COMPONENTS OF OILS SANDS PROCESS WATER INVOLVED IN TOXICITY, ESTROGENICITY AND BIODEGRADATION

by

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Abstract

To process one m^3 of oil sands, four m^3 of oil sands process wastewater (OSPW) is produced. OSPW is a mixture of compounds including hundreds of different naphthenic acids (NAs) which are considered to the major toxicity contributor of OSPW. Effect-directed analysis was used to establish the link between toxicity/estrogenicity and components in OSPW. OSPW and biologically treated OSPW were separated by solid phase extraction and reverse phase HPLC into "toxic and nontoxic" or "estrogenic and nonestrogenic" fractions as detected by the Microtox® and a yeast estrogenic screening assay respectively. The composition of fractions was determined by ultrahigh resolution mass spectrometry. Multivariate statistical methods were used to analyze the relationship between composition and toxicity/estrogenicity. C15-C18 O2-NAs ($C_nH_{2n+z}O_2$) with a double bond equivalent (DBE) of 4, as well as C14-C17 with DBE=3 were found to be the most likely contributors to toxicity, while O2 ($C_nH_{2n+z}O_2$), O3 ($C_nH_{2n+z}O_3$) and O4 ($C_nH_{2n+z}O_4$) C17 to C20 compounds with a DBE 6-10 likely cause estrogenicity. From these studies, the exact formulae and masses of possible estrogenic/toxic compounds in OSPW were identified. These findings will help to focus study on the most environmentally significant components in OSPW.

In an earlier published study, OSPW was treated with an immobilized soil bioreactor (ISBR) at a 7 day residence time, which achieved NA reduction by 30-40%. The present study compared untreated and ISBR-treated OSPW, which showed that while DBE=3, 4 were the most recalcitrant NAs and DBE=5 were biodegradable NAs. The presence of aromatic and diamondoid NA structures was confirmed by tandem MS. These results may help to better understand NA biodegradation, the recalcitrant components and improve ISBR performance. Bacteria belonging to the *Ochrobactrum*, *Brevundimonas* and *Bacillus* genera were isolated from the ISBR and tested for their biodegradation of aliphatic, cyclic and aromatic NA surrogates in 96-well plates by tetrazolium redox dyes as the metabolic activity indicator. *Ochrobactrum* grew best on the most surrogates and both *Ochrobactrum* and *Bacillus* were found to degrade recalcitrant tricyclic NAs. This approach can be used to understand the role of individual

microorganisms in the mixed microbial population that are involved in the NAs biodegradation in the ISBR.

Co-Authorship

The author wishes to acknowledge the contribution of Dr. Bruce A Ramsay and Dr. Juliana A. Ramsay in the supervision of and the preparation of all manuscripts presented in this thesis. All experiments were performed by the author.

The thesis is comprised of the following manuscripts:

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My contributions: searched literature, designed and conducted experiments, processed and analyzed data, and in writing paper

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My contributions: searched literature, designed and conducted experiments, processed and analyzed data, and in writing paper

"Probable link between toxicity and composition profiles of fractions from solid phase extraction of oil sands process water" Yue, S., B. A. Ramsay, J. Wang, and J.A. Ramsay. (Preparing to submit)

My contributions: searched literature, designed and conducted experiments, processed and analyzed data, and in writing paper

"Characterizing the biodegradation of oil sands process water by immobilized soil reactor based on fractionation, MicrotoxTM acute assay and yeast estrogenic screening (YES) assay"Yue, S., B. A. Ramsay, J. Wang, and J.A. Ramsay. (Preparing to submit)

My contributions: searched literature, designed and conducted experiments, processed and analyzed data, and in writing paper

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List of Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance
9-FCA	9-fluorenecarboxylic acid
AEO	Acid Extractable Organics
ANOVA	Analysis Of Covariance
AOF	Acid Extractable Fraction
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photoionization
BOD	Biological Oxygen Demand
C18	octade-cylsilane
CCA	Cyclohexane Carboxylic Acid
CI	Confidence Interval
c-NAs	Classical NAs
COD	Chemical Oxygen Demand
CPRG	Chlorophenol Red-B-D-Galactopyranoside
CT	Consolidated Tailings
DBE	Double Bond Equivalents
DCM	Dichloromethane
DHNA	Decahydro-2-Naphthoic Acid
E2	Endogenous Estrogen
ECOSAR	Ecological Structure Activity Relationships
EDA	Effect-Directed Analysis
EE2	Estrogen Ethynylestradiol
EI	Electron Impact

ER	Estrogen Receptor
ERCB	Energy Resources Conservation Board
EREs	Estrogen Response Elements
ESI-MS	Electrospray Ionization-Mass Spectrometry
FTICRMS	Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
FTIR	Fourier transform infrared spectroscopy
FWHM	Full width at half maximum
GC×GC	Two-dimensional gas chromatography
GC-EI-MS	Gas chromatography–Electron ionization-mass spectrometry
HPLC	High Pressure Liquid Chromatography
HPLC-HRMS	High Performance Liquid Chromatography-High Resolution Mass Spectrometry
HRT	Hydraulic Retention Time
ISBR	Immobilized Soil Bioreactor
LLE	Liquid-Liquid Extraction
LTQ	Linear Ion Trap Quadropole
MCF -7	Michigan Cancer Foundation-7
MLSB	Mildred Lake Settling Basin
MS/MS	Tandem Mass Spectrometry
MTBSTFA	N-methyl-N-(t-butyldimethylsilyl) trifluoroacteamide
MW	Molecular Weight
NA	Naphthenic Acid
ONPG	o-nitrophenol-β-D-galactopyranoside
OSPW	Oil Sands Process Water
PA	Palmitic Acid
PAH	Polycyclic Aromatic Hydrocarbons

PAH	Polycyclic Aromatic Hydrocarbons
PCA	Principal Component Analysis
PCBs	Polychlorinated Biphenyls
ECP	Entachlorophenol
PCP	Pentachlorophenol
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Regression-Discriminant Analysis
PSDVB	Polystyrene Divinylbenzene
QSAR	Quantitative Structure Activity Relationship
RP	Reversed Phase
SFS	Synchronous Fluorescence Spectra
SPE	Solid Phase Extraction
SSU	Small Subunit
TAN	Total Acid Number
TCE	Trichloroethylene
TOC	Total Organic Carbon
TOF-MS	Time Of Flight Mass Spectrometry
USEPA	United States Environmental Protection Agency
YES	Yeast Estrogenic Screening

Nomenclature

EC_{50}	Effective Concentration resulting in 50% decrease in bioluminescence
m/z	mass to charge ratio
K_{ow}	Octanol-water partition coefficient
K_{mw}	membrane vesicle-water partition coefficient
pKa	acid dissociation constant
ppm	part per million
TU_{50}	To express toxicity in a proportion to effect, Toxic Units $TU_{50}=1/EC_{50}$

Chapter 1

Introduction

1.1 Oil Sands and Oil Sands Process Water

The oil sands industry is very important to the economy of Canada, especially the province of Alberta. However, the extraction of bitumen from oil sands by the Clark's hot water extraction method (MacKinnon et al. 2005) has resulted in about 720 million m^3 of tailings water covering an area of ~130 km^2 in 2011 (ERCB 2011). Generally, OSPW is moderately hard (15–25 mg L^{-1} Ca^{2+}, 5–10 mg L^{-1} Mg^{2+}) with a pH of 8.0–8.4 and an alkalinity of ~800–1000 m L^{-1} HCO_3^- (Burchfield et al. 1979). Organic compounds in tailing pond waters include bitumen, naphthenic acids (NAs), asphaltenes, benzene, phenols, and polycyclic aromatic hydrocarbons (PAH) (Allen 2008a) with high concentrations of NAs and dissolved inorganic ions, low concentrations of polyaromatic compounds, and some metals (Allen 2008a).

1.2 Aquatic Toxicity

Long term exposure to OSPW is toxic to various organisms, especially aquatic organisms such as aquatic plants (Armstrong et al. 2008), invertebrates (McCormick 2000), and fish (Colavecchia et al. 2004, Colavecchia et al. 2006). Under the Alberta Environmental Protection and Enhancement Act (1993), OSPW cannot be released until properly treated (Madill et al. 2001). NAs have been identified as the major contributor to OSPW toxicity (Madill et al. 2001). NAs are defined as a mixture of naturally occurring alicyclic and alkyl-substituted carboxylic acids with the structural formula $C_nH_{2n+z}O_x$, where "z" refers to the hydrogen deficiency or double bond equivalent and is zero or a negative, even integer. Classical NAs (c-NAs, x=2) and oxidized NAs (oxy-NAs, x =3-5) comprise less than 50% of the acid extractable organics (AEO) from OSPW (Grewer et al. 2010). NAs include alicyclic and aromatic carboxylic acids, as well as

non-NA AEO that may also contain sulfur, nitrogen, and other elements with up to 10 O groups (Headley et al. 2012).

Various methods have been tested to treat OSPW. Physical methods include settling, filtration, centrifugation and freezing; chemical methods include coagulation, flocculation, and ozonation (Allen 2008b). Compared to physical and chemical treatment methods, biological treatments using microbes to remove the toxic contaminants can be simpler and more economical. Biological treatments have been demonstrated in shake flasks (Han et al. 2008, Herman et al. 1994, Scott et al. 2008) and in simulated wetlands (Toor et al. 2013a) using naturally occurring microorganisms, which feed on the organic pollutants to degrade and detoxify them. However, half-life of NAs ranges from 44 to 240 days in shake flask (Han et al. 2008) and 11-13 years in the tailings pond (Han et al. 2009).

The Microtox® acute assay is a widely used standard toxicity test and uses *Vibrio fisheri* as the test organism (Johnson 2005). The test measures the reduction in the light emitted by this bioluminescent bacterium when exposed to a sample and compared to a control to obtain the EC_{50}, the concentration that reduces 50% of light intensity. A low EC_{50} means the sample has a high toxicity. Other toxicity assays with eukaryotes are more expensive and time consuming and are not suitable for large-scale analysis or regular monitoring. Microtox® has thus become a standard toxicity assay in OSPW and demonstrates good correlations with other assays (Bulich et al. 1981, Cronin et al. 1991, Klinkow et al. 1998).

1.3 Estrogenicity

Endocrine disruptors are chemicals that can mimic the natural hormone actions and cause abnormal hormone modulation. Some synthetic chemicals, such as bisphenol A, polychlorinated biphenyls, function as estrogens and are thus referred to as xenoestrogens. Estrogens are compounds which bind to and activate an estrogen receptor, thus regulating the activity of

estrogen-specific gene transcription. Exposure to estrogens may lead to viability reduction, gonadal malformations or feminization of genetically male fish (Wedekind 2014).

OSPW was also found to be estrogenic and anti-androgenic in the human H295R cell line (He et al. 2010), and goldfish (Lister et al. 2008). After goldfish were exposed to OSPW, *in vitro* production of plasma T and E2 by ovarian and testicular tissues significantly decreased (Lister et al. 2008). Siwik et al. (2000) reported that, after exposure to OSPW tailings pond water, fathead minnows took significantly longer to produce their first eggs, and the amount of eggs was half that at a reference site. Reinardy et al. (2013) also showed that there was weak estrogenicity in aromatic NAs that were extracted from OSPW. Although each xenoestrogen may have much less estrogenicity (10^4-10^5 less) compared to the natural hormone 17β-estradiol, their combined additive effect can dramatically enhance steroid hormone action (Rajapakse et al. 2002). Previous studies implicated steroidal aromatic NAs as OSPW compounds which cause estrogenicity, but the exact components in OSPW that are responsible for the estrogenic effect are not yet confirmed (Rowland et al. 2011).

1.4 Immobilized Soil Bioreactor

The immobilized soil bioreactor (ISBR) uses plates of a highly porous geotextile material to entrap soil particles with associated microorganisms, providing a surface for growth, and reducing the hydrodynamic shear stress on the microorganisms. ISBRs have been shown to achieve high degradation rates of pentachlorophenol (PCP) (Karamanev et al. 1998), trichloroethylene (TCE) (Ramsay et al. 2001). In a previous study, ISBRs removed NAs at a rate of 2.32 mg L^{-1} d^{-1} in treating OSPW (McKenzie et al. 2014).

Identification of the key or environmentally relevant pollutants in OSPW is important to assess the environmental risk and improve the treatment. Effect-directed analysis has been used as a tool to reduce the complexity of study and has been applied to other complex contaminant

mixtures such as tannery wastewater (Fiehn et al. 1997), sewage-treatment works (Desbrow et al. 1998), and creosote-contaminated groundwater (Hartnik et al. 2007). Effect-directed analysis is comprised of chemical analysis and toxicity testing. The most probable contributors to the total toxicity can be identified from analyzing the composition of the bioactive fraction(s). In the present study, it could also be very useful in characterizing the selective degradation in an ISBR and to determine which recalcitrant components that might contribute to the residual toxicity.

1.5 Microbial Degradation

Microorganisms are detected in untreated tailings pond water (Foght et al. 1985, Lai et al. 1996, Quagraine et al. 2005) and they are known to be able to degrade some toxic OSPW components (Kannel et al. 2012, Toor et al. 2013b). It is important to identify the OSPW-degrading microorganisms in order to know how to manipulate environmental factors in order to induce or enhance biodegradation. NA surrogates, commercial NAs, and OSPW have been used to study NA biodegradation. Studies have focused on consortia degrading OSPW NAs (Clemente et al. 2004, Scott et al. 2008), rhizosphere microorganisms not previously exposed to NAs (Biryukova et al. 2007), and cultures enriched from OSPW using commercially available NAs as the carbon substrate (Clemente et al. 2004). Because of the complexity of NAs and OSPW, no single organism can degrade all OSPW or NAs. The wide variety of chemical structures make substrate utilization difficult to monitor so biodegradation has usually been evaluated by measurement of microbial activity, but the degradation of NA surrogates is useful to better understand NA biodegradation and controlling factors. For example, Paslawski et al. (2009) tested the biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid and found that the maximum specific growth rate (1.69 day^{-1}) and biomass yield (0.41 mg mg^{-1}) at pH 10. However, there are relatively few NA surrogates commercially available and they are typically only available in small quantities. Also, some NAs are poorly water-soluble and bioavailable. All

these factors make degradation testing in standard shake flasks challenging. A high throughput and small scale method that can detect the bacterial growth on poorly water soluble compounds is needed.

The microtiter plate can provide high throughput assays with little substrate usage. Tetrazolium redox dyes have been used to evaluate the microbial use of hydrophobic compounds such as toluene and o-xylene (Strong-Gunderson et al. 1994), and polyaromatic hydrocarbons (Johnsen et al. 2002). In this study, hydrophobic compounds were dissolved in an organic solvent such as methanol and added to microtiter plate wells. After methanol evaporation, the hydrophobic compounds form a coat on the well walls. Then the inoculum and mineral salts were added to test the degradation by adding the tetrazolium redox dye to detect microbial respiration.

1.6 Research Objectives

Although NAs are considered to be the toxic component(s) in OSPW, the connection between composition and toxicity and/or estrogenicity has not been thoroughly investigated because of its complexity. Effect-directed analysis can narrow down the targeted components in the OSPW. In a previous study, the ISBR was demonstrated to achieve a 30%-40% NAs reduction based on GC/MS data (McKenzie et al. 2014). However, more information concerning the degradation and detoxification of OSPW in the ISBR is needed to improve the future treatment. The aims of the research presented in this thesis were as follows:

1) Establish which components in OSPW are associated with toxicity by effect-directed analysis (EDA)
2) Establish which components in OSPW are associated with estrogenicity by effect-directed analysis (EDA)

3) Determine which types of NAs are recalcitrant and which are more readily biodegradable by comparing the composition of OSPW with that of OSPW which has been treated by ISBRs

4) Assess growth on a range of aliphatic, cyclic and aromatic NA surrogates by bacterial cultures isolated from ISBRs

1.7 References

Alberta Environmental Protection and Enhancement Act (1993) Edmonton: Alberta Environmental Protection http://www.qp.Alberta.ca/documents/Regs/1993_118.pdf (cite date:2014-12-01)

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

Allen, E. (2008b). "Process water treatment in Canadas oil sands industry: II. A review of emerging technologies." Journal of Environmental Engineering and Science **7**(5): 499-524.

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

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

Bulich, A., M. Greene and D. Isenberg (1981). "Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents." Aquatic Toxicology and Hazard Assessment **4**(737): 338-347.

Burchfield, T. E. and L. G. Hepler (1979). "Some chemical and physical properties of tailings water from oil sands extraction plants." Fuel **58**(10): 745-747.

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

Colavecchia, M. V., S. M. Backus, P. V. Hodson and J. L. Parrott (2004). "Toxicity of oil sands to early life stages of fathead minnows (*Pimephales promelas*)." Environmental Toxicology and Chemistry **23**(7): 1709-1718.

Colavecchia, M. V., P. V. Hodson and J. L. Parrott (2006). "CYP1A induction and blue sac disease in early life stages of white suckers (*Catostomus commersoni*) exposed to oil sands." Journal of Toxicology and Environmental Health, Part A **69**(10): 967-994.

Cronin, M., J. Dearden and A. Dobbs (1991). "QSAR studies of comparative toxicity in aquatic organisms." Science of the Total Environment **109**(0): 431-439.

Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter and M. Waldock (1998). "Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening." Environmental Science & Technology **32**(11): 1549-1558.

ERCB, Energy Resources Conservation Board (ERCB). EnerFAQs 12: Oil Sands. **2011.** (citation date:2014-12-01) http://www.lica.ca/attachments/077_12%20Oil%20Sands.pdf

Fiehn, O., L. Vigelahn, G. Kalnowski, T. Reemtsma and M. Jekel (1997). "Toxicity-directed fractionation of tannery wastewater using solid-phase extraction and luminescence inhibition in microtiter plates." Acta Hydrochimica Et Hydrobiologica **25**(1): 11-16.

Foght, J., P. Fedorak, D. Westlake and H. Boerger (1985). "Microbial content and metabolic activities in the syncrude tailings pond." AOSTRA Journal of Research **1**(8): 139-146.

Grewer, D. M., R. F. Young, R. M. Whittal and P. M. Fedorak (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.

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

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

Hartnik, T., H. R. Norli, T. Eggen and G. D. Breedveld (2007). "Bioassay-directed identification of toxic organic compounds in creosote-contaminated groundwater." Chemosphere **66**(3): 435-443.

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

Headley, J. V., K. M. Peru, M. H. Mohamed, L. Wilson, D. W. McMartin, M. M. Mapolelo, V. V. Lobodin, R. P. Rodgers and A. G. Marshall (2012). "Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry characterization of tunable carbohydrate-based materials for sorption of oil sands naphthenic acids." Energy & Fuels **27**(4): 1772-1778.

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

Johnsen, A. R., K. Bendixen and U. Karlson (2002). "Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1." Applied and Environmental Microbiology **68**(6): 2683-2689.

Johnson, B. (2005). Microtox® acute toxicity test. Small-scale freshwater toxicity investigations. C. Blaise and J.-F. Férard, Springer Netherlands**:** 69-105.

Kannel, P. R. and T. Y. Gan (2012). "Naphthenic acids degradation and toxicity mitigation in tailings wastewater systems and aquatic environments: A review." Journal of Environmental Science and Health, Part A **47**(1): 1-21.

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

Klinkow, N., J. Oleksy-Frenzel and M. Jekel (1998). "Toxicity-directed fractionation of organic compounds in tannery wastewater with regard to their molecular weight and polarity." Water Research **32**(9): 2583-2592.

Lai, J. W. S., L. J. Pinto, E. Kiehlmann, L. I. BendellYoung and M. M. Moore (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.

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

MacKinnon, M., G. Kampala, B. Marsh, P. Fedorak and S. Guigard (2005). "Indicators for assessing transport of oil sands process-affected waters." IAHS Publication **297**: 71-80.

Madill, R. E., M. T. Orzechowski, G. Chen, B. G. Brownlee and N. J. Bunce (2001). "Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water." Environmental Toxicology **16**(3): 197-208.

McCormick, J. K. (2000). The effects of oil sands tailings on zooplankton communities in northern Alberta, PhD thesis, University of Waterloo.

McKenzie, N., S. Yue, X. Liu, B. A. Ramsay and J. A. Ramsay (2014). "Biodegradation of naphthenic acids in oils sands process waters in an immobilized soil/sediment bioreactor." Chemosphere **109**: 164-172.

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

Quagraine, E. K., H. G. Peterson and J. V. Headley (2005). "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 & Environmental Engineering **40**(3): 685-722.

Rajapakse, N., E. Silva and A. Kortenkamp (2002). "Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action." Environmental Health Perspectives **110**(9): 917-921.

Ramsay, B., D. Karamanev, S. Pierre, C. Lafontaine, T. Sakamoto and J. Ramsay (2001). "Efficient TCE mineralization in a novel methanotrophic bioreactor system." In Situ Aeration and Aerobic Remediation **6**(10): 171-178

Reinardy, H. C., A. G. Scarlett, T. B. Henry, C. E. West, L. M. Hewitt, R. A. Frank and S. J. Rowland (2013). "Aromatic naphthenic acids in oil sands process-affected water, resolved by GCxGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (*Danio rerio*) larvae." Environmental Science & Technology **47**(12): 6614-6620.

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

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

Siwik, P. L., T. Van Meer, M. D. MacKinnon and C. A. Paszkowski (2000). "Growth of fathead minnows in oil sand-processed wastewater in laboratory and field." Environmental Toxicology and Chemistry **19**(7): 1837-1845.

Strong-Gunderson, J. M. and A. V. Palumbo (1994). "Alternative method for rapidly screening microbial isolates for their potential to degrade volatile contaminants." Journal of Industrial Microbiology **13**(6): 361-366.

Toor, N. S., E. D. Franz, P. M. Fedorak, M. D. MacKinnon and K. Liber (2013a). "Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands." Chemosphere **90**(2): 449-458.

Toor, N. S., X. Han, E. Franz, M. D. MacKinnon, J. W. Martin and K. Liber (2013b). "Selective biodegradation of naphthenic acids and a probable link between mixture profiles and aquatic toxicity." Environmental Toxicology and Chemistry **32**(10): 2207-2216.

Wedekind, C. (2014). "Fish populations surviving estrogen pollution." BMC Biology **12**(1): 10.

Chapter 2

Literature Review

2.1 Oil Sands and Bitumen Extraction

Canada has the largest oil sands deposits in the world (Chan et al. 2012). Oil sands contain sand, bitumen, clay and water (Allen 2008). The total reserve of recoverable bitumen is 167.2 billion barrels; in 2013 Alberta's oil sands industry produced 357 million barrels from surface mining (Alberta Energy Regulator 2014). Syncrude Canada Ltd. and Suncor Energy Inc. are the two major oil sands companies that use surface mining methods that extract bitumen by Clark's hot water extraction (Mackinnon et al. 2005).

In Clark's hot water extraction, the surface mined oil sands are mixed with hot water (80-90 ℃) and NaOH (50-200 g ton^{-1} of oil sands) (Yen et al. 2004) to produce a slurry and separate bitumen by density differences. Recovered bitumen is sent to a refinery while the clay, sand and residual hydrocarbons are released in the water and stored in the tailings pond as oil sand process water (OSPW). Two to four m^3 of water per m^3 of oil is used and has resulted in 720 million m^3 of tailings water, covering an area of ~130 km^2 by 2011 (ERCB 2011). Under the Alberta Environmental Protection and Enhancement Act (1993), the OSPW must be held on site (Madill et al. 2001). Detoxification of the OSPW is required to reduce tailings volume and reclaiming the disturbed lands.

2.2 Oil Sands Process Water (OSPW)

2.2.1 General Characterization of OSPW

OSPW composition varies with ore quality, extraction methods and storage times. Generally, OSPW is moderately hard (15–25 mg L^{-1} Ca^{2+}, 5–10 mg L^{-1} Mg^{2+}) with a pH of 8.0–8.4 and an alkalinity of ~800–1000 mg L^{-1} HCO_3^- (Burchfield et al. 1979). Organic compounds detected in

tailing pond waters include bitumen, naphthenic acids, asphaltenes, benzene, phenols, and polycyclic aromatic hydrocarbons (PAH) (Allen 2008). Although there are many different classes of compounds that could be toxic, the polar acidic fraction of the dissolved organic matter had been tentatively identified as the main toxic components (MacKinnon et al. 1986, Lo et al. 2006). Eighty percent of the dissolved organic acids are naphthenic acids (NAs) (Nelson et al. 1993) and their natural attenuation half-life is 12.8 to 13.6 years (Han et al. 2009). Because of the potential toxicity and the persistence of the NAs, they are of particular concern.

2.2.2 Naphthenic Acids (NAs)

NAs are defined as a mixture of naturally occurring alicyclic and alkyl-substituted carboxylic acids with the structural formula $C_nH_{2n+z}O_x$. Classical NAs (c-NAs, x=2) and oxidized NAs (oxy-NAs, x =3-5) comprise less than 50% of the acid extractable organics (AEO) from OSPW (Grewer et al. 2010). NA compounds include alicyclic and aromatic carboxylic acids while non-NA AEO may also contain sulfur, nitrogen, and other elements with up to 10 O groups (Headley et al. 2012). In alicyclic NAs, "z" reflects the degree of cyclicity (Han et al. 2008). However, hydrogen deficiency may also be due to aromaticity (Reinardy et al. 2013). Aromatic NAs are also included in the general chemical formula. The "x" is the number of oxygens and ranges from 2-5 (Clemente et al. 2005). Typical O2-NAs structures are shown in Figure 2-1.

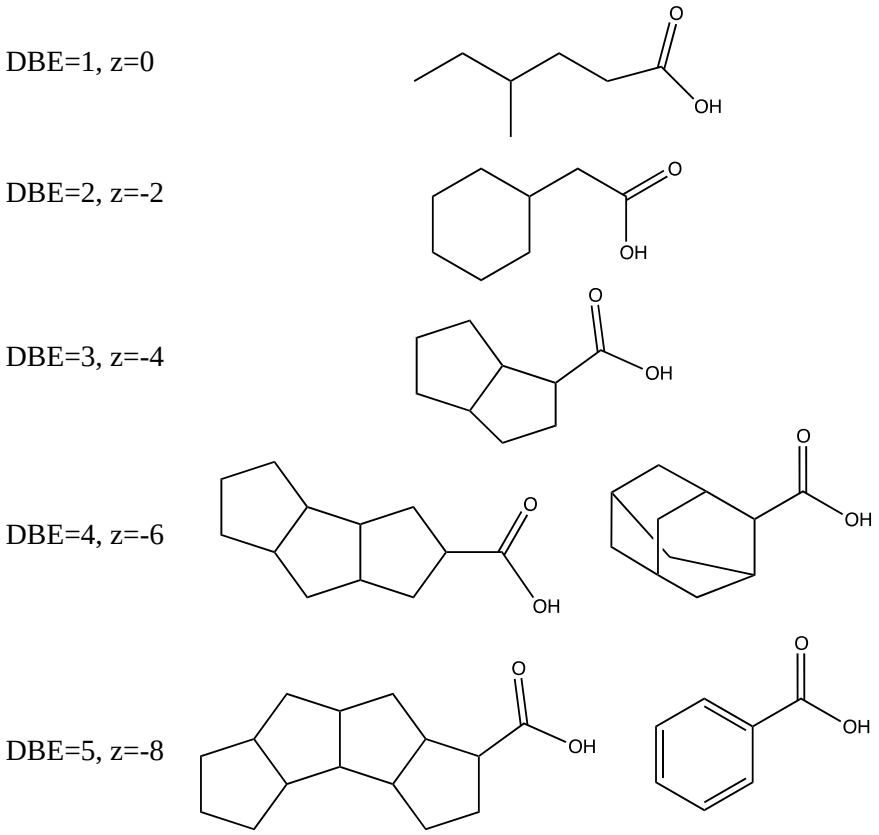

Figure 2-1 Possible NAs structures of DBE groups

Commercial NAs mixtures have been prepared by caustic extraction of petroleum distillates which boil at 200–370 ℃ and have numerous applications including wood and textile preservation, as well as promoting vulcanization of rubber used to manufacture tires (Brient et al. 2000). Some studies used commercially prepared NAs mixtures (Kodak Salts and Merichem) to study biodegradation (Biryukova et al. 2007, Videla et al. 2009), although the Fourier transform infrared spectroscopy (FTIR) spectra of commercial NAs and the NAs extracted from OSPW were found to be very similar, numerous studies have shown that the OSPW NAs are more complex, more recalcitrant (Scott et al. 2005), and they differ in toxicity (West et al. 2011).

Some model NAs were synthesized to better understand the relationship between structure and toxicity of NAs, and also the biodegradation pathways of NAs. Generally, the model NAs are used as NA surrogates that can be classified into groups including aliphatic n-acids,

monocyclic acids, bicyclic acids, tricyclic acids, tetracyclic acids, monoaromatic acids, and polycyclic aromatic acids.

2.2.3 Analysis of NAs

The concentration of NAs in petroleum are often referred to as total acid number (TAN), which is determined by titration with KOH (Brient et al. 2000) and expressed as mg KOH g^{-1} sample. However, TAN only provides information about the acidity.

FTIR spectroscopy is the industry's standard method to quantify NAs. Based on Syncrude's standard analytical method using FTIR spectroscopy, the concentration of NAs in OSPW ranged from 20-150 $mg \cdot L^{-1}$ (Holowenko et al. 2001,Yen et al. 2004, Martin et al. 2008). In the FTIR quantification method, suspended solids are removed by a 0.45 μm filter, acidified to pH 2.5 and extracted by dichloromethane (DCM). The spectrum is obtained from 1850 to 1650 cm^{-1} and compared to a standard curve of Kodak standard P2388 for quantitative analysis. Because carboxylic acids have an absorbance peak between 1740 to 1750 cm^{-1} for monomers and 1700-1715 cm^{-1} for dimers, peak heights between 1743 and 1706 cm^{-1} are summed and compared with the standard to calculate the NA concentration in OSPW (Holowenko et al. 2001). This method also detects natural carboxylic acids, and thus overestimates the NA concentration and generates no composition data (Scott et al. 2008).

Mass spectrometric techniques have been also used to analyze NAs. Both GC and HPLC chromatographic methods were applied for partial separation. For example, a gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) technique was applied to characterize a commercial NA mixture (St. John et al. 1998) and has since been widely used (Clemente et al. 2004a, Del Rio et al. 2006, Chen et al. 2010). In this method, 9-fluorenecarboxylic acid (9-FCA) dissolved in DCM is used as a surrogate standard. It is added to aqueous samples, and the solution is acidified to pH 2 and extracted with DCM. The carboxylic acids are derivatized into their tertbutyldimethylsilyl esters by adding N-methyl-N-(t-

butyldimethylsilyl) trifluoroacteamide (MTBSTFA). After fragmentation, NAs produce $[M+57]^+$ ions, where M is the mass of the NAs. The peak integration of ion chromatograms for m/z = 267 (NA: $C_{13}H_{22}O_2$) is used to calculate the presence of NAs by comparison to the Merichem commercial NAs (8% of $C_{13}H_{22}O_2$). GC-EI-MS is more selective than FTIR, but the assumption of similarity in the $C_{13}H_{22}O_2$ content between commercial NAs and OSPW NAs causes problems in quantification.

Although GC-EI-MS has been widely used, the complexity of the NAs makes an unresolved hump in the chromatographic profile. The use of low-resolution mass spectrometry with derivatization in GC/MS analysis can lead to misclassification of NAs. The application of HPLC coupled with high resolution MS has shown that double-derivatized hydroxylated NAs could be mistaken for high molecular weight NAs (n>22) and the fragmentation of [naphthenate +dimethylsilyl]$^+$ ions may be mistaken as low molecular weight NAs (Clemente et al. 2004a, Bataineh et al. 2006). HPLC with high resolution MS has the advantage over GC/MS because there is no need for derivatization. This approach has revealed the presence of C-81 and C-82, 7- and 8-ring analogs, which show the importance of HPLC in NA separation and analysis (Smith et al. 2007).

Direct analysis without derivatization requires soft ionization that can produce little fragmentation; atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) can provide molecular or pseudo-molecular ions to determine the molecular distribution of NAs. Hsu et al. (2000) described the negative-ion and positive-ion APCI application in NA characterization. Negative-ion APCI using acetonitrile as the solvent is a good choice for NAs but the interfering background ions were hard to eliminate (Hsu et al. 2000). Lo et al. (2006) compared APCI-MS with ESI-MS in the analysis of OSPW NAs and NA surrogates, which showed that APCI-MS had a wider range for the quantification of NAs, but it may form $[M+32]^-$ that were found in NA surrogates APCI-MS spectra. Additionally, compared to ESI-MS, the

signal-to-noise ratio of APCI-MS was relatively lower and the detection limit was poorer (Lo et al. 2006). ESI seems to be most widely used for environmental sample analysis due to minimal fragmentation, less adduct formation and greater sensitivity (detection limit: 0.01 mg L^{-1}) (Headley et al. 2002).

Two-dimensional gas chromatography (GC×GC) has also been used to improve the chromatographic peak separation of NAs. Hao et al. (2005) used GC×GC-MS to analyze commercial and OSPW NAs as methyl esters. Specific patterns were found for acyclic and monocyclic NAs and future development of congener-specific analysis of some NAs may be possible (Hao et al. 2005). However, interpretation of EI-MS data remains a challenge due to the lack of individual authentic NAs.

2.2.4 Identification of Individual Naphthenic Acids

Although ultrahigh resolution MS can determine a NA formula with high confidence and tandem MS could provide some structural information, numerous isomers make the identification of individual NAs a big challenge. Rowland et al. (2011a) applied GCxGC, coupled with time of flight mass spectrometry (TOF-MS) to study methyl ester derivatives of OSPW acid-extractable compounds. By comparing to reference acids, they identified a number of diamondoid tricyclic acids, including adamantine-1-carboxylic acid and 3-ethyl adamantine-1-carboxylic acid (Rowland et al. 2011a). Some tetra- and penta-cyclic such as diamantine-1-carboxylic acid and diamantine-3-carboxylic acid were also identified (Rowland et al. 2011a). Because of the known estrogenic effect of the OSPW, the presence of the steroidal aromatic NAs is a concern. Synchronous fluorescence spectra of OSPW suggested the possible presence of aromatic carboxylic acids and GCxGC-TOF MS showed the potential existence of C19 and C20 C-ring monoaromatic hydroxy steroid acids. Ultraviolet absorption spectra also indicated benzenoid moieties (Rowland et al. 2011b). The possible steroid-like components in OSPW may be

potential estrogenic components but confirmation of these structures needs the synthesis of the actual compounds.

2.3 Toxicity of OSPW

2.3.1 Acute Toxicity

According to previous chemical analysis of OSPW, it is high in NAs and dissolved inorganic ions, and has a low concentration of PAHs, and some metals (Allen 2008). As OSPW is stored in tailing ponds, some organisms may directly be affected, such as microorganisms, aquatic plants (Armstrong et al. 2008), invertebrates (McCormick 2000), and fish (Colavecchia et al. 2004, Colavecchia et al. 2006).

The Microtox® acute toxicity assay is a widely used standard toxicity test. It employs a bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism (Johnson 2005). The bacteria are exposed to a range of concentrations of the material being tested. The reduction in light emitted from the bacteria is related to the inhibition of respiration, and can be measured against control samples. The dose-response relationship is generated from a plot of the amount of light emitted versus the toxicant concentration. The results are normalized and the EC_{50}, the concentration that reduces 50% of light intensity, is calculated (Johnson 2005). A low EC_{50} means high toxicity. Besides being convenient and fast, many studies and published data have compared the Microtox® assay with toxicity values to fish (Bulich et al. 1981), crustaceans (Cronin et al. 1991), and algae (Cheung et al. 1997) for a wide range of organic, and inorganic chemicals with excellent correlation (Klinkow et al. 1998). The toxicity assays with eukaryotes are expensive and time consuming, and are not suitable for large-scale analysis or regular monitoring. The Microtox® thus became a standard toxicity assay for OSPW, and surrogate NAs (Jones et al. 2011, Mistiti et al. 2012, Toor et al. 2013).

Among NA compounds, different structures are associated with widely varying toxicities. Previous Microtox® assays on OSPW, on commercially available NAs, and NAs extracted from OSPW are summarized in Table 2-1, which show that the Microtox® toxicity (EC_{50}) were not correlated with the total NA concentration. Holowenko et al. (2002) tested the acute toxicity of OSPW from nine sources with total NA concentration ranging from 24-68 mg L^{-1}, but the EC_{50} of MLSBF (NAs: 49 mg L^{-1}) was 32 while the Suncor 1 and Suncor 5 with higher NAs concentrations were much less toxic. Commercial NA mixtures were also different in toxicity compared to OSPW NAs that may result from their differences in the chemical compositions (Clemente et al. 2004a, Herman et al. 1994).

Additionally, the reduction of Microtox® toxicity did not correlate with the total NA concentrations observed in previous studies using wetlands (Toor et al. 2013). Jones et al. (2011) conducted Microtox® assays on 35 model NAs that have different structures, and determined that the EC_{50} of individual NAs varied from 0.004 to 0.784 mM (Jones et al. 2011). Other studies have shown that NAs with lower molecular weight were more toxic to *V. fisheri* but degraded faster, while NAs with higher molecular weight, such as high carbon number NAs with multi-ring structures, were less toxic but degraded slower (Holowenko et al. 2001, Lo et al. 2006).

