized soil bioreactor." Biochemical Engineering Journal **27**(3):204–211.

Khunjar, W. O., Mackintosh, S.A., Skotnicka-Pitak, J., Baik, S., Aga, D.S., and Love, N.G. (2011). "Elucidating the relative roles of ammonia oxidizing and heterotrophic bacteria during the biotransformation of 17α-ethinylestradiol and trimethoprim." Environmental Science & Technology **45**(8): 3605-3612.

Knight, R. L., Kadlec, R. H. and Ohlendorf, H. M. (1999). "The use of treatment wetlands for petroleum industry effluents." Environmental Science & Technology **33**(7): 973-980.

Lai, J. W. S., Pinto, L. J., Kiehlmann, E., Bendell-Young, L. I. and Moore, M. M. (1996). "Factors that affect the degradation of naphthenic acids in oil sands wastewater by indigenous microbial communities." Environmental Toxicology and Chemistry **15**(9): 1482-1491.

Lanzetta, P. A., Alvarez, L. J., Reinach, P.S. and Candia, O.A. (1979). "An improved assay for nanomole amounts of inorganic phosphate." Analytical Biochemistry **100**(1): 95.

Liang, X., Zhu, X. and Butler, E. C. (2011). "Comparison of four advanced oxidation processes for the removal of naphthenic acids from model oil sands process water." Journal of Hazardous Materials **190**(1-3): 168-176.

Lister, A., Nero, V., Farwell, A., Dixon, D. G. and Van der Kraak, G. (2008). "Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water." Aquatic Toxicology **87**(3): 170-177.

Lo, C. C., Brownlee, B. G. and Bunce, N. J. (2003). "Electrospray-mass spectrometric analysis of reference carboxylic acids and Athabasca oil sands naphthenic acids." Analytical Chemistry **75**(23): 6394-6400.

Lo, C. C., Brownlee, B. G. and Bunce, N. J. (2006). "Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids." Water Research **40**(4): 655-664.

MacKinnon, M. D., Matthews, J. G., Shaw, W. H. and Cuddy, R. G. (2001). "Water quality issues associated with composite tailings (CT) technology for managing oil sands tailings." International Journal of Surface Mining, Reclamation and Environment **15**(4): 235-256.

MacKinnon, M. D. and Boerger, H. (1986). "Description of two treatment methods for detoxifying oil sands tailings pond water." Water Quality Research Journal of Canada **21**(4): 17.

Martin, J. W., Han, X., Peru, K. M. and Headley, J. V. (2008). "Comparison of high- and low-resolution electrospray ionization mass spectrometry for the analysis of naphthenic acid mixtures in oil sands process water." Rapid Communications in Mass Spectrometry **22**(12): 1919-1924.

Martin, J. W., Barri, T., Han, X., Fedorak, P. M., El-Din, M. G., Perez, L., Scott, A. C. and Jiang, J. T. (2010). "Ozonation of oil sands process-affected water accelerates microbial bioremediation." Environmental Science and Technology **44**(21): 8350-8356.

Masliyah, J., Zhou, Z. J., Xu, Z. H., Czarnecki, J., and Hamza, H. (2004). "Understanding water-based bitumen extraction from Athabasca oil sands." Canadian Journal of Chemical Engineering **82**(4): 628-654.

McMartin, D.W., Headley, J. V., Friesen, D. A., Peru, K. M. and Gillies, J. A. (2004). "Photolysis of naphthenic acids in natural surface water." Journal of Environmental Science and Health. Part A: Toxic/Hazardous Substances & Environmental Engineering **39**(6): 1361-1383.

Mehrotra, A. K. and Banerjee, A. (1986). "Evaluation of reverse osmosis for the treatment of oil sands produced water." Water Pollution Research Journal of Canada **21**: 11-21.

Merlin, M., Guigard, S. E. and Fedorak, P. M. (2007). "Detecting naphthenic acids in waters by gas chromatography–mass spectrometry." Journal of Chromatography A **114**(1-2): 225-229.

Mishra, S., Meda, V., Dalai, A. J., McMartin, D. W., Headley, J. V. and Peru, K. M. (2010). "Photocatalysis of naphthenic acids in water." Journal of Water Resource and Protection **2**(7): 644.

Misiti, T., Tandukar, M., Tezel, U. and S.G. Pavlostathis. (2013). "Inhibition and biotransformation potential of naphthenic acids under different electron accepting conditions." Water Research **47**(1): 406-418.