Table 2-1 The Microtox® toxicity assay results of OSPW, fractions of NAs, and NAs (CT wetland - Composite tails wetland)

Sample	**Description of sample including fractionation methods used for some samples**	**NAs (mg L^{-1})**	**EC_{50}(volume percent)**	**Reference**
MLSBF	fresh tailing release waters	49	32	Holowenko et al. 2002
MLSB7	OSPW has naturally aged for 7 years	36	100	
Pit 5	Mature fine tails under naturally aged for 11 years	24	100	
Suncor 1	OSPW treated with gypsum	68	64	
Suncor 2	Runoff water	24	100	
Suncor 3	Runoff waters(dyke seepage waters)	52	100	

Suncor 4	Outlet flow from CT wetland	42	100	
Suncor 5	Waters from wetlands receiving a mixture of dyke seepage water, CT release water, and natural runoff waters	55	98	
Suncor 6	OSPW from settling basin used to water cap mature fine tailings pond (south pond, filled in 1991)	44	100	
Kodak salts	Kodak NAs sodium salts	98	10-13%	Clemente et al. 2004a
Merichem NAs	Merichem NAs mixture	102	10%	
TEX	tailing water extract	1:50(21mg L^{-1} dissolved carbon)	43	Herman et al. 1994
		1:20(50mg L^{-1} dissolved carbon)	25	
Kodak salts	Kodak NAs sodium salts	100	30	
130 ℃ Fraction	Fractionation of the methylated NA mixture at distillation temperatures of 130, 160, 190, and 220 ℃ and demethylation. The methylated NAs that distilled at each temperature step were condensed in a collection vessel over ice water.	141.4	41.9 ± 5.5	Frank et al. 2008
160 ℃ Fraction		167.6	58.1 ± 9.0	
190 ℃ Fraction		162.9	43.5 ± 7.4	
220 ℃ Fraction		153.7	54.7 ± 9.6	
>220 ℃ Residue		151.8	64.9 ± 14.5	
NAs stock	HCl precipitation, dissolved in methanol and dried	176.1	52.7 ± 7.8	
Particulate NAs	Fractionation on the basis of solubility did not differ significantly in congener distribution		37	Lo et al. 2006
Aqueous NAs			35	
Fraction 1 pH=8	Fractionation by anion exchange		34	
Fraction 3 pH=5			48	
Fraction 8 pH=4			82	
Fresh fine tailings			20-40	Madill et al. 2001
Fraction I	Neutral/base fraction	200 × concentrated	48	
Fraction II	Acid organics fraction	200 × concentrated	NO survival	
	10 × dilution	21 × concentrated	19	
	25 × dilution	8 × concentrated	58	

The toxicity of OSPW components has been related to molecular structure. NAs with longer alkyl chains and fewer carbon rings were predicted to have greater toxicity using United States

Environmental Protection Agency (US EPA) Ecological Structure Activity Relationships (ECOSAR) quantitative structure activity relationship (QSAR) model (Frank et al. 2010). QSAR models have shown that NAs have a narcosis mechanism of action (MOA) in the acute toxicity of *V. fisheri* (Cronin et al. 1997). Theoretical QSARs can be established based on narcosis and toxicity data (EC_{50}) to *V. fischeri* (Zhao et al. 1998). Zhao et al. (1998) constructed the equation: $\log EC_{50}$ (M) = -0.79 $\log K_{mw}$ - 1.54 (where K_{mw} is the membrane vesicle-water partition coefficient). The K_{mw} for nonpolar and polar compounds is different, for nonpolar compounds: $\log K_{mw}$= 1.05 $\log K_{ow}$ - 0.32, and for polar compounds: $\log K_{mw}$= 0.90 $\log K_{ow}$ + 0.521 (Vaes et al. 1997). This suggests that increasing hydrophobicity is correlated to higher toxicity in general. A correlation plot is generated based on hydrophobicity and toxicity of NAs as shown in Figure , which compares the toxicity data ($\log EC_{50}$) (data from Jones et al. 2011) of model NAs with their hydrophobicity ($\log K_{ow}$).

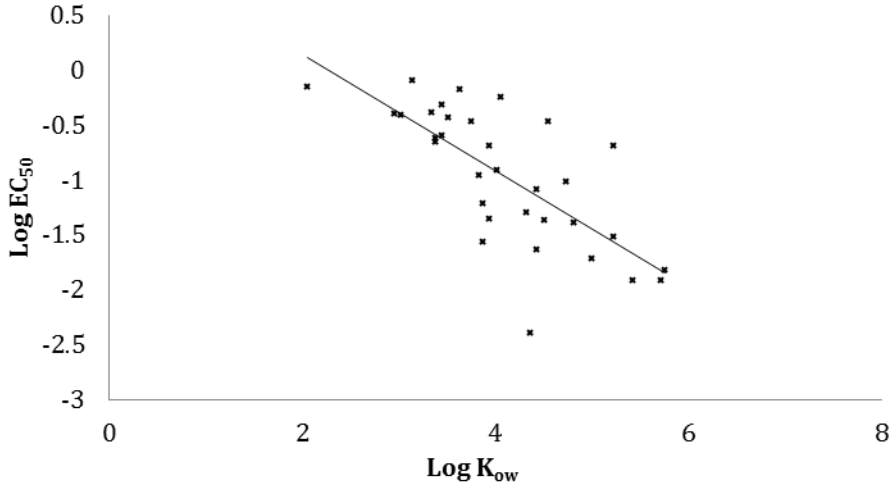

Figure 2-2 Relationship between model NAs toxicity in *Vibrio fisheri* (EC_{50}) and hydrophobicity ($\log K_{ow}$) (data from Jones et al. 2011)

As shown in Table 2-1, Madill et al. (2001) fractionated fresh tailings by precipitation based on pH into neutral/base fraction and acid fractions, the acute toxicity (as determined by Microtox®) of the acid fraction was 200 times more than that of the neutral/basic fraction. Lo et

al. (2006) used acid/base fractionation and anion exchange chromatography to separate the components of OSPW based on pKa differences and also found that the acid fraction was more toxic. OSPW is a complex mixture, but its acute toxicity was mostly attributed to NAs even though the total NA concentration does not seem to correlate to acute toxicity, as shown in the Table 2-1.

2.3.2 Chronic Toxicity and Endocrine Disrupt

NAs may contribute to an increase of relative liver weight, hepatic glycogen and blood amylase, and decrease hypochlosterolemia in rodents (Rogers et al. 2002). While acute toxicity seems to decrease significantly after 1-2 years in wetland, chronic toxicity may not be completely removed (Toor et al. 2013).

The assessment of endocrine disruptive effects leading to feminization of the fish is also important. An "endocrine disruptor" can mimic the function of a natural hormone then cause various negative health effects. "Endocrine disruptors" have been associated with degenerative effects such as reduced fertility, changes in the sex ratio, impacts on fish population, developmental degeneration in both animal and human embryos (Cardin et al. 1994, Guillette et al. 2000, Knorr and Braunbeck, 2002), as well as an increase in breast and testicular cancer in humans (Carlsen et al. 1995, Colborn et al. 1993). Kidd et al. (2007) used a low concentration (5-6 ng L^{-1}) of the synthetic steroid (17α-ethynylestradiol) at the "experimental lakes" area in Ontario and caused the near extinction of fathead minnows within two years. Endocrine disruptive effects, such as an estrogenic effect, have rarely been used in the standard toxicity tests of OSPW. Estrogen can specifically bind to the receptor and activate the transcription of target genes. A compound with estrogenic activity may exert its effects through several mechanisms, such as promotion of estrogen-dependent processes through binding to the nuclear estrogen receptor (ER). Estrogenic compounds can bind to and activate the transcription factor estrogen receptor by dissociating the heat shock protein complex and then form a ligand:receptor dimer.

The dimer interacts with estrogen response elements (EREs), which are located in the regulatory region of estrogen-inducible genes, resulting in increased gene transcription and synthesis of proteins to express hormonal action (Joyeux et al. 1997), which then disrupts the hormone system of vertebrates and invertebrates. Exposure to estrogens may lead to viability reduction, gonadal malformations or feminization of genetic male fish (Wedekind 2014).

OSPW was found to be estrogenic and anti-androgenic using the human H295R cell line (He et al. 2010), and goldfish (Lister et al. 2008). After goldfish were exposed to OSPW, *in vitro* production of testosterone and 17β-estradiol (E2) significantly decreased (Lister et al. 2008). Siwik et al. (2000) reported that after exposure to OSPW tailings pond water, fathead minnows took significantly longer to produce their first eggs, and the amount of eggs was half that at a reference site. Reinardy et al. (2013) showed that aromatic NAs extracted from OSPW had a weak estrogenicity. Although xenoestrogens may have a much lower estrogenicity (by a factor of 10^4-10^5) compared to the natural hormone, 17β-estradiol, the combined additive effect can dramatically enhance steroid hormone action (Rajapakse et al. 2002). Previous studies implicate steroidal aromatic NAs as possible compounds which cause estrogenicity, but the exact components in OSPW that are responsible for the estrogenic effect have not yet been confirmed (Rowland et al. 2011b).

2.3.3 Effect of Mixtures on Toxic Modes

Environment samples are usually contaminated with a variety of toxicants. In those mixtures, the effects can be different depending on different toxic modes. The probable acute toxicity mode of NAs is narcosis (Frank et al. 2008, Frank et al. 2010). Narcosis is a nonspecific toxicity mode that disturbs the membrane integrity and function, and occurs if the effective concentration is achieved. Usually the effect of mixtures is additive (Niederlehner et al. 1998).

An estrogenic effect is a receptor-mediated mechanism, which is mediated by the estrogenic compound binding to the estrogen receptor and may occur at low concentrations (Gaido et al. 1997) and may be prone to interactive effects in mixtures. Arnold et al. (1997) found that synergism of estrogens may be involved in the reproductive process. Rajapakse et al. (2002) tested 11 xenoestrogens below their individual no-observed-effect concentration and 17β-estradiol. They found the arithmetic sum of their estrogenicity led to dramatic underestimation, about 50% of observed estrogenicity that produced by the mixture.

2.4 Effect-Directed Analysis

2.4.1 General Scheme of Effect-Directed Analysis

Millions of kilograms of toxicants are released into the environment to form complex mixtures. Detection and characterization of these complex mixtures is an essential part of understanding their potential toxicity, however, the ecological relevance of contaminants as mixtures is difficult to assess, because the generally applied approach for risk assessment is based on targeted chemical analysis and individual toxicity data. Non-targeted screening is time-consuming, and relatively expensive (Bester et al. 1998). Not all compounds in the environment have toxicity data and possible interactions in a mixture, antagonism and synergism for example, is unknown. We need to identify the components that are responsible for the observed toxic effects to establish the causal link between specific chemicals and the toxic effect. Effect-directed analysis involves fractionation and is based on toxicity test results. It is used to reduce the complexity of analysis. The goal is to separate the compounds responsible for toxicity from those that are not causing toxicity before carrying out chemical analysis. In targeted analysis, the highly toxic but less bioavailable (i.e. very hydrophobic) compounds may be misleading and the more bioavailable toxicants may be ignored. Compared to two conventional wastewater analyses, target-chemical analysis and non-target analysis, effect-directed fractionation analysis avoids the

bias in target-chemical analysis and the lack of guidance in non-target analysis. Standard target-chemical analyses can only detect chemicals that have toxicity data and cannot reflect the interaction effects in the mixture while non-target analysis has a broader range of compounds but the results are difficult to interpret. Effect-directed analysis has been successfully applied to tannery wastewater (Klinkow et al. 1998), marine surface sediment (Kammann et al. 2005), multi-contaminated sediment (Creusot et al. 2013), creosote-contaminated groundwater (Hartnik et al. 2007), and industrial wastewater (Reemtsma et al. 1999).

On the whole, effect-directed fractionation analysis is comprised of chemical analysis and toxicity testing, but is not done in parallel. The general steps are as follows: 1) concentrate active components of sample; 2) fractionate the sample, then based on the results of the bioassay(s) to further fractionate the bioactive fraction(s) and at the same time analyze the composition of the bioactive fraction(s) to determine the most probable contributors to the total toxicity; 3) confirm tentatively the identified toxicants to establish a cause-effect relationship. The biological effect directs all steps of the analytical process, from sample selection, extraction, and fractionation to the identification.

In the remediation of contaminated tailing ponds, it is useful to confirm the target contaminants in order to develop a suitable treatment plan. As discussed above, OSPW is a complex mixture. Chemical analysis alone cannot provide toxicity data while toxicity tests can only give a generic response of all the compounds present in OSPW. By combining a fractionation procedure, toxicity tests, and chemical analyses, it is possible to generate links between toxicity and OSPW components.

2.4.2 Fractionation to Reduce the Complexity of Mixtures

Mixtures can be fractionated by molecular weight, fugacity, hydrophilicity, pH or reactivity. Methods that can be used include acid/base/neutral partitioning and fractionation based on

normal/reverse phase HPLC, molecular weight, ion-exchange chromatography, etc. Because narcosis is related to the hydrophobicity, it is common to fractionate samples based on hydrophobicity. Hydrophobicity levels are expressed numerically using K_{ow}. K_{ow} is the octanol/water participation coefficient and measures differential solubility of a compound between octanol and water (Mitra et al. 1977, Huwyler et al. 1990, Allen et al. 1992). However, toxicity assessment cannot be based solely or simply on the separation of hydrophobic components. In bioassay-directed fractionation schemes, liquid-liquid extraction (LLE) can be used to separate compounds using two immiscible liquids such as octanol and water but has been proven to be unsuccessful (Metcalfe et al. 1988) as complete solvent removal is needed since the solvents themselves may be toxic. Artificial toxicity may also occur because of the loss of volatile toxicants during LLE (Burkhard et al. 1991). Due to the limitations of biological detection systems that are sensitive to the toxicity of LLE organic solvents, traditional LLE with water immiscible organic solvents has been rarely used for toxicity-directed effluent analysis (Schuetzle et al. 1986).

The use of solid media and water-soluble solvents avoids the introduction of toxicity during fractionation. Solid phase extraction (SPE) uses water-soluble, nontoxic solvents. SPE using C18 column is recommended in the extraction of compounds with $K_{ow} > 2$ (Ho et al. 1993) but is not suitable for polar wastewater constituents. Use of C18 or C8 phases is limited to a small range of hydrophobic compounds (Amato et al. 1992). Recent studies have used polystyrene-divinylbenzene polymers as the solid phase to extract a wider range of hydrophobic compounds. A combination of four sorbents greatly improved the recovery rate in extracting polar organics from large volumes of samples (Fiehn et al. 1996). First, the samples were passed through C18 cartridges at pH 7 to extract neutral to slightly basic hydrophobic organic compounds. In the next step, (aromatic) compounds with a little higher polarity such as simple phenols or carboxyindoles were extracted with EnviChrom at pH 7. After acidification to pH 4.5, the samples were extracted

with LiChrolut EN to extract even more polar compounds such as hydroxybenzoic acids. The fourth step was an extraction with LiChrolut EN-sorbents at pH 2.5, where highly polar compounds containing several acidic functionalities were retained.

Fractionation based on HPLC involves the use of normal phase, reverse stationary phase, and a combination of phases. Normal phase HPLC typically uses cyanopropyl as the sorbent to fractionate extracts according to their polarity (Demarini et al. 1992, DeMarini et al. 1996, Marvin et al. 2007). Demarini et al. (1992) used cyanopropyl-bonded silica in HPLC for partitioning organic extracts from incinerator emissions and determined mutagenicity of the fractions by a micro-suspension mutagenicity assay with *Salmonella* TA98.

Environmental samples usually have relatively low levels of contamination, so high enrichment factors and sufficient fractionation is required to make a causal relationship between compounds at low concentrations and toxicity. Therefore, reverse phase (RP) procedures with less separation power have been applied instead of normal phase separation (Reemtsma et al. 1999, Thomas et al. 1999, Reineke et al. 2002). Reineke et al. (2002) used SPE followed by RP-HPLC fractionation and subfractionation to reduce the complexity of Elbe river surface water contaminants. By applying a luminescent bacteria test for acute toxicity and GC-MS analysis of the respective fractions, they identified several toxicants including pesticides and pharmaceuticals.

Thomas et al. (1999) used RP-HPLC to isolate causal compounds according to the Crustacea (*Tisbe battagliai*) toxicity test. To obtain a reduction in the complexity of the extracts, SPE was conducted, followed by fractionation by RP-HPLC and candidate toxic contaminants were identified by GC-MS. Several compounds which have not been traditionally selected for monitoring, such as chlorinated phenols and alkylphenol surfactant metabolites, were successfully identified as the probable cause of detrimental environmental effects (Thomas et al. 1999). In Snyder et al (2001), water samples from three municipal wastewater treatment plants in south

central Michigan, four point source locations on the Trenton Channel of Detroit River and five locations in Lake Mead were analyzed. They were first extracted using SPE, then separated into three fractions based on polarity using normal phase HPLC. Associated with the *in vitro* bioassay based on a bioluminescent MCF-7 (Michigan Cancer Foundation-7)-derived cell line (called MVLN) for estrogenic receptor (ER) agonist potency, fractions contain ER agonist were further fractionated using RP-HPLC. According to their results, the F1 fraction that contained nonpolar compounds, such as PAHs and polychlorinated biphenyls (PCBs), did not show a significant response in the MVLN assay. The fractions that showed greatest ER mediated responses were further fractionated by RP-HPLC and the analysis showed that the major estrogencity was associated with two known chemicals: endogenous estrogen (E2) and synthetic estrogen ethynylestradiol (EE2). This bioassay-directed fractionation results supported that the E2 and EE2 were the dominant environmental estrogens in water samples from mid-Michigan and Lake Mead.

2.4.3 Fractionation of OSPW

Previous studies have fractionated OSPW. For example, Beg et al. (2001) fractionated OSPW based on polarity. Samples were first extracted with pentane then NaOH, to separate into two fractions: organic (neutral and basic) and aqueous (acidic) fractions. The organic fraction was further partitioned with HCl to obtain neutral and basic fractions. From the results of the Microtox® conducted on the three fractions, the neutral fraction contained the most toxic components (Beg et al. 2001). Frank et al. (2008) fractionated methylated NAs based on boiling point, which showed slight differences among fractions (Table 2-1). Lo et al. (2006) used acid/base/neutral partitioning and anion exchange chromatography to fractionate the OSPW. In anion exchange chromatography, they separated NA mixtures over a range of pH 8-4, eluting NA fractions on the basis of pKa differences to get eight fractions with which to conduct Microtox®

acute toxicity and GC/MS analysis. EC_{50} values of Fractions 1, 3, and 8 by the Microtox® acute toxicity assay were 34, 48 and 82 mg L^{-1}, respectively. The acid fraction was 200 times more toxic than the neutral/base fraction (Table 2-1). Combined with the congener structure analysis by GC/MS, it seems that fractions having a higher proportion of multi-ring structures exhibited less toxic potency. Argentation SPE was used to separate non-alicyclic (e.g. aromatic) acidic compounds of methyl esters of the acids of OSPW (Jones et al. 2012). This led to the conclusion that the higher molecular weight aromatic acid fraction was more toxic than alicyclic acids (Scarlett et al. 2013) and that the aromatic acid fraction was weakly estrogenic because of increased vitellogenin production in zebrafish (*Danio rerio*) larvae (Reinardy et al. 2013).

2.4.4 Selection of Appropriate Toxicity Methods

To select an appropriate toxicity test, the primary concern is the scale of the test. Large-scale assays can be very laborious and require large volumes to be processed, especially when multiple levels of fractionation are to be conducted. Because of the small volume of fractions and large number of samples, microscale assays are the best choice. For example, luminescence inhibition of *V. fischeri* is advantageous because of its sensitivity, rapidity, reproducibility, and small sample volume required. Microtox® employs the bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism. The bacteria are exposed to a range of concentrations of the material being tested. The reduction in light emitted from the bacteria is related to the inhibition of respiration and can be measured against control samples. The dose-response relationship is generated from a plot of the amount of light emitted versus the toxicant concentration. The results are normalized and the EC_{50}, the concentration that reduces 50% of light intensity, is calculated. There are many comparison studies and published data assessing the Microtox® assay with toxicity values to fish (Bulich et al., 1981), crustaceans (Cronin et al., 1991) and algae (Cheung et al., 1997) for a wide range of organic and inorganic chemicals with excellent correlation

(Klinkow et al.,1998). When combined with SPE-HPLC fractionation, Reemtsma et al. (1999) identified that benzothiazoles was the major contributor to the toxicity of tannery wastewater.

Another concern in toxicity testing is the mode of toxicity that can be directly analyzed by microscale toxicity tests. For example, the estrogenic effects of chemicals can be measured by the estrogen receptor (ER)-mediated activity using recombinant yeast cells instead of observing the hormone disruptive effects in the whole live organisms (Heisterkamp et al. 2004). One such test uses yeast in which an estrogen-responsive element containing plasmid has been introduced (Gaido et al. 1997) or integrated into the yeast chromosome (Routledge et al. 1996).

Modifications were made to enhance the level of detection by using chlorophenol red-β-D-galactopyranoside (CPRG) as the target substrate instead of o-nitrophenol-β-D-galactopyranoside (ONPG) as CPRG is reported to be ten times more sensitive than ONPG (Eustice et al. 1991). Recombinant yeast have been modified to link estrogen-responding elements to the *lac*Z gene, which codes for β-galactosidase. Estrogenic substances, which bind to the estrogen receptor will bind to the estrogen responding elements and result in β-galactosidase production. The amount of β-galactosidase produced is quantified after the cells have been made more permeable with sodium dodecyl sulfate to allow the enzyme to react with CPRG (yellow color) to form galactose and cholorophenol red (red color quantified at 562 nm). There is a direct correlation between β-galactosidase activity and estrogenic effect. Estrogenic activity expressed as estradiol equivalency can be determined by interpolation from E2 standard curves.

2.4.5 Pattern Recognition

A mass spectrum of OSPW usually contains thousands of m/z ratios which mean thousands of measurements or variables in one sample, generating “high-dimensional” data. It is hard to discriminate samples when there are so many variables. Techniques that can select fewer discriminative variables to describe differences among samples become important. Chemometrics

provides several tools for pattern recognition and has found success in reducing the dimensionality of the data while still preserving most of the relevant information.

To get an unsupervised view of the data, principal component analysis (PCA) can be used to extract the systematic variation in the multivariate dataset, reduce the data dimensionality, and obtain a preliminary visualization of the samples (Wiklund et al. 2008). PCA has been widely used among biologists, geologist and chemists since the 1970s. It is a data reduction method that can transform a multi-dimensional interrelated data into a much lower dimensional data set while retaining most of the information. PCA can determine how similar sample groupings are based on their variables, and identify the important variables without giving any classification data. The PCA results are usually described as "scores" and "loadings". The scores are the original data representation in the new coordinate system and the loadings are the original data's weights.

Partial least squares (PLS, also known as projections to latent structures) regression is a method to model data sets and estimate the parameters in the models. The partial least squares regression-discriminant analysis (PLS-DA, also known as projections to latent structures discriminate analysis) is a variant of PLS and can be used for classification of samples. It is possible to determine the important discriminate variables that can separate different sample groups. Compared to unsupervised methods such as PCA, the classification of the samples is given to better reveal the between-group variability. In PCA and PLS-DA, a plot of 2D or 3D scores identifies the separation between different groups.

PCA/PLS-DA allows analysis of the similarities and differences in toxicity relative to the chemical components, explaining the profile-response relationship. Both PCA and PLS-DA can be applied to analyze the similarities and differences of toxicity relative to the chemical composition of samples, and furthermore to reveal the potential toxicants in a mixture. For example, they have been used successfully to determine the relationship between composition

and toxicity of motor vehicle emission (McDonald et al. 2004) and found that the nitro-polycyclic aromatic hydrocarbons of diesel exhaust particles cause mutations in bacteria (Eide et al. 2002).

2.5 Treatment of OSPW

Because of the zero discharge policy requirements that industry has not been able to meet, OSPW is held in tailings ponds. Various methods have been tested in the lab to treat OSPW. Physical and chemical treatments such as adsorption (El-Din et al. 2011), membrane filtration (Peng et al. 2004), ozonation (Anderson et al. 2012) and advanced oxidation (Liang et al. 2011) have been applied to investigate degradation and detoxification. Other physical methods include settling, centrifugation (MacKinnon et al. 1986), and freezing (Gao et al. 2004). Settling and centrifugation cannot remove dissolved pollutants and thus have demonstrated little effect on reducing the toxicity (MacKinnon et al. 1986). Absorbents such as activated carbon, zeolites, clays and synthetic polymers can remove a variety of pollutants, including naphthenic acids, oil, and heavy metals (Allen, 2008). Membrane filtration can achieve 95% removal of NAs (Peng et al. 2004) but the cost and disposal can be problematic because of the huge volumes involved. Micro-, nano-, and ultrafiltration are able to remove pollutants and toxicity efficiently (Mehrotra et al. 1986, Peng et al. 2004) but fouling problems can arise. Spray freezing uses ice crystals to reject impurities (or contaminants) from the ice structure and leave the concentrated contaminants in the remaining liquid phase (Gao et al. 2004) and have resulted in 40-50% reduction in total organic carbon (TOC).

Chemical methods include coagulation, flocculation, and ozonation (Scott et al. 2008). Chemical oxidation processes use chlorine, hydrogen peroxide, and/or ozone to degrade highly toxic pollutants or ones that were too recalcitrant to be biologically removed. Scott et al. (2008) found that more than 95% NAs were removed when OSPW was treated with ozone and that the treated effluent was nontoxic based on the Microtox® assay. TOC was not reduced and the

biological oxygen demand (BOD) increased (Scott et al. 2008). Photocatalysis (Bessa et al. 1999) removed 40% of the TOC and 100% of the acute toxicity to *Artemia sp.* Most chemical oxidation processes require high-energy input and generate by-products and radical scavengers. Furthermore, the chemicals used may cause toxicity. For example, the cationic polyelectrolytes used in flocculation can be a source of toxicity (Rowland et al. 2000, de Rosemond et al. 2004).

2.5.1 Biological Treatment of OSPW

Biological treatments include cultivation using microbial populations indigenous to oil sands tailings (Herman et al. 1994, Scott et al. 2005, Han et al. 2008) and simulated wetlands (Toor et al. 2012) using naturally occurring microorganisms, which feed on the organic pollutants to degrade and detoxify them. This can be a cost-efficient method. However, the half-lives for OSPW NAs range from 44 to 240 days in shake flasks (Han et al. 2008) to 11-13 years in the tailings pond (Table 2-1). Conventional biological treatments including activated sludge (Tellez et al. 2002), and membrane bioreactor (Melin et al. 2006) can have modest footprints and effectively degrade some organic components but some NAs are recalcitrant.

Natural aging of tailing waters has shown reduction of the total NAs and acute toxicity (Holowenko et al. 2002). Constructed wetlands were built to investigate its potential and it was found that the approach needs a very long hydraulic retention time (HRT). Toor et al. (2012) used simulated wetlands and achieved 64-74% NA reduction with an HRT of 400 days. The degradation of NAs may require long HRT because they are toxic and inhibitory to the microbes.

Bioreactors such as membrane bioreactors could be effective in removing contaminants but can be very expensive when such a large quantity of wastewater is involved (Viero et al. 2008). Constructed wetland is a natural and inexpensive biological method (Del Rio et al. 2006) that can partially degrade NAs and decreased the acute toxicity as determined by Microtox® (MacKinnon et al. 1986). Compared to a single organism, a consortium is more adaptable to the degradation of

mixtures of compounds and has been used to remove NAs in OSPW and detoxification (Lai et al. 1996, Whitby, 2010). Lai et al. (1996) inoculated 2.2 L oil sand process water from different ponds in 4 L flasks with air and with or without phosphate, monitored the degradation rate of two ^{14}C-naphthenic acids: palmitic acid and dechydro-2-naphthoic acid and the toxicity by Microtox®. They found that dissolved oxygen, temperature and phosphate were factors that affected degradation and detoxification. A membrane reactor was also used to improve chemical oxygen demand and TOC removal efficiencies by 17 and 20%, respectively (Viero et al., 2008). In 2008, Alberta's oil sands industry produced 4,736,000 barrels of OSPW per day (Giesy et al. 2010) and because of the high cost of scaling up reactors, constructed wetlands have been evaluated (Knight et al. 1999) with total extractable hydrocarbon removal efficiencies ranging from 35 to 70% (Knight et al. 1999) and Microtox® acute toxicity decreased by 98% in one study (MacKinnon et al., 1986).The immobilized soil bioreactor (ISBR) uses plates of a highly porous geotextile material entrapping microorganisms, providing a surface for growth, and reducing the hydrodynamic shear stress on the microorganisms. The ISBR was first used to degrade pentachlorophenol (PCP) (Karamanev et al. 1998) at a volumetric rate of up to 950 $mg \cdot L^{-1} \cdot h^{-1}$, which is 100 times higher than suspension culture. It has also been successfully applied to degrading trichloroethylene (Ramsay et al. 2001), and xylene and naphthalene (Jajuee et al. 2007). In a previous study, two ISBRs were connected in series and operated continuously for over 2 years with OSPW as the sole carbon source and achieved 30-40% removal of NAs at an HRT of 7 days consistently (McKenzie et al. 2013).

2.6 Characterization of NA-Degrading Microbial Community

Microorganisms are detected in untreated OSPW (Lai et al. 1996, Quagraine et al. 2005b) and these may have the potential of degrading NAs, which means that microorganisms could play a very important role in the remediation of OSPW. To enhance the bioremediation of OSPW, it is

important to identify the OSPW-degrading microorganisms to know how to manipulate the environmental factors to induce biodegradation. Microorganisms that are capable of metabolizing OSPW or NAs are summarized in Table 2-2. Generally, studies of NAs or OSPW biodegradation may be classified into degradation of: surrogate NAs, commercial NAs including Merichem, Kodak, and Fluka, NAs extracted from OSPW, and OSPW itself. In the surrogate NA degrading studies, monocyclic NAs are most commonly used because of their commercial availability. Bacterial isolates including various *Actinobacteria*, *β-*, and *γ-proteobacteria* are able to metabolize monocyclic NAs. Several studies have shown that *Mycobacterium,* and *Pseudomonas putida* can metabolize aromatic NAs. The eukaryotes, *Dunaliella tertiolecta* and *Trichosporon cutaneum* (a fungus) are also capable of degrading NAs. In the biodegradation of OSPW, many microorganisms have been found to be associated with NAs degradation, always as part of a microbial consortium (Del Rio et al. 2006). For instance, *Acaligenes*, *Acinetobacter*, *Pseudomonas* sp and *Thauera* were shown to be involved in aerobic NAs degradation ((Biryukova et al. 2007,Whitby, 2010). Blakley et al. (1982a) demonstrated that *Alcaligenes faecalis* can metabolize cyclohexanecarboxylic acid, 3-cyclohexenecarboxylic acid supplemented with gluconate. Rontani et al. (1992) used a marine *Alcaligenes* to degrade n-alkyl-substituted cyclohexanes via cyclohexylcarboxylic acid which was further degraded by alpha- or beta-oxidation. *Alcanivorax* sp. strain MBIC 4326 has also been found to degrade long-chain n-alkylbenzenes and n-alkylcyclohexanes with cyclohexanecarboxylic acid as an intermediate which was further transformed to benzoic acid via 1-cyclohexene-1-carboxylic acid (Dutta et al. 2001).

Table 2-2 Bacteria that can degrade NAs

Classification		Substrate	Reference
Bacteria			
Actinobacteria			
	Arthrobacter sp.	Monocyclic NAs	(Blakley 1974, Ougham et al. 1982)
	Corynebacterium cyclohexanicum	Monocyclic NAs	(Rho and Evans 1975)
	Nocardia	Monocyclic NAs	(Hasegawa et al. 1982a)
	Dietzia	Monocyclic NAs	(Iwaki et al. 2008)
	Rhodococcus	Monocyclic NAs	(Iwaki et al. 2008)
	Mycobacterium	Aromatic NAs	(Johnson et al. 2012b)
Proteobacteria			
	beta		
	Alcaligenes faecalis	Monocyclic NAs	(Blakley et al. 1982a)
	Cupriavidus	Monocyclic NAs	(Iwaki et al. 2008)
	gamma		
	Acinetobacter anitratum	Monocyclic NAs	(Rho and Evans 1975)
	Pseudomonas putida	Monocyclic, aromatic NAs	(Blakley et al. 1982b, Johnson et al. 2012a)
	Alcanivorax sp.	Monocyclic NAs	(Dutta et al. 2001)
Eukaryota			
	Dunaliella tertiolecta	Monocyclic, aromatic NAs	(Quesnel et al. 2011)
Fungi			
	Trichosporon cutaneum	Monocyclic NAs	(Hasegawa et al. 1982b)
Consortia			
	Circulating packed-bed Bioreactor	Monocyclic NAs	(Huang et al. 2012)
	Batch culture	monocyclic NAs	(Paslawski et al. 2009)
	Enriched soil	Aromatic NAs	(Johnson et al. 2011)
	Sediment	Monocyclic NAs	(Smith et al. 2008)
	MLSB Syncrude Canada	Commercial NAs mixture, OSPW NAs extract	(Videla et al. 2009)
	Rhizosphere microorganisms	Commercial NAs mixture	(Biryukova et al. 2007)
	Sediment microorganism	Commercial NAs mixture	(Del Rio et al. 2006)
	Pseudomonas putida and *Pseudomonas fluorescens*	Commercial NAs mixture	(Del Rio et al. 2006)
	Aerobic cultures from OSPW	Commercial NAs mixture	(Clemente et al. 2004b)
	Oil sands tailings microcosms	Monocyclic NAs, commercial NAs, OSPW NAs extract	(Herman et al. 1993, Herman et al. 1994)
	Simulated wetland	OSPW	(Toor et al. 2013)
	Activated sludge	OSPW	(Misiti et al. 2013)
	Algae	Monocyclic NA, OSPW NAs	(Headley et al. 2008)
	Aerobic culture from tailings pond	Commercial NAs, OSPW	(Scott et al. 2005)
	Microbial consortia in TPW	Linear NA, bicyclic NA	(Lai et al. 1996)

Classical phenotypic classification methods based on morphological and physiological traits are not accurate. DNA-DNA hybridization and chemotaxonomic methods including cell wall and lipid composition analysis have proved to effectively differentiate taxa but lack comprehensive phylogenetic relationship insight. Because of improvement in molecular sequencing techniques, comparison of the primary structures of macromolecules such as DNA or protein to obtain phylogenetic information is now readily available. Cytochrome and ferredoxins were the first molecules to construct phylogenetic trees (Fitch et al. 1967). In the mid-1970s, small subunit (SSU) rRNA was used as a "molecular chronometer" and has advantages over cytochromes or ferredoxins for several reasons. From the functional aspect, ribosomal RNA combines protein to form a nucleoprotein called a ribosome. The ribosome binds mRNA and carries out protein synthesis. Because of the essential function of the ribosomal RNA, it is one of the only genes present in all cells. The gene for rRNA can be used to identify an organism's taxonomic group that in bacteria include genes that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes. The 16S rRNA gene (i.e. 16S rDNA) is now the gene most commonly used for taxonomic purposes for bacteria (Bulgari et al. 2009). The length of 16S rDNA is ~1550 bp containing highly conserved regions that can be used to design universal primers for amplification as well as variable regions to do comparisons. The 16S rDNA is selected to study the sequence-based phylogenetic diversity of microbial communities because it is (1) universally distributed, (2) functionally constant, (3) conserved, (4) an adequate length to provide sufficient information, and (5) there is a large database of 16S rDNA sequences.

Besides classifying bacterial isolates, 16S rDNA can also be used to characterize the microbial community composition. To characterize the microbial community composition, dilution, and cultivation on agar plates was the technique most widely used before the 1990s. Although straightforward, this gives an incomplete picture. Torsvik et al. (1990) quantified the

microbial community structure from the DNA of the entire community and compared this to the isolates that were obtained from cultivation methods. They showed that cultivation methods greatly underestimated the diversity of the microbial community since the cultivability of the environmental samples was very low (0.001 to 15%). Also, different culture media gave different results so that the cultivable bacteria did not clearly represent the real community. Golby et al. (2012) used 16S rDNA pyrosequencing to study the composition in oil sands sludge, and found several most abundant genera including *Brachymonas* (17.2%), *Acidovorax* (6.2%), *Rhodoferax* (3.7%), and *Thioalkalispira* (3.7%).

Although cultivation-independent methods are advantageous in characterizing the composition of the whole microbial population, telling which microorganisms are present and their respective abundance, it cannot give physiological or enough functional information about the microorganisms. To compensate, cultivation techniques are still very important when it comes to the study of the population's function. It can provide the functional sequence information in designing functional specific primers to monitor the population's composition and function. On the other hand, cultivation-independent methods can be used to guide the choice of cultivation techniques. For example, when predominant groups are detected in the cultivation-independent method, it is easier to predict the members' function in the community and to design a specific isolation method to get pure cultures to do further metabolic, and function analyses.