Muyzer, G., E. C. De Waal, and A.G. Uitterlinden (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-

amplified genes coding for 16S rRNA." Applied and Environmental Microbiology **59**(3): 695-700.

Nero, V., Farwell, A., Lister, A., Van der Kraak, G., Lee, L. E., Van Meer, T., MacKinnon, M. D., and Dixon, D. G. (2006). "Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to oil sands process-affected water." Ecotoxicology and Environmental Safety **63**(3): 365-377.

Otte, M. P., Gagnon, J., Comeau, Y., Matte, N., Greer, C. W. and Samson, R. (1994). "Activation of an indigenous microbial consortium for bioaugmentation of pentachlorophenol/creosote contaminated soils." Applied Microbiology and Biotechnology **40**(6): 926-932.

Paslawski, J., Nemati, M., Hill, G. and Headley, J. V. (2009). "Biodegradation kinetics of -4-methyl-1-cyclohexane carboxylic acid in continuously stirred tank and immobilized cell bioreactors." Journal of Chemical Technology and Biotechnology (1986) **84**(7): 992-1000.

Peng, H., Volchek, K., MacKinnon, M., Wong, W.P. and C.E. Brown (2004). "Application on to nanofiltration to water management options for oil sands operation." Desalination **170**(2): 137-150.

Pérez-Estrada, L. A., Han, S., Drzewicz, P., El-Din, M. G., Fedoark, P. M. and Martin, J. W. (2011). "Structure–reactivity of naphthenic acids in the ozonation process." Environmental Science & Technology **45**(17): 7431-7437.

Province of Alberta (2010). *Environmental Protection and Enhancement Act*. http://environment.alberta.ca/01530.html

Quagraine, E. K., Peterson, H. G. and Headley, J. V. (2005A). "In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region--demonstrated field studies and plausible options: A review." Journal of Environmental Science and Health, Part A: Toxic / Hazardous Substances and Environmental Engineering **40**(3): 685-722.

Quagraine, E. K., Headley, J. V. and Peterson, H. G. (2005B). "Is biodegradation of bitumen a source of recalcitrant naphthenic acid mixtures in oil sands tailing pond waters?" Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering **40**(3): 671-684.

Rogers, V. V., Wickstrom, M. M., Liber, K. and MacKinnon, M. D. (2002). "Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings." Toxicological Sciences **66**(2): 347-355.

Rontani, J. F. and Bonin, P. (1992). "Utilization of n-alkyl-substituted cyclohexanes by a marine *Alcaligenes*." Chemosphere **24**(10): 1441-1446.

Rowland, S. J., West, C. E., Scarlett, A. G., Jones, D. and Frank, R. A. (2011). "Identification of individual tetra- and pentacyclic naphthenic acids in oil sands process water by comprehensive two-dimensional gas chromatography/mass spectrometry: Identification of tetra- and pentacyclic NA in OSPW." Rapid Communications in Mass Spectrometry **25**(9): 1198-1204.

Rowland, S. J., Scarlett, A. G., Jones, D., West, C. E. and Frank, R. A. (2011). "Diamonds in the rough: Identification of individual naphthenic acids in oil sands process water." Environmental Science & Technology **45**(7): 3154-3159.

Rowland, S. J., West, C. E., Jones, D., Scarlett, A. G., Frank, R. A. and Hewitt, L. M. (2011). "Steroidal aromatic 'naphthenic acids' in oil sands process-affected water: Structural comparisons with environmental estrogens." Environmental Science & Technology **45**(22): 9806-9815.

Sarkar, D., Ferguson, M., Datta, R., and Birnbaum, S. (2005). "Bioremediation of petroleum hydrocarbons in contaminated soils: comparison of biosolids addition, carbon supplementation, and monitored natural attenuation." Environmental Pollution **136**(1): 187-195.

Scott, A. C., MacKinnon, M. D. and Fedorak, P. M. (2005). "Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids." Environmental Science & Technology **39**(21): 8388-8394.

Scott, A. C., Young, R. F. and Fedorak, P. M. (2008A). "Comparison of GC–MS and FTIR methods for quantifying naphthenic acids in water samples." Chemosphere **73**(8): 1258-1264.

Scott, A. C., Zubot, W., MacKinnon, M. D., Smith, D. W. and Fedorak, P. M. (2008B). "Ozonation of oil sands process water removes naphthenic acids and toxicity." Chemosphere **71**(1): 156-160.

Shi, J., Fujisawa, S., Nakai, S. and Hosomi, M. (2004). "Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*." Water Research **38**(9): 2323-2330.

Smith, B. E., Lewis, C. A., Belt, S. T., Whitby, C. and Rowland, S. J. (2008). "Effects of alkyl chain branching on the biotransformation of naphthenic acids." Environmental Science & Technology **42**(24): 9323-9328.

St. John, W. P., Rughani, J., Green, S. A. and McGinnis, G. D. (1998). "Analysis and characterization of naphthenic acids by gas chromatography–electron impact mass spectrometry of tert.-butyldimethylsilyl derivatives." Journal of Chromatography A **807**(2): 241-251.

Syncrude Canada Ltd. (2009). "2008/09 Sustainability report." http://www.syncrude.ca/pdf/Syncrude-SD-report.pdf

Taylor, D. G. and Trudgill, P. W. (1978). "Metabolism of cyclohexane carboxylic acid by *Alcaligenes* strain W1." Journal of Bacteriology **134**(2): 401-411.

Toor, N. S., Franz, E. D., Fedorak, P. M., MacKinnon, M. D. and Liber, K. (2012). "Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands." Chemosphere **90**(2): 449-458.

van den Heuvel, M. R., Power, M., MacKinnon, M. D., and Dixon, D. G. (1999). "Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters." Canadian Journal of Fisheries and Aquatic Sciences **56**(7): 1226-1233.

Volk, W. A. and J. C. Brown (1998). Basic Microbiology. Menlo Park, California, Benjamin Cummings.

Weatherburn, M. W. (1967). "Phenol-hypochlorite reaction for determination of ammonia." Analytical Chemistry **39**(8): 971-974.

Yi, T. and Harper, W. F. J. (2007). "The link between nitrification and biotransformation of 17α-ethinylestradiol." Environmental Science and Technology **41**(12): 4331-4316.

Young, R. F., Orr, E. A., Goss, G. G. and Fedorak, F. M. (2007). "Detection of naphthenic acids in fish exposed to commercial naphthenic acids and oil sands process-affected water." Chemosphere **68**(3): 518-527.

Zhou, S., Huang, H. and Liu, Y. (2008). "Biodegradation and origin of oil sands in the Western Canada Sedimentary Basin." Petroleum Science **5**(2): 87-94.

Appendix A: Chapter 3 supplementary material

Table A1: Composition of WIP OSPW used in experimentation

Parameter	Concentration (mg/L)
Chloride	379
Nitrate	<0.5
Nitrite	<0.5
Sulphate	425
Ag	<0.01
Al	<1.0
As	0.049
B	2.9
Ba	0.24
Ca	39
Cd	<0.025
Co	<0.02
Cr	<0.04
Cu	<0.2
Fe	0.15
K	17
Mg	17
Mn	<0.05
Mo	0.15
Na	612
Ni	<0.3
P	<1.0
Pb	<0.03
S	139
Sb	<0.1
Se	<0.1
Sn	<0.01
Sr	1.1
Ti	<0.01
Tl	<0.025
U	<0.2
V	<0.02
Zn	0.011
TOC	54
TC	215
TIC (estimated)	161
Naphthenic Acids	49

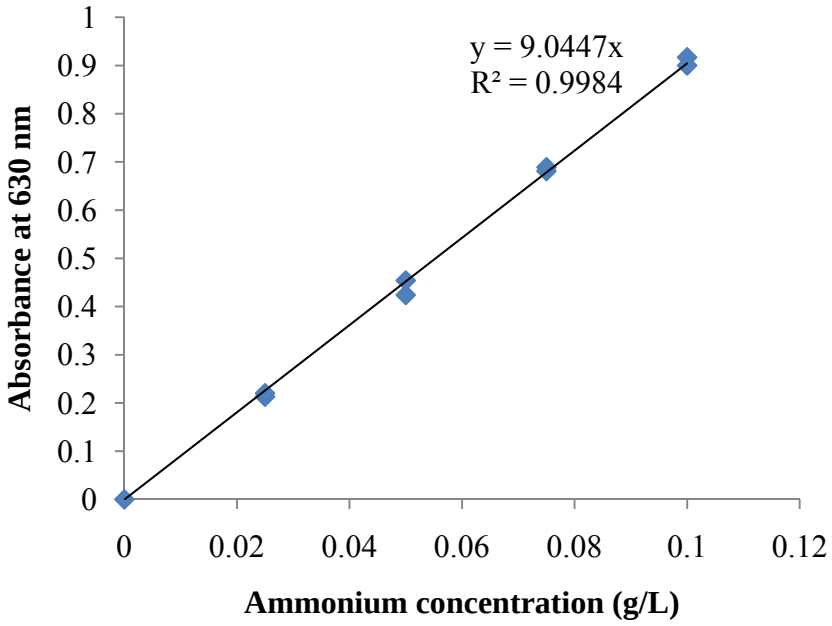

Figure A1: Ammonium assay calibration curve generated from analysis of duplicate standards ranging from 0 to 0.1 g/L ammonium