Because of their complexity and the lack of NA surrogates, little is known about the range of NAs that these bacteria may metabolize. Assessing the range of NAs metabolized by specific strains is desirable in the characterization of isolates. Traditionally, the degradation tests have been done by using radioactive tracers (Del Rio et al. 2006) and shaking flasks (Herman et al. 1993, Holowenko et al. 2001). However, OSPW NAs are a complex mixture, and only very few of them have been identified and are commercially available. Chemically synthesized NAs are also only available in small amounts. In addition, some NAs are low in water solubility and

bioavailability, which makes them hard to investigate by using shaking flasks. In the present study, a high throughput and sensitive microtiter plate method for detection of bacterial growth on crystalline NAs was used as a tool for screening NA-degrading bacteria. The method is based on the respiratory reduction of the tetrazolium salt, biology redox dye H, to a colored formazan by active cells (Johnsen et al. 2002). When the isolates are added to mineral salt medium and NAs surrogates in the microtiter plate wells, the cells proliferate only in wells amended with the NAs that they can metabolize. Biolog redox dye H serve as indicators of cell respiratory and the production of the formazan can be measured by colorimetric assay to determine the isolates' degradation potentials.

2.7 Biodegradation Pathways

An understanding of NA biodegradation pathways is important in the development of OSPW bioremediation techniques. In previous studies, differences in NA degradation were observed. Herman et al. (1993) found six carbons NAs with single-ring (cyclopentane carboxylic acid) was degraded faster than seven carbons NAs (cyclohexane carboxylic acid). Han et al. (2008) suggested that a decreasing NAs degradation rate was also associated with the increasing cyclization (higher DBE value). Lai et al. (1996) monitored the biodegradation of (linear, DBE=1) U-^{14}C-palmitic acid (PA) and (bicyclic, DBE=3) decahydro-2-naphthoic acid-8-^{14}C (DHNA) by oil sands microcosms: PA was degraded 10-15% in 4 weeks while DHNA was degraded 2-4% in 8 weeks. Herman et al. (1994) monitored the degradation rate of several linear NAs (palmitic acid, DBE=1) and monocyclic NAs (cyclohexane carboxylic acid, methylcyclohexane carboxylic acid, cyclohexane pentanoic acid and pentylcyclohexane carboxylic acid DBE=2), where 64% PA was degraded compared to 24%-67% monocyclic NAs in 6 weeks in microcosms using oil sands tailings. Del Rio et al. (2006) also found a bicyclic NA surrogate [^{14}C-decahydronaphthoic acid (DHNA)] was degraded significantly slower than a

monocyclic NA surrogate [^{14}C-cyclohexane carboxylic acid (CCA)]. Besides carbon number and DBE, the differences in the extent of alkyl branching may also contribute to differences in the degradation rate (Bataineh et al. 2006). This may due to the preferred β-oxidation metabolism pathway during the degradation of aliphatic carboxylic acids by aerobic microorganisms (Taylor et al. 1978, Quagraine et al. 2005a, Han et al. 2008). The β-oxidation process occurs at a β carbon (Figure 2-3) to the carboxyl group and forms new carboxylic acid with a loss of two carbons until hindered by the quaternary or tertiary carbons. This explained why OSPW NAs that are more branched were degraded more slowly than commercial NAs (Bataineh et al. 2006) and degradation was more associated with the number of rings than carbon number (Han et al. 2008).

Besides aliphatic acyclic or cyclic NAs, some OSPW NAs contain aromatic functional groups, which can be detected by UV absorption (Mohamed et al. 2008). NAs with DBE=5 might contain one aromatic ring in the structure or multiple aliphatic cyclic rings, i.e. tetracyclic. Both aromatic (Rowland et al. 2011d) and tetracyclic structures (Rowland et al. 2011e) with the same DBE=5 have been found in OSPW, which means that more than one type of structure may be present in the same DBE group. Although previous studies found NAs with low carbon number and low DBE value were generally more rapidly degraded, many insignificant degradation rate differences based on grouping by carbon number or cyclization (DBE value) were also observed (Toor et al. 2013). This may due to complexity of the NAs structure requiring different degradation pathways, which makes the chemical analysis a big challenge.

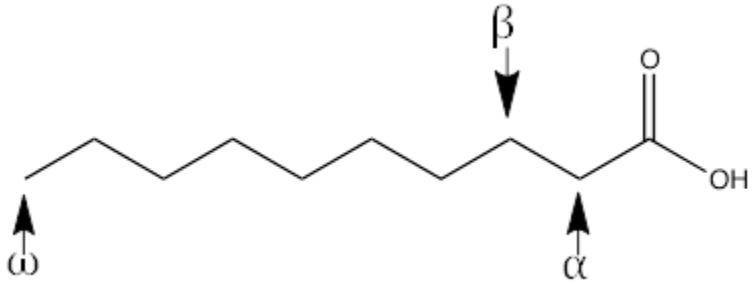

Figure 2-2 α, β, and ω carbons in a carboxylic acid

In summary, NAs were found to be the primary toxicity contributors of OSPW, however, the OSPW after treatment still contain residual toxicity and estrogenicity. Because of the complexity of NAs, the specific NAs causing toxicity and estrogenicity need to be identified to set water treatment priorities, and recalcitrant NAs also need to be identified to determine the limits of biological water treatment.

2.8 References

Alberta Energy Regulator (2014). Alberta's energy reserves 2013 and supply/demand outlook 2014–2023 http://www.aer.ca/documents/sts/ST98/ST98-2014.pdf (citation date:2014-12-01)

Alberta Environmental Protection and Enhancement Act (1993) Edmonton: Alberta Environmental Protection http://www.qp.Alberta.ca/documents/Regs/1993_118.pdf (citation date:2014-12-01)

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

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

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

Arnold, S. F., J. M. Bergeron, D. Q. Tran, B. M. Collins, P. M. Vonier, D. Crews, W. A. Toscano Jr and J. A. McLachlan (1997). "Synergistic responses of steroidal estrogens *in vitro* (yeast) and *in vivo* (turtles)." Biochemical and Biophysical Research Communications **235**(2): 336-342.

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

Beg, M. U., T. Saeed, S. Al-Muzaini, K. R. Beg, T. Al-Obaid and A. Kurian (2001). "Extraction, fractionation, and toxicity determination of organic contaminants in sediment from coastal area receiving industrial effluents in Kuwait." Bulletin of Environmental Contamination and Toxicology **67**(6): 881-888.

Bessa, E., S. Anna, G. Lippel and M. Dezotti (1999). "Photocatalysis: An approach to the treatment of oil field produced waters." Journal of Advanced Oxidation Technologies **4**(2): 196-202.

Bester, K., S. Biselli, R. Gatermann, H. Huhnerfuss, W. Lange and N. Theobald (1998). "Results of non-target screening of lipophilic organic pollutants in the German bight III: Identification and quantification of 2,5-dichloroaniline." Chemosphere **36**(9): 1973-1983.

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

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

Blakley, E. and B. Papish (1982a). "The metabolism of 3-cyclohexenecarboxylic acid by *Alcaligenes faecalis*." Canadian Journal of Microbiology **28**(9): 1037-1046.

Blakley, E. and B. Papish (1982b). "The metabolism of cyclohexanecarboxylic acid and 3-cyclohexenecarboxylic acid by *Pseudomonas putida*." Canadian Journal of Microbiology **28**(12): 1324-1329.

Brient, J. A., P. J. Wessner and M. N. Doyle (2000). Naphthenic acids. Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons, Inc.

Bulgari, D., P. Casati, L. Brusetti, F. Quaglino, M. Brasca, D. Daffonchio and P. A. Bianco (2009). "Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR." The Journal of Microbiology **47**(4): 393-401.

Bulich, A., M. Greene and D. Isenberg (1981). "Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents." Aquatic Toxicology and Hazard Assessment **4**(737): 338-347.

Burchfield, T. E. and L. G. Hepler (1979). "Some chemical and physical properties of tailings water from oil sands extraction plants." Fuel **58**(10): 745-747.

Chan, G., J. M. Reilly, S. Paltsev and Y. H. H. Chen (2012). "The Canadian oil sands industry under carbon constraints." Energy Policy **50**(0): 540-550.

Chen, C. Y., C. K. Wang and Y. H. Shih (2010). "Microbial degradation of 4-monobrominated diphenyl ether in an aerobic sludge and the DGGE analysis of diversity." Journal of Environmental Science and Health, Part B. Pesticides, Food Contaminants, and Agricultural Wastes **45**(5): 379-385.

Cheung, Y. H., A. Neller, K. H. Chu, N. F. Tam, C. K. Wong, Y. S. Wong and M. H. Wong (1997). "Assessment of sediment toxicity using different trophic organisms." Archives of Environmental Contamination and Toxicology **32**(3): 260-267.

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

Colavecchia, M. V., S. M. Backus, P. V. Hodson and J. L. Parrott (2004). "Toxicity of oil sands to early life stages of fathead minnows (*Pimephales promelas*)." Environmental Toxicology and Chemistry **23**(7): 1709-1718.

Colavecchia, M. V., P. V. Hodson and J. L. Parrott (2006). "Cyp1a induction and blue sac disease in early life stages of white suckers (*Catostomus commersoni*) exposed to oil sands." Journal of Toxicology and Environmental Health, Part A **69**(10): 967-994.

Cronin, M., J. Dearden and A. Dobbs (1991). "QSAR studies of comparative toxicity in aquatic organisms." Science of the Total Environment **109**(0): 431-439.

Cronin, M. T. and T. W. Schultz (1997). "Validation of *Vibrio fisheri* acute toxicity data: Mechanism of action-based QSARs for non-polar narcotics and polar narcotic phenols." Science of the Total Environment **204**(1): 75-88.

de Rosemond, S. J. C. and K. Liber (2004). "Wastewater treatment polymers identified as the toxic component of a diamond mine effluent." Environmental Toxicology and Chemistry **23**(9): 2234-2242.

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

DeMarini, D., R. Williams, L. Brooks and M. Taylor (1992). "Use of cyanopropyl-bonded HPLC column for bioassay-directed fractionation of organic extracts from incinerator emissions." International Journal of Environmental Analytical Chemistry **48**(3-4): 187-199.

DeMarini, D. M., M. L. Shelton and D. A. Bell (1996). "Mutation spectra of chemical fractions of a complex mixture: Role of nitroarenes in the mutagenic specificity of municipal waste incinerator emissions." Mutation Research **349**(1): 1-20.

Dutta, T. K. and S. Harayama (2001). "Biodegradation of n-alkylcycloalkanes and n-alkylbenzenes via new pathways in *Alcanivorax sp* strain mbic 4326." Applied and Environmental Microbiology **67**(4): 1970-1974.

Eide, I., G. Neverdal, B. Thorvaldsen, B. Grung and O. M. Kvalheim (2002). "Toxicological evaluation of complex mixtures by pattern recognition: Correlating chemical fingerprints to mutagenicity." Environmental Health Perspectives **110**(Suppl 6): 985.

El-Din, M. G., H. J. Fu, N. Wang, P. Chelme-Ayala, L. Perez-Estrada, P. Drzewicz, J. W. Martin, W. Zubot and D. W. Smith (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.

ERCB, Energy Resources Conservation Board (ERCB). EnerFAQs 12: Oil Sands. **2011.** (citation date:2014-12-01) http://www.lica.ca/attachments/077_12%20Oil%20Sands.pdf

Eustice, D., P. Feldman, A. Colberg-Poley, R. Buckery and R. Neubauer (1991). "A sensitive method for the detection of β-galactosidase in transfected mammalian cells." Biotechniques **11**(6): 739-740, 742-733.

Fitch, W. M. and E. Margoliash (1967). "Construction of phylogenetic trees." Science **155**(760): 279-284.

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

Gaido, K. W., L. S. Leonard, S. Lovell, J. C. Gould, D. Babai, C. J. Portier and D. P. McDonnell (1997). "Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay." Toxicology and Applied Pharmacology **143**(1): 205-212.

Gentes, M. L., C. Waldner, Z. Papp and J. E. Smits (2007). "Effects of exposure to naphthenic acids in tree swallows (*Tachycineta bicolor*) on the Athabasca oil sands, Alberta, Canada." Journal of Toxicology and Environmental Health, Part A **70**(14): 1182-1190.

Giesy, J. P., J. C. Anderson and S. B. Wiseman (2010). "Alberta oil sands development." Proceedings of the National Academy of Sciences **107**(3): 951-952.

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

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

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

Hao, C., J. V. Headley, K. M. Peru, R. Frank, P. Yang and K. R. Solomon (2005). "Characterization and pattern recognition of oil-sand naphthenic acids using comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry." Journal of Chromatography A **1067**(1-2): 277-284.

Hartnik, T., H. R. Norli, T. Eggen and G. D. Breedveld (2007). "Bioassay-directed identification of toxic organic compounds in creosote-contaminated groundwater." Chemosphere **66**(3): 435-443.

Hasegawa, Y., K. Hamano, H. Obata and T. Tokuyama (1982a). "Microbial degradation of cycloheptanone." Agricultural and Biological Chemistry **46**(5): 1139.

Hasegawa, Y., K. Higuchi, H. Obata, T. Tokuyama and T. Kaneda (1982b). "A cyclohexanecarboxylic acid utilizing yeast: Isolation, identification, and nutritional characteristics." Canadian Journal of Microbiology **28**(8): 942-944.

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

Headley, J. V., K. M. Peru, M. H. Mohamed, L. Wilson, D. W. McMartin, M. M. Mapolelo, V. V. Lobodin, R. P. Rodgers and A. G. Marshall (2012). "Electrospray ionization fourier transform ion cyclotron resonance mass spectrometry characterization of tunable carbohydrate-based materials for sorption of oil sands naphthenic acids." Energy & Fuels **27**(4): 1772-1778.

Headley, J. V., J. L. Du, K. M. Peru, N. Gurprasad and D. W. McMartin (2008). "Evaluation of algal phytodegradation of petroleum naphthenic acids." Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering **43**(3): 227-232.

Headley, J. V., K. M. Peru, D. W. McMartin and M. Winkler (2002). "Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry." Journal of AOAC International **85**(1): 182-187.

Heisterkamp, I., J. Gandrass and W. Ruck (2004). "Bioassay-directed chemical analysis utilizing LC-MS: A tool for identifying estrogenic compounds in water samples?" Analytical and Bioanalytical Chemistry **378**(3): 709-715.

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

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

Holowenko, F. M., M. D. Mackinnon and P. M. Fedorak (2001). "Naphthenic acids and surrogate naphthenic acids in methanogenic microcosms." Water Research **35**(11): 2595-2606.

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

Houtman, C., J. Legler and K. Thomas (2011). Effect-directed analysis of endocrine disruptors in aquatic ecosystems Effect-Directed Analysis of Complex Environmental Contamination. W. Brack, Springer Berlin / Heidelberg. **15:** 237-265.

Hsu, C. S., G. J. Dechert, W. K. Robbins and E. K. Fukuda (2000). "Naphthenic acids in crude oils characterized by mass spectrometry." Energy & Fuels **14**(1): 217-223.

Huang, J., M. Nemati, G. Hill and J. Headley (2012). "Batch and continuous biodegradation of three model naphthenic acids in a circulating packed-bed bioreactor." Journal of Hazardous Materials **201**(2012): 132-140.

Iwaki, H., E. Nakai, S. Nakamura and Y. Hasegawa (2008). "Isolation and characterization of new cyclohexylacetic acid-degrading bacteria." Current Microbiology **57**(2): 107-110.

Jajuee, B., A. Margaritis, D. Karamanev and M. A. Bergougnou (2007). "Kinetics of biodegradation of p-xylene and naphthalene and oxygen transfer in a novel airlift immobilized bioreactor." Biotechnology and Bioengineering **96**(2): 232-243.

Johnsen, A. R., K. Bendixen and U. Karlson (2002). "Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1." Applied and Environmental Microbiology **68**(6): 2683-2689.

Johnson, B. (2005). Microtox® acute toxicity test. Small-scale freshwater toxicity investigations. C. Blaise and J.-F. Férard, Springer Netherlands**:** 69-105.

Johnson, R. J., B. E. Smith, S. J. Rowland and C. Whitby (2012a). "Biodegradation of alkyl branched aromatic alkanoic naphthenic acids by *Pseudomonas putida* KT2440." International Biodeterioration & Biodegradation **81** (2013): 3-8.

Johnson, R. J., C. E. West, A. M. Swaih, B. D. Folwell, B. E. Smith, S. J. Rowland and C. Whitby (2012b). "Aerobic biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two different pathways by a new isolate of *Mycobacterium*." Environmental Microbiology **14**(4): 872-882.

Johnson, R. J., B. E. Smith, P. A. Sutton, T. J. McGenity, S. J. Rowland and C. Whitby (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., A. G. Scarlett, C. E. West and S. J. Rowland (2011). "Toxicity of individual naphthenic acids to *Vibrio fischeri*." Environmental Science & Technology **45**(22): 9776-9782.

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

Joyeux, A., P. Balaguer, P. Germain, A. M. Boussioux, M. Pons and J. C. Nicolas (1997). "Engineered cell lines as a tool for monitoring biological activity of hormone analogs." Analytical Biochemistry **249**(2): 119-130.

Kammann, U., S. Biselli, N. Reineke, W. Wosniok, D. Danischewski, H. Huhnerfuss, A. Kinder, A. Sierts-Herrmann, N. Theobald, H. H. Vahl, M. Vobach, J. Westendorf and H. Steinhart (2005). "Bioassay-directed fractionation of organic extracts of marine surface sediments from the north and baltic sea - part II: Results of the biotest battery and development of a biotest index." Journal of Soils and Sediments **5**(4): 225-232.

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

Kidd, K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans, J. M. Lazorchak and R. W. Flick (2007). "Collapse of a fish population after exposure to a synthetic estrogen." Proceedings of the National Academy of Sciences **104**(21): 8897-8901.

Klinkow, N., J. Oleksy-Frenzel and M. Jekel (1998). "Toxicity-directed fractionation of organic compounds in tannery wastewater with regard to their molecular weight and polarity." Water Research **32**(9): 2583-2592.

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

Lai, J. W. S., L. J. Pinto, E. Kiehlmann, L. I. BendellYoung and M. M. Moore (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.

Liang, X. M., X. D. Zhu and E. C. Butler (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., V. Nero, A. Farwell, D. G. Dixon and G. Van Der Kraak (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., B. G. Brownlee and N. J. Bunce (2006). "Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids." Water Research **40**(4): 655-664.

MacKinnon, M. and H. Boerger (1986). "Description of two treatment methods for detoxifying oil sands tailings pond water." Water Quality Research Journal of Canada **21**(4): 496-512.

Mackinnon, M., G. Kampala, B. Marsh, P. Fedorak and S. Guigard (2005). "Indicators for assessing transport of oil sands process-affected waters." Bringing Groundwater Quality Research to the Watershed Scale **297**(2005): 71-80.

Madill, R. E., M. T. Orzechowski, G. Chen, B. G. Brownlee and N. J. Bunce (2001). "Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water." Environmental Toxicology **16**(3): 197-208.

Martin, J. W., X. M. Han, K. M. Peru and J. V. Headley (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.

Marvin, C. H. and L. M. Hewitt (2007). "Analytical methods in bioassay-directed investigations of mutagenicity of air particulate material." Mutation Research **636**(1-3): 4-35.

McCormick, J. K. (2000). The effects of oil sands tailings on zooplankton communities in northern Alberta, University of Waterloo.

McDonald, J. D., I. Eide, J. Seagrave, B. Zielinska, K. Whitney, D. R. Lawson and J. L. Mauderly (2004). "Relationship between composition and toxicity of motor vehicle emission samples." Environmental Health Perspectives **112**(15): 1527-1538.

Mehrotra, A. and A. Banerjee (1986). "Evaluation of reverse osmosis for the treatment of oil sands produced water." Water Pollution Research Journal of Canada **21**(2):141-152.

Melin, T., B. Jefferson, D. Bixio, C. Thoeye, W. De Wilde, J. De Koning, J. Van der Graaf and T. Wintgens (2006). "Membrane bioreactor technology for wastewater treatment and reuse." Desalination **187**(1): 271-282.

Misiti, T., U. Tezel and S. G. Pavlostathis (2013). "Fate and effect of naphthenic acids on oil refinery activated sludge wastewater treatment systems." Water Research **47**(1): 449-460.

Mohamed, M. H., L. D. Wilson, J. V. Headley and K. M. Peru (2008). "Screening of oil sands naphthenic acids by UV-Vis absorption and fluorescence emission spectrophotometry." Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering **43**(14): 1700-1705.

Nelson, L., M. MacKinnon and J. Gulley (1993). "Application of toxicity testing in the evaluation of reclamation options for oil sands fine tails." Oil Sands: Our Petroleum Future Conference **25** (2):1-11

Niederlehner, B., J. Cairns Jr and E. P. Smith (1998). "Modeling acute and chronic toxicity of nonpolar narcotic chemicals and mixtures to *Ceriodaphnia dubia*." Ecotoxicology and Environmental Safety **39**(2): 136-146.

Ougham, H. and P. Trudgill (1982). "Metabolism of cyclohexaneacetic acid and cyclohexanebutyric acid by *Arthrobacter* sp. Strain CA1." Journal of Bacteriology **150**(3): 1172-1182.

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

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

Peterman, S. M. and J. J. Mulholland (2006). "A novel approach for identification and characterization of glycoproteins using a hybrid Linear Ion Trap/FT-ICR mass spectrometer." Journal of the American Society for Mass Spectrometry **17**(2): 168-179.

Quagraine, E. K., J. V. Headley and H. G. Peterson (2005). "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.

Quesnel, D. M., I. M. Bhaskar, L. M. Gieg and G. Chua (2011). "Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*." Chemosphere **84**(4): 504-511.

Rajapakse, N., E. Silva and A. Kortenkamp (2002). "Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action." Environmental Health Perspectives **110**(9): 917-921.

Ramsay, B., D. Karamanev, S. Pierre, C. Lafontaine, T. Sakamoto and J. Ramsay (2001). "Efficient TCE mineralization in a novel methanotrophic bioreactor system." In Situ Aeration and Aerobic Remediation **6**(10): 171-178

Reemtsma, T., O. Fiehn and M. Jekel (1999). "A modified method for the analysis of organics in industrial wastewater as directed by their toxicity to *Vibrio fischeri*." Fresenius Journal of Analytical Chemistry **363**(8): 771-776.

Reinardy, H. C., A. G. Scarlett, T. B. Henry, C. E. West, L. M. Hewitt, R. A. Frank and S. J. Rowland (2013). "Aromatic naphthenic acids in oil sands process-affected water, resolved by GCXGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (*Danio rerio*) larvae." Environmental Science & Technology **47**(12): 6614-6620.

Rho, E. and W. Evans (1975). "The aerobic metabolism of cyclohexanecarboxylic acid by *Acinetobacter anitratum*." Biochemical Journal **148**(1): 11.

Rogers, V. V., K. Liber and M. D. MacKinnon (2002). "Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water." Chemosphere **48**(5): 519-527.

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

Routledge, E. J. and J. P. Sumpter (1996). "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen." Environmental Toxicology and Chemistry **15**(3): 241-248.

Rowland, C. D., G. A. Burton and S. M. Morrison (2000). "Implication of polymer toxicity in a municipal wastewater effluent." Environmental Toxicology and Chemistry **19**(8): 2136-2139.

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

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

Rowland, S. J., C. E. West, A. G. Scarlett and D. Jones (2011c). "Identification of individual acids in a commercial sample of naphthenic acids from petroleum by two-dimensional comprehensive gas chromatography/mass spectrometry." Rapid Communications in Mass Spectrometry **25**(12): 1741-1751.

Rowland, S. J., C. E. West, A. G. Scarlett, D. Jones, M. Boberek, L. Pan, M. Ng, L. Kwong and A. Tonkin (2011d). "Monocyclic and monoaromatic naphthenic acids: Synthesis and characterisation." Environmental Chemistry Letters **9**(4): 525-533.

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

Scarlett, A. G., H. C. Reinardy, T. B. Henry, C. E. West, R. A. Frank, L. M. Hewitt and S. J. Rowland (2013). "Acute toxicity of aromatic and non-aromatic fractions of naphthenic acids extracted from oil sands process-affected water to larval zebrafish." Chemosphere **93**(2): 415-420.

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

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

Siwik, P. L., T. Van Meer, M. D. MacKinnon and C. A. Paszkowski (2000). "Growth of fathead minnows in oil sand processed wastewater in laboratory and field." Environmental Toxicology and Chemistry **19**(7): 1837-1845.

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

Speight, J. G. (2000). Tar sands. Kirk-Othmer encyclopedia of chemical technology, John Wiley & Sons, Inc.

St. John, W. P., J. Rughani, S. A. Green and G. D. McGinnis (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.

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

Tellez, G. T., N. Nirmalakhandan and J. L. Gardea-Torresdey (2002). "Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oilfield produced water." Advances in Environmental Research **6**(4): 455-470.

Thomas, K. V., R. E. Benstead, J. E. Thain and M. J. Waldock (1999). "Toxicity characterization of organic contaminants in industrialized UK estuaries and coastal waters." Marine Pollution Bulletin **38**(10): 925-932.

Thomas, K. V., J. E. Thain and M. J. Waldock (1999). "Identification of toxic substances in United Kingdom estuaries." Environmental Toxicology and Chemistry **18**(3): 401-411.

Toor, N. S., E. D. Franz, P. M. Fedorak, M. D. MacKinnon and K. Liber (2013). "Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands." Chemosphere **90**(2): 449-458.

Toor, N. S., X. Han, E. Franz, M. D. MacKinnon, J. W. Martin and K. Liber (2013). "Selective biodegradation of naphthenic acids and a probable link between mixture profiles and aquatic toxicity." Environmental Toxicology and Chemistry **32**(10): 2207-2216.

Torsvik, V., K. Salte, R. Sorheim and J. Goksoyr (1990). "Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria." Applied and Environmental Microbiology **56**(3): 776-781.

Vaes, W. H., E. Urrestarazu Ramos, C. Hamwijk, I. van Holsteijn, B. J. Blaauboer, W. Seinen, H. J. Verhaar and J. L. Hermens (1997). "Solid phase microextraction as a tool to determine membrane/water partition coefficients and bioavailable concentrations in in vitro systems." Chemical Research in Toxicology **10**(10): 1067-1072.

Videla, P., A. Farwell, B. Butler and D. G. Dixon (2009). "Examining the microbial degradation of naphthenic acids using stable isotope analysis of carbon and nitrogen." Water, Air, and Soil Pollution **197**(1-4): 107-119.

Viero, A. F., T. M. de Melo, A. P. R. Torres, N. R. Ferreira, G. L. Sant'Anna Jr, C. P. Borges and V. M. J. Santiago (2008). "The effects of long-term feeding of high organic loading in a submerged membrane bioreactor treating oil refinery wastewater." Journal of Membrane Science **319**(1-2): 223-230.

Wedekind, C. (2014). "Fish populations surviving estrogen pollution." BMC Biology **12**(1): 10.

West, C. E., D. Jones, A. G. Scarlett and S. J. Rowland (2011). "Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays." Science of the Total Environment **409**(19): 4125-4131.

Whitby, C. (2010). "Microbial naphthenic acid degradation." Advances in Applied Microbiology **70**(2010): 93-125.

Wiklund, S., E. Johansson, L. Sjostrom, E. J. Mellerowicz, U. Edlund, J. P. Shockcor, J. Gottfries, T. Moritz and J. Trygg (2008). "Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models." Analytical Chemistry **80**(1): 115-122.

Yen, T. W., W. P. Marsh, M. D. MacKinnon and P. M. Fedorak (2004). "Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography." Journal of Chromatography A **1033**(1): 83-90.

Zhao, Y. H., M. T. Cronin and J. C. Dearden (1998). "Quantitative structure - activity relationships of chemicals acting by nonpolar narcosis - theoretical considerations." Quantitative Structure - Activity Relationships **17**(2): 131-138.

Chapter 3

Probable Link between Toxicity and Composition Profiles of Solid Phase Extracts of Oil Sands Process Water

This chapter will be submitted to Chemosphere

3.1 Abstract

Using effect-directed analysis (EDA), the presence of toxic components in oil sands process water (OSPW) was detected by the Microtox® acute toxicity assay after fractionation using sequential solid phase extraction (SPE). The composition of each fraction was determined by high-resolution electrospray ionization-Orbitrap mass spectrometry. A combination of principal component analysis (PCA) and partial least-squares regression discriminant analysis (PLS-DA; also known as projection to latent structures discriminant analysis) was used to evaluate the relationships between chemical composition of the fractions and toxicity. The PLS-DA analysis revealed the chemical constituents covarying most strongly with toxicity. Although O2 compounds with double bond equivalence (DBE) between 3 and 9 positively correlated to toxicity, C15-C18 O2-NAs with DBE=4 (tricyclic structure), , as well as C14-C17 O2-NAs with DBE=3 (bicyclic structure), were found to be most likely associated with OSPW toxicity, consistent with published toxicity studies using surrogate NAs. Many O3 and a few O2 compounds were found to negatively correlate with toxicity. The results demonstrate the utility of the PCA/PLS-DA approach for evaluating composition-response relationships in a complex mixture and also contribute to a better understanding of the toxic compounds in OSPW. These findings will help to focus study on the most environmentally significant components in OSPW.

3.2 Introduction

A large quantity of wastewater known as oil sands process water (OSPW) is produced in the recovery of bitumen from surface mined oil sands from the Clark's caustic hot water extraction process (Romanova et al. 2006). To produce one barrel of oil, three barrels of OSPW are produced. Since OSPW has been shown to be toxic to a wide range of organisms such as *Daphnia magna*, *Vibrio fischeri* (Holowenko et al. 2002, MacKinnon et al. 1986), and rainbow trout (*Oncorhynchus mykiss*) (Gagne et al. 2012), its release into the environment is not permitted and it is stored in large tailings ponds. By the end of 2011, the size of the tailing ponds was estimated to be 720 million m^3 (ERCB 2011).

Although there are many classes of toxic compounds such as polyaromatic hydrocarbons and alkylphenols in OSPW, the acidic fraction of OSPW containing families of naphthenic acids (NAs) has been shown to contain a majority of the toxicity (Lo et al. 2006, MacKinnon et al. 1986). The acid extractable fraction (AOF) includes classical NAs (c-NAs) with two oxygen atoms ($C_nH_{2n+z}O_2$) and oxidized NAs (oxy-NAs). Both can be represented by $C_nH_{2n+z}O_x$ (Clemente et al. 2005) and may include elements such as S and/or N where "z" refers to the hydrogen deficiency or double bond equivalent (DBE) and is zero or a negative, even integer and "x" ranges from 2-10.

The complexity of the composition of NAs makes identification of toxicants challenging. Fractionation can reduce the complexity, and has been applied using acid-base extraction (Madill et al. 2001), differences in solubility based on the pK_a (Lo et al. 2006), boiling point (Frank et al. 2008), or anion exchange (Lo et al. 2006). Keeping in mind that most earlier studies only considered the c-NAs, OSPW with a higher proportion of NAs with $n < 21$ were found to be more toxic (Holowenko et al. 2002). Although acute toxicity of individual NA surrogates increases with increasing molecular weight (Frank et al. 2009, Jones et al. 2011) , the lowest molecular weight fraction obtained by distillation was found to be more toxic than the highest molecular

weight fraction (Frank et al. 2008). Frank et al. (2009) attributed this to the presence of a second carboxylic acid group on higher molecular weight NAs decreasing hydrophobicity and hence toxicity. Scarlett et al. (2013) found that an aromatic NA fraction containing higher MW were more toxic than an alicyclic NA fraction containing adamantane and diamantane carboxylic acids. However, no direct link between toxicity and specific NAs in OSPW has been made.

Seward et al. (1999) developed highly significant quantitative structure-activity relationship [(Q)SAR] models to predict the acute toxicity of saturated aliphatic carboxylic acids and their salts by using hydrophobicity (log K_{ow}) as the single descriptor (Seward et al. 1999). Based on the ECOSAR model, Frank et al. (2010) suggested that narcosis may be the mechanism of acute toxic of NAs and is related to the compound's hydrophobicity. Since elution time in a C18 reversed phase column was shown to correlate with hydrophobicity (log K_{ow}) of pure compounds (Braumann 1986), OSPW fractionation based on hydrophobicity may better correlate with toxicity than fractionation based on other principles such as solubility, boiling point, or anion exchange but has not been previously studied.

The objective of this paper is to better identify the toxicants in OSPW. To achieve this, OSPW was fractionated by reverse phase solid phase extraction (SPE), the acute Microtox® toxicity evaluated for each fraction and their composition determined by ESI-Orbitrap high-resolution MS. Finally, using multivariate analysis such as principal component analysis (PCA) and partial least-squares regression discriminate analysis (PLS-DA), compounds associated with toxicity may be identified (McDonald et al. 2004). These results may provide information on which compounds to target during a treatment and to evaluate the effectiveness of detoxification methods of OSPW.

3.3 Materials and Methods

3.3.1 OSPW

OSPW was collected from the West In Pit (WIP) at the discharge inlet to the active settling basin on the Syncrude Canada site in Fort McMurrary, AB.

3.3.2 Solid Phase Extraction (SPE)

The pH of OSPW was adjusted to 10 with 1 M NaOH, filtered through a 0.45 μm nylon filter (Millipore Corporation, Billerica, MA, USA) then passed through two C18 (octade-cylsilane) columns connected in series to a polystyrene-divinylbenzene (PSDVB) column (J.T. Baker, Phillipsburg, NJ, USA). Each column contained 1 g of sorbent. Both C18 and PSDVB columns are reverse phase columns with the C18 retaining the hydrophobic or weakly polar compounds and PSDVB retaining a wider range of compounds including the more polar ones (Reemtsma et al. 1999). Columns were pre-conditioned by passing 25 mL methanol followed by 25 mL high-purity water (18 megaohms.cm, Millipore water) before passing 5 L of OSPW. Fractions were eluted from both C18 (F1 to F4) or the PSVD column (F5 to F8) with an increasing amount of methanol (20, 60, 80, and 100%) for a total of eight fractions such that F1 and F5 were recovered with 20% methanol, F2 and F6 with 60% etc. Of the fractions collected from the same type of column, hydrophobicity of components should increase with increasing methanol concentration.

3.3.3 Microtox®

The toxicity of (i) the eight SPE fractions, (ii) the original, unfractionated OSPW and (iii) the flow through after passage of OSPW through the SPE columns was measured by the Microtox® assay (Azur Environmental, Fairfax, CA, USA) modified to 96-well plates (Fiehn et al. 1997). Each SPE fraction was concentrated by purging with N_2. An appropriate volume of the

concentrate was evaporated to almost dryness then solubilized in 2.5 mL of 4% NaCl (pH 7) to achieve a 50-fold concentration of the original sample. Triplicate samples of a dilution series were added to reconstituted *Vibrio fischeri* NRRL B-11177. All samples were analyzed using a Synergy^{TM} HT microtiter plate reader (Biotek Instruments Inc., Winnoski, VT) and the effective concentration resulting in 50% decrease in bioluminescence (EC_{50}) was calculated as L equivalent of original OSPW L^{-1} test solution by taking into account the concentration factor of each fraction (Reemtsma et al. 1999) and expressed as $1/EC_{50,}$ or TU_{50}, where a high TU_{50} corresponds to a high toxicity. Glucose (1 g L^{-1}) was used as a negative control and $ZnSO_4$ as the positive control. The latter was found to be within the parameters set by Microtox® (EC_{50} at 15 min was between 3 to 10 mg L^{-1}). Procedural blanks were performed in parallel, tested in duplicate and showed no toxicity.

3.3.4 LTQ Orbitrap Velos Pro Mass Spectrometry Analysis

Based on the Microtox® assay, three toxic and the four non-toxic fractions were selected for MS analysis using a linear ion trap quadropole (LTQ)-Orbitrap high resolution mass spectrometer equipped with a heated electrospray ionization (ESI) source (LTQ-Orbitrap Velos Pro Hybrid MS, Thermo Fisher Scientific, Waltham, MA, USA). Samples were injected directly by a Hamilton syringe (250 μL) into the ESI-MS using direct infusion by the LTQ-Orbitrap MS (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 5 μL min^{-1}. The MS system was operated using the Xcalibur software (Thermo Fisher Scientific). The electrospray voltage was 2.7 kV, nebulizing gas (N_2) flow of 1.5 L min^{-1}, drying gas (N_2) flow of 3.0 L min^{-1}, and the ion transfer tube temperature was 270 ℃. Prior to sample analysis, the LTQ and Orbitrap were tuned to optimize conditions for the detection of ions in the m/z range of 150–1000 and calibrated according to the manufacturers' calibration mixture. Full MS scans in negative ion mode used over 400 scans for each acquisition in the Orbitrap mass analyzer at a mass resolution of 100,000

(FWHM defined at m/z 400) and a mass accuracy better than 5 ppm. MS/MS scans in the negative mode were acquired by collision induced fragmentation m/z were analyzed in the linear ion trap alone and ultrahigh resolution mass spectra were acquired with the Orbitrap. Xcalibur software was also used for data visualization, processing, and calculation of elemental composition. The percent relative abundance of each mass peak was calculated as the percent of the total intensity of all masses.

Calculations were limited to formulae containing less than 100 ^{12}C, 2 ^{13}C, 200 ^{1}H, 5 ^{14}N, 10 ^{16}O, 3 ^{32}S, and 1 ^{34}S. If more than one possible formula was generated for a specific mass, one or more could almost always be confirmed or eliminated by the presence/absence of a corresponding ion containing ^{13}C, ^{18}O, or ^{34}S as described in Headley et al. (2012).

3.3.5 Pattern Recognition by Multivariate Data Analysis

Multivariate data analysis was used to correlate Microtox® acute toxicity with compounds detected by ESI-LTQ-Orbitrap MS. The compositional data were structured in an X-matrix with one row per SPE fraction of m/z values for which the signal to noise ratio was greater than 5. The acute Microtox® data were structured in a Y-matrix with one column per response variable using 0 for “non-toxic” and 1 for “toxic”. Multivariate data analysis was performed with Matlab R2013 for Mac. PCA (Jackson et al. 1993) was performed on the X-matrix to find in what respect one sample is different from another. PLS-DA (Lundstedt-Enkel et al. 2005) used a classical PLS regression to correlate the responses to the compositional parameters (Wold et al. 1984) where the response variable is a categorical one (Y-matrix) to express the class membership. The objective of PLS-DA in this study is to find a model that separates the samples on the basis of the toxicity evaluated by Microtox® acute toxicity assay, and by quantifying the eventual discriminating variables (m/z values) that contributed to class separation.

3.4 Results and Discussion

The aim of the SPE fractionation was to separate the components of OSPW based on their hydrophobicity. There were eight fractions, four from the two C18 columns (F1-F4) and four from the PSDVB column (F5-F8) (Figure 3-1), recovered with increasing amounts of methanol (20, 60, 80, and 100%). Fractions recovered with 100% methanol contain the most hydrophobic components.

3.4.1 Microtox® Acute Toxicity

The Microtox® acute toxicity test was conducted and the TU_{50} values are shown in Figure 3-1. No toxicity was detected in the SPE flow through water indicating that all toxic components were captured on the SPE columns. Most of the toxicity (>94%) was recovered from the SPE columns since the TU_{50} of the unfractionated OSPW was 4197 L L^{-1} (95% confident interval: 3064~5749) compared to the sum of all SPE fractions (3959 L L^{-1} (95% confident interval: 2534~5876)). Less than 5% of the toxicity was recovered from the PSDVB column which is likely to trap aromatic compounds. Most of the toxicity was recovered in fractions F1 to F3 (i.e. low to moderately hydrophobic fractions) with more than 70% of the overall toxicity in the F2 fraction. Although increasing hydrophobicity has been implicated in the toxicity of OSPW (Frank et al. 2010, Seward and Schultz 1999), F2 was not the most hydrophobic fraction indicating that mechanisms other than narcosis may cause toxicity because of the complex composition of OSPW.

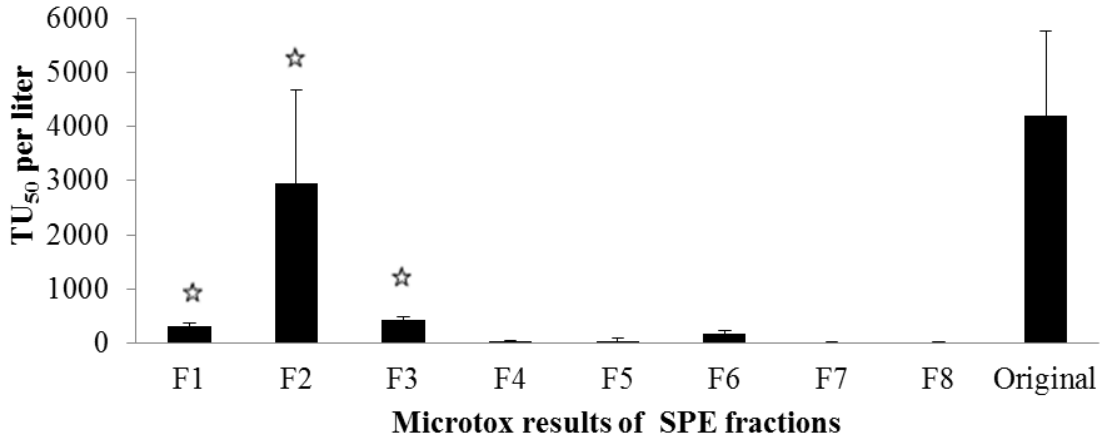

Figure 3-1 Microtox® acute toxicity of SPE fractions of OSPW. F1-F4 were recovered from two C18 columns with fractions F1,F 2, F3, and F4 recovered with 20, 60, 80 and 100% methanol respectively. F5-F8 were similarly recovered from the PSDVB column with 20%, 60%, 80% and 100% methanol. Each fraction was analyzed in triplicate according to the Microtox® protocol and data shown with the 95% confidence intervals. (Toxic fractions are marked with ☆)

3.4.2 ESI-FTICR MS of SPE fractions

Mass formulae can be assigned with high confidence because mass peaks are mostly molecular peaks as ESI uses soft ionization and the Orbitrap MS has ultra-high resolution and high mass accuracy (<5 ppm rms). Less than 5% of the peaks did not meet the criteria specified in the Materials and Method and were not assignable. It is difficult to quantify the components in each fraction because the mixtures are very complex and no authentic standards are available, hence they are compared in terms of their relative abundance (Headley et al. 2009). Since the toxicity of fractions F5 to F8 was negligible, F7 was not analyzed to reduce the quantity of data and would likely not compromise the analysis.

All compound classes containing oxygen (O) observed in the SPE fractions are shown in Figure 3-2. The O2 group is likely c-NAs as MS/MS fragmentation of five random O2 compounds resulted in the most abundant neutral losses of 44, 38 and 18, consistent with neutral losses of CO_2, CO, and H_2O respectively from carboxylic acid moieties (Rudzinski et al. 2002).

The c-NAs was dominant in most fractions, especially in F2 (most toxic), F3 (toxic), and F4 (nontoxic) with 81, 95, and 78% O2 compounds respectively. Among the more polar but much less toxic fractions (F5-F8), F8 contained the highest relative amount of O2 compounds (~55%) while O3 compounds were more dominant in F5 and F6 (insignificant toxicity) containing 43% and 38% O3 compounds respectively.

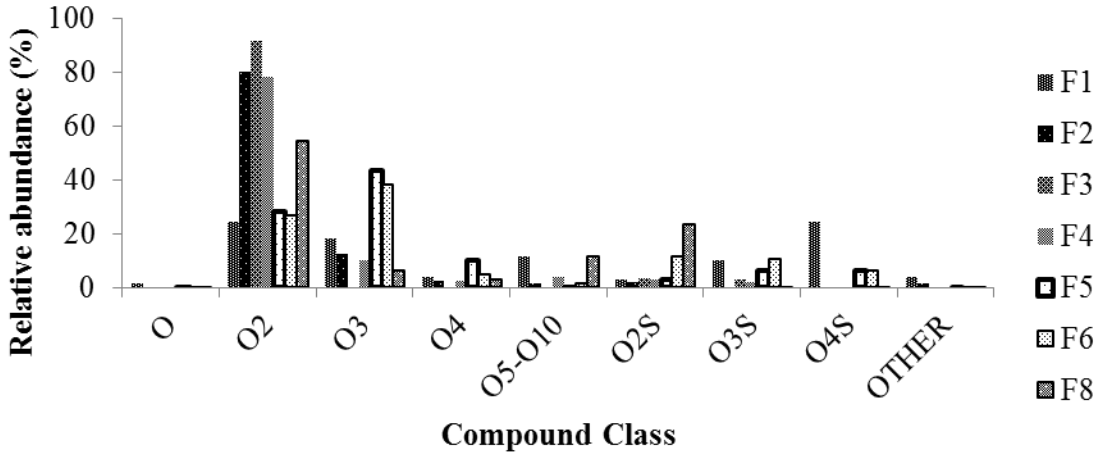

Figure 3-2 Distribution of major compound classes observed in ESI-FTICR mass spectra of F1, F2, F3, F4, F5, F6 and F8 fractions.

The plots of the double bond equivalents (DBE) versus carbon number (n) for the O2 species in the SPE fractions are given in Fig. 3-3. For each type of SPE column (fractions F1-F4 vs F5-F6, F8), compounds with higher DBE values were recovered with increasing methanol concentration, and at a given DBE value, the carbon number of the compounds recovered increased with increasing methanol concentration. More hydrophobic compounds are eluted with increasing methanol concentration. The more toxic fractions, F1-F3, contained more O2 species than other O species (Figure 3-2) and more O2 species with DBE < 5 (Figure 3-3).

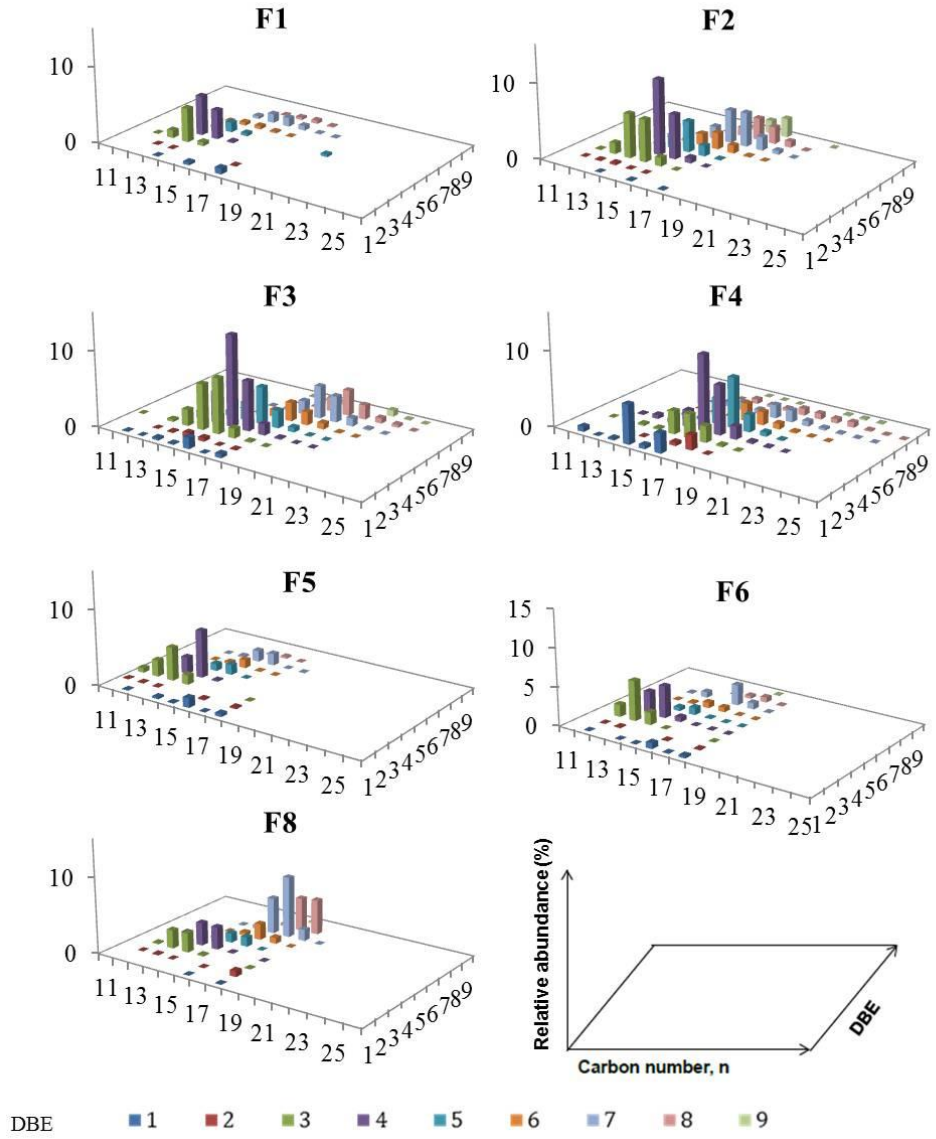

Figure 3-3 Distribution of O2 species present in seven SPE fractions as a function of carbon number and DBE. The percentage of each mass peak was calculated as intensity over the total intensity.

3.4.3 Relationship between Composition and Toxicity

To examine a possible link between the "composition" of a fraction and toxicity, PCA was performed on the spectral data of the seven fractions. A 3-D plot of the first three components (PC1, PC2, and PC3) is shown in Figure (a). These three components accounted for about 84% of the total variance (PC1=40.32%, PC2=24.65%, and PC3=18.88%) with the more

toxic F1-F3 fractions forming a separate group from the nontoxic fractions. Since three PCAs were needed to achieve this separation, PLS-DA was applied in an attempt to enhance the separation between toxic and non-toxic fractions. This is a classical PLS regression in which the data is categorized ("0" for non-toxic and "1" for toxic) to enhance the separation. Using the first two components, the most toxic F1-F3 fractions were separated from nontoxic fractions by PLS1 as shown in Figure (b). The goodness fit of the model is R2X=58.43%, and R2Y=94.08%, which represents the fraction of the variation of the X (chemical species) or Y (toxic vs nontoxic) variables in the PLS-DA model.

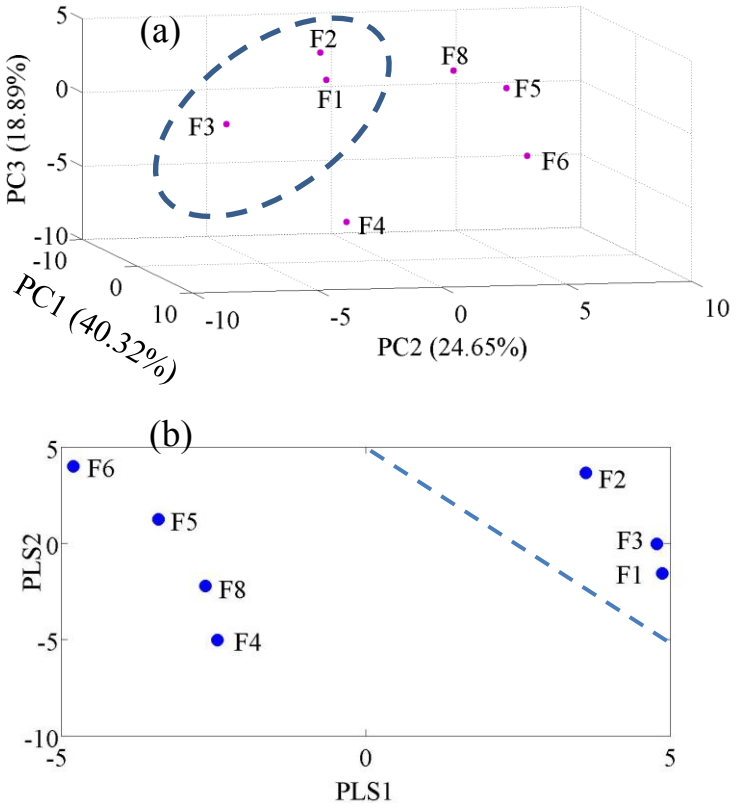

Figure 3-4 (a) 3-D plot of the scores of the first three components in PCA of all SPE fractions, (b) plot PLS-DA scores first two components based on Microtox® results and ESI-FTICR MS data from all SPE fractions.

Figure 3-5 shows a heat map of the distribution of O2 and O3 species with the PLS1 loading scores correlating each component with toxicity. In general, a majority of O_2 species positively correlated with toxicity, and most O_3 compounds correlated negatively and other chemical classes had poor or no association. The greater a positive (or negative) loading value of a species, the higher the possible positive (or negative) correlation with toxicity. Some O2 species are more likely associated with the toxicity. For instance, O2 species with 15-18 carbons and DBE=4 have the highest positive correlation with toxicity and are more likely to be toxic. Other O2 species, such as saturated aliphatic O2 species with a DBE=1 have a low or negative loading value and are unlikely to be associated with the toxicity. Generally, the species which are more likely to be toxic are the O2 species.

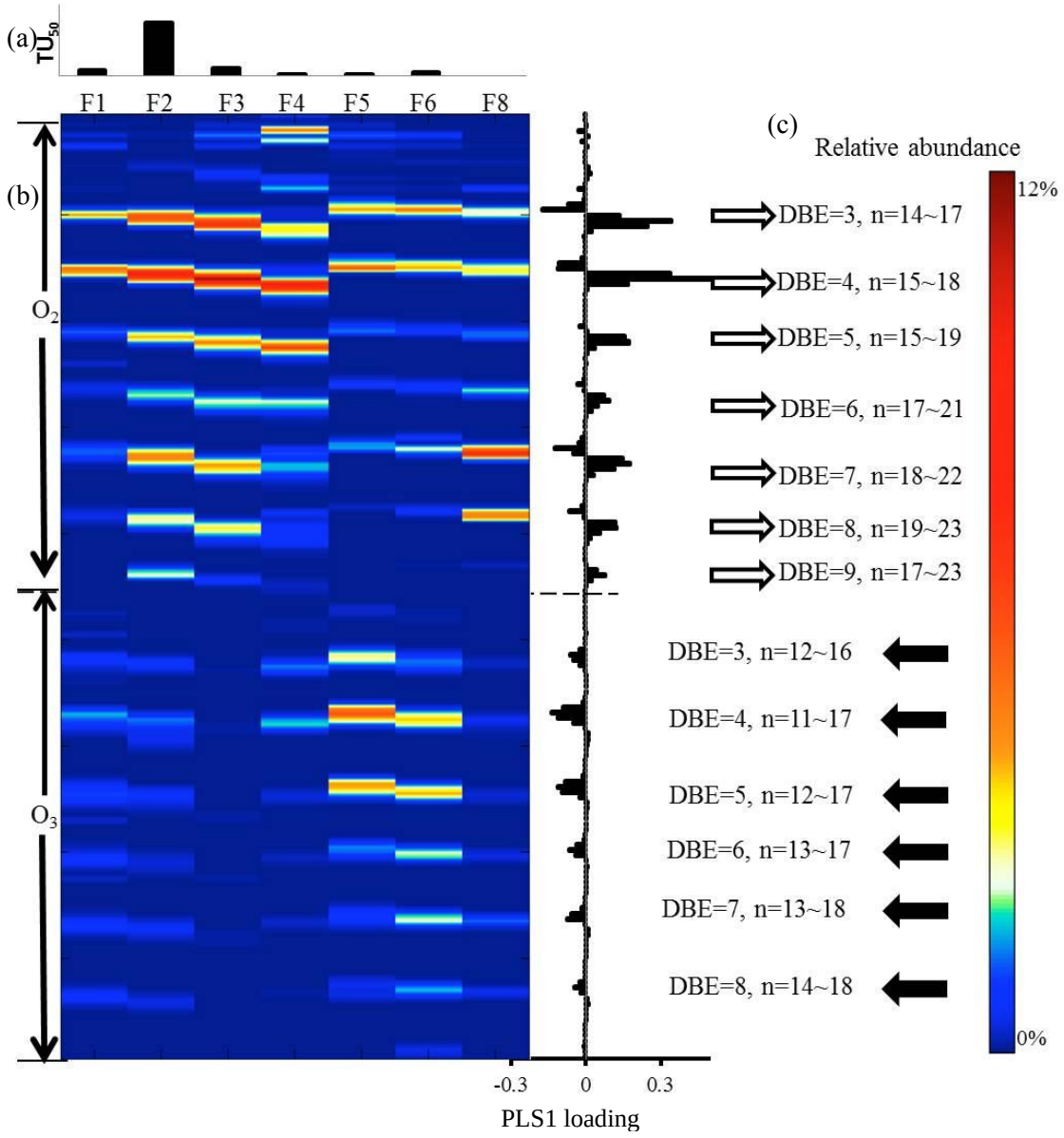

Figure 3-4 (a) Toxicity of all fractions with (b) corresponding heatmap of O2 and O3 species distribution. In the heatmap, each band represents an individual species and the intensity of its color indicates relative abundance. Double bond equivalence (DBE) and carbon number (n) increases from the top to the bottom within each O group. (c) The PLS1 loading plot (on right of heat map) shows the positive (clear arrows) and negative (black arrows) association between species and toxicity.

3.4.4 Compounds That Positively Correlate to Toxicity

Based on the PLS-DA analysis, O2-NAs, which positively correlated with toxicity, had DBE values between 3 and 9 and were present in greater relative abundance than those with DBE<3. O2 compounds with DBE=3 and 4 are likely aliphatic bi-, and tri-cyclic compounds which are the most abundant NAs with similar relative abundance in OSPW (Barrow et al. 2010, Martin et al. 2008, Rowland et al. 2011a, Rowland et al. 2011e). The C14 to C17 bicyclics (DBE=3), and C15 to C18 tricyclics (DBE=4) positively correlated with toxicity (PLS1 loading in Figure 3-4). The presence of these compounds is consistent with Barrow et al. (2010), Martin et al. (2008), Rowland et al. (2011a), and Rowland et al. (2011e) who detected C10-C19 DBE=3 compounds with C14 and C15 having the highest relative abundance and C11-C20 DBE=4 compounds with C15 and C16 being the most abundant. Measured EC_{50} for C11-C13 bicyclic and C11-C14 tricyclic NA surrogates (Jones et al. 2011) are in the range of 0.218-0.004 mM and 0.784-0.337 mM respectively (i.e. tenths to tens of mg L^{-1} range). Since experimental results (Jones et al. 2011) and the ADMET predictor (Scarlett et al. 2012) show that toxicity generally increased with carbon number, the higher carbon numbered bicyclics (C14-C17) and tricyclics (C15-C18) in the F2 fraction should be more toxic.

Of the bicyclic (DBE=3) compounds that positively correlated to toxicity, $C_{14}H_{23}O_2$ and $C_{15}H_{25}O_2$ were the most abundant in the most toxic F2 fraction (Table 3-1). Rowland et al. (2011c) tentatively identified several bicyclic isomers in commercially available NAs including those of perhydroindane carboxylic acids and > C11 isomers that may be methyl substituted decalin moiety with a carboxylic acid group like ethanoic or propanoic acid. The mass formulae and DBE values of bicyclic (DBE=3) $C_{14}H_{23}O_2$ and $C_{15}H_{25}O_2$ which positively correlated to toxicity are consistent with decalin carboxylic acids that may also be methylated. Jones et al. (2011) found that 3-decalin-1-yl-propanoic (C13) was the most toxic (EC_{50}=0.004 mM) of the bicyclic compounds tested and $C_{14}H_{23}O_2$ and $C_{15}H_{25}O_2$ are likely more toxic.

Table 3-1 A summary of the 29 most abundant O components identified by ESI- LTQ-Orbitrap MS with the highest positive correlation (P1) to acute Microtox® toxicity. Since ESI resulted in a loss of one proton, an adjustment to the molecular formulae was made in the determination of DBE. ND: not detected

P1	formula	DBE	m/z	Δ(ppm)	F1	F2	F3	F4	F5	F6	F8
					% relative abundance						
O2 compound class											
0.02	$C_{15}H_{27}O_2$	2	239.20156	-0.391	ND	0.03	0.58	0.03	ND	ND	ND
0.13	$C_{14}H_{23}O_2$	3	223.17025	-0.463	4.44	5.84	2.15	0.25	1.24	1.63	2.52
0.34	$C_{15}H_{25}O_2$	3	237.18606	0.239	0.50	5.61	6.04	0.20	ND	0.01	0.17
0.25	$C_{16}H_{27}O_2$	3	251.20140	-1.009	ND	1.16	7.30	3.04	ND	ND	ND
0.02	$C_{17}H_{29}O_2$	3	265.21735	0.175	ND	0.01	1.28	3.16	ND	ND	ND
0.34	$C_{15}H_{23}O_2$	4	235.17032	-0.142	3.82	10.01	4.17	0.46	0.16	0.62	2.93
0.53	$C_{16}H_{25}O_2$	4	249.18613	0.508	0.19	5.90	12.06	2.19	ND	0.02	0.07
0.17	$C_{17}H_{27}O_2$	4	263.20137	-1.077	ND	0.71	6.54	10.02	ND	0.02	ND
0.16	$C_{16}H_{23}O_2$	5	247.17019	-0.661	0.37	4.10	2.31	0.28	0.01	0.08	1.21
0.17	$C_{17}H_{25}O_2$	5	261.18592	-0.319	ND	1.40	4.89	3.46	ND	0.02	0.01
0.04	$C_{18}H_{27}O_2$	5	275.20183	0.642	ND	0.11	2.31	6.68	ND	0.01	ND
0.07	$C_{17}H_{23}O_2$	6	259.17020	-0.591	0.20	2.20	0.96	0.19	0.01	0.05	0.78
0.09	$C_{18}H_{25}O_2$	6	273.18577	0.563	0.03	1.05	2.46	2.35	ND	0.01	0.02
0.05	$C_{19}H_{27}O_2$	6	287.20164	-0.047	ND	0.12	1.73	1.76	ND	ND	ND
0.02	$C_{20}H_{29}O_2$	6	301.21730	-0.012	ND	0.01	0.81	0.75	ND	ND	ND
0.15	$C_{18}H_{23}O_2$	7	271.16991	-0.207	0.60	4.52	1.78	0.59	0.01	0.04	1.49
0.18	$C_{19}H_{25}O_2$	7	285.18576	-0.853	0.07	1.78	4.27	1.76	ND	ND	0.01
0.12	$C_{20}H_{27}O_2$	7	299.20168	0.089	0.04	0.48	3.45	1.58	ND	ND	ND

0.03	$C_{21}H_{29}O_2$	7	313.21735	0.149	ND	0.01	1.07	0.68	ND	ND	ND
0.12	$C_{19}H_{23}O_2$	8	283.17043	0.271	0.14	2.25	1.82	0.36	ND	ND	ND
0.12	$C_{20}H_{25}O_2$	8	297.18571	-0.987	ND	0.80	3.34	0.89	ND	ND	ND
0.06	$C_{21}H_{27}O_2$	8	311.20172	0.214	ND	0.06	1.89	0.76	ND	ND	ND
0.05	$C_{18}H_{19}O_2$	9	267.13918	0.475	ND	1.73	0.01	0.03	ND	ND	ND
0.08	$C_{19}H_{21}O_2$	9	281.15480	0.344	ND	2.51	0.36	0.01	ND	ND	ND
0.09	$C_{20}H_{23}O_2$	9	295.17029	-0.214	ND	1.44	1.64	0.04	ND	ND	ND
0.07	$C_{21}H_{25}O_2$	9	309.18609	0.280	ND	0.27	1.86	0.10	ND	ND	ND
0.03	$C_{22}H_{27}O_2$	9	323.20183	0.546	ND	0.01	0.81	0.14	ND	ND	ND
O3S compound class											
0.02	$C_{17}H_{27}O_3S$	4	311.1684	-0.799	1.74	ND	1.02	0.48	0.41	0.14	0.07
0.03	$C_{18}H_{29}O_3S$	4	325.1844	0.250	0.52	ND	1.09	0.42	0.16	0.06	0.07

Among NAs that positively correlated to toxicity, the tricyclic (DBE=4) compounds, $C_{15}H_{24}O_2$ (MW=236) and $C_{16}H_{26}O_2$ (MW=250) were the most abundant in the most toxic F2 fraction (Table 3-1). Based on the mass formulae and DBE values, these tricyclic compounds are likely a methyl and/or ethyl substituited adamantane moiety with an ethanoic group. Such compounds were tentatively identified in OSPW (Rowland et al. 2011a). Not previously shown to correlate with toxicity were two tricyclic (DBE=4) sulfur containing compounds, $C_{17}H_{27}O_3S$ and $C_{18}H_{29}O_3S$ (Table 3-1).

C16 to C18 compounds with DBE=5 also positively correlated with toxicity (PLS1 loading in Figure 3-4). DBE=5 compounds may be monoaromatic or tetracyclic (diamantane) NAs. The relative abundance of DBE=5 compounds have been shown to be about one third that of DBE=3 or 4 compounds (Barrow et al. 2010, Martin et al. 2008, Rowland et al. 2011a, Rowland et al. 2011e). Although the distribution of monoaromatic vs tetracyclic NAs is

unknown, aromatic NAs are much less abundant than alicyclic NAs (Jones et al. 2012). $C_{16}H_{23}O_2$ is the most abundant DBE=5 compound in the F2 fraction (Table 3-1) and its mass formula is consistent with an "abundant" C16 tetracyclic acid (a diamantane carboxylic acid) tentatively identified in OSPW (West et al. 2013). It is more likely to be a methyl diamantane carboxylic acid than the much less abundant diamantane ethanonic acid (Rowland et al. 2011e). Monoaromatic NAs identified in commercial NAs have been a methyl or ethyl substituited phenyl group with a carboxylic acid such as ethanoic, butanoic or propanoic (Rowland et al. 2011d).

DBE=6 compounds may be pentacyclic (diamantane) NAs or a monoaromatic compound with an alicyclic group. $C_{17}H_{23}O_2$ (DBE=6) which positively correlated to toxicity (Table 3-1) is likely dimethyl diamantane carboxylic acid or methyl diamantane ethanoic acid, tentatively identified in OSPW by Rowland et al.(2011e).

Scarlett et al. (2013) has shown that an alicyclic as well as an aromatic esterifiable fraction of OSPW was toxic to larval zebrafish. Their aromatic fraction contained mainly 258-300 Da compounds and was potentially more toxic on a molar basis than the alicyclic fraction, and although dominated by naphtheno-monoaromatic acids (~300 Da, DBE=7 (z =-12)), no specific components were correlated with toxicity. Dehydroabietic acids (DHAAs,) and >C17 diaromatic monocyclic acids (possible diaromatic analogues of the monoaromatic octahydrophenanthroic acids) have been previously identified in the same aromatic fraction (Jones et al. 2012, Rowland et al. 2011b). Within experimental error in the current study, a compound consistent with DHAA positively correlated with toxicity. It is $C_{20}H_{27}O_2$ (MW=299.20168, DBE=7) (Table 3-1) and given that ESI^- results in the loss of one proton, this compound may be DHAA ($C_{20}H_{28}O_2$ with MW = 300.435 and DBE =7 (z = -12). Although DHAA in pulp and paper effluents is known to be toxic (Oikari et al. 1983) and in studying NA surrogates, Scarlett et al. (2012) predicted that DHAA has a toxicity similar to octadecanoic acid (which had an LC_{50} of 0.56 μM i.e. ~ 0.17 mg

DHAA • L^{-1}), the link between toxicity and DHAA in OSPW has not been previously shown. There are also possible >C17 diaromatic monocyclic acids that positively correlated to toxicity (Table 3-1) such as the DBE=9 (z=-16) O2 C18-C22 compounds. No studies examining the toxicity of this group of compounds were found in the literature and they have not been previously implicated in toxicity.

3.4.5 Compounds That Negatively Correlate to Toxicity

In the toxic fractions (F1-F3), alicyclic NAs with a DBE < 3 (z < -6) (i.e. linear and branched aliphatic acids and monocyclic acids) were either negatively or poorly correlated with toxicity (Figure 3-4). Furthermore, the poorly toxic F4 fraction had the highest relative abundance of O2 compounds with DBE=1 (linear and branched aliphatic acids) and with DBE=2 (monocyclic acids) at ~8 and 3.4% respectively of the total relative abundance while the most toxic F2 fraction had only 0.14 and 3.4% respectively, indicating that these groups of compounds were not contributing to toxicity. Using NA surrogates, Jones et al. (2011) showed that linear and branched aliphatic acids and monocyclic acids achieved EC_{50} values in the Microtox® assay at concentrations of 0.700 to 0.012 mM (equivalent to ~ ones to tens of mg L^{-1}) with toxicity increasing as carbon numbers increased. Although Jones et al. (2011) did not test compounds with more than 12-14 carbons because of insolubility, Scarlett et al. (2012) predicted that toxicity would further increase at higher carbon numbers for linear (to C18) and branched (to C15) aliphatic acids which were more toxic than monocyclic NAs using the ADMET predictor. Since total NAs is 20-120 mg L^{-1} (Frank et al. 2008) and c-NAs with DBE < 3 have been shown to have low (~3-4%) relative abundance in OSPW (Barrow et al. 2010), even concentrated ~50 times by SPE fractionation in this study, the concentration of these compounds were likely too low to have a toxic effect.

While NAs comprise ~30-50% of the acid extractable fraction of OSPW, ~30-70% of these may be O2 NAs and 15-40% O3-NAs with some O4 and O5 compounds (Grewer et al. 2010). O3 compounds are likely hydroxylated (i.e. oxidized) O2 NAs. In this study, all O3 NAs (C13 to C17, DBEs=3 to 8) correlated negatively with toxicity and were usually present at higher relative abundance in poorly toxic fractions (e.g. ~5.99% $C_{14}H_{21}O_3$ (DBE=4) and 4% $C_{14}H_{19}O_3$ (DBE=5) in F5) than the most toxic F2 fraction (0.55% $C_{14}H_{21}O_3$ (DBE=4) and 0.01% $C_{14}H_{19}O_3$ (DBE=5), Figure 3-4 and Table 3-2). Although the toxicity of O3 NAs has not been previously studied, they have been suggested to be linked to estrogenicity (He et al. 2011). Rowland (2011b) has shown the presence of O3 C19 and C20 (DBE=7) aromatic steroidal hydroxy acids similar to estrone and estradiol in OSPW (Rowland et al. 2011b). However, they were not among the O3 compounds that positively (Table 3-1) or negatively (Table 3-2) correlated to toxicity.

There were a few O2S compounds that negatively correlated to toxicity. These were more abundant in the non-toxic or poorly toxic fractions.

Table 3-2 A summary of the 21 most abundant O components identified by ESI- LTQ-Orbitrap MS with the highest negative correlation (P1) to Microtox® acute toxicity. Since ESI resulted in a loss of one proton, an adjustment to the molecular formulae was made in the determination of DBE. ND: not detected.

P1	formula	DBE	m/z	Δ(ppm)	F1	F2	F3	F4	F5	F6	F8
					% relative abundance						
O3 compound class											
-0.06	$C_{13}H_{21}O_3$	3	225.14981	0.854	0.72	0.13	0.01	0.10	2.91	0.77	0.04
-0.05	$C_{14}H_{23}O_3$	3	239.16544	0.720	0.85	0.72	0.02	0.61	2.32	1.33	0.21
-0.09	$C_{13}H_{19}O_3$	4	223.13415	0.817	0.61	0.02	ND	0.02	4.68	0.58	0.01
-0.14	$C_{14}H_{21}O_3$	4	237.14961	-0.033	1.70	0.55	0.01	0.31	5.99	2.33	0.16
-0.11	$C_{15}H_{23}O_3$	4	251.16510	-0.668	1.18	1.33	0.01	1.43	3.37	3.83	0.53
-0.05	$C_{16}H_{25}O_3$	4	265.18095	0.121	0.83	1.09	0.06	1.92	1.07	2.60	0.32
-0.08	$C_{14}H_{19}O_3$	5	235.17045	0.411	0.46	0.01	ND	ND	4.12	0.65	ND
-0.11	$C_{15}H_{21}O_3$	5	249.14977	0.611	0.73	0.32	0.01	0.08	3.90	2.77	0.10
-0.10	$C_{16}H_{23}O_3$	5	263.16535	0.312	1.05	0.66	0.01	0.31	1.87	4.09	0.30
-0.07	$C_{16}H_{21}O_3$	6	261.14957	-0.183	0.34	0.09	ND	0.08	1.22	2.24	0.32
-0.06	$C_{16}H_{19}O_3$	7	259.13393	-0.146	0.21	0.06	ND	0.06	0.91	1.85	0.49
-0.08	$C_{17}H_{21}O_3$	7	273.14941	-0.761	0.61	0.26	ND	0.27	0.65	2.41	1.31
O2 compound class											
-0.07	$C_{12}H_{19}O_2$	3	195.13893	-0.631	0.04	0.07	ND	0.02	2.16	1.58	0.16
-0.17	$C_{13}H_{21}O_2$	3	209.15468	0.111	0.99	1.45	0.29	0.27	4.38	5.23	2.40
-0.11	$C_{13}H_{19}O_2$	4	207.13902	-0.160	0.26	0.28	ND	0.10	2.20	2.91	1.21
-0.11	$C_{14}H_{21}O_2$	4	221.15481	0.483	5.19	3.38	1.02	0.51	6.24	4.16	2.99
-0.13	$C_{16}H_{19}O_2$	7	243.13894	-0.465	1.14	1.64	0.01	0.61	1.55	2.70	4.70
-0.07	$C_{17}H_{19}O_2$	8	255.13940	1.477	0.32	1.13	ND	0.60	0.02	0.66	4.34
O2S compound class											
-0.11	$C_{14}H_{21}O_2S$	4	253.12681	0.142	0.22	ND	ND	0.05	1.12	2.27	2.53
-0.28	$C_{15}H_{23}O_2S$	4	267.14254	0.434	0.67	ND	ND	0.51	0.30	2.76	11.42
-0.10	$C_{16}H_{25}O_2S$	4	281.15776	-1.117	0.69	ND	ND	0.21	ND	0.13	5.13

3.6 References

Barrow, M. P., M. Witt, J. V. Headley and K. M. Peru (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.

Braumann, T. (1986). "Determination of hydrophobic parameters by reversed-phase liquid chromatography: Theory, experimental techniques, and application in studies on quantitative structure-activity relationships." Journal of Chromatography **373**(2): 191-225.