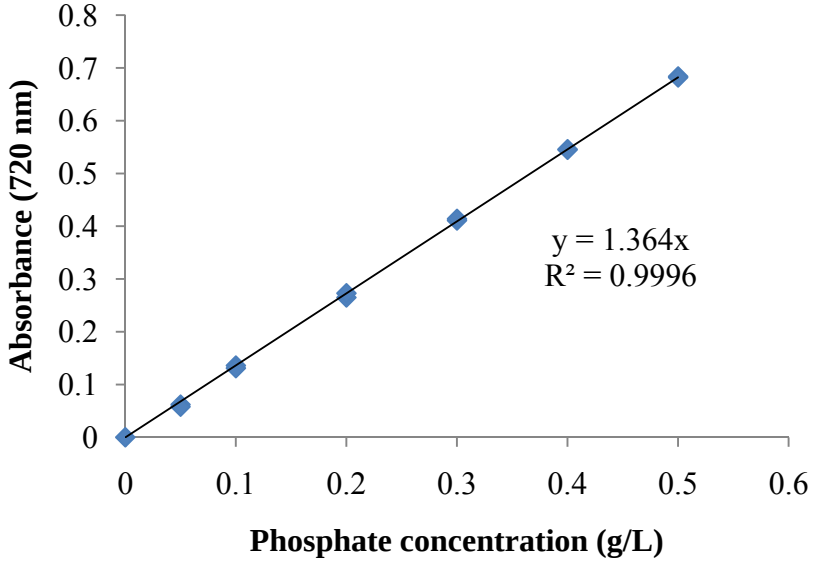

Figure A2: Phosphate assay calibration curve generated from analysis of duplicate standards ranging from 0 to 0.5 g/L of phosphate

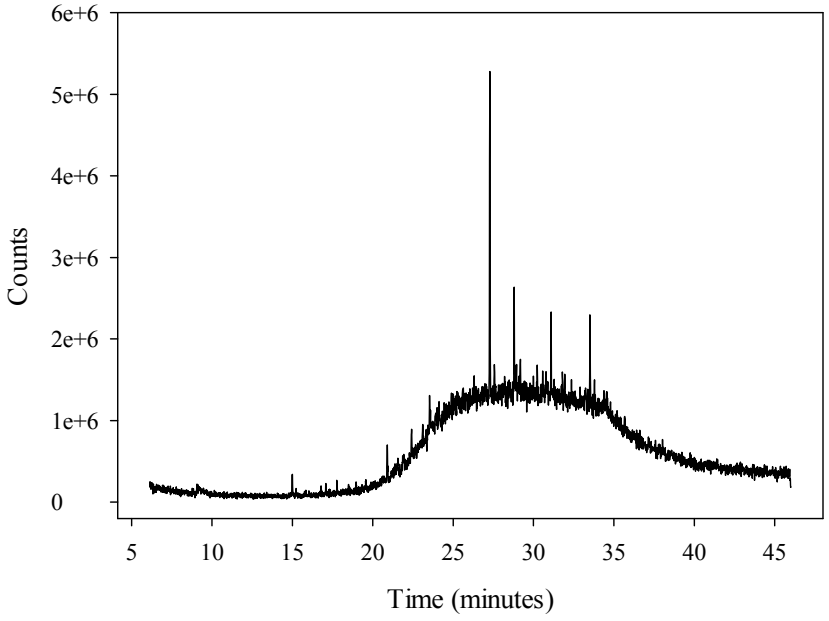

Figure A3: GC-MS total ion chromatogram (m/z = 60 to 550) of a derivatized OSPW extract