Clemente, J. S. and P. M. Fedorak (2005). "A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids." Chemosphere **60**(5): 585-600.

ERCB, Energy Resources Conservation Board (ERCB). EnerFAQs 12: Oil Sands. **2011.** (citation date:2014-12-01) http://www.lica.ca/attachments/077_12%20Oil%20Sands.pdf

Fiehn, O., L. Vigelahn, G. Kalnowski, T. Reemtsma and M. Jekel (1997). "Toxicity-directed fractionation of tannery wastewater using solid phase extraction and luminescence inhibition in microtiter plates." Acta Hydrochimica et Hydrobiologica **25**(1): 11-16.

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

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

Frank, R. A., H. Sanderson, R. Kavanagh, B. K. Burnison, J. V. Headley and K. R. Solomon (2010). "Use of a (quantitative) structure-activity relationship [(Q)SAR] model to predict the toxicity of naphthenic acids." Journal of Toxicology and Environmental Health-Part A-Current Issues **73**(4): 319-329.

Gagne, F., M. Douville, C. Andre, T. Debenest, A. Talbot, J. Sherry, L. M. Hewitt, R. A. Frank, M. E. McMaster, J. Parrott and G. Bickerton (2012). "Differential changes in gene expression in rainbow trout hepatocytes exposed to extracts of oil sands process-affected water and the Athabasca river." Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **155**(4): 551-559.

Grewer, D. M., R. F. Young, R. M. Whittal and P. M. Fedorak (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.

He, Y. H., S. B. Wiseman, M. Hecker, X. W. Zhang, N. Wang, L. A. Perez, P. D. Jones, M. G. El-Din, J. W. Martin and J. P. Giesy (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., K. M. Peru and M. P. Barrow (2009). "Mass spectrometric characterization of naphthenic acids in environmental samples: A review." Mass Spectrometry Reviews **28**(1): 121-134.

Headley, J. V., K. M. Peru, M. H. Mohamed, L. Wilson, D. W. McMartin, M. M. Mapolelo, V. V. Lobodin, R. P. Rodgers and A. G. Marshall (2012). "Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry characterization of tunable carbohydrate-based materials for sorption of oil sands naphthenic acids." Energy & Fuels **27**(4): 1772-1778.

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

Jackson, A., J. B. Fitzgerald, R. W. J. Hartley, A. Leonard and J. Yates (1993). "Ct appearances of hematomas in the corpus-callosum in patients with subarachnoid hemorrhage." Neuroradiology **35**(6): 420-423.

Jones, D., A. G. Scarlett, C. E. West and S. J. Rowland (2011). "Toxicity of individual naphthenic acids to *Vibrio fischeri*." Environmental Science & Technology **45**(22): 9776-9782.

Jones, D., C. E. West, A. G. Scarlett, R. A. Frank and S. J. Rowland (2012). "Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract." Journal of Chromatography A **1247**(0): 171-175.

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

Lundstedt-Enkel, K., M. Tysklind, J. Trygg, P. Schuller, L. Asplund, U. Eriksson, L. Haggberg, T. Odsjo, M. Hjelmberg, M. Olsson and J. Orberg (2005). "A statistical resampling method to calculate biomagnification factors exemplified with organochlorine data from herring (*Clupea harengus*) muscle and guillemot (*Uria aalge*) egg from the Baltic sea." Environmental Science & Technology **39**(21): 8395-8402.

MacKinnon, M. D. and H. Boerger (1986). "Description of two treatment methods for detoxifying oil sands tailings pond water." Water Quality Research Journal of Canada **21**(4): 496-512.

Madill, R. E. A., M. T. Orzechowski, G. S. Chen, B. G. Brownlee and N. J. Bunce (2001). "Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water." Environmental Toxicology **16**(3): 197-208.

Martin, J. W., X. M. Han, K. M. Peru and J. V. Headley (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.

McDonald, J. D., I. Eide, J. Seagrave, B. Zielinska, K. Whitney, D. R. Lawson and J. L. Mauderly (2004). "Relationship between composition and toxicity of motor vehicle emission samples." Environmental Health Perspectives **112**(15): 1527-1538.

Oikari, A., B.-E. Lönn, M. Castrén, T. Nakari, B. Snickars-Nikinmaa, H. Bister and E. Virtanen (1983). "Toxicological effects of dehydroabietic acid (DHAA) on the trout, *Salmo gairdneri* Richardson, in fresh water." Water Research **17**(1): 81-89.

Reemtsma, T., O. Fiehn and M. Jekel (1999). "A modified method for the analysis of organics in industrial wastewater as directed by their toxicity to *Vibrio fischeri*." Fresenius Journal of Analytical Chemistry **363**(8): 771-776.

Romanova, U. G., M. Valinasab, E. N. Stasiuk and H. W. Yarranton (2006). "The effect of oil sands bitumen extraction conditions on froth treatment performance." Journal of Canadian Petroleum Technology **45**(9): 36-45.

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

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

Rowland, S. J., C. E. West, A. G. Scarlett and D. Jones (2011c). "Identification of individual acids in a commercial sample of naphthenic acids from petroleum by two-dimensional comprehensive gas chromatography/mass spectrometry." Rapid Communications in Mass Spectrometry **25**(12): 1741-1751.

Rowland, S. J., C. E. West, A. G. Scarlett, D. Jones, M. Boberek, L. Pan, M. Ng, L. Kwong and A. Tonkin (2011d). "Monocyclic and monoaromatic naphthenic acids: Synthesis and characterisation." Environmental Chemistry Letters **9**(4): 525-533.

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

Rudzinski, W. E., L. Oehlers and Y. Zhang (2002). "Tandem mass spectrometric characterization of commercial naphthenic acids and a maya crude oil." Energy & Fuels **16**(5): 1178-1185.

Scarlett, A. G., H. C. Reinardy, T. B. Henry, C. E. West, R. A. Frank, L. M. Hewitt and S. J. Rowland (2013). "Acute toxicity of aromatic and non-aromatic fractions of naphthenic

acids extracted from oil sands process-affected water to larval zebrafish." Chemosphere **93**(2): 415-420.

Scarlett, A. G., C. E. West, D. Jones, T. S. Galloway and S. J. Rowland (2012). "Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints." Science of the Total Environment **425**(2012): 119-127.

Seward, J. R. and T. W. Schultz (1999). "QSAR analyses of the toxicity of aliphatic carboxylic acids and salts to *Tetrahymena pyriformis*." SAR and QSAR in Environmental Research **10**(6): 557-567.

West, C. E., A. G. Scarlett, J. Pureveen, E. W. Tegelaar and S. J. Rowland (2013). "Abundant naphthenic acids in oil sands process‐affected water: Studies by synthesis, derivatisation and two‐dimensional gas chromatography/high-resolution mass spectrometry." Rapid Communications in Mass Spectrometry **27**(2): 357-365.

Wold, S., C. Albano, W. J. Dunn, K. Esbensen, S. Hellberg, E. Johansson, W. Lindberg and M. Sjostrom (1984). "Modeling data tables by principal components and PLS - class patterns and quantitative predictive relations." Analusis **12**(10): 477-485.

Chapter 4

Identification of Estrogenic Compounds in Oil Sands Process Waters by Effect-Directed Analysis

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4.1 Abstract

Using effect-directed analysis, the presence of estrogenic components in untreated and biologically treated oil sands process water (OSPW) was detected with the yeast estrogenic screening assay after fractionation with solid phase extraction followed by reversed phase HPLC. Comparison of the composition, as determined by electrospray ionization combined with high-resolution Linear Trap Quadropole (LTQ)-Orbitrap Velos Pro hybrid mass spectrometry (negative ion) of selected estrogenic and non-estrogenic fractions identified compounds that were uniquely present in the estrogenic samples, biologically treated and untreated. Of the 30 most abundant compounds, there were 14 possible non-aromatic structures and 16 possible aromatic structures. Based on the published literature, the latter are the most likely to cause estrogenicity and were O2, O3 and O4 C17 to C20 compounds with double bond equivalents between 6 and 10 and chemical formulae similar to estrone- and estradiol-like compounds. This study shows exact formulae and masses of possible estrogenic compounds in OSPW. These findings will help to focus study on the most environmentally significant components in OSPW.

4.2 Introduction

Caustic hot water extraction of bitumen from oil sands produces a wastewater known as oil sands process water (OSPW) which is toxic to aquatic organisms, such as rainbow trout (*Oncorhynchus mykiss*) (MacDonald et al. 2013), *Daphnia magna* (Clemente et al. 2005), and

Vibrio fischeri (Holowenko et al. 2002, MacKinnon et al. 1986). OSPW is not released into the environment under Alberta's Environmental Protection and Enhancement Act, and therefore is held in storage ponds that have a total volume exceeding 720 million m^3 in 2011 (ERCB 2011). Exposure to OSPW has caused endocrine disruption in fathead minnows (Kavanagh et al. 2011), goldfish (Lister et al. 2008) and T47D-kbluc and MDAkb2 cells (He et al. 2011). Starting with an OSPW concentrate as described by Frank et al. (2008), Reinardy et al. (2013) used acidification, solvent extraction, esterification then argentation solid phase fractionation to recover "classical" alicyclic naphthenic acid (NA) fractions and "aromatic" NA fractions. At the concentrations tested, the de-esterified aromatic NA fraction was shown to have an estrogenic effect, consistent with a weak estrogenic substance. Using effect-directed analysis (EDA), Thomas et al. (2009) showed that fractions of off-shore North Sea produced waters containing petrogenic naphthenic acids (C7 to C14 with 1 to 4 rings) and short chain alkyl phenols (C1 to C5) were responsible, respectively, for 65 and 35% of the estrogen receptor agonist activity, while fractions containing polycyclic aromatic hydrocarbons were androgen receptor antagonists. Rowland et al. (2011a) have shown that some aromatic NAs in OSPW have a structural similarity to sex steroid hormones like estrone and estradiol, but they did not test for estrogenicity (Rowland et al. 2011a).

Oil sands NAs are a complex mixture of carboxylic acids with the structural formula $C_nH_{2n+z}O_x$ (Grewer et al. 2010), where n is the number of carbons, "z" is related to the hydrogen deficiency or double bond equivalent and is zero or a negative, even integer, and "x" is the number of oxygen and ranges from 2-5. Classical NAs (c-NAs, x=2) and oxidized NAs (oxy-NAs, x=3-5) comprise less than 50% of the acid extractable organics (AEO) from OSPW (Grewer et al. 2010). NA compounds include alicyclic and aromatic carboxylic acids and non-NA AEO may also contain sulfur, nitrogen, and other elements with up to 10 O groups (Headley et al. 2012). Specific NAs or their chemical formulae in OSPW implicated in estrogenicity have not been identified because of the complexity of the mixtures and limitations of the analytical

methods used. Many methods have been used to characterize the components in OSPW, including GC-MS, HPLC-HRMS, and GC×GC-MS (Headley et al. 2013b). Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) analysis of OSPW has demonstrated advantages over the other methods because its ultra-high resolution (450,000−650,000 at m/z 500) enables the isolation of mass peaks which may differ by less than 3 mDa. This high mass accuracy (better than 300 ppb mass error) allows for unambiguous assignment of a molecular formula (Headley et al. 2013b). Barrow et al. (2004) was the first to apply FTICR-MS to characterize commercially available NAs and OSPW. When coupled with electrospray ionization (ESI), a wider range of compounds were shown to be presence including O_x, O_xS, O_xS_2, and NO_x species that were not seen using other methods (Barrow et al. 2010, Headley et al. 2011a, Headley et al. 2011b, Headley et al. 2009). Comparison of mass spectra obtained from combining ESI or atmospheric pressure photoionization (APPI) with positive- or negative-ion mode showed a greater number of peaks in the positive- than negative-ion mode as well as when APPI was used instead of ESI (Barrow et al. 2010). Some compound classes were better detected by certain modes of operation eg O_x, O_xS, O_xS_2, and NO_x species were better characterized by ESI in the positive-ion mode while ESI in the negative mode was better for NAs. Ultrahigh resolution (>100,000 at m/z 400) and high mass accuracy (< 5 ppm) can also be achieved with the Linear Trap Quadropole (LTQ)-Orbitrap MS. When used to analyze OSPW NAs, the presence of SO_x and NO_x compounds were also shown (Headley et al. 2013a, Headley et al. 2011c). Pereira et al. (2013) showed that O2 species detected in ESI negative mode are chemically distinct from those detected in the positive-ion mode using HPLC LTQ-Orbitrap-MS.

The objective of this paper is to establish which components in OSPW are associated with estrogenicity by effect-directed analysis (EDA). To achieve this, an approach was used that is similar to Thomas et al. (2009), in which the estrogenic components were first isolated by multistep fractionation using a combination of solid phase extraction (SPE) and reverse phase

(RP) HPLC fractionation, then the fractions with endocrine disrupting potential were identified by the yeast estrogenic (YES) assay, and their composition evaluated by LTQ-Orbitrap Velos Pro MS with ultrahigh-resolution. Finally, the compounds present in only certain estrogenic sub-fractions were identified. Applying our current knowledge of the possible estrogenic nature of components in OSPW, several chemical formulae of aromatic structures are suggested as potential estrogenic compounds. Such knowledge is useful in targeting the more important pollutants in OSPW to achieve cleanup or to assess environmental risk.

4.3 Experimental Section

4.3.1 OSPW

OSPW was collected from the West In Pit, WIP, at the entrance of an active settling basin on the Syncrude Canada site in Fort McMurray, AB. OSPW was biologically treated using two immobilized soil bioreactors (ISBRs) connected in series and operated as described by McKenzie et al. (2014) at a removal rate of NAs of 2.32 mg L^{-1} d^{-1} by microorganisms immobilized in a biofilm.

4.3.2 Fractionation Strategy

Treated and untreated OSPW were fractionated into less complex mixtures using the procedure outlined in Figure 4-1.

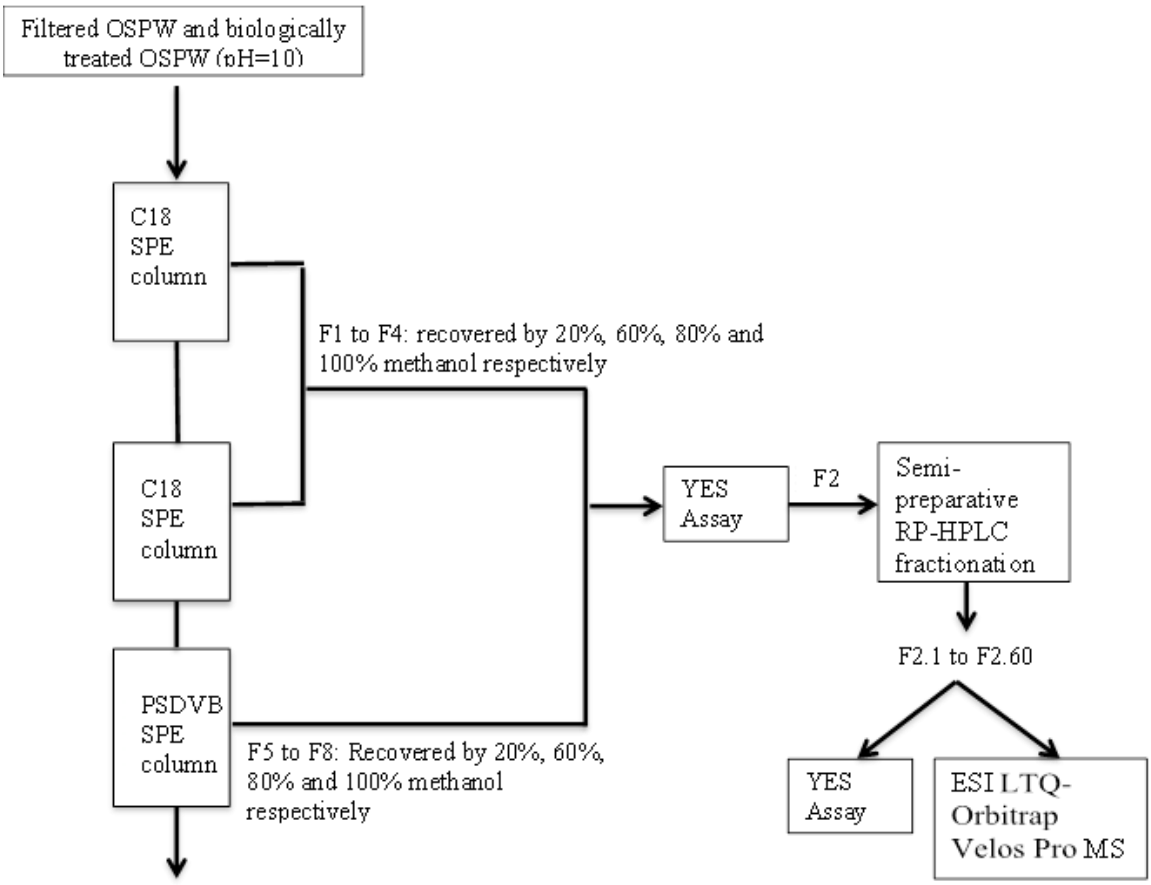

Figure 4-1 Fractionation strategy in the EDA evaluation of NAs in untreated and biologically treated OSPW. Estrogenic samples from SPE fractionation were selected for further RP-HPLC fractionation. Selected estrogenic and non-estrogenic RP-HPLC fractions were then analyzed by LTQ-Orbitrap Velos Pro Hybrid MS.

4.3.3 Solid Phase Extraction (SPE)

The pH of treated or untreated OPSW was adjusted to 10 with 1M NaOH, filtered through a 0.45 μm nylon filter (Millipore Corporation, Billerica, MA, USA) then passed through two C18 (octade-cylsilane) SPE columns and one polystyrene-divinylbenzene (PSDVB) SPE column (J.T.Baker, Phillipsburg, NJ, USA) connected in series (adapted from Reemtsma et al. (1999) and Thomas et al. (2009)). Two C18 columns were needed to ensure that all adsorbable compounds were removed. Each column contained 1 g of sorbent. Columns were pre-conditioned

by passing 25 mL methanol followed by 25 mL high-purity water (18 megaohms.cm, Millipore water) before passing 5 L of treated or untreated OSPW. Fractions were eluted with an increasing amount of methanol (20, 60, 80, and 100%) and achieved a 50-fold concentration of the SPE-retained components from the OSPW samples. Fractions F1 to F4 were from the two C18 SPE columns recovered with 20, 60, 80 and 100% methanol respectively and F5 to F8 are from the PSDVB column recovered with 20, 60, 80 and 100% methanol respectively.

4.3.4 Semi-Preparative Reverse-Phase HPLC

The F2 fraction from the SPE fractionation had the highest estrogenic activity and the greatest decrease in estrogenicity after biological treatment, so it was selected for further fractionation by semi-preparative RP-HPLC (Hewlett-Packard 1050, Agilent Technologies, Bracknell, UK) equipped with a UV detector (230 nm). Four one mL samples (each equivalent to 250 mL of the original OSPW) were injected onto a silica guard column (5 cm×10 mm× 5 μm) connected to a ZORBAX Eclipse XDB-C8 semi-preparative HPLC column (25 cm×10 mm×5 μm, Agilent). The mobile phase consisted of HPLC-grade methanol (Fisher Scientific Inc., Ottawa, CA) (eluant A) and water containing 5% formic acid and 20 mM NH_4Ac at pH=7 (eluant B) pumped at a total flow rate of 1.5 mL min^{-1} using a gradient: 60% A for 10 min, then to 80% A in 10 min and maintained for 15 min, then to 100% A in 10 min and maintained for 15 min, for a total run-time of 60 min. Fractions were collected at 1 min intervals using a fraction collector (Dynamax model FC-4, Varian) obtaining a total of 60 1.5 mL samples which were designated as samples F2.1 to F2.60. Separation and collection were controlled by a Varian Star Chromatography Workstation. Samples were evaporated to dryness under nitrogen gas and redisolved in methanol to achieve a 200-fold concentration of the OSPW sample.

4.3.5 Yeast Estrogenic Screening Assay (YES)

The procedure followed was as described by Routledge et al. (1996). A culture of the recombinant yeast, *Saccharomyces cerevisiae* BJ3505 hER/2ERE (Gaido et al. 1997), provided by H. Engelhardt, University of Waterloo, was added to a 96-well plate (Corning Inc., Tewksbury, MA) and exposed to triplicate samples for 3 days. 17*β*-estradiol (E2, 98% pure, Sigma) in methanol was used as the positive control, methanol only as the negative control, and an E2 standard curve was made from a serial dilution of 17β-estradiol. Absorbance of the yellow chromogen, chlorophenol red-*β*-D-galactopyranoside (CPRG) (>96% purity, cat no. 10884308001, Roche Diagnostics, Indianapolis, IN), transformed to a red product by β-galactosidase, was measured using a Synergy^{TM} HT microtiter plate reader (Biotek Instruments Inc., Winnoski, VT). The absorbance readings were corrected for color (A_{540}) (i.e. background color without yeast) and for yeast growth (A_{630}) and the sample's estrogenic activity quantified by using E2 standard curves and expressed as equivalent E2 values (i.e. ng EEQ L^{-1}). Procedural blanks were performed in parallel, tested in triplicate and showed no estrogenicity.

4.3.6 LTQ-Orbitrap Velos Pro MS and MS/MS Analysis

The LTQ-Orbitrap Velos Pro Hybrid MS with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA) was used to analyze selected semi-preparative RP-HPLC fractions. Samples were injected directly by a Hamilton syringe (250 μL) into the ESI-MS using direct infusion by the inbuilt syringe pump of the LTQ-Orbitrap Velos Pro at a flow rate of 5 μL min^{-1}. The MS system was operated using Xcalibur software (Thermo Fisher Scientific). The electrospray voltage was 2.7 kV, with a nebulizing gas (N_2) flow of 1.5 L min^{-1}, drying gas (N_2) flow of 3.0 L min^{-1}, and the ion transfer tube temperature was 270 ℃ for the LTQ-Orbitrap Velos Pro. Prior to sample analysis, the LTQ and Orbitrap were tuned to optimize conditions for the detection of ions in the range m/z 150–1000 and calibrated according to the

manufacturers' calibration mixture. Full MS scans in negative ion mode used over 400 scans for each acquisition in the Orbitrap mass analyzer at a mass resolution of 100 000 (FWHM defined at m/z 400) and mass accuracy better than 5 ppm. MS/MS scans in the negative mode were performed by collision induced fragmentation and m/z analyzed using the ion trap alone. Ultrahigh resolution mass spectra were acquired using the Orbitrap region. Xcalibur software was used for data visualization, processing, and calculation of elemental composition.

Calculations were limited to formulas containing less than 100 ^{12}C, 2 ^{13}C, 200 ^{1}H, 5 ^{14}N, 10 ^{16}O, 3 ^{32}S, and 1 ^{34}S. If more than one possible formula was generated for a specific mass, one or more could almost always be confirmed or eliminated by the presence/absence of a corresponding ion containing ^{13}C, ^{18}O, or ^{34}S as described in Headley et al. (2012) using data acquired by negative ESI FTICR mass spectrometry.

4.4 Results and Discussion

4.4.1 OSPW

A portion of OSPW was subjected to biological treatment as described by McKenzie et al. (2014). Estrogenic activity of SPE fractions of untreated and biologically treated OSPW showed that the overall estrogenicity had decreased by 22% with treatment (Figure 4-2).

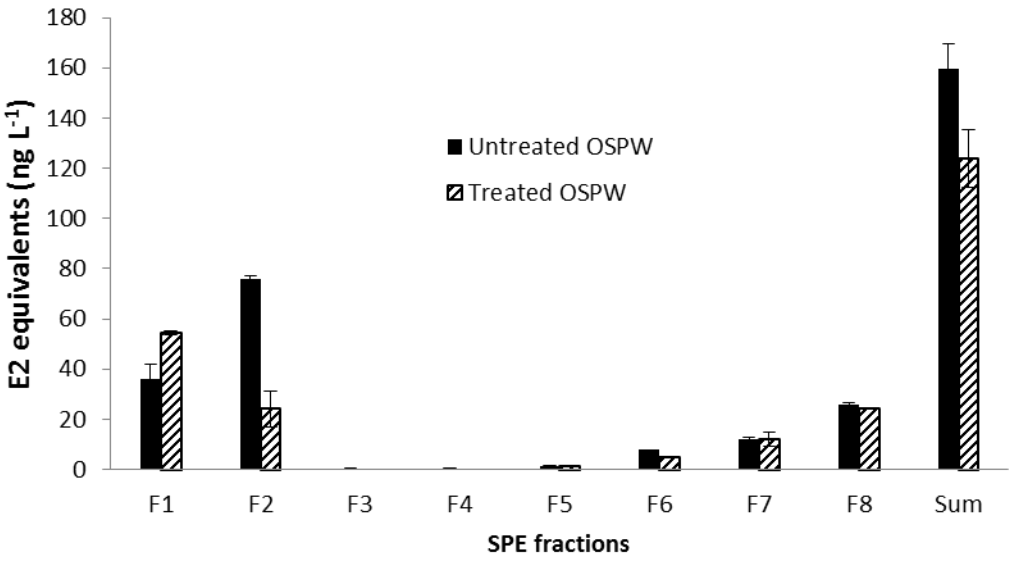

Figure 4-2 Estrogenic activity of SPE fractions of OSPW and biologically treated OSPW. F1 to F4 are from two C18 SPE columns recovered with 20, 60, 80 and 100% methanol respectively and F5 to F8 are from a PSDVB column recovered with 20, 60, 80 and 100% methanol respectively, as shown in Figure 4-1. Each SPE fraction was tested in triplicate and the data represent mean±95% confidence interval. The sum represents the total of untreated or treated SPE fractions.

4.4.2 Estrogenic Activity of SPE Fractions

A similar fractionation scheme had been used to identify toxic components in tannery wastewaters (Reemtsma et al. 1999) and produced waters from off-shore petroleum recovery (Thomas et al. 2009). The aim of the SPE fractionation was to prepare initial fractions, then select the most potent fraction(s) for further fractionation by RP-HPLC. There were eight fractions from each water sample, four fractions from the C18 columns (F1-F4) and four fractions from the PSDVB column (F5-F8) (Figure 4-1), recovered with increasing amounts of methanol (20, 60, 80, and 100%). It was difficult to determine how much estrogenicity was recovered in the fractions compared to OSPW as the estrogenicity of OSPW was too low to measure accurately. However, in a related study, at least 94% of Microtox® toxicity of OSPW was recovered in the eight fractions.

The majority of the estrogenicity in both untreated and treated OSPW was recovered from the C18 columns with 20% (F1) and 60% (F2) methanol (Figure 4-2). The 60% methanol (F2, solid bar) fraction of untreated OSPW had the highest estrogenicity (76 ng EEQ L^{-1}) representing 48.3% of the total estrogenicity of OSPW (157.5 ng EEQ L^{-1}). The estrogenicity of the same fraction for the biologically-treated OSPW (F2, diagonal hatching) decreased to 24 ng EEQ L^{-1}. This was the largest decrease in estrogenicity of all samples tested. Therefore, these two fractions were selected for further fractionation by semi-preparative RP-HPLC.

4.4.3 Estrogenic Activity in RP-HPLC Subfractions

The estrogenic activity of the RP-HPLC sub-fractions is shown in Figure 4-3. The sub-fractions were collected every minute for a total of 60 minutes and designated as F2.1 to F2.60. Estrogenic activity of both treated and untreated OSPW clustered between sub-fractions F2.28 to F2.41. Since RP-HPLC separates compounds based on hydrophobicity, this clustering implies that estrogenic activity may arise from a family of compounds. Nine fractions (F2.31, F2.31T, F2.41T, F2.11, F2.11T, F2.19, F2.19T, F2.20, and F2.20T) were selected for ESI- LTQ-Orbitrap Velos Pro MS analysis to identify compounds that may contribute to estrogenic activity.

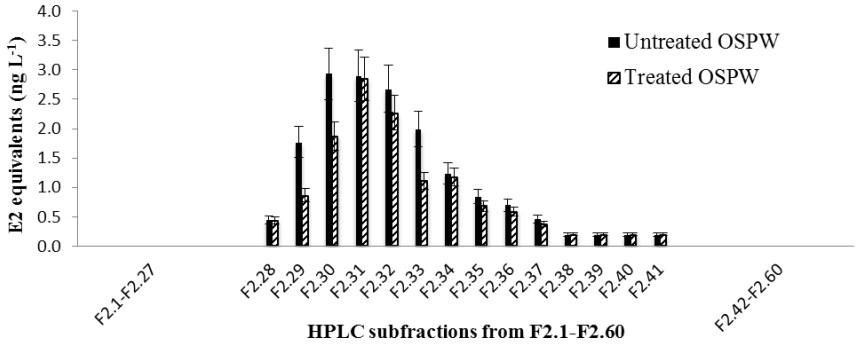

Figure 4-3 Estrogenic activity of 60 sub-fractions collected from semi-preparative RP-HPLC fractionation of the F2 fraction from SPE fractionation of OSPW and biologically treated OSPW. Fractions F2.1-F2.27 and F2.42-F2.60 had no detectable estrogenicity. Each sub-fraction was tested in triplicate and the data represent mean ± 95% confidence interval.

4.4.4 ESI-LTQ Orbitrap Velos Pro MS of Estrogenic and Nonestrogenic Fractions

Since the ESI technique uses soft ionization, the mass peaks are mostly molecular peaks and the mass formula can be assigned with high confidence because of the ultra-high resolution of the Orbitrap Velos Pro MS. After calculating the mass formula, the distribution of the different classes of NA compounds can be plotted as 3D graphs from the mass spectral data (See Appendix A, Figure S1). Figure 4 shows typical results of a subfraction with little estrogenicity (Figure (a), F2.11) and one that is highly estrogenic (Figure (b), F2.31). The components of all selected sub-fractions are shown in the heat map of Figure 4-4.

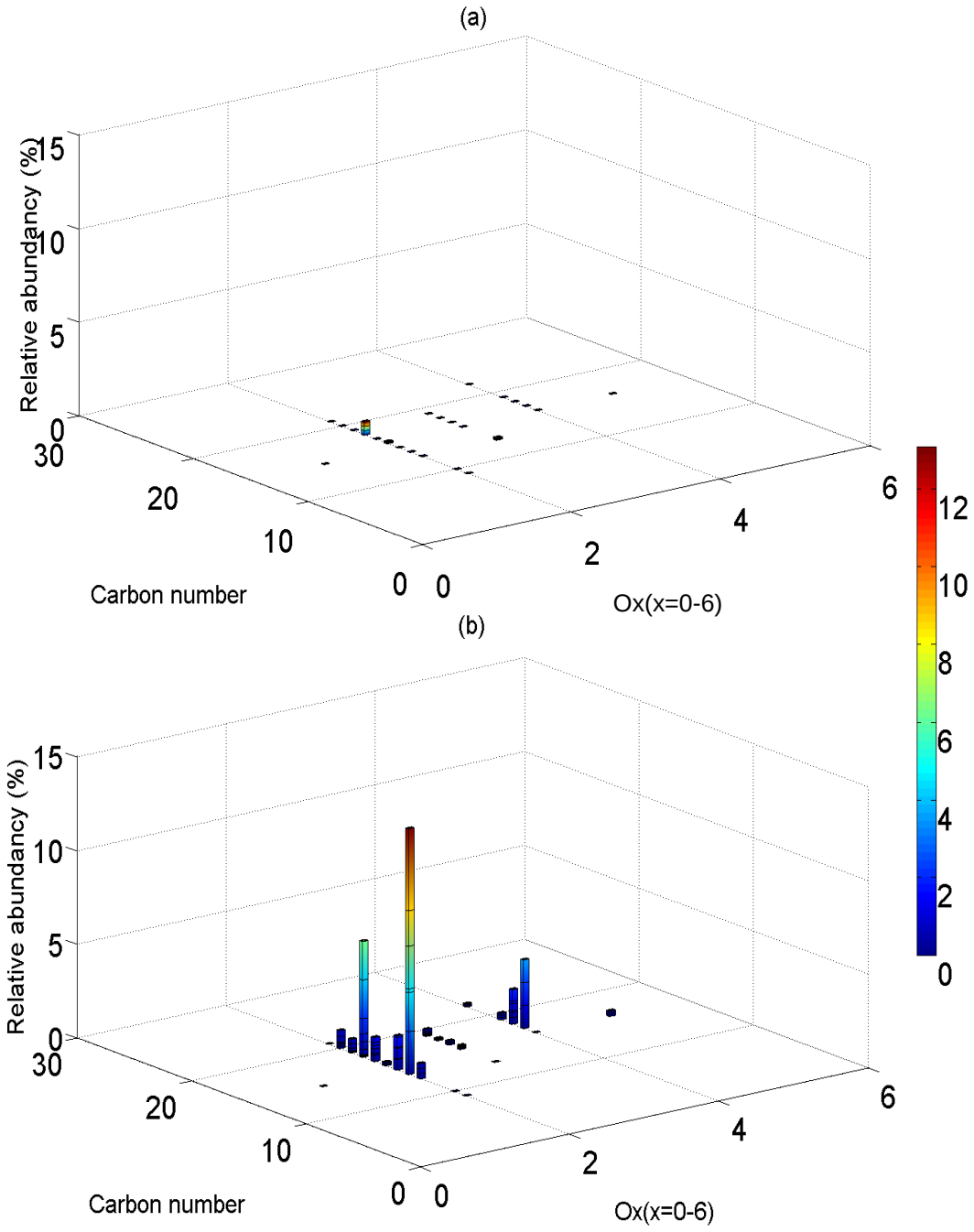

Figure 4-4 Representative 3D plots of the distribution of O_x (x=0-6) classes of compounds observed by LTQ-Orbitrap Velos Pro MS of HPLC sub-fractions with (a) little estrogenicity (F2.11) and (b) high estrogenicity (F2.31).

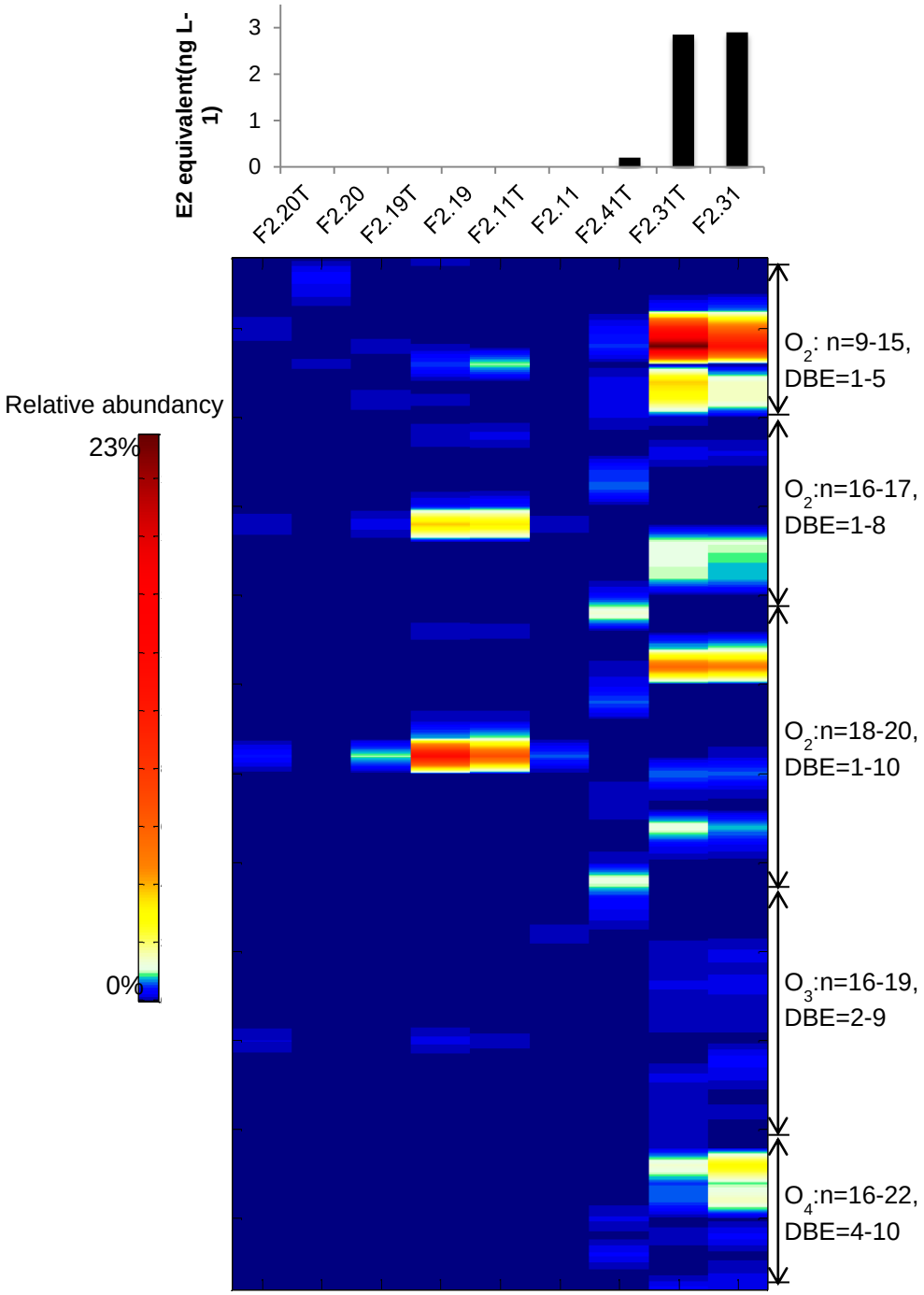

Figure 4-4 Estrogenicity of sub-fractions shown in bar graph (above) and corresponding class distribution of O_x (x=1-5) compounds in heat map (below) as detected by LTQ-Orbitrap Velos Pro MS of selected HPLC sub-fractions of the F2 SPE fraction. T indicates fractions from biologically treated OSPW. Within each arrow the compounds are grouped according to carbon number with the lowest carbon number at the top. Within each carbon number, those with the highest DBE are at the top. The details of each O group are shown in Figure 4-4.

The highly estrogenic sub-fraction (Figure 4-4, F2.31) of untreated OSPW had a higher relative abundance of compounds with O groups, especially ones with the O2 group. The corresponding biologically treated sub-fraction (F2.31T) had a similar distribution, while non-estrogenic sub-fractions had much fewer compounds with O groups. Since F2.31 and F2.31T had strong estrogenic activity, it is likely that some compounds with O groups have an impact on the estrogenic activity. To examine this relationship, principal component analysis (PCA) of ESI spectral data of the 9 samples was performed. PC1 and PC2 accounted for more than 88% of the data, showing good separation in PC1. PC1 clearly separated the strong estrogenic fractions from the non-estrogenic and weakly estrogenic fractions (Figure 4-5).

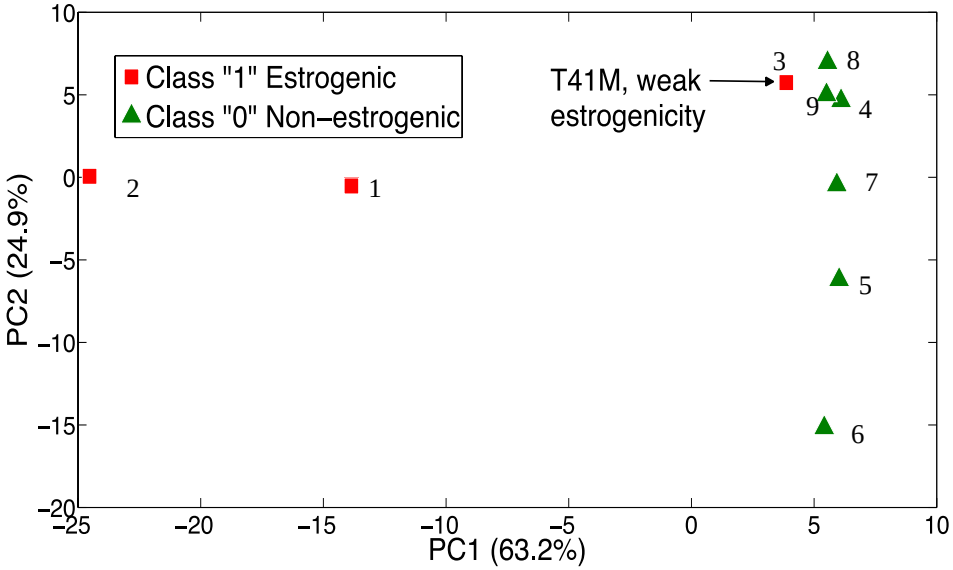

Figure 4-5 Principal component analysis based on all mass peaks observed by LTQ-Orbitrap Velos Pro MS in selected HPLC subfractions. 1 and 2 are the most estrogenic samples (F2.31 and F2.31T respectively), 3 is a weakly estrogenic sample (F2.41T) and 4-9 are the non-estrogenic samples (F2.11, F2.11T, F2.19, F2.19T, F2.20, and F2.20T respectively).

Table 3-1 summarizes the 30 most abundant compounds identified by Orbitrap Velos Pro MS and present in estrogenic but not in the non-estrogenic HPLC sub-fractions. Because of the ultrahigh resolution of this MS and its high mass accuracy (<5 ppm rms), unambiguous

assignments of their molecular formula were made. With electrospray ionization, there was a loss of one proton for each compound detected in negative ion mode, so an adjustment to the molecular formula was made to calculate the double bond equivalence (DBE). Although O1 compounds were not present in the estrogenic samples, C_{15}, C_{27}, C_{29} and C_{30} O1 compounds were detected in some of the non-estrogenic samples. Their total relative abundance was as high as ~ 2% in the F2.19 fraction of untreated OSPW, 0.2% in the F2.20T fraction from treated OSPW, and zero in estrogenic samples. O1 compounds may be alkylphenols and have been shown to be present in OSPW at ppb to low ppm concentrations (Hargesheimer et al. 1984). The formula of the C_{15} O1 compound ($C_{15}H_{24}O$) is consistent with that of nonylphenol. Alkylphenols are thought to contribute significantly to endocrine disruption. Using a similar fractionation scheme, Thomas et al. (2009) showed that 35% of the estrogenic activity could be attributed to petrogenic alkylphenols in off-shore North Sea discharge waters. Since the HPLC sub-fractions with O1 compounds were not estrogenic, it is possible that they were either not alkylphenols or were present at concentrations too low to cause an estrogenic effect. The latter is more likely as 245 to 35,000 μg L^{-1} total phenols have been measured in off-shore produced waters in the North and Norwegian Seas (Boitsov et al. 2007) compared to much lower values of 97-332 μg L^{-1} in OSPW (Hargesheimer et al. 1984).

Table 4-1 A summary of the 30 most abundant O components identified by ESI- LTQ-Orbitrap Velos Pro MS and present in estrogenic samples but not in non-estrogenic samples. Subfractions F2.11, F2.11T and F2.21T had no detectable amounts of these components (T refers to sub-fractions from biologically treated OSPW). ”*” indicates the compound with the same formula as estradiol. Since ESI resulted in a loss of one proton, an adjustment to the molecular formulae was made in the determination of DBE. ND: not detected.

Mass	Formula	Δ(ppm)	DBE	F2.31	F2.31T	F2.41T	F2.19	F2.19T	F2.20
				% Relative abundance					
Non-aromatic structures									
223.17034	$C_{14}H_{23}O_2$	-0.07	3	13.07	23.15	0.57	0.07	0.10	ND
209.15441	$C_{13}H_{21}O_2$	-1.41	3	0.81	0.50	ND	ND	ND	ND
267.19602	$C_{16}H_{27}O_3$	-2.04	3	0.16	0.17	ND	ND	ND	ND
237.18659	$C_{15}H_{25}O_2$	2.48	3	ND	0.11	0.18	ND	ND	ND
221.15467	$C_{14}H_{21}O_2$	-0.16	4	6.82	12.07	0.34	ND	ND	0.18
235.17010	$C_{15}H_{23}O_2$	-1.09	4	1.82	3.03	0.26	0.09	0.13	ND
279.19567	$C_{17}H_{27}O_3$	-3.21	4	0.22	0.19	ND	ND	ND	ND
245.15423	$C_{16}H_{25}O_3$	-2.50	4	0.21	0.17	ND	ND	ND	ND
293.21121	$C_{18}H_{29}O_3$	-3.44	4	0.12	0.13	ND	ND	ND	ND
281.17549	$C_{16}H_{25}O_4$	-1.22	4	ND	0.11	ND	ND	ND	ND
307.22676	$C_{19}H_{31}O_3$	-3.60	4	ND	0.08	ND	ND	ND	ND
295.15525	$C_{16}H_{23}O_5$	0.52	4	0.29	0.35	ND	ND	ND	ND
Possible aromatic structures									
233.15445	$C_{15}H_{21}O_2$	-1.09	5	1.73	4.44	0.25	ND	ND	ND
305.21115	$C_{19}H_{29}O_3$	-3.51	5	0.11	0.13	ND	ND	ND	ND
291.19564	$C_{18}H_{27}O_3$	-3.18	5	0.12	0.12	ND	ND	ND	ND
291.16055	$C_{17}H_{23}O_4$	1.26	6	3.65	1.67	ND	ND	ND	ND
259.17076	$C_{17}H_{23}O_2$	1.57	6	1.01	1.16	ND	ND	ND	ND
245.15423	$C_{16}H_{21}O_2$	-1.92	6	0.20	0.28	ND	ND	ND	ND
303.19564	$C_{19}H_{27}O_3$	-3.07	6	ND	0.11	ND	ND	ND	ND

271.17002*	$C_{18}H_{23}O_2$	-1.25	7	6.16	6.80	0.09	ND	ND	ND
255.13866	$C_{17}H_{19}O_2$	-1.55	7	1.28	1.30	ND	ND	ND	ND
303.16044	$C_{18}H_{23}O_4$	0.85	7	1.84	0.73	ND	ND	ND	ND
301.18124	$C_{19}H_{25}O_3$	1.07	7	0.23	0.22	ND	ND	ND	ND
285.18624	$C_{19}H_{25}O_2$	0.83	7	0.10	0.13	0.14	ND	ND	ND
269.15402	$C_{18}H_{21}O_2$	-2.55	8	1.23	1.04	ND	ND	ND	ND
301.14476	$C_{18}H_{21}O_4$	0.76	8	1.09	0.65	ND	ND	ND	ND
283.16916	$C_{19}H_{23}O_2$	-4.20	8	0.73	0.80	ND	ND	ND	ND
315.16035	$C_{19}H_{23}O_4$	0.53	8	0.36	0.13	ND	ND	ND	ND
299.16564	$C_{19}H_{23}O_3$	1.24	8	0.37	ND	ND	ND	ND	ND
293.15323	$C_{20}H_{21}O_2$	-5.04	10	0.96	1.48	ND	ND	ND	

Five of the O2 compounds were fragmented by MS/MS. The most common neutral losses were 44, 28 and 18 and are consistent with neutral losses of CO_2, CO, and H_2O from carboxylic acid moieties (Rudzinski et al. 2002). Therefore, the O2 group of compounds is likely carboxylic acids. In the estrogenic sub-fractions, among the alicyclic NAs there were no compounds with a DBE < 3 ($z < -6$), indicating the absence of aliphatic linear, branched and monocyclic acids. Monocyclic acids are not abundant in OSPW (Martin et al. 2008) and these three acid groups are not predicted to have an estrogenic effect (Scarlett et al. 2012). Compounds with DBE=3, 4 or 5 are likely aliphatic bi-, tri- and tetra-cyclic compounds with the possible bicyclic $C_{14}H_{23}O_2$ (DBE=3), tricyclic $C_{14}H_{21}O_2$ and $C_{15}H_{23}O_2$ (DBE=4), and tetracyclic $C_{15}H_{21}O_2$ (DBE=5) compounds among the more abundant. Some DBE=6 compounds may be pentacyclic. Although bi-, tri-, tetra-, and penta-cyclic acids are known to be common in OSPW (Rowland et al. 2011b, Rowland et al. 2011c), esterifiable alicyclic NAs from OSPW have been shown not to induce estrogenic activity (Reinardy et al. 2013)

The O3 compounds, $C_{17}H_{27}O_3$, $C_{16}H_{25}O_3$, $C_{18}H_{29}O_3$, and $C_{19}H_{31}O_3$, are likely oxy-NAs with one hydroxyl group and the O4 compound, $C_{16}H_{25}O_4$, likely has two hydroxyl groups consistent with oxy-NAs identified by Wang et al. (2013). Since ozonation of OSPW decreased NA concentration and toxicity but not estrogenicity, He et al. (2011) has suggested that hydroxylated NAs may be implicated in estrogenicity since they are degraded and formed during ozonation (Han et al. 2008).

For polycyclic aromatic compounds, an aromatic group accounts for a DBE of four, C=O for one and each alicyclic group can account for one DBE. Polycyclic aromatic compounds with a DBE > 5 may contain at least one aromatic group (DBE=4) as well as alicyclic groups (DBE=1 per ring). $C_{17}H_{23}O_4$ (DBE=6, z=-10), $C_{17}H_{23}O_2$ (DBE=6, z=-10), $C_{17}H_{19}O_2$ (DBE=8, z=-14), $C_{18}H_{23}O_4$ (DBE=7, z=-12), $C_{18}H_{21}O_2$ (DBE=8, z=-14), and $C_{18}H_{21}O_4$ (DBE=8, z=-14) were among the most abundant possible aromatic structures uniquely present in estrogenic sub-fractions (Table 4-1). Dehydroabietic acids ($C_{20}H_{28}O_2$) are polycyclic aromatic compounds whose identity has been confirmed in OSPW (Jones et al. 2012). However, using the ADMET predictor, they were predicted not to have estrogenic activity (Scarlett et al. 2012), and this was experimentally confirmed (Reinardy et al. 2013). Rowland et al. (Rowland et al. 2011a) have shown that C19 and C20 compounds (DBE=6 to 8, z=-12 to -16) in OSPW have structures that are similar to estrone and estradiol (monoaromatic hydroxyl steroid acids). Some formulae of possible aromatic structures (Table 4-1) are consistent with this and similar to estradiol ($C_{18}H_{23}O_2$). Furthermore, although no compounds had been identified, Reinardy et al.(2013) have shown that esterifiable aromatic NAs of OSPW had estrogenic activity, and Scarlett et al.(2012) predicted that several polycyclic monoaromatic compounds with DBE $\geq$ 4 (z $\geq$ -8) had endocrine and reproductive effects.

The above compounds represent only some of the estrogenic compounds that are present in OSPW. Although this study focused on the most estrogenic F2 fraction, other fractions i.e. F1

and F6-8 also had an estrogenic effect. These fractions likely contain compounds different from those that are in F2. One might expected related compounds with varying levels of alkylation to be involved in estrogenicity (Rowland et al. 2011a). However, this was not observed. To reduce the complexity of the analysis, F2 was further fractionated by semipreparative HPLC and subfractions collected every minute for a total of 60 mins. This may have resulted in fewer related compounds with varying levels of alkylation being in the same subfractions as those with higher or lower levels of aklylation are now likely to be in neighbouring subfractions. This is consistent with estrogenicity clustering in the HPLC subfractions F2.28 to F2.41.

4.4.5 Potential Degradation Products

When comparing the relative abundance of compounds in the untreated HPLC sub-fraction (Figure 4-4, F2.31) with the biologically treated sub-fraction (F2.31T), four new compounds ($C_{15}H_{25}O_2$ (DBE=3, possible bicyclic NA), $C_{16}H_{25}O_4$, $C_{19}H_{31}O_3$ (DBE=4, possible tricyclic NAs) and $C_{19}H_{27}O_3$ (DBE=6, possible pentacyclic or aromatic NA) were more abundant in the biologically treated subfraction. These are potentially metabolites or degradation products, with the O3 and O4 products representing possible hydroxylation (Han et al. 2008) or carboxylation products (Johnson et al. 2012). Major compounds that show greater abundance in the treated OSPW subfraction (F2.31T) compared to the untreated sub-fraction are primarily possible alicyclic NAs (such as $C_{14}H_{23}O_2$, $C_{14}H_{21}O_2$, $C_{15}H_{23}O_4$ and $C_{15}H_{21}O_2$), indicating that these compounds may accumulate as dead-end products that cannot be further biodegraded. Furthermore, since the relative proportion of these compounds increases but the estrogenicity of the treated sample is unchanged, they are also unlikely to contribute to estrogenicity. On the other hand, among the more likely estrogenic aromatic compounds, there are no major ones which change between untreated and treated samples and all are present at low relative abundance.

Of the 30 most abundant compounds uniquely present in the estrogenic subfractions, there were 16 possible aromatic structures that are the most likely to cause estrogenicity. These were O2, O3 and O4 C17 to C20 compounds with DBEs between 6 and 10 and chemical formulae similar to estrone- and estradiol-like compounds. This is the first time that exact masses of possible estrogenic compounds in OSPW have been determined and used to suggest formulae of the estrogenic compounds. This will aid future studies in developing rational methods for identifying specific estrogenic compounds and can help optimize treatment methods to remediate OSPW.

4.5 References

Barrow, M. P., J. V. Headley, K. M. Peru and P. J. Derrick (2004). " Fourier transform ion cyclotron resonance mass spectrometry of principal components in oilsands naphthenic acids." Journal of Chromatography A **1058**(1-2): 51-59.

Barrow, M. P., M. Witt, J. V. Headley and K. M. Peru (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.

Boitsov, S., S. A. Mjos and S. Meier (2007). "Identification of estrogen-like alkylphenols in produced water from offshore oil installations." Marine Environmental Research **64**(5): 651-665.

Clemente, J. S. and P. M. Fedorak (2005). "A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids." Chemosphere **60**(5): 585-600.

ERCB, Energy Resources Conservation Board (ERCB). EnerFAQs 12: Oil Sands. **2011**(citation date:2014-12-01) http://www.lica.ca/attachments/077_12%20Oil%20Sands.pdf

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

Gaido, K. W., L. S. Leonard, S. Lovell, J. C. Gould, D. Babai, C. J. Portier and D. P. McDonnell (1997). "Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay." Toxicology and Applied Pharmacology **143**(1): 205-212.

Grewer, D. M., R. F. Young, R. M. Whittal and P. M. Fedorak (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.

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

Hargesheimer, E. E., R. T. Coutts and M. D. MacKinnon (1984). "Characterization of simple phenols in oil sands extraction ‐ process water." Environmental Technology **5**(1-11): 433-440.

He, Y., S. B. Wiseman, M. Hecker, X. Zhang, N. Wang, L. A. Perez, P. D. Jones, M. G. El-Din, J. W. Martin and J. P. Giesy (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., M. P. Barrow, K. M. Peru and P. J. Derrick (2011a). "Salting-out effects on the characterization of naphthenic acids from Athabasca oil sands using electrospray ionization." Journal of Environmental Science and Health Part A-toxic/Hazardous Substance **46**(8): 844-854.

Headley, J. V., M. P. Barrow, K. M. Peru, B. Fahlman, R. A. Frank, G. Bickerton, M. E. McMaster, J. Parrott and L. M. Hewitt (2011b). "Preliminary fingerprinting of Athabasca oil sands polar organics in environmental samples using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry." Rapid Communications in Mass Spectrometry **25**(13): 1899-1909.

Headley, J. V., K. M. Peru, S. A. Armstrong, X. Han, J. W. Martin, M. M. Mapolelo, D. F. Smith, R. P. Rogers and A. G. Marshall (2009). "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., K. M. Peru, B. Fahlman, A. Colodey and D. W. McMartin (2013a). "Selective solvent extraction and characterization of the acid extractable fraction of Athabasca oils sands process waters by orbitrap mass spectrometry." International Journal of Mass Spectrometry **345**(0): 104-108.

Headley, J. V., K. M. Peru, A. Janfada, B. Fahlman, C. Gu and S. Hassan (2011c). "Characterization of oil sands acids in plant tissue using orbitrap ultra-high resolution mass spectrometry with electrospray ionization." Rapid Communications in Mass Spectrometry **25**(3): 459-462.

Headley, J. V., K. M. Peru, M. H. Mohamed, R. A. Frank, J. W. Martin, R. R. Hazewinkel, D. Humphries, N. P. Gurprasad, L. M. Hewitt, D. C. Muir, D. Lindeman, R. Strub, R. F. Young, D. M. Grewer, R. M. Whittal, P. M. Fedorak, D. A. Birkholz, R. Hindle, R. Reisdorph, X. Wang, K. L. Kasperski, C. Hamilton, M. Woudneh, G. Wang, B. Loescher, A. Farwell, D. G. Dixon, M. Ross, S. Pereira Ados, E. King, M. P. Barrow, B. Fahlman, J. Bailey, D. W. McMartin, C. H. Borchers, C. H. Ryan, N. S. Toor, H. M. Gillis, L. Zuin, G. Bickerton, M. McMaster, E. Sverko, D. Shang, L. D. Wilson and F. J. Wrona (2013b). "Chemical fingerprinting of naphthenic acids and oil sands process waters-a review of analytical methods for environmental samples." Journal of Environmental Science and Health, Part A **48**(10): 1145-1163.

Headley, J. V., K. M. Peru, M. H. Mohamed, L. Wilson, D. W. McMartin, M. M. Mapolelo, V. V. Lobodin, R. P. Rodgers and A. G. Marshall (2012). "Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry characterization of tunable carbohydrate-based materials for sorption of oil sands naphthenic acids." Energy & Fuels **27**(4): 1772-1778.

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

Johnson, R. J., C. E. West, A. M. Swaih, B. D. Folwell, B. E. Smith, S. J. Rowland and C. Whitby (2012). "Aerobic biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two different pathways by a new isolate of *Mycobacterium*." Environmental Microbiology **14**(4): 872-882.

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

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

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

MacDonald, G. Z., N. S. Hogan, B. Kollner, K. L. Thorpe, L. J. Phalen, B. D. Wagner and M. R. van den Heuvel (2013). "Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout." Aquatic Toxicology **126**(2013): 95-103.

MacKinnon, M. and H. Boerger (1986). "Description of two treatment methods for detoxifying oil sands tailings pond water." Water Quality Research Journal of Canada **21**(4): 496-512.

Martin, J. W., X. M. Han, K. M. Peru and J. V. Headley (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.

McKenzie, N., S. Q. Yue, X. D. Liu, B. A. Ramsay and J. A. Ramsay (2014). "Biodegradation of naphthenic acids in oils sands process waters in an immobilized soil/sediment bioreactor." Chemosphere **109**(0): 164-172.

Pereira, A. S., S. Bhattacharjee and J. W. Martin (2013). "Characterization of oil sands process-affected waters by liquid chromatography orbitrap mass spectrometry." Environmental Science & Technology **47**(10): 5504-5513.

Reemtsma, T., O. Fiehn and M. Jekel (1999). "A modified method for the analysis of organics in industrial wastewater as directed by their toxicity to *Vibrio fischeri*." Fresenius Journal of Analytical Chemistry **363**(8): 771-776.

Reinardy, H. C., A. G. Scarlett, T. B. Henry, C. E. West, L. M. Hewitt, R. A. Frank and S. J. Rowland (2013). "Aromatic naphthenic acids in oil sands process-affected water, resolved by GCxGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (*Danio rerio*) larvae." Environmental Science & Technology **47**(12): 6614-6620.

Routledge, E. J. and J. P. Sumpter (1996). "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen." Environmental Toxicology and Chemistry **15**(3): 241-248.

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

Rowland, S. J., C. E. West, A. G. Scarlett and D. Jones (2011b). "Identification of individual acids in a commercial sample of naphthenic acids from petroleum by two-dimensional comprehensive gas chromatography/mass spectrometry." Rapid Communications in Mass Spectrometry **25**(12): 1741-1751.

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

Rudzinski, W. E., L. Oehlers, Y. Zhang and B. Najera (2002). "Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil." Energy & fuels **16**(5): 1178-1185.

Scarlett, A. G., C. E. West, D. Jones, T. S. Galloway and S. J. Rowland (2012). "Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints." Science of the Total Environment **425**(0): 119-127.

Thomas, K. V., K. Langford, K. Petersen, A. J. Smith and K. E. Tollefsen (2009). "Effect-directed identification of naphthenic acids as important *in vitro* xeno-estrogens and anti-androgens in north sea offshore produced water discharges." Environmental Science & Technology **43**(21): 8066-8071.

Wang, B., Y. Wan, Y. Gao, M. Yang and J. Hu (2013). "Determination and characterization of oxy-naphthenic acids in oilfield wastewater." Environmental Science & Technology **47**(16): 9545-9554.

Chapter 5

Biodegradable Naphthenic Acids in Oil Sands Process Waters

This chapter will be submitted to Science for the Total Environment

5.1 Abstract

The oil sands industry in Canada is rapidly growing. To process each cubic metre of oil sands, three cubic meters of oil sands process wastewater (OSPW) is produced by Clark's hot water extraction. OSPW is a mixture of hundreds of organic compounds and its major toxicity has been attributed to the naphthenic acids (NAs) about which little is known. Because OSPW is toxic and estrogenic, more than 1 billion cubic metres of OSPW must be held on site under a zero discharge policy. An immobilized soil bioreactor (ISBR) reduced 40% total NAs in previous studies. To further characterize the biodegradation, fractionation and toxicity assays (Microtox® Acute Toxicity test and Yeast Estrogenic Screening (YES) assay), were conducted. The present study showed the concomitant decrease in NAs and toxicity, and further chemical analysis by ESI-LTQ-Orbitrap MS found that most degradation was observed with NAs with DBE=5 while NA with DBE=3 (bicyclic) and 4 (tricyclic) seemed to be the most persistent groups. MS/MS and MS/MS/MS analysis showed the presence of an aromatic structure in NAs with DBE 5 and 6. These observations may be related to the degradation differences between alicyclic NAs with DBE >5 and aromatic structures. These results may help to better understand the biodegradation of NAs in OSPW and identify recalcitrant components of OSPW.

5.2 Introduction

Oil sands are a type of petroleum deposit, which consists of sand, clay, water, bitumen, etc. (Attanasi et al. 2007). Over 70% of the total global natural bitumen reserves are found in

Canada (Attanasi et al. 2007). To extract bitumen, the Clark's hot water extraction is used and has resulted in an enormous volume of tailings and oil sand process water (OSPW) (Giesy et al. 2010). To extract one barrel of oil, approximately three barrels of liquid tailings are produced and the total volume was approximately 720 m^3 by 2011 (ERCB, 2011).

OSPW is a large, complex mixture, which contains naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs), phenols and benzene (Giesy et al. 2010). The concentration of NAs in OSPW is usually 20-120 mg L^{-1}. Acute and chronic toxicity has been attributed to the acid fraction containing NAs in OSPW (Madill et al. 2001). NAs have the formula $C_nH_{2n+z}O_x$, which includes classical NAs (c-NAs, x=2) and oxidized NAs (oxy-NAs, x=3-5), where "n" is the number of carbons, "z" is related to the hydrogen deficiency or double bond equivalents (DBE) that includes ringed structures plus double bonds containing carbon. Since a carboxyl group contains one C=O double bond, z=-2 is equivalent to DBE=2, which indicate a NA with one alicyclic ring (DBE=1) and a carboxylic acid group (DBE=1). For a NA with DBE $\geq$ 5, aromaticity may also contribute to the hydrogen deficiency as an aromatic ring accounts for DBE=4, and aromatic NAs have been identified in the OSPW (Reinardy et al. 2013).

Currently, OSPW cannot be released into the environment under a zero policy, and to release OSPW an appropriate treatment that can degrade/remove and detoxify OSPW is needed. Microorganisms were effective in degrading model NAs (Del Rio et al. 2006, Johnson et al. 2012), commercial NAs mixtures (Clemente et al. 2004, Videla et al. 2009), and OSPW NAs (Scott et al. 2005). Previous studies found that biodegradation rates decreased when the number of rings increased (Clemente et al. 2004, Del Rio et al. 2006). For example, bicyclic NA surrogates degraded 60 times slower than monocyclic NAs (Del Rio et al. 2006). Tricyclic NAs are major NAs in OSPW (Grewer et al. 2010, Han et al. 2009). Some diamondoid NAs, including methyl, dimethyl and ethyladamantane carboxylic acids and adamantane ethanoic acid, were identified in OSPW (Rowland et al. 2011a). Diamondoid hydrocarbons are generally considered

very difficult to biodegrade and only adamantane has been shown to be metabolized by *Pseudomonas putida* to adamantanone then to a dead-end product, 1-hydroxy-4-oxohomoadamantan-5-one, which accumulated in the cell (Selifonov 1992). Although in general, the biodegradation rate decreased as DBE values increased, the half-life of NAs with DBE values of 5, 6, 7, and 8 were similar (Toor et al. 2013).

Because of the complexity of OSPW, fractionation techniques can be very useful in separating complex mixtures. Microtox® and yeast estrogenic screening (YES) assays can be used as indicators in fractionation to evaluate the biodegradation performance. Chemical composition of treated and untreated OSPW can be analyzed by linear ion trap Quadropole (LTQ)-Orbitrap Velos Pro hybrid mass spectrometer which combines a linear ion trap with an Orbitrap analyzer that has high resolution (>100,000 at m/z 400) and high mass accuracy (< 5 ppm). In addition to the molecular formula, the structure of the NAs which affect toxicity and biodegradation is also very important and may be elucidated by tandem MS that fragments the molecular mass peaks to produce distinctive daughter ions.

The aim of this study was to evaluate the detailed compositional change and detoxification of NAs in the biological treatment of OSPW by an immobilized soil reactor (ISBR). OSPW (before and after biological treatment) was fractionated, and detoxification assessed using two bioassays (Microtox® Acute Toxicity test and YES assay) and the changes in the NAs composition associated with biodegradation or persistence was determined. This study will help better understand the biodegradation and detoxification of the OSPW and give useful information to improve the treatment performance.

5.3 Methods and Materials

5.3.1 Untreated and Treated OSPW

Untreated OSPW was obtained from the active settling basin (WIP) of Syncrude Canada in Fort McMurray, Alberta. OSPW was treated by two ISBRs connected in series as described by McKenzie et al. (2014) in which about 40% NAs was degraded by microorganisms immobilized in the ISBR, and the effluent was collected as treated OSPW for analysis.

5.3.2 Fractionation Strategy

Fractionation of untreated and treated OSPW was performed using solid phase extraction (SPE) columns which separated components based on their hydrophobicity. Based on toxicity results, fractions were selected for further fractionation by semi-preparative reverse phase HPLC to achieve fractions that have less complex compositions.

5.3.3 Solid Phase Extraction (SPE)

The pH of treated or untreated OPSW was adjusted to 10 and filtered through a 0.45 μm nylon filter (Millipore Corporation, Billerica, MA, USA). Every 1 L of the samples was extracted by two C18 (octade-cylsilane) and a polystyrene-divinylbenzene (PSDVB) column (J.T.Baker, Phillipsburg, NJ, USA) connected in series. Each column contained 1 g of sorbent. Columns were pre-conditioned by passing 25 mL methanol followed by 25 mL high-purity water before passing 1 L of treated or untreated OSPW. Fractions were eluted with an increasing amount of methanol (20, 60, 80, and 100%) and achieving a 50-fold concentration compared to the original sample.

5.3.4 Semi-Preparative Reverse-Phase HPLC

Since the F2 fraction from the SPE fractionation had the highest estrogenic activity and the greatest decrease in estrogenicity upon biological treatment, it was selected for further

fractionation by semi-preparative reverse-phase, high-performance liquid chromatography (RP-HPLC; Hewlett-Packard 1050, Agilent Technologies, Bracknell, UK). A one mL sample was injected four times (total equivalent of 1 L of original OSPW) onto a silica guard column (5 cm×10 mm× 5 µm) connected to a ZORBAX Eclipse XDB-C8 semipreparative HPLC column (25 cm×10 mm×5 µm; Agilent) equipped with a UV detector (230 nm). The mobile phase consisted of HPLC-grade methanol (Fisher Scientific Inc., CA) (eluent A) and water containing 5% formic acid and 20 mM NH_4Ac at pH=7 (eluent B) pumped at a total flow rate of 1.5 mL/min using a gradient: 60% A for 10 min, then to 80% A in 10 min and maintained for 15 min, then to 100% A in 10 min and maintained for 15 min for a total run-time of 60 min. Fractions were collected at 1 min intervals using a fraction collector (Dynamax model FC-4, Varian) obtaining a total of 60 1.5 mL samples. Separation and collection were controlled by a Varian Star Chromatography Workstation. Fractions were evaporated to dryness under nitrogen gas and redisolved in methanol to achieve a 200-fold concentration compared to the original sample.

5.3.5 Microtox® and YES Assay

The toxicity of fractions was measured by Microtox® assay (Azur Environmental, Fairfax, CA, USA). Triplicates samples were analyzed, 1 g L^{-1} glucose was used as a negative control and $ZnSO_4$ was tested as a positive control and found to be within the parameters set by Microtox® (EC_{50} in 15 min was between 3 to 10 mg L^{-1}). All samples were analyzed in a dilution series in order to determine the EC_{50} values and calculated as TU_{50} [per liter of sample] =1/EC_{50}. The inhibition of the bioluminescence of *Vibrio fischeri* was analyzed determined by the Synergy™ HT microtiter plate reader (Biotek Instruments Inc., Winnoski, VT).

The YES assay was conducted as described by Routledge et al. (1996). Briefly, the assay uses recombinant yeast, *Saccharomyces cerevisiae* BJ3505 hER/2ERE (Gaido et al. 1997), provided by H. Engelhardt, University of Waterloo to cultivate with the samples and the

estrogenicity was determined by comparing responses with that of the E2 standard and calculated as E2 equivalent.

5.3.6 MS, MS/MS, and MS/MS/MS Analysis

The LTQ-Orbitrap Velos Pro Hybrid MS with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Waltham, MA) was used to analyze selected semi-preparative RP-HPLC fractions. Samples were injected directly by a Hamilton syringe (250 μL) into the HESI-MS using direct infusion by the inbuilt syringe pump of the LTQ-Orbitrap Velos Pro at a flow rate of 5 μL min^{-1}. The MS system was operated using Xcalibur software (Thermo Fisher Scientific). The electrospray voltage was 2.7 kV, with a nebulizing gas (N_2) flow of 1.5 L min^{-1}, drying gas (N_2) flow of 3.0 L min^{-1}, and the ion transfer tube temperature was 270 ℃ for the LTQ-Orbitrap Velos Pro. Prior to sample analysis, the LTQ and Orbitrap were tuned to optimize conditions for the detection of ions in the range m/z 150–1000 and calibrated according to the manufacturers' calibration mixture. Full MS scans in negative ion mode used over 400 scans for each acquisition in the Orbitrap mass analyzer at a mass resolution of 100 000 (FWHM defined at m/z 400) and mass accuracy better than 5 ppm. MS/MS and MS/MS/MS scans in the negative mode were performed acquired in the linear ion trap by collision induced fragmentation and mass spectra acquired using the ion trap alone. Xcalibur software was used for data visualization, processing, and calculation of elemental composition.

Calculations were limited to formulae containing less than 100 ^{12}C, 2 ^{13}C, 200 ^{1}H, 5 ^{14}N, 10 ^{16}O, 3 ^{32}S, and 1 ^{34}S. If more than one possible formula was generated for a specific mass, one or more could almost always be confirmed or eliminated by the presence/absence of a corresponding ion containing ^{13}C, ^{18}O, or ^{34}S as described in Headley et al. (2012).

5.4 Results and Discussion

The present study combined fractionation of OSPW with degradation, toxicity, and estrogenicity with MS/MS and MS/MS/MS to reveal structural information of relevant components.

5.4.1 Microtox® and YES Assay of SPE Fractions

To reduce the complexity of OSPW NAs and better characterize biodegradation, fractionation was conducted of both untreated and treated OSPW. Eight fractions were collected from each of the treated or untreated OSPW: F1-F4 from two C18 columns and F5-F8 from the PSDVB column were recovered with increasing amounts of methanol. The toxicity test was conducted and TU_{50} (per liter of sample) values are shown as a bar graph in Figure 5-1 and estrogenicity as a line graph. The toxicity and estrogenicity of the F2 fraction decreased the most with biological treatment, 76% and 68% respectively.

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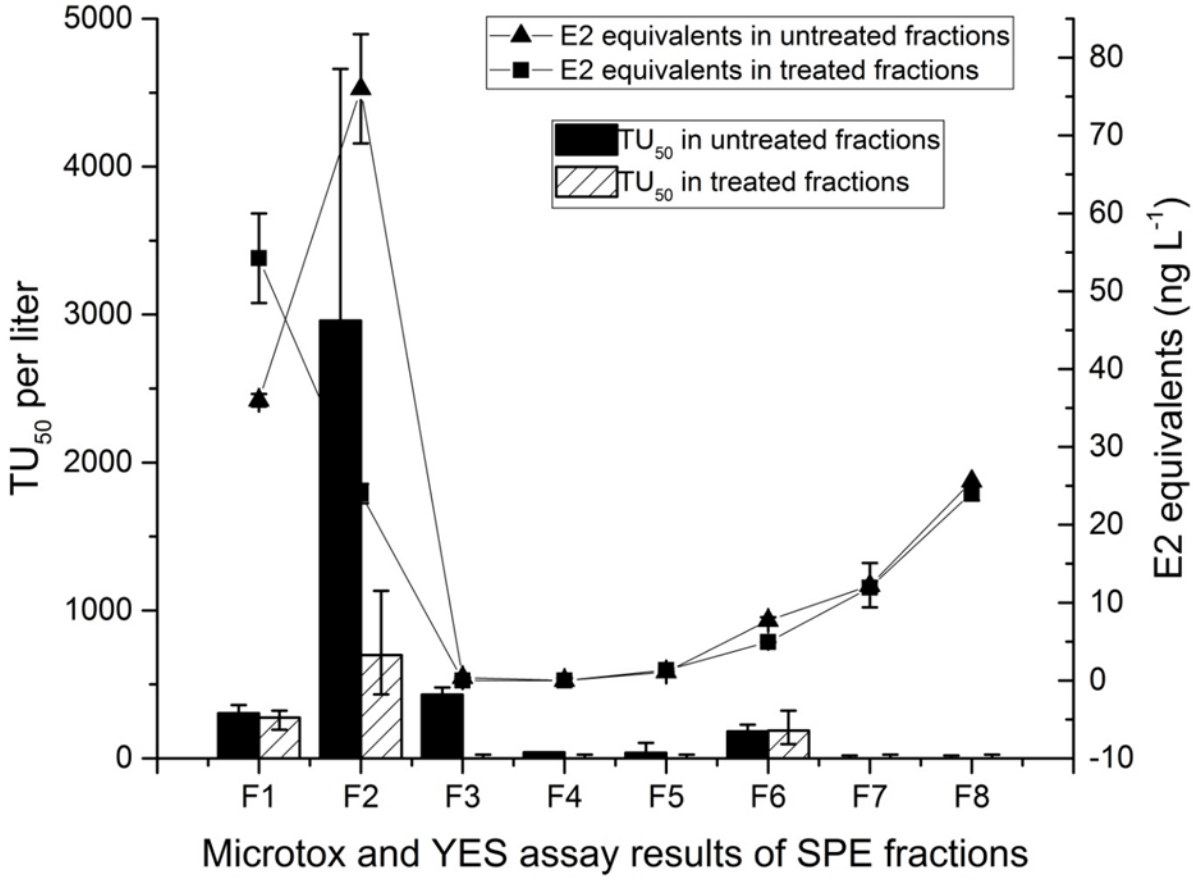


Figure 5-1 Measured Microtox® acute toxicity and estrogenic activity of untreated and treated OSPW collected as SPE fractions from two C18 (F1-F4) and one PSDVB (F5-F8) column with increasing amounts of methanol (20, 60, 80, and 100%). F1 and F5 were recovered with 20% methanol, F2 and F6 with 60% and so on. Each fraction was tested in triplicate and the data for the YES represent mean ± 95% confidence interval.