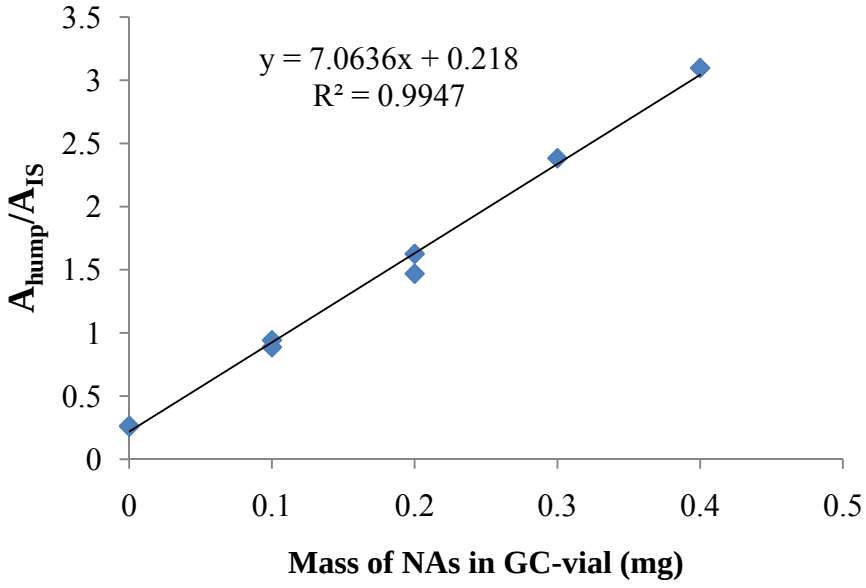

Figure A4: Merichem NA calibration curve

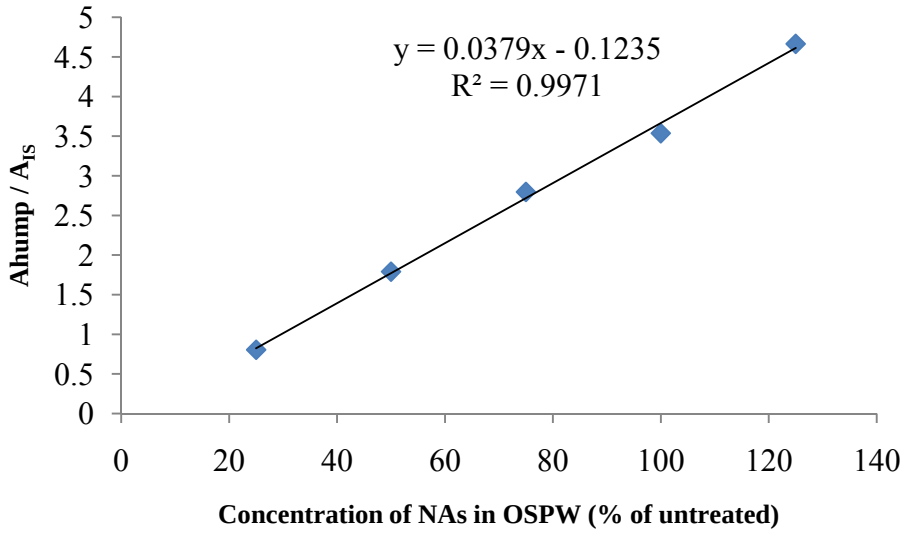

Figure A5: OSPW calibration curve generated from extracted OSPW samples of varying volume