5.4.2 ESI LTQ-Orbitrap MS of F2 Fraction From Untreated and Treated OPSW

Since F2 was found to be the major toxic and estrogenic fraction of untreated OSPW (Figure 5-1) and showed the largest decrease after biological treatment, the chemical composition of F2 was analyzed by ESI LTQ-Orbitrap MS. Since ESI uses a soft ionization and the Orbitrap Velos Pro MS is an ultra-high resolution instrument, the mass formulae obtained can be assigned

with high confidence. The relative abundance of the different DBE classes of O2-NA compounds are plotted as 3D graphs from the mass spectral data (Figure 5-2). The data are considered to be semi-quantitative due to unknown response factors and matrix effects (Headley et al. 2013a).The relative abundance of the predominant O2 species increased from 81.5 to 92% and O3 decreased from14.1 to 3.1%.

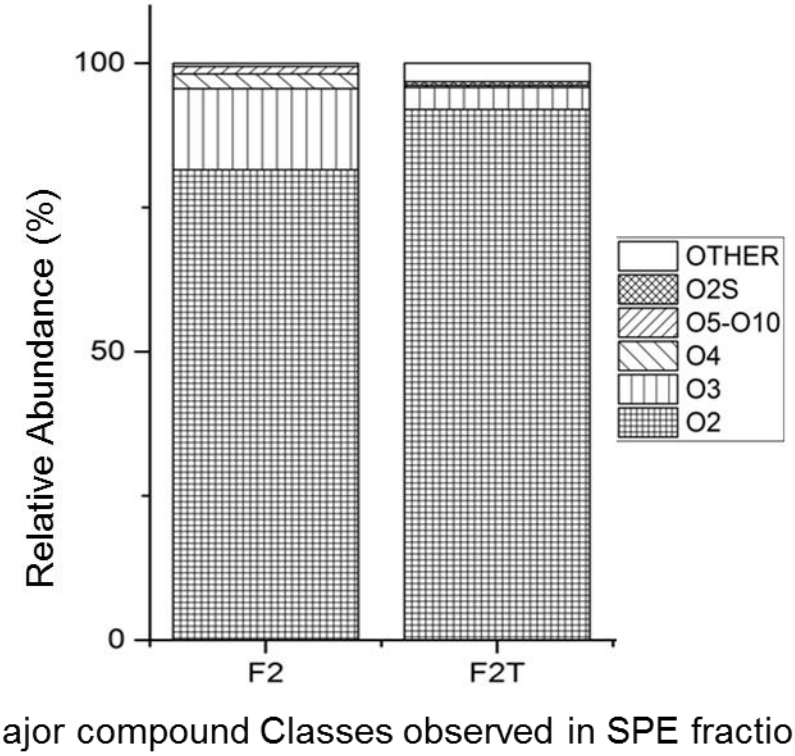


Figure 5-2 The major compound classes observed in untreated (F2) and treated (F2T) SPE fractions of OSPW

Since the O2 species class was dominant in both fractions, its composition was further investigated and plots of the DBE and carbon number distribution are shown in Figure 5-3. Among them, the change of representative O2-NAs by relative abundance after treatment is shown in Table 5-1.

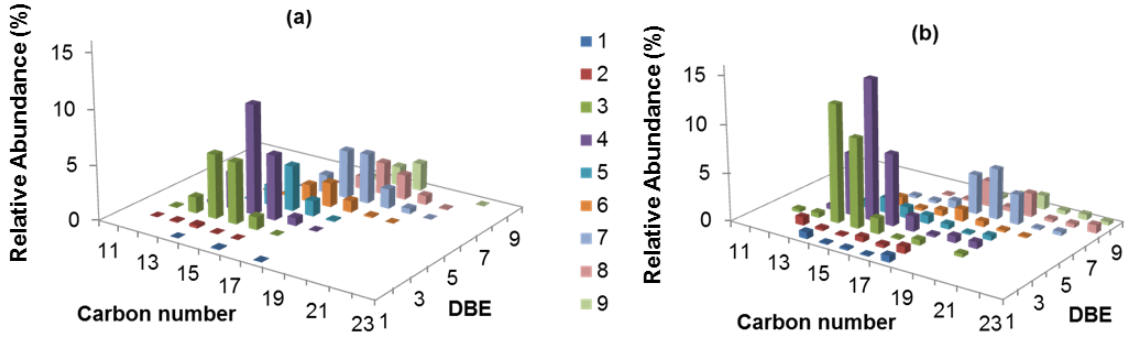


Figure 5-3 The distribution of O2 species according to carbon number and DBE values of the F2 fraction from (a) untreated (F2) and (b) treated OSPW (F2T). The bars represent the relative percentage in the samples. Different colors represent DBE groups.

Table 5-1 Comparison of representative O2-NAs change in relative abundance after treatment. F2 is from untreated OSPW and F2T is from biologically treated OSPW (Standard deviations: 0.3-5.9)

	m/z	Δ (ppm)	Formula	DBE	F2	F2T
Components which increased	223.17029	-0.284	$C_{14}H_{23}O_2$	3	5.84	12.26
	237.18570	-1.279	$C_{15}H_{25}O_2$	3	5.61	9.30
	221.15483	0.573	$C_{14}H_{21}O_2$	4	3.38	6.47
	235.17035	-0.014	$C_{15}H_{23}O_2$	4	10.01	14.58
	249.18574	-1.057	$C_{16}H_{25}O_2$	4	5.90	7.45
	285.18637	1.286	$C_{19}H_{25}O_2$	7	1.78	5.25
	299.20183	0.590	$C_{20}H_{27}O_2$	7	0.48	3.01
Components which decreased	247.17022	-0.539	$C_{16}H_{23}O_2$	5	4.10	2.36
	245.15451	-0.788	$C_{16}H_{21}O_2$	6	1.54	0.28
	259.16993	-1.633	$C_{17}H_{23}O_2$	6	2.20	0.60
	243.13894	-0.465	$C_{16}H_{19}O_2$	7	1.64	0.28
	257.15445	-0.985	$C_{17}H_{21}O_2$	7	4.36	0.82
	283.17075	1.401	$C_{19}H_{23}O_2$	8	2.25	0.40

Previous studies characterized NA degradation grouping them by carbon number and DBE and found that the DBE value plays a more important role in the degradation rate than the carbon number (Han et al. 2008), as NA degradation rates was found to decrease with increasing cyclization (i.e. compounds with higher DBE values) in OSPW. This was likely due to the

preferred β-oxidation metabolism of aliphatic carboxylic acids by aerobic microorganisms (Han et al. 2008, Quagraine et al. 2005, Taylor et al. 1978). β-Oxidation occurs at a β carbon relative to the carboxyl group and forms a new carboxylic acid with a loss of carbons until hindered by the quaternary or tertiary carbons.

In the present study, the relative abundance of both $C_{14}H_{21}O_2$ (DBE=4) and $C_{14}H_{23}O_2$ (DBE=3) increased from 3.38 to 6.47% and 5.84 to 12.26%, respectively (Table 5-1), which were much higher than the less than 1% increase of DBE 1 and 2 compounds. This indicates that the DBE 3 and 4 components were more recalcitrant. Previous studies have shown that bicyclic and tricyclic NAs were more recalcitrant by using NA surrogates (Del Rio et al. 2006, Herman et al. 1994, Lai et al. 1996). For example, Lai et al. (1996) monitored the biodegradation of U-^{14}C-palmitic acid (PA, a linear aliphatic compound with DBE=1) and decahydro-2-naphthoic acid-8-^{14}C (DHNA, a bicyclic compound with DBE=3) in oil sands microcosms and found that 10-15% PA was degraded in 4 weeks compared to 2-4% DHNA degradation in 8 weeks. Herman et al. (1994) monitored the degradation rate of palmitic acid (PA, linear with DBE=1) and several monocyclic NAs (cyclohexane carboxylic acid, methylcyclohexane carboxylic acid, cyclohexane pentanoic acid and pentylcyclohexane carboxylic acid with DBE=2), and found that 64% PA was degraded compared to 24-67% of the monocyclic NAs in 6 weeks in microcosm studies using microbial inoculum indigenous to oil sands tailings. Del Rio et al. (2006) also found that bicyclic NA surrogate [^{14}C-decahydronaphthoic acid (DHNA)] was degraded significantly slower than a monocyclic NA surrogate [^{14}C-cyclohexane carboxylic acid (CCA)].

However, in present study (Figure 5-3), most NAs with DBE 5-9 were more biodegradable compared to components with DBE<5. This is consistent with Kim et al. (2005), who found that the NAs with DBE 6 decreased significantly while NAs with DBE between 2 and 6 increased during the biodegradation of OSPW. Insignificant differences in degradation rates between low DBE NAs and high DBE NAs were also observed by Toor et al. (2013) in treating

OSPW in wetlands. However based on β-oxidation theory, NAs with low DBE values should be more rapidly degraded. Such observations may due to the limitation of using just the mass spectrometer which cannot distinguish isomers and may oversimplify the characterization of NA structures when using only carbon number and DBE. This is also supported by biodegradation studies of Bataineh et al. (2006) who examined the effect of alkyl branching in OSPW and commercially available NAs and by Johnson et al. (2011) who studied aromatic alkanoic isomers with different alky substuients with same DBE and carbon numbers.

5.4.3 HPLC Subfractions

F2 was further fractionated by semi-preparative HPLC and Microtox® and YES assay were conducted for each fraction. Of these, the HPLC sub-fractions of untreated and treated OSPW F2.8 and F2.8T (30% decrease in acute toxicity), F2.11 and F2.11T (100% decrease in acute toxicity) were selected for further chemical characterization and results are shown in Figure 5-4.

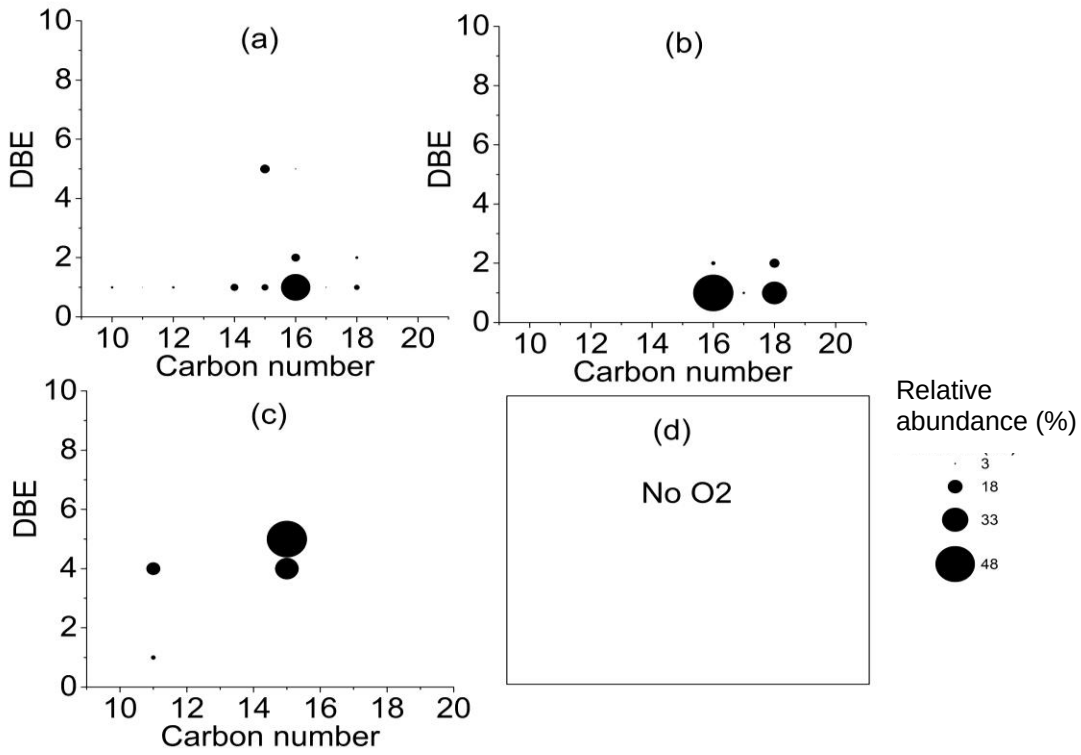


Figure 5-4 Representative bubble plots of the DBE distribution of O2 species observed in the ESI LTQ-Orbitrap mass spectra of selected HPLC subfractions (a) F2.8 (from untreated OSPW) (b) F2.8T (from biologically treated OSPW) (c) F2.11 (from untreated OSPW) (d) F2.11T (from treated OSPW). Filled circles sized by the relative abundance. Disappeared in F2.8: $C_{10}H_{20}O_2$, $C_{12}H_{24}O_2$, $C_{14}H_{28}O_2$, $C_{15}H_{30}O_2$, and $C_{15}H_{22}O_2$. Disappeared in F2.11: $C_{11}H_{22}O_2$, $C_{11}H_{16}O_2$, $C_{15}H_{24}O_2$ and $C_{15}H_{22}O_2$.

The concomitant degradation of O2-NAs and detoxification were observed in F2.8 and F2.11 (Figure 5-2), after biological treatment the distribution of the O2 species changed significantly in the F2.8 sub-fraction (30% decrease in acute toxicity) and was undetectable in the F2.11 sub-fraction (100% decrease in acute toxicity) (Figure 5-4). In F2.8 and F2.11, the lower carbon numbered NAs were preferrentially degraded. Most NAs except those with higher carbon numbers (C16-C18) in F2.8, and (C12-C15) in F2.11 decreased to undetectable levels. This is consistent with Herman et al. (1993) who found that monocyclic model NAs with 6 carbons degraded faster than 7 carbons monocyclic NA surrogates in shake flask studies.

5.4.4 MS/MS and MS/MS/MS of m/z 221, 233 and 259

Since the relative abundance of $C_{14}H_{21}O_2$ (DBE=4, m/z= 233.15486, Δ=0.672ppm) fraction increased in the F2 from 3.8 to 6.5% after biological treatment, it was selected for MS/MS and MS/MS/MS analysis and was compared to a tricyclic (diamondoid) NA surrogate, 3,5-dimethyladamantane-1-acetic acid ($C_{14}H_{22}O_2$), that was previously identified as one of the diamondoid isomers in OSPW by Rowland et al. (2011a). The results are shown in Figure 5-5.

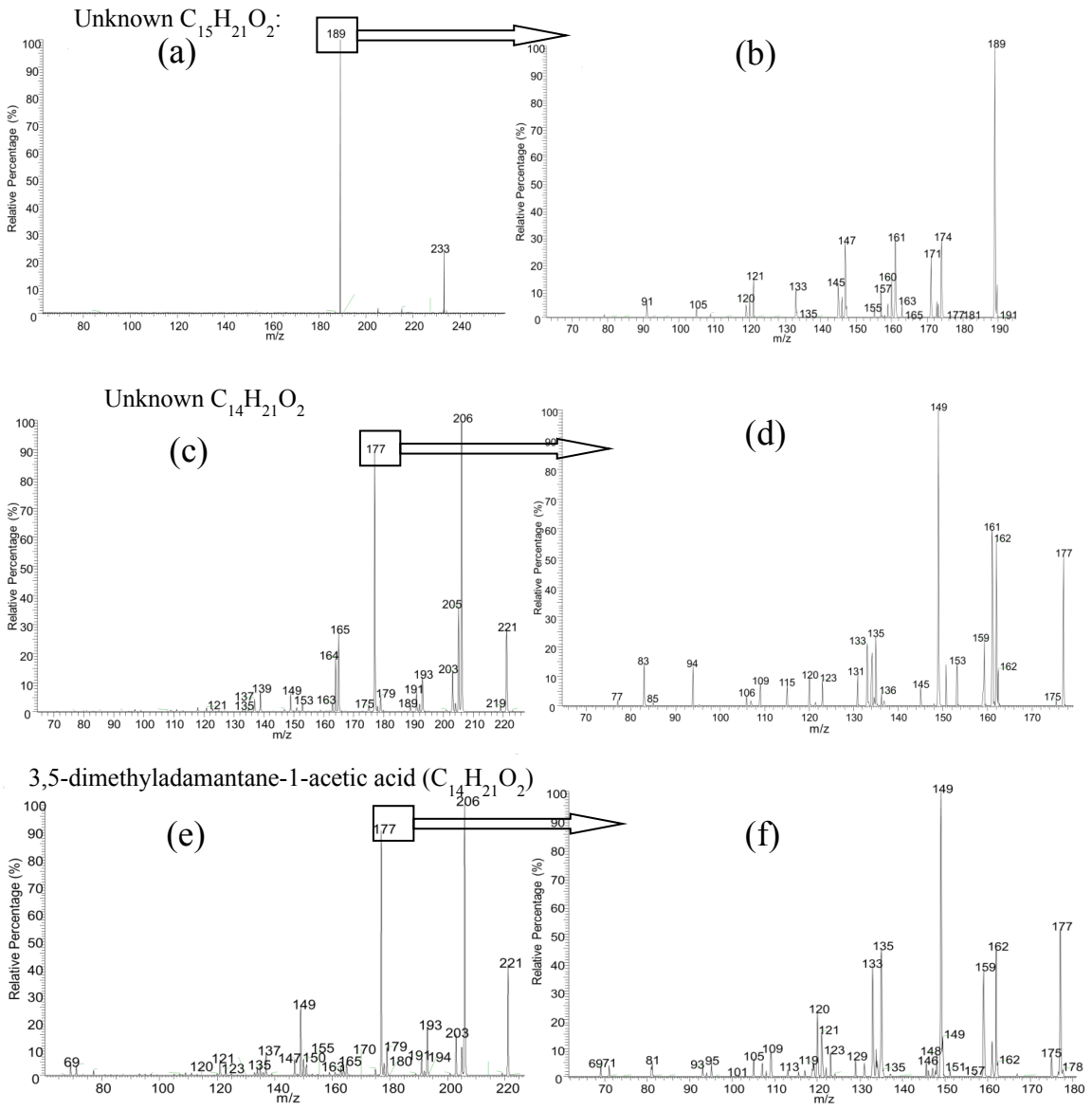


Figure 5-5 ESI^- MS/MS and MS/MS/MS spectra of unknown $C_{14}H_{21}O_2$ and $C_{15}H_{21}O_2$ in HPLC fractions, and 3,5-dimethyladamantane-1-acetic acid ($C_{14}H_{21}O_2$) : (a) MS/MS spectra of unknown $C_{15}H_{21}O_2$ (m/z 233) in F2.31 ; (b) Further fragmentation of m/z 189 from unknown $C_{15}H_{21}O_2$ (m/z 233) in HPLC fraction; (c) MS/MS spectra of unknown $C_{14}H_{21}O_2$ in HPLC fraction; (d) Further fragmentation of m/z 177 from unknown $C_{14}H_{21}O_2$ (m/z 221) in HPLC fraction; (e) MS/MS spectra of 3,5-dimethyladamantane-1-acetic acid ($C_{14}H_{21}O_2$); (f) Further fragmentation of m/z 177 from 3,5-dimethyladamantane-1-acetic acid ($C_{14}H_{21}O_2$) (m/z 221)

The MS/MS and MS/MS/MS results of [M-H]-= 221.15557, $C_{14}H_{22}O_2$ in the F2 fraction were very similar to those of the NA surrogate 3,5-dimethyladamantane-1-acetic acid ($C_{14}H_{22}O_2$), and suggests that $C_{14}H_{22}O_2$ in F2 could be a diamondoid structure. Previous studies showed that the diamondoid hydrocarbons are generally considered very difficult to biodegrade and only adamantane has been shown to be metabolized by *Pseudomonas putida* to adamantanone then to a dead-end product, 1-hydroxy-4-oxohomoadamantan-5-one, which accumulated in the cell (Selifonov 1992).

In the present study, the aromatic structure was found by ESI-LTQ-Orbitrap MS/MS analysis of both $C_{15}H_{21}O_2$ (DBE=5, m/z=233.15493, δ=0.973ppm), and $C_{17}H_{23}O_2$ (DBE=6, 259.17021, δ=-0.553ppm). Both $C_{15}H_{21}O_2$ and $C_{17}H_{23}O_2$ showed a m/z 44 loss that suggests a carboxyl group, and further fragmentation resulted in a typical m/z 91 peak (tropylium ion) that provides the evidence of an aromatic structure. Both aromatic (Rowland et al. 2011b) and tetracyclic structures (Rowland et al. 2011c) with DBE 5 have been confirmed in OSPW. Johnson et al. (2012) studied the biodegradation of aromatic alkanoic NAs (DBE=5), and showed they can be mostly degraded within 7 weeks, much faster than alicyclic NAs (Lai et al. 1996). Williams et al. (1986) monitored the biodegradation in south Texas eocene oils and also observed that aromatics were more biodegradable than bicyclic, tricyclic and tetracyclic. Other than the biodegradation differences, the aromatic fraction of OSPW NAs could contribute to the estrogenicity in OSPW (Reinardy et al. 2013), which may indicate the need to monitor the biodegradation of aromatic NAs in treatment studies.

In the present study, toxicity reduction and concomitant degradation of O2-NAs was observed as well as the uneven degradation of different groups of NAs in OSPW. Although both of the toxicity and estrogenicity decreased significantly, there was a persistent toxicity after treatment. The residual toxicity may come from the recalcitrant components and/or degradation products. The identification of recalcitrant components suggested that O2-NAs with DBE 3

containing diamondoid structure may associate with residual toxicity. Future targeted treatment study may provide an optimized method of removing the recalcitrant components and toxicity. This needs more details on NA structures to improve the degradation performance with the possibility of targeting the recalcitrant and toxic components.

5.6 References

Attanasi, E. D. and R. F. Meyer (2007). "Natural bitumen and extra-heavy oil." Survey of Energy Resources **22** (2010): : 123-140.

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

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

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

Gaido, K. W., L. S. Leonard, S. Lovell, J. C. Gould, D. Babai, C. J. Portier and D. P. McDonnell (1997). "Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay." Toxicology and Applied Pharmacology **143**(1): 205-212.

Giesy, J. P., J. C. Anderson and S. B. Wiseman (2010). "Alberta oil sands development." Proceedings of the National Academy of Sciences **107**(3): 951-952.

Grewer, D. M., R. F. Young, R. M. Whittal and P. M. Fedorak (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.

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

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

Headley, J. V., K. M. Peru, M. H. Mohamed, R. A. Frank, J. W. Martin, R. R. Hazewinkel, D. Humphries, N. P. Gurprasad, L. M. Hewitt, D. C. Muir, D. Lindeman, R. Strub, R. F.

Young, D. M. Grewer, R. M. Whittal, P. M. Fedorak, D. A. Birkholz, R. Hindle, R. Reisdorph, X. Wang, K. L. Kasperski, C. Hamilton, M. Woudneh, G. Wang, B. Loescher, A. Farwell, D. G. Dixon, M. Ross, S. Pereira Ados, E. King, M. P. Barrow, B. Fahlman, J. Bailey, D. W. McMartin, C. H. Borchers, C. H. Ryan, N. S. Toor, H. M. Gillis, L. Zuin, G. Bickerton, M. McMaster, E. Sverko, D. Shang, L. D. Wilson and F. J. Wrona (2013a). "Chemical fingerprinting of naphthenic acids and oil sands process waters-a review of analytical methods for environmental samples." Journal of Environmental Science and Health, Part A **48**(10): 1145-1163.

Headley, J. V., K. M. Peru, M. H. Mohamed, L. Wilson, D. W. McMartin, M. M. Mapolelo, V. V. Lobodin, R. P. Rodgers and A. G. Marshall (2012). "Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry characterization of tunable carbohydrate-based materials for sorption of oil sands naphthenic acids." Energy & Fuels **27**(4): 1772-1778.

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

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

Johnson, R. J., C. E. West, A. M. Swaih, B. D. Folwell, B. E. Smith, S. J. Rowland and C. Whitby (2012). "Aerobic biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two different pathways by a new isolate of *Mycobacterium*." Environmental Microbiology **14**(4): 872-882.

Kim, S., L. A. Stanford, R. P. Rodgers, A. G. Marshall, C. C. Walters, K. Qian, L. M. Wenger and P. Mankiewicz (2005). "Microbial alteration of the acidic and neutral polar nso compounds revealed by Fourier transform ion cyclotron resonance mass spectrometry." Organic Geochemistry **36**(8): 1117-1134.

Lai, J. W. S., L. J. Pinto, E. Kiehlmann, L. I. Bendell Young and M. M. Moore (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.

Madill, R. E., M. T. Orzechowski, G. Chen, B. G. Brownlee and N. J. Bunce (2001). "Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water." Environmental Toxicology and Chemistry**16**(3): 197-208.

McKenzie, N., S. Yue, X. Liu, B. A. Ramsay and J. A. Ramsay (2014). "Biodegradation of naphthenic acids in oils sands process waters in an immobilized soil/sediment bioreactor." Chemosphere **109**(0): 164-172.

Quagraine, E. K., J. V. Headley and H. G. Peterson (2005). "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.

Reinardy, H. C., A. G. Scarlett, T. B. Henry, C. E. West, L. M. Hewitt, R. A. Frank and S. J. Rowland (2013). "Aromatic naphthenic acids in oil sands process-affected water, resolved by GCxGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (danio rerio) larvae." Environmental Science & Technology **47**(12): 6614-6620.

Routledge, E. J. and J. P. Sumpter (1996). "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen." Environmental Toxicology and Chemistry **15**(3): 241-248.

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

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

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

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

Selifonov, S. A. (1992). "Microbial oxidation of adamantanone by *Pseudomonas putida* carrying the camphor catabolic plasmid." Biochemical and Biophysical Research Communications **186**(3): 1429-1436.

Taylor, D. G. and P. W. Trudgill (1978). "Metabolism of cyclohexane carboxylic acid by *Alcaligenes* strain w1." Journal of Bacteriology **134**(2): 401-411.

Toor, N. S., X. Han, E. Franz, M. D. MacKinnon, J. W. Martin and K. Liber (2013). "Selective biodegradation of naphthenic acids and a probable link between mixture profiles and aquatic toxicity." Environmental Toxicology and Chemistry **32**(10): 2207-2216.

Videla, P., A. Farwell, B. Butler and D. G. Dixon (2009). "Examining the microbial degradation of naphthenic acids using stable isotope analysis of carbon and nitrogen." Water, Air, and Soil Pollution **197**(1-4): 107-119.

Williams, J., M. Bjorøy, D. Dolcater and J. Winters (1986). "Biodegradation in south Texas eocene oils - effects on aromatics and biomarkers." Organic Geochemistry **10**(1): 451-461.

Chapter 6

A Microtiter Study of the Biodegradation of Naphthenic Acid Surrogates by Axenic Cultures

This chapter has been submitted to Biodegradation

6.1 Abstract

This is the first study to report that bacteria from the genera *Ochrobactrum*, *Brevundimonas* and *Bacillus* can be isolated by growth on naphthenic acids (NAs) extracted from oil sands process water (OSPW). These pure cultures were screened for their ability to use a range of aliphatic, cyclic and aromatic NA surrogates in 96-well microtiter plates. Water-soluble tetrazolium redox dyes (Biolog Redox Dye H) was used as the indicator of metabolic activity. Of the three cultures, *Ochrobactrum* grew best on the widest range of NA surrogates. *Brevundomonas* and especially *Ochrobactrum* grew better on the polycyclic aromatic compounds than other classes of NA surrogates. *Bacillus* also grew on a wide range of NA surrogates but not as well as *Ochrobactrum*. Using this method to characterize NA utilisation, one can identify which NAs or NA classes in OSPW are more readily degraded. Since aromatic NAs have been shown to have an estrogenic effect and polycyclic monoaromatic compounds have been suggested to pose the greatest environmental threat among the NAs, these bacterial genera may play an important role in detoxification of OSPW. Furthermore, this study demonstrates that bacteria belonging to the genera *Ochrobactrum* and *Bacillus*, can also degrade surrogates of tricyclic NAs.

6.2 Introduction

Canada has the second largest global oil reserves of 1.7 trillion barrels, most of which can be produced from oil sands (Allen 2008). To separate bitumen from the oil sands, caustic soda is used in the Clark's alkaline hot (79-93 ℃) water extraction process and generates a wastewater known as oil sands process water (OSPW). Although a variety of organic compounds such as bitumen, naphthenic acids (NAs), asphaltenes, phenols, and polyaromatic hydrocarbons are present in OSPW (Giesy et al. 2010), toxicity is generally considered to be due to the NAs (Frank et al. 2009, Herman et al. 1994).

NAs are a complex mixture of alkyl-substituted acyclic, monocyclic, polycyclic aliphatic, aromatic, and polycyclic aromatic carboxylic acids. Currently, NAs are considered to fit the chemical formula $C_nH_{2n+Z}O_x$ (Grewer et al. 2010) where n indicates the carbon number, and Z is zero or a negative, even integer that specifies a family of homologous compounds. For classical NAs (c-NAs), x equals 2 and for the oxidized NAs (oxy-NAs) that may arise from the oxidation of c-NAs, $3 < x < 5$. NAs may also contain sulfur and/or nitrogen atoms (Barrow et al. 2010). They are toxic to fish (Allen 2008, Nero et al. 2006b, Siwik et al. 2000), phytoplankton (Hayes 2005, Leung et al. 2001), other aquatic organisms (Rogers et al. 2007), mammals, and plants (Kamaluddin and Zwiazek 2002). Endocrine disruption has been demonstrated in fathead minnows (Kavanagh et al. 2011, Siwik et al. 2000), goldfish (Lister et al. 2008) and T47D-kbluc and MDAkb2 cells (He et al. 2011) with exposure to OSPW and the aromatic NAs have been implicated (Reinardy et al. 2013, Rowland et al. 2011b). Because of the potential adverse environmental impact, the Alberta Environmental Protection and Enhancement Act (1993) has imposed a zero discharge policy on OSPW, resulting in > 720 million m^3 of it being currently stored in tailing ponds. Hence, there is a need to remove and/or detoxify NAs in OSPW. Biodegradation is generally considered to be the most cost effective treatment for wastewaters (Scott et al. 2008).

Little is known about NA-degrading microorganisms and the range of NAs that they may metabolize. Some studies have focused on understanding the biodegradation of NAs by consortia obtained from centrifuged OSPW (Clemente et al. 2004, Scott et al. 2005) rhizosphere microorganisms not previously exposed to NAs (Biryukova et al. 2007), and cultures enriched from OSPW using commercially available NAs as the carbon substrate (Clemente et al. 2004). Although the degradation of certain NA surrogates by isolates and mixed cultures have been determined (reviewed by Whitby (2010) and summarized in Table 6-1), no screening method has been previously applied to evaluate a range of NA surrogates that could be metabolized. Assessing the range of NAs metabolized by isolated strains is desirable in their characterization. However, the NA composition of OSPW is complex and there are relatively few NA surrogates commercially available. Some NAs are poorly water soluble and hence poorly bioavailable. In addition, those which are chemically synthesized are typically available only in small amounts. This makes assessing growth or substrate utilization by standard assays challenging as growth rates and cell yields may be low. Water soluble tetrazolium redox dyes (well known for their use in Biolog taxonomic and other assays) have been widely used as a tool to measure metabolic activity in mammalian cells in drug screening (Berridge et al. 2005), and a few studies have been done with microbial cells to evaluate the use of electron mediators (Tsukatani et al. 2008), or to assess growth on organic carbon compounds such as toluene and o-xylene (Strong-Gunderson et al. 1994), and polyaromatic hydrocarbons (Johnsen et al. 2002).

In this paper, an easy-to-use, high throughput and sensitive microtiter plate method is adapted to assess growth on a range of aliphatic, cyclic and aromatic NA surrogates by three bacterial cultures isolated on agar containing NAs extracted from OSPW as the sole carbon source. The method is based on the respiratory reduction of a water soluble tetrazolium salt, Biolog redox dye H. The cells proliferate only in wells amended with a carbon substrate that they can metabolize. Electrons from the oxidation of a NA surrogate reduce the dye to produce colored formazan, which has been correlated to C14 mineralization (Johnsen et al. 2002) and viable cell density (Tsukatani et al. 2008). The application of this assay is of great potential value not only in characterizing NA-degrading microorganisms but also in determining the treatment of OSPW and its detoxification. It can help to determine which NAs are readily biodegradable and requires small amounts of substrate which is important as pure NAs or NA surrogates may be expensive or only synthesized in limited amounts.

6.3 Materials and Method

6.3.1 OSPW and Extracted NAs

OSPW was obtained from an active settling basin (West-In-Pit, WIP) of Syncrude Canada in Fort McMurray, AB. The procedure to extract NAs from OSPW was adopted from Nero et al. (2006a). OSPW was acidified with 2 M H_2SO_4 to pH 2 to precipitate the NAs, and the overlying process water discarded. After redissolving the NAs in 0.1 M NaOH and a second precipitation with H_2SO_4 to pH 2, NAs were extracted into dichloromethane (Fisher Scientific ACS stabilized) followed by rotary evaporation at 39 ℃ then redissolved in 0.1 M NaOH.

6.3.2 Source and Isolation of Cultures

The isolates were obtained from planktonic samples from an immobilized soil bioreactor (ISBR) used to treat OSPW as described by McKenzie et al. (2014). A biofilm had been

established on a nonwoven textile in the ISBR from microorganisms associated with soil particles from an OSPW contaminated wetland. After 22 months of continuous operation with OSPW as the sole source of carbon and energy, about 40% NAs were degraded. Planktonic samples from the bioreactor were streaked onto dilute nutrient agar plates (0.8 g L^{-1} nutrient broth, 15 g L^{-1} agar). Colonies were transferred to agar plates containing extracted NAs as the sole source of carbon. These agar plates contained: 5 g L^{-1} Na_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 5 g L^{-1} NH_4NO_3, 6.6 mg L^{-1} $MgSO_4 \cdot 7H_2O$, and 5 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 16 g L^{-1} Noble agar and extracted OSPW NAs, pH 8.0. The quantity of NAs added was extracted from 133 L of OSPW as described above. Incubation was at room temperature (22 ± 2 ℃) until visible growth was observed and isolates were re-streaked to obtain pure cultures.

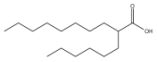
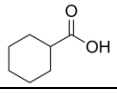
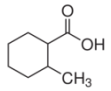
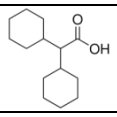
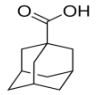
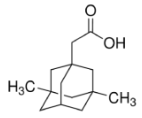
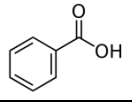
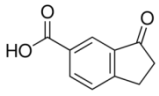
6.3.3 16S rDNA Identification of Isolates

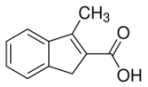
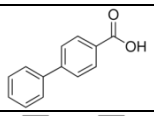
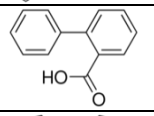
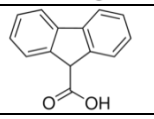
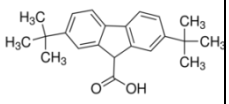
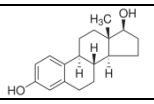
Near full length of 16S rDNA fragments were amplified using universal bacterial primers 27f (5'AGAGTTTGATCCTGGCTCAG-3'), and 1391R (5'-GACGGGCGGTGWGTRCA-3') (de la Torre et al. 2003), which were designed on the basis of the conserved bacterial regions at the positions 27f and 1391r respectively, on the *Escherichia coli* rRNA gene sequence. A 50 µL reaction mixture contained 1U Taq DNA polymerase and 5 µL of the corresponding 10X buffer, 4 mL of 25 mM dNTP mixture (Invitrogen Co., Carlsbad, CA USA), 25 pmol of each primer and 10 ng of genomic DNA. Amplification was performed using the following program: initial denaturation at 94 ℃ for 4 min, 20 cycles consisting of 94 ℃ for 45 s, 55 ℃ for 1 min and 72 ℃ for 1 min, and final extension at 72 ℃ for 6 min. The PCR products were purified and concentrated with HiYield™ DNA UltraPurification kit (RBC Inc., New Taipei City, Taiwan). Fragments were sequenced by Genome Québec and were blasted using NCBI-BLAST (http://www.ncbi.nlm.nih.gov). The 16S rRNA sequence reported in this study has been deposited in GenBank under Accession No. KM017733-KM017735.