Mathematica program code used to assign detected masses to an isomeric group of the form $C_nH_{2n+Z}O_2$

```
MSi=27.976926;   (* Silicon 28 *)
MO=15.9949;    (* Oxygen 16 *)
MH=1.0078;     (* Hydrogen 1 *)
MN=14.003074;  (* Nitrogen 14 *)
MC=12.0000;  (* Carbon 12 *)
MS=31.97207100; (* Sulfur 32 *)

Carbons=33;
Hdef=12;

numcarb=Table[n,{n,6,Carbons}];
Z=Table[-z,{z,0,Hdef,2}];
numoxy=Table[m,{m,1,5}];

Structures=Table[If[((2*numcarb[[a]]+2)+Z[[b]])<numcarb[[a]]/2,Null,If[numcarb[[a]]<Abs[-
Z[[b]]-2],Null,
    {-Z[[b]],numcarb[[a]],"C"numcarb[[a]] "H"((2*numcarb[[a]])+Z[[b]])
"O"numoxy[[c]],SetPrecision[(MC*numcarb[[a]]+MH*((2*numcarb[[a]])+Z[[b]])+MO*numoxy[[c]]),
6]}]],
  {c,Length[numoxy]},{b,Length[Z]},{a,Length[numcarb]}];

O1=Sort[DeleteCases[Flatten[Structures[[1]],1],Null]];
O2=Sort[DeleteCases[Flatten[Structures[[2]],1],Null]];
O3=Sort[DeleteCases[Flatten[Structures[[3]],1],Null]];
O4=Sort[DeleteCases[Flatten[Structures[[4]],1],Null]];
O5=Sort[DeleteCases[Flatten[Structures[[5]],1],Null]];

Clear[str1,str2];
str1=OpenRead["L:\\a ozone spectra\\r2 824 for analysis.txt"];
str2=OpenRead["C:\\Users\\Istok\\Naphthenic Acids Project\\GC-MS Analysis Notebooks and
Files\\base pairs for O2.txt"];

AA=str1;
Find[AA,"* Pair Count"];
Lst=DeleteCases[Table[Read[AA,{Real,Real}],{n,1,5000}],EndOfFile];
LstA=DeleteCases[Table[If[Lst[[n,2]]≥10,Lst[[n]],Null],{n,1,Length[Lst]}],Null];

Basepairs=str2;
Bp=Table[Read[Basepairs,{Real,Real,Real,Real,Real}],{n,1,156}];
ListofBp=Table[Round[Bp[[n,4]],.1],{n,1,Length[Bp]}];

resol=0.25;
```

```
Base=Flatten[Table[{{a,b,0},{a,b,0}},{b,0,Hdef,2},{a,0,30,1}],1];

Requiredfit=1000;
min=50;
Matches2=DeleteCases[Flatten[Table[If[Round[Lst[[n,1]],resol]==Round[O2[[m,4]],resol],{{O2[
[m,2]],O2[[m,1]],0},{O2[[m,2]],O2[[m,1]],Lst[[n,2]]}},Null],{m,1,Length[O2]},{n,1,Length[Lst
]}],1],Null];

MaxPeak=1;
Matches2=Table[{Matches2[[n,1]],{Matches2[[n,2,1]],Matches2[[n,2,2]],Matches2[[n,2,3]]/Max
Peak}},{n,1,Length[Matches2]}];
MatchesB=DeleteCases[Flatten[Table[If[Round[Lst[[n,1]],resol]==Round[O2[[m,4]],resol],{O2[[
m,2]],O2[[m,1]],2,O2[[m,4]],(Lst[[n,1]]-
O2[[m,4]])/O2[[m,4]]*1000000,Lst[[n,2]]},Null],{m,1,Length[O2]},{n,1,Length[Lst]}],1],Null];
MatchesB1=DeleteCases[Table[If[Abs[MatchesB[[m,5]]]<Requiredfit,MatchesB[[m]],Null],{m,
1,Length[MatchesB]}],Null];
MatchesB2=Table[{MatchesB1[[m,1]],MatchesB1[[m,2]],MatchesB1[[m,3]],MatchesB1[[m,4]],
MatchesB1[[m,5]],MatchesB1[[m,6]]},{m,1,Length[MatchesB1]}];
CorrectB2=DeleteCases[Table[If[(MatchesB2[[m,2]]+min)<MatchesB2[[m,1]],MatchesB2[[m]],
Null],{m,1,Length[MatchesB2]}],Null];

Massmatches=Table[Round[MatchesB2[[m,4]],.1],{m,1,Length[MatchesB1]}];

Missingmasses=Complement[ListofBp,Massmatches];
Missingmasses2=DeleteCases[Flatten[Table[If[Round[Bp[[m,4]],.1]==Missingmasses[[n]],Bp[[
m]],Null],{n,1,Length[Missingmasses]},{m,1,Length[Bp]}],1],Null];

CompleteTable=Flatten[{Missingmasses2,CorrectB2},1];
Export["C:\\Users\\Istok\\Naphthenic Acids Project\\nitrate experiments\\output for t-tests.xls",
CompleteTable];
```

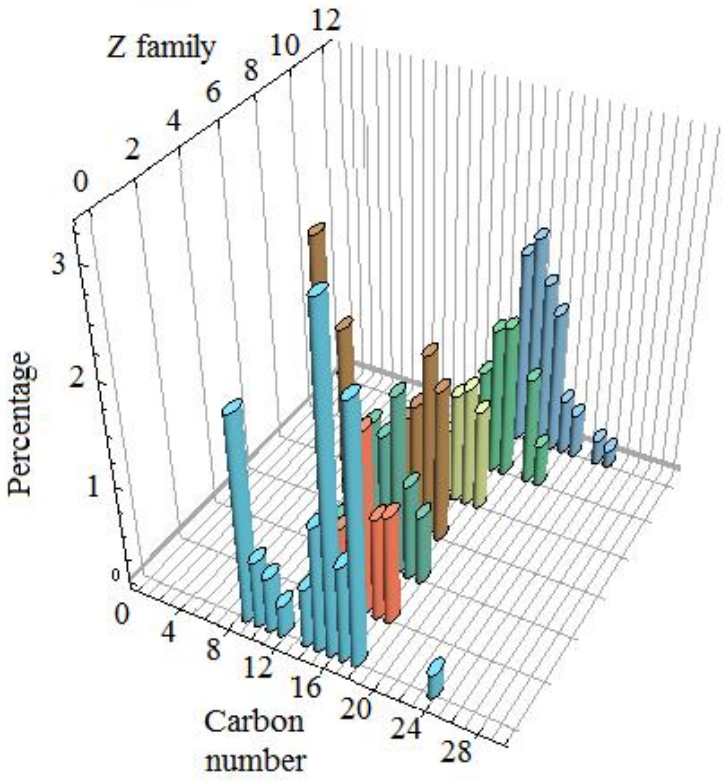

Figure A6: NA profile of untreated OSPW generated by HPLC-HRMS

Welch's t-tests

Welch's t-test was chosen to compare the Z-distribution and average n-number of treated and untreated OSPW samples, because equal variance could not be assumed, as treated samples are expected to have higher variance than OSPW. Two sided t-tests were used, and the test statistic compared to values obtained in t-tables (Johnson, 2011).

The test statistic was calculated using equation A1, where X_i is the sample mean, s_i^2 is the sample variance, and N_i is the sample size:

$$t = \frac{\overline{X_1 - X_2}}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}}$$

The degrees of freedom were calculated using the Welch-Satterthwaite equation, equation A2:

$$\upsilon = \frac{\left(\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}\right)^2}{\frac{s_1^4}{N_1^2(N_1 - 1)} + \frac{s_2^4}{N_2^2(N_2 - 1)}}$$

Table A 2: Change in average n-number for each Z-series with ISBR treatment

	Z-series													
	0		-2		-4		-6		-8		-10		-12	
Untreated OSPW														
ave carbon number	12.8	± 0.2	14.7	± 0.1	14.1	± 0.1	15.1	± 0.1	16.7	± 0.2	18.9	± 0.2	20.7	± 0.1
Treated OSPW														
ave carbon number	13.1	± 0.2	15.2	± 0.1	14.6	± 0.2	15.3	± 0.1	16.8	± 0.3	19.1	± 0.4	20.9	± 0.2
Test for statistical significance														
t-statistic	-2.12		-7.07		-4.47		-2.83		-0.55		-0.89		-1.79	
degrees of freedom	6		6		4.41		6		5.23		4.41		4.41	
Confidence level	0.9		1		0.98		0.95		<0.5		0.5		0.8	

Table A 3: Change in average Z-series distribution with ISBR treatment

	Z-series													
	0		-2		-4		-6		-8		-10		-12	
Untreated OSPW														
Proportion (%)	21.5	± 0.8	14.4	± 0.4	24.2	± 0.5	22.1	± 0.5	8.6	± 0.3	4.9	± 0.1	4.2	± 0.2
Treated OSPW														
Proportion (%)	24.4	± 0.7	14.2	± 0.2	21.3	± 0.2	21.4	± 0.5	9.1	± 0.4	4.9	± 0.4	4.7	± 0.3
Test for statistical significance														
t-statistic	-5.46		0.89		10.8		1.98		-2		0		-2.77	
degrees of freedom	5.9		4.41		3.94		6		5.56		3.37		5.23	
Confidence level	1		0.5		1		0.9		0.8		0		0.95	

Appendix B: Chapter 4 supplementary material

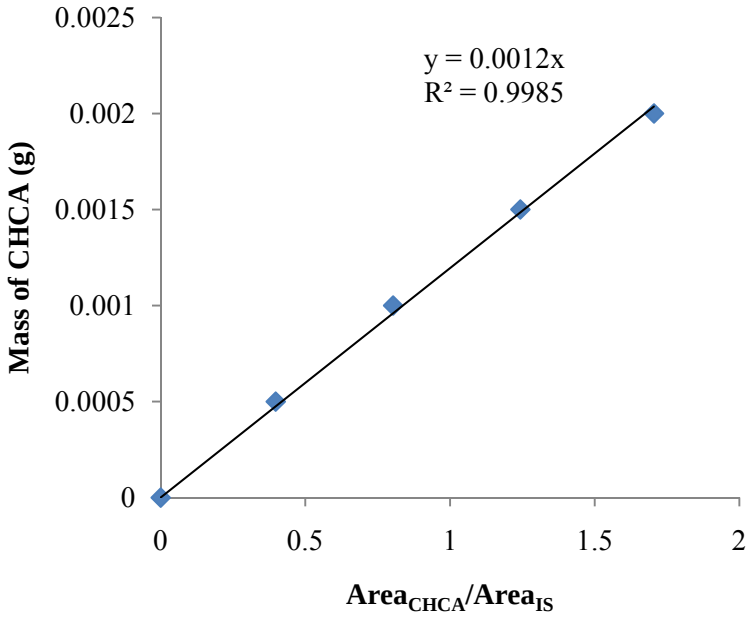

Figure B1: CHCA calibration curve

Calculation of OUR required for metabolism of NAs

$$OUR = X \cdot \frac{156\ mg\ C}{210\ mg\ NAs} \cdot \frac{1\ mmol\ C}{12\ mg\ C} \cdot \frac{1\ mmol\ O_2}{1\ mmol\ CO_2} \cdot F$$

Where OUR is in mmol/h, X is the mg/L of NAs removed with ISBR treatment, and F is the flow rate through the reactor in L/h (0.06 L/h). The most abundant NA acid $C_{13}H_{22}O_2$ was used to estimate the mass of carbon per mass of NAs (156 mg C per 210 mg of NA).

Appendix C: Chapter 5 supplementary material

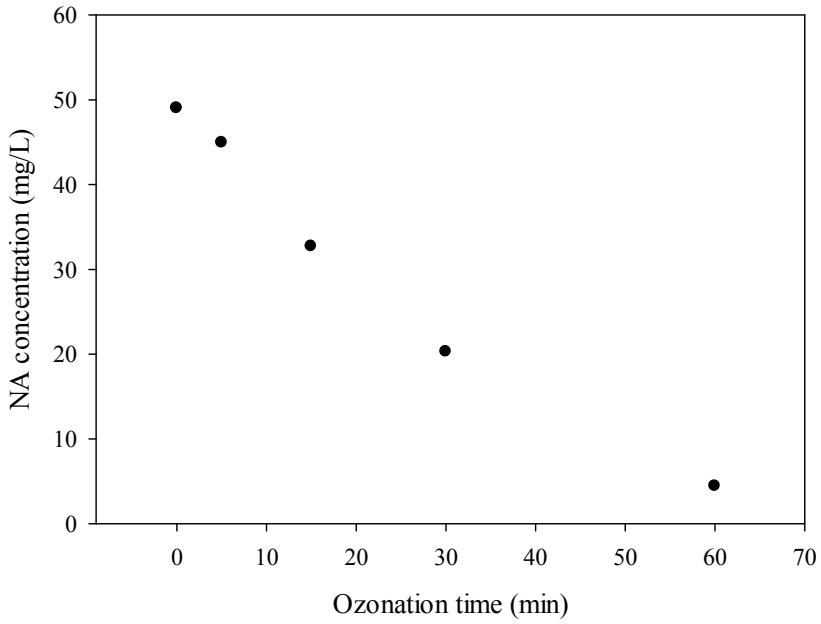

Figure C 1: NA concentration with ozone treatment time

Table C1: Change in Z-number distribution and average carbon number with ozonation

	Z-number						
	0	**-2**	**-4**	**-6**	**-8**	**-10**	**-12**
Z-distribution (%)							
OSPW	17.4 (0.2)*	14.3 (0.1)	28.4 (0.5)	23.6 (0.1)	9.3 (0.2)	4.2 (0.1)	2.90 (0.04)
O_3-treated	23 (3)	16.1 (0.2)	35 (3)	16.2 (0.5)	4.5 (0.6)	2.74 (0.08)	2.59 (0.01)
Average carbon number							
OSPW	13.9 (0.01)	14.3 (0.01)	13.2 (0.02)	14.5 (0.03)	16.1 (0.03)	17.9 (0.04)	19.7 (0.02)
O_3-treated	14.4 (0.3)	13.6 (0.08)	12.6 (0.03)	13.8 (0.2)	16.7 (0.2)	19.9 (0.5)	20.1 (0.04)

**standard deviation*