6.3.4 Naphthenic Acid Surrogates

NA surrogates (Table 1) were selected to represent a range of NA structures, and were purchased from Sigma-Aldrich Canada (Oakville, ON Canada). Estradiol was also selected because it has the same chemical formula as some NAs and similar structures have been shown to be present in OSPW (Rowland et al. 2011b).

Table 6-1 Physical properties and EC_{50} Microtox® results of NA surrogates used in microtiter respiratory test

Group	**Name**	**Chemical formula**	**Water Solubility at 25 ℃ (mg L^{-1}) pH7.4**	**LOG K_{ow}**	**EC_{50}**		**Structure**
					g L^{-1}	**mM**	
Aliphatic n-acids (ali_)	Palmitic acid (ali_1)	$C_{16}H_{32}O_2$	0.04073	7.17	0.04	0.17	
	Stearic acid (ali_2)	$C_{18}H_{36}O_2$	0.003512	8.23	0.14	0.48	
	2-hexyldecanoic acid (alic_3)	$C_{16}H_{32}O_2$	0.07083	6.89	0.35	1.38	
Monocyclic acids (cyc_)	Cyclohexanecarboxylic acid (cyc_1)	$C_7H_{12}O_2$	4919	1.96	0.17	1.29	
	2-Methyl-1-cyclohexanecarboxylic acid (cyc_2)	$C_8H_{14}O_2$	870.3	2.77	0.02	0.16	
Bicyclic acids (bi_)	Dicyclohexylacetic acid (bi_1)	$C_{14}H_{24}O_2$	1.521	5.53	0.02	0.11	
Tricyclic acids (tri_)	1-amantanecarboxylic acid (tri_1)	$C_{11}H_{16}O_2$	276.3	3.15	0.87	4.8	
	3,5-Dimethyladamantane-1-acetic acid (tri_2)	$C_{14}H_{22}O_2$	10.77	4.55	0.03	0.13	
Monoaromatic acids (ma_)	Benzoic acid (ma_1)	$C_7H_6O_2$	2493	1.87	0.04	0.37	
	Indanone-6-carboxylic acid (ma_2)	$C_{10}H_8O_3$	1148	1.99	0.07	0.38	

	3-Methylindene-2-carboxylic acid (ma_3)	$C_{11}H_{10}O_2$	322.6	3.11	2.00	11.48	
Polycyclic aromatic acids (pa_)	Biphenyl-4-carboxylic acid (pa_1)	$C_{13}H_{10}O_2$	28.03	3.75	0.22	1.10	
	Biphenyl-2-carboxylic acid (pa_2)	$C_{13}H_{10}O_2$	152.1	2.89	0.05	0.22	
	Fluorene-9-carboxylic acid (pa_3)	$C_{14}H_{10}O_2$	30.34	2.80	0.05	0.26	
	2,7-Di-tert-butylfluorene-9-carboxylic acid (pa_4)	$C_{22}H_{26}O_2$	0.002318	6.88	0.19	0.60	
Estradiol	Estradiol	$C_{18}H_{24}O_2$	1.51	4.13	1.15	4.24	

6.3.5 Microtiter Respiratory Assay for Growth on NA Surrogates and Estradiol

The protocol developed was based on a Biolog Technical Bulletin (Bulletin 2007) and Johnsen et al. (2002). To prepare the inocula for the experiment, each isolate was grown in Luria-Bertani medium with 0.5 g L^{-1} cyclohexane carboxylic acid (CHCA) for 24 h. The cells were recovered by centrifugation at 5000 × g for 10 minutes, washed once in sterile mineral salt medium (5 g L^{-1} Na_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 5 g L^{-1} NH_4NO_3, 6.6 mg L^{-1} $MgSO_4 \cdot 7H_2O$, and 5 mg L^{-1} $CaCl_2 \cdot 2H_2O$), and the optical density at 630 nm adjusted to 0.1 with sterile mineral salt medium containing Biolog Redox Dye H (Biolog Inc., Hayward CA, USA), a mixture of water soluble tetrazolium dyes. Stock solutions of NA surrogates were prepared in methanol, diluted to an appropriate concentration and 10 µL aliquots of each concentration were added to a 96-well microtiter plate (VWR International, Mississauga, Ontario). Wells containing only methanol were used as a control. After methanol was allowed to evaporate to dryness, 200 µL of the medium containing an inoculum of each isolate was added to wells such that the final concentration of individual surrogates was 0, 0.5, 1 or 2 g L^{-1}. To minimize evaporation, plates were covered with

a silicone cap (VWR International, Mississauga, ON), wrapped in plastic bags and incubated for 21 days at 30 ℃ in an Innova® 44/44R incubator shaker (Fisher Scientific, Mississauga, ON) at 150 rpm. Absorbance was measured with a Synergy™HT microtiter plate reader (Biotek Instruments Inc., Winnoski,VT) at 590 nm with a reference wavelength of 750 nm. Substrate utilisation was evaluated as the absorbance of formazan produced and calculated as follows：

Substrate utilisation = $(A_{590}-A_{750})_{day21}$ -$(A_{590}-A_{750})_{initial}$ -$(A_{590}-A_{750})_{control}$

Each test was done in triplicate and the average was calculated as an indication of substrate utilisation.

6.3.6 Microtox® Assay

The Microtox® acute toxicity test was adapted to be performed in 96-well plates (Reemtsma et al. 1999). Triplicates of increasing concentrations of each NA solubilized in methanol were added to individual wells and the methanol solution reduced to almost dryness then diluted with 2% NaCl solution to keep the methanol content below 2% (v/v). Reconstituted *Vibrio fischeri* NRRL B-11177 cells were added and using the liquid-phase test protocol, the effective concentration resulting in 50% decrease in bioluminescence (EC_{50}) was calculated as described by the manufacturer (Azur Environmental, Fairfax, CA, USA). Lower EC_{50} values indicate a greater toxicity. 1 g L^{-1} glucose was used as a negative control and $ZnSO_4$ was tested as a positive control and found to be within the parameters set by Microtox® (15 minute EC_{50} was between 3 to 10 mg L^{-1}). The inhibition of *V.fischeri*'s bioluminescence was analyzed with a Synergy ™ HT microtiter plate reader (Biotek Instruments Inc., Winnoski, VT). Procedural blanks were also performed in parallel, tested in duplicate and showed no toxicity.

6.4 Results and Discussion

6.4.1 Isolates from Extracted Naphthenic Acid Agar Plates

Three isolates were obtained as pure cultures from planktonic samples of an ISBR fed with OSPW as the sole source of carbon and energy. They were isolated on Noble agar plates containing extracted NAs as the sole carbon source. After their near full length 16S rDNA fragments were amplified, sequenced, aligned and blasted, they were identified (Table 6-2). Although several pure cultures (Table 2-2) have been shown to degrade model alicyclic NAs and even fewer (*Burkholderia, Pseudomonas* and *Sphingomonas* spp.) shown to transform mono-aromatic alkanoic NAs (Johnson et al. 2011), this is the first time that isolates belonging to the genera *Ochrobactrum*, *Brevundimona*s, or *Bacillus* have been isolated on extracted NAs as the sole carbon source and evaluated for NA degradation.

Table 6-2 Identity of isolates

Isolates	Accession no.	Closest match	Similarity
isolate1	KM017733	*Ochrobactrum anthropi* ATCC 49188 (NR_074243)	99%
isolate2	KM017734	*Bacillus thuringiensis* ATCC 10792 (NR_114581)	99%
isolate3	KM017735	*Brevundimonas diminuta* NBRC 12697 (NR_113602)	98%

6.4.2 Toxicity of NAs Surrogates and Estradiol

The Microtox® toxicity assay of the NA surrogates was performed and the results summarized in Table 2-2. The majority of these compounds had a similar level of toxicity except for 3-methylindene-2carboxylic acid (ma_3), estradiol and 1-amantanecarboxylic acid (tri_1) which were the least toxic with much higher EC_{50} values. Jones et al. (2011) evaluated the toxicity of families of linear, cyclic and monoaromatic NAs, many of which were chemically synthesized and some commercially available, to *V. fisheri*. Although the compounds in this study were different from Jones et al. (2011), when comparing our EC_{50} data within compound families

there was general agreement except for the tricyclic NA, 1-amantanecarboxylic acid. Although toxicity may inhibit microbial degradation (Brient et al. 2000), there was no consistent relationship between toxicity to *V. fischeri* (i.e. test culture for the Microtox® assay) and growth of the isolates on the NA surrogates. For example, fluorene-9-carboxylic acid (pa_3) was among the most toxic (EC50 < 0.05 g L^{-1} (0.26 mM)) but all three cultures grew well on it (Figure 6-1) while 3-methylindene-2-carboxylic acid (ma_3) was the least toxic (EC_{50}> 2.00 g L^{-1} (11.48 mM)) but produced poor growth of all three cultures.

6.4.3 NA Surrogates and Estradiol Utilisation in Microtiter Respirometry Assay

The growth of each isolate was evaluated on mineral salt medium containing tetrazolium salt (Biolog Redox Dye H) as a redox indicator of microbial growth and individual NA surrogates or estradiol as the sole carbon source at 0.5, 1, and 2 g L^{-1}. Controls were prepared with the same amount of methanol without a NA surrogate and dried as with the surrogates. No significant color developed in the control wells, indicating that residual methanol did not contribute to growth. The cultures proliferated in wells amended with NA surrogates that they could metabolize and the formazan produced was measured by absorbance with the results shown in Figure 6-1. NA surrogates are grouped by families and absorbance values above the dashed line indicate growths that are statistically significant ($p < 0.05$) compared to the methanol only control from a one-way ANOVA analysis.

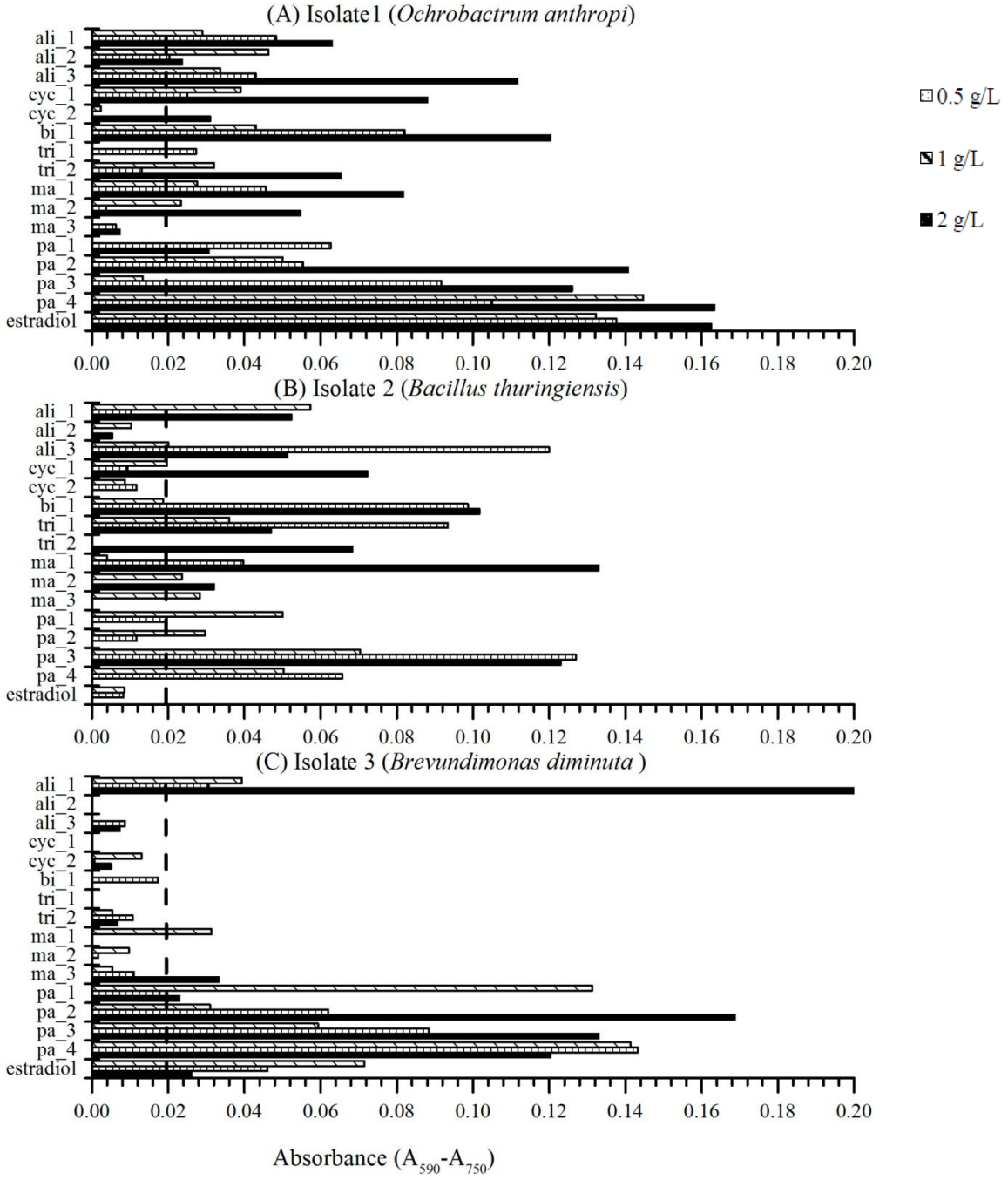


Figure 6-1 NA surrogate and estradiol utilisation as sole sources of carbon and energy in 96-well microtiter plates after 21 days of incubation. The identity of each surrogate is listed in column 2 of Table 6-2. e.g. ali_1 is palmitic acid, ali_2 is stearic acid, etc. Absorbances for the methanol only control has already been substrated, and based on an ANOVA analysis, absorbance values above the dashed line are statistically significant ($p < 0.05$) compared to the methanol only control.

In OSPW, major carbon substrates which can support bacterial growth are oil and grease (25 ± 15 mg L^{-1}) (MacKinnon and Boerger 1986) and NAs (20-120 mg L^{-1}) (Frank et al. 2008) with minor amounts of alkylphenols (< 0.3 mg L^{-1}) (Hargesheimer et al. 1984) and PAHs (10 µg L^{-1}) (Rogers et al. 2002). Although NAs, alkylphenols and PAHs are known to be toxic, the latter two are at relatively low concentrations; hence this study has focused on the potential of the three isolates to degrade NAs. The NA composition in OSPW is complex with NA definition evolving as new information is obtained. Advances in the characterization of NAs in OSPW and commercial preparations have identified aliphatic, alicyclic (mono-to penta-cyclic), and aromatic (mono-and poly-aromatic) NAs.

C8-C18 aliphatic NAs have been identified in commercially available NAs (Rowland et al. 2011c) and in OSPW (Scarlett et al. 2012). Although low amounts of mostly even numbered fatty acids are found in OSPW (Martin et al. 2008), biodegradation of NAs produced a significant accumulation of fatty acids such as palmitic (C16) and stearic (C18) acids (Biryukova et al. 2007, Clemente et al. 2004). In evaluating the toxicity of NAs to *V. fisheri*, Jones et al. (2011) did not test these fatty acids. We found that palmitic and stearic acids (**错误!未找到引用源。**) had similar toxicity to the shorter (C6-C12) linear aliphatic acids tested by Jones et al. (2011). None of the isolates grew well on stearic acid, but all, especially *Brevundimona*s, could metabolize palmitic acid and *Ochrobactrum* and *Bacillus* metabolized 2-hexyldecanoic acid (alic_3).

Alicyclic NAs comprise about 70% of the NAs in OSPW (Jones et al. 2012). Monocyclic NAs are present but not abundant (Barrow et al. 2004, Martin et al. 2008), while bi-, tri-, tetra-, and pentacyclic NAs are known to be common (Rowland et al. 2011a, Rowland et al. 2011d, Rowland et al. 2011e). A few tri-cyclic NAs are commercially available but since tetra-and penta-cyclic NAs are not, the latter were not studied. *Brevundimona*s did not metabolize any mono-, bi- or tri-cyclic surrogates. The *Ochrobactrum* and *Bacillus* isolates metabolized cyclohexanecarboxylic acid (cyc_1) but not 2methyl-1-cyclohexanecarboxylic acid (cyc_2), most

likely because of the presence of a methyl group in the ortho position of the latter compound (Han et al. 2008, Quagraine et al. 2005). Several pure cultures in the genera belonging to the group of *Actinobacteria* and *Proteobacteria* have been shown to grow on monocyclic NAs (**错误! 未找到引用源。**) and sediment microorganisms have been shown to aerobically degrade a bicyclic surrogate (decahydro-2-naphthoic acid) (Del Rio et al. 2006). Both *Ochrobactrum* and *Bacillus* showed good metabolic activity with an increasing concentration of the bicyclic surrogate, dicyclohexylacetic acid (bi_1).

As the number of rings in alicyclic NAs increase, the biodegradation rate decreased (Clemente et al. 2003, Del Rio et al. 2006). Mineralization rates were found to be slower by as much as 60 times with a bicyclic NA surrogate compared to a monocyclic one (Del Rio et al. 2006). Although few studies have directly examined the biodegradation of NAs with three or more rings, the biodegradation of such diamondoid hydrocarbons in crude oil has been determined by analyzing the composition of various crude oils (Grice et al. 2000, Wei et al. 2007). Diamondoid hydrocarbons are generally considered to be significantly more difficult to biodegrade and among them, only adamantane has been shown to be metabolized by *Pseudomonas putida* to adamantanone then to a dead-end product, 1hydroxy-4-oxohomoadamantan-5-one, which accumulates in the cell (Selifonov 1992). No study demonstrating the biodegradation of the carboxylic acid of adamantane (1-amantanecarboxylic acid (tri_1) or 3,5-dimethyladamantane-1-acetic acid (tri_2)) has been found. This study shows that an *Ochrobactrum* and *Bacillus* species can also degrade surrogates of tricyclic NAs.

Aromatic NAs are present in highly complex mixtures with alicyclic NAs and may comprise >30% of the NAs in OSPW (Jones et al. 2012). Although few of them have been identified, some monoaromatic structures such as dehydroabietic acid (Jones et al. 2012), and C_{19} and C_{20} hydroxyl steroid acids similar to estrone and estradiol (Rowland et al. 2011b) were shown to be present. Polyaromatic carboxylic acids are present in crude oil (Seifert and Teeter 1970) and

OSPW (Jones et al. 2012) but specific structures in OSPW have not yet been determined and are known to co-elute with fluorene-9-carboxylic acid (Jones et al. 2012). Aromatic NAs have been shown to have an estrogenic effect (Reinardy et al. 2013) and while polycyclic monoaromatic compounds have been suggested by Scarlett et al. (2012) to pose the "greatest environmental threat" because they were predicted to be among the most toxic NAs, the toxicity of polyaromatic NAs is unknown. It is promising that among the three isolates that *Ochrobactrum* was able to degrade well estradiol, poly-and mono-aromatic NAs except for 3-methylindene-2-carboxylic acid (ma_3). The latter compound may have been difficult to degrade because of stearic hindrance of the methyl group in the ortho position (Han et al. 2008, Quagraine et al. 2005).

It is likely that the isolates *Ochrobactrum* and *Brevundimona*s may play a more important role in detoxification of OSPW than *Bacillus*. Of the three cultures, *Ochrobactrum* grew best on the widest range of NA surrogates. Like *Ochrobactrum*, *Bacillus* grew on a range of NA surrogates albeit less well and on fewer of them, and not on estradiol. However, *Brevundomonas* like *Ochrobactrum* showed similar growth patterns on the polycyclic aromatic compounds but did not grow well on other surrogate classes except for palmitic acid.

Although *Ochrobactrum anthropi* has been shown to degrade dioxin (Chen et al. 2010), polycyclic aromatic hydrocarbon, oil refinery wastewater (Calvo et al. 2008) and aromatic amides (Sonke et al. 2005), this is the first study showing its potential in NA degradation. *B. thuringiensis* is not well known for the degradation of organic pollutants but it can degrade fipronil (Mandal et al. 2013), and bis (2-ethylhexyl) phthalate (Pham et al. 2011). Apart from noting the survival of a species similar to *Brevundimonas sp*. following ozonation of OSPW (Hwang et al. 2013), no role was assigned to it. Our study shows that our Isolate 3, *Brevundimona*s, can degrade a wide range of NAs clearly showing the potential of the screening assay to assess the role of these types of isolates in NA degradation and hence in remediating OSPW.

In conclusion, this study reports that bacteria, (*Ochrobactrum*, *Brevundimonas* and *Bacillus* isolated on NAs extracted from OSPW as the sole carbon substrate) were screened for their ability to use a range of different classes of NA surrogates using water soluble tetrazolium redox dyes to measure metabolic activity in a 96-well microtiter plate. From the results of this assessment, it appears that these isolates play a role in the degradation of the NAs in OSPW and since they can metabolize aromatic NAs, which have been shown to be estrogenic and toxic; these cultures may also contribute to detoxification of OSPW.

6.6 References

Alberta Environmental Protection and Enhancement Act (1993) Edmonton: Alberta Environmental Protection http://www.qp.Alberta.ca/documents/Regs/1993_118.pdf (citation date:2014-12-01)

Allen, E. (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.

Barrow, M. P., J. V. Headley, K. M. Peru and P. J. Derrick (2004). "Fourier transform ion cyclotron resonance mass spectrometry of principal components in oil sands naphthenic acids." Journal of Chromatography A **1058**(1-2): 51-59.

Barrow, M. P., M. Witt, J. V. Headley and K. M. Peru (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.

Berridge, M. V., P. M. Herst and A. S. Tan (2005). "Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction." Biotechnology Annual Review **11**(2005): 127-152.

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

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

Blakley, E. and B. Papish (1982). "The metabolism of cyclohexanecarboxylic acid and 3-cyclohexenecarboxylic acid by *Pseudomonas putida*." Canadian Journal of Microbiology **28**(12): 1324-1329.

Blakley, E. R. and B. Papish (1982). "The metabolism of 3-cyclohexenecarboxylic acid by *Alcaligenes faecalis*." Canadian Journal of Microbiology **28**(9): 1037-1046.

Brient, J. A., P. J. Wessner and M. N. Doyle (2000). Naphthenic acids. Kirk-Othmer encyclopedia of chemical technology, John Wiley & Sons, Inc.

Calvo, C., G. Silva-Castro, I. Uad, C. G. Fandiño, J. Laguna and J. González-López (2008). "Efficiency of the EPS emulsifier produced by *Ochrobactrum anthropi* in different hydrocarbon bioremediation assays." Journal of Industrial Microbiology & Biotechnology **35**(11): 1493-1501.

Chen, H.-J., C. Fan, M.-S. Lee, H.-C. Gong, J.-M. Fanchiang and D.-H. Tseng (2010). Screening of dioxin biotransformation bacteria from the contaminated soil. Bioinformatics and Biomedical Engineering (iCBBE), 2010 4th International Conference on, IEEE.

Clemente, J. S. and P. M. Fedorak (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., Y. Tin-Win and P. M. i. Fedorak (2003). "Development of a high performance liquid chromatography method to monitor the biodegradation of naphthenic acids." Journal of Environmental Engineering & Science **2**(3): 177-186.

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

Dutta, T. K. and S. Harayama (2001). "Biodegradation of n-alkylcycloalkanes and n-alkylbenzenes via new pathways in *Alcanivorax* sp strain MBIC4326." Applied and Environmental Microbiology **67**(4): 1970-1974.

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

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

Giesy, J. P., J. C. Anderson and S. B. Wiseman (2010). "Alberta oil sands development." Proceedings of the National Academy of Sciences **107**(3): 951-952.

Grewer, D. M., R. F. Young, R. M. Whittal and P. M. Fedorak (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.

Grice, K., R. Alexander and R. I. Kagi (2000). "Diamondoid hydrocarbon ratios as indicators of biodegradation in Australian crude oils." Organic Geochemistry **31**(1): 67-73.

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

Hargesheimer, E. E., R. T. Coutts and M. D. MacKinnon (1984). "Characterization of simple phenols in oil sands extraction-process water." Environmental Technology **5**(1-11): 433-440.

Hasegawa, Y., K. Hamano, H. Obata and T. Tokuyama (1982a). "Microbial degradation of cycloheptanone." Agricultural and Biological Chemistry **46**(5): 1139.

Hasegawa, Y., K. Higuchi, H. Obata, T. Tokuyama and T. Kaneda (1982b). "A cyclohexanecarboxylic acid utilizing yeast: Isolation, identification, and nutritional characteristics." Canadian Journal of Microbiology **28**(8): 942-944.

Hayes, T. M. (2005). "Examining the ecological effects of naphthenic acids and major ions on phytoplankton in the Athabasca oil sands region." Unpublished PhD Thesis, University of Waterloo, Waterloo, ON.

He, Y., S. B. Wiseman, M. Hecker, X. Zhang, N. Wang, L. A. Perez, P. D. Jones, M. G. El-Din, J. W. Martin and J. P. Giesy (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., J. L. Du, K. M. Peru, N. Gurprasad and D. W. McMartin (2008). "Evaluation of algal phytodegradation of petroleum naphthenic acids." Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering **43**(3): 227-232.

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

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

Huang, J., M. Nemati, G. Hill and J. Headley (2012). "Batch and continuous biodegradation of three model naphthenic acids in a circulating packed-bed bioreactor." Journal of Hazardous Materials **201**(2012): 132-140.

Hwang, G., T. Dong, M. S. Islam, Z. Sheng, L. A. Pérez-Estrada, Y. Liu and M. Gamal El-Din (2013). "The impacts of ozonation on oil sands process-affected water biodegradability and biofilm formation characteristics in bioreactors." Bioresource Technology **130**(2013): 269-277.

Iwaki, H., E. Nakai, S. Nakamura and Y. Hasegawa (2008). "Isolation and characterization of new cyclohexylacetic acid-degrading bacteria." Current Microbiology **57**(2): 107-110.

Johnson, B. (2005). Microtox® acute toxicity test. Small-scale freshwater toxicity investigations. C. Blaise and J.-F. Férard, Springer Netherlands**:** 69-105.

Johnson, R. J., B. E. Smith, S. J. Rowland and C. Whitby (2012a). "Biodegradation of alkyl branched aromatic alkanoic naphthenic acids by *Pseudomonas putida* KT2440." International Biodeterioration & Biodegradation **81** (2013): 3-8.

Johnson, R. J., C. E. West, A. M. Swaih, B. D. Folwell, B. E. Smith, S. J. Rowland and C. Whitby (2012b). "Aerobic biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two different pathways by a new isolate of *Mycobacterium*." Environmental Microbiology **14**(4): 872-882.

Johnson, R. J., B. E. Smith, P. A. Sutton, T. J. McGenity, S. J. Rowland and C. Whitby (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., A. G. Scarlett, C. E. West and S. J. Rowland (2011). "Toxicity of individual naphthenic acids to *Vibrio fischeri*." Environmental Science & Technology **45**(22): 9776-9782.

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

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

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

Lai, J. W. S., L. J. Pinto, E. Kiehlmann, L. I. BendellYoung and M. M. Moore (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.

Leung, S. S., M. D. MacKinnon and R. E. Smith (2001). "Aquatic reclamation in the Athabasca, Canada, oil sands: Naphthenate and salt effects on phytoplankton communities." Environmental Toxicology and Chemistry **20**(7): 1532-1543.

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

MacKinnon, M. and H. Boerger (1986). "Description of two treatment methods for detoxifying oil sands tailings pond water." Water Quality Research Journal of Canada **21**(4): 496-512.

Mandal, K., B. Singh, M. Jariyal and V. K. Gupta (2013). "Microbial degradation of fipronil by *Bacillus thuringiensis*." Ecotoxicology and Environmental Safety **93**(2012): 87-92.

Martin, J. W., X. Han, K. M. Peru and J. V. Headley (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.

McKenzie, N., S. Yue, X. Liu, B. A. Ramsay and J. A. Ramsay (2014). "Biodegradation of naphthenic acids in oils sands process waters in an immobilized soil/sediment bioreactor." Chemosphere **109** (2014): 164-172..

Misiti, T., U. Tezel and S. G. Pavlostathis (2013). "Fate and effect of naphthenic acids on oil refinery activated sludge wastewater treatment systems." Water Research **47**(1): 449-460.

Nero, V., A. Farwell, L. E. J. Lee, T. Van Meer, M. D. MacKinnon and D. G. Dixon (2006). "The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology." Ecotoxicology and Environmental Safety **65**(2): 252-264.

Nero, V., A. Farwell, A. Lister, G. Van der Kraak, L. E. Lee, T. Van Meer, M. D. MacKinnon and D. G. Dixon (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.

Ougham, H. and P. Trudgill (1982). "Metabolism of cyclohexaneacetic acid and cyclohexanebutyric acid by *Arthrobacter* sp. strain CA1." Journal of Bacteriology **150**(3): 1172-1182.

Paslawski, J. C., J. V. Headley, G. A. Hill and M. Nemati (2009). "Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid." Biodegradation **20**(1): 125-133.

Pham, T. T. H., R. D. Tyagi, S. K. Brar and R. Y. Surampalli (2011). "Effect of ultrasonication and Fenton oxidation on biodegradation of bis(2-ethylhexyl) phthalate (DEHP) in wastewater sludge." Chemosphere **82**(6): 923-928.

Quesnel, D. M., I. M. Bhaskar, L. M. Gieg and G. Chua (2011). "Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*." Chemosphere **84**(4): 504-511.

Reemtsma, T., O. Fiehn and M. Jekel (1999). "A modified method for the analysis of organics in industrial wastewater as directed by their toxicity to *Vibrio fischeri*." Fresenius Journal of Analytical Chemistry **363**(8): 771-776.

Reinardy, H. C., A. G. Scarlett, T. B. Henry, C. E. West, L. M. Hewitt, R. A. Frank and S. J. Rowland (2013). "Aromatic naphthenic acids in oil sands process-affected water, resolved by GCXGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (*Danio rerio*) larvae." Environmental Science & Technology **47**(12): 6614-6620.

Rho, E. and W. Evans (1975). "The aerobic metabolism of cyclohexanecarboxylic acid by *Acinetobacter anitratum*." Biochemical Journal **148**(1): 11.

Rogers, V., M. MacKinnon and B. Brownlee (2007). "Analytical approaches to characterizing fish tainting potential of oil sands process waters." Water Science and Technology **55**(5): 311-318

Rogers, V. V., K. Liber and M. D. MacKinnon (2002). "Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water." Chemosphere **48**(5): 519-527.

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

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

Rowland, S. J., C. E. West, A. G. Scarlett and D. Jones (2011c). "Identification of individual acids in a commercial sample of naphthenic acids from petroleum by two-dimensional comprehensive gas chromatography/mass spectrometry." Rapid Communications in Mass Spectrometry **25**(12): 1741-1751.

Rowland, S. J., C. E. West, A. G. Scarlett, D. Jones, M. Boberek, L. Pan, M. Ng, L. Kwong and A. Tonkin (2011d). "Monocyclic and monoaromatic naphthenic acids: Synthesis and characterization." Environmental Chemistry Letters **9**(4): 525-533.

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

Scarlett, A. G., C. E. West, D. Jones, T. S. Galloway and S. J. Rowland (2012). "Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints." Science of the Total Environment **425**(2012): 119-127.

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

Seifert, W. K. and R. M. Teeter (1970). "Identification of polycyclic aromatic and heterocyclic crude oil carboxylic acids." Analytical Chemistry **42**(7): 750-758.

Selifonov, S. A. (1992). "Microbial oxidation of adamantanone by *Pseudomonas putida* carrying the camphor catabolic plasmid." Biochemical and Biophysical Research Communications **186**(3): 1429-1436.

Siwik, P. L., T. Van Meer, M. D. MacKinnon and C. A. Paszkowski (2000). "Growth of fathead minnows in oil sand-processed wastewater in laboratory and field." Environmental Toxicology and Chemistry **19**(7): 1837-1845.

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

Sonke, T., S. Ernste, R. F. Tandler, B. Kaptein, W. P. Peeters, F. B. van Assema, M. G. Wubbolts and H. E. Schoemaker (2005). "L-selective amidase with extremely broad substrate specificity from *Ochrobactrum anthropi* NCIMB 40321." Applied and Environmental Microbiology **71**(12): 7961-7973.

Strong-Gunderson, J. M. and A. V. Palumbo (1994). "Alternative method for rapidly screening microbial isolates for their potential to degrade volatile contaminants." Journal of Industrial Microbiology **13**(6): 361-366.

Tsukatani, T., H. Suenaga, T. Higuchi, T. Akao, M. Ishiyama, K. Ezoe and K. Matsumoto (2008). "Colorimetric cell proliferation assay for microorganisms in microtiter plate using water-soluble tetrazolium salts." Journal of Microbiological Methods **75**(1): 109-116.

Videla, P., A. Farwell, B. Butler and D. G. Dixon (2009). "Examining the microbial degradation of naphthenic acids using stable isotope analysis of carbon and nitrogen." Water, Air, and Soil Pollution **197**(1-4): 107-119.

Wei, Z., J. M. Moldowan, K. E. Peters, Y. Wang and W. Xiang (2007). "The abundance and distribution of diamondoids in biodegraded oils from the San Joaquin valley: Implications for biodegradation of diamondoids in petroleum reservoirs." Organic Geochemistry **38**(11): 1910-1926.

Whitby, C. (2010). "Microbial naphthenic acid degradation." Advances in Applied Microbiology **70**(0): 93-125.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions and Scientific Contributions

1) The NAs that are most likely to be associated with toxicity in OSPW are O2-NAs with DBE=4, and carbon numbers between 15-18, as well as O2-NAs with DBE=3; while NAs that are less likely to be associated with toxicity of OSPW are O3-NAs with carbon numbers between 14-17. For the first time, the exact chemical formulae of possible major toxicity contributors in OSPW were identified.

2) The 16 compounds most likely to cause estrogenicity were O2, O3, and O4 species with carbon numbers between C17 to C20 and DBEs between 6 and 10 with mass formulae consistent with estrone- and estradiol-like compounds. For the first time, exact formulae and masses of possible estrogenic OSPW compounds have been identified.

3) ISBR treatment of OSPW operated at a 7-day HRT decreased Microtox^{TM} acute toxicity by 75% and estrogenicity by 25% based on the YES assay.

4) NAs with DBE 3 and 4 were more resistant to biodegradation and among them, $C_{14}H_{22}O_2$ was found to have a diamondoid structure.

5) NAs with DBE 5 and 6 were more biodegradable than other NAs. Among these compounds $C_{15}H_{21}O_2$ and $C_{17}H_{23}O_2$ were found to have an aromatic structure.

6) Bacteria belonging to the genera *Ochrobactrum, Brevundomonas* and *Bacillus* were isolated from a bioreactor fed with OSPW as the sole carbon and energy source. Of the three cultures, *Ochrobactrum* grew best on the widest range of NA surrogates and *Brevundomonas* and *Ochrobactrum* grew better on polycyclic aromatic compounds than other classes of NA surrogates. This is of potential importance as polycyclic aromatic compounds have been shown to be estrogenic. Surrogates of diamondoid NAs have also been

shown to be degraded and No previous study has shown the capability of axenic cultures to degrade diamondoid NAs.

7.2 Recommendations

The high complexity of OSPW presents chemical and biological challenges in the treatment of OSPW. The correlation of chemical composition and toxicity is essential to set the priority for targeted removal of compounds.

In present study, the fractionation of OSPW successfully separated OSPW into significant different fractions by toxicity and estrogenicity. The relative percentages of components in the fractions were found to be very different. However, the absolute quantity of each component is not available because of the lack of standard quantification methods. Due to unknown response factors and matrix effects (Headley et al. 2013), the accurate quantification method is a big challenge since OSPW is a complex mixture with many unknown components. Future quantification work could begin with one or several external/internal standard mixture to set up a standard quantification test.

To reduce the complexity of OSPW is important to better understand the toxicity and degradation. The present study used SPE and semi-preparative HPLC based on hydrophobicity to fractionate OSPW. The identification of some alicyclic acids and aromatic compounds in OSPW suggests that OSPW should first be separated into non-aromatic and aromatic fractions followed by fractionation based on hydrophobicity to determine which is most responsible for toxicity and estrogenicity.

The present study successfully isolated several NA-degrading bacteria and tested for the biodegradation of model NA compounds. Two strains showed the capability of degrading diamondoid NAs. Because of the persistence of the diamondoid NAs, further biodegradation experiments are needed to reveal their biodegradation pathway and improve the biodegradation

performance. Future work can mix known isolates to study the degradation of various NAs and NAs in OSPW, as no single isolate can degrade all NAs. Future work can also combine cultivate-independent techniques such as sequencing of the biofilm to further study the function of the isolates in the reactor.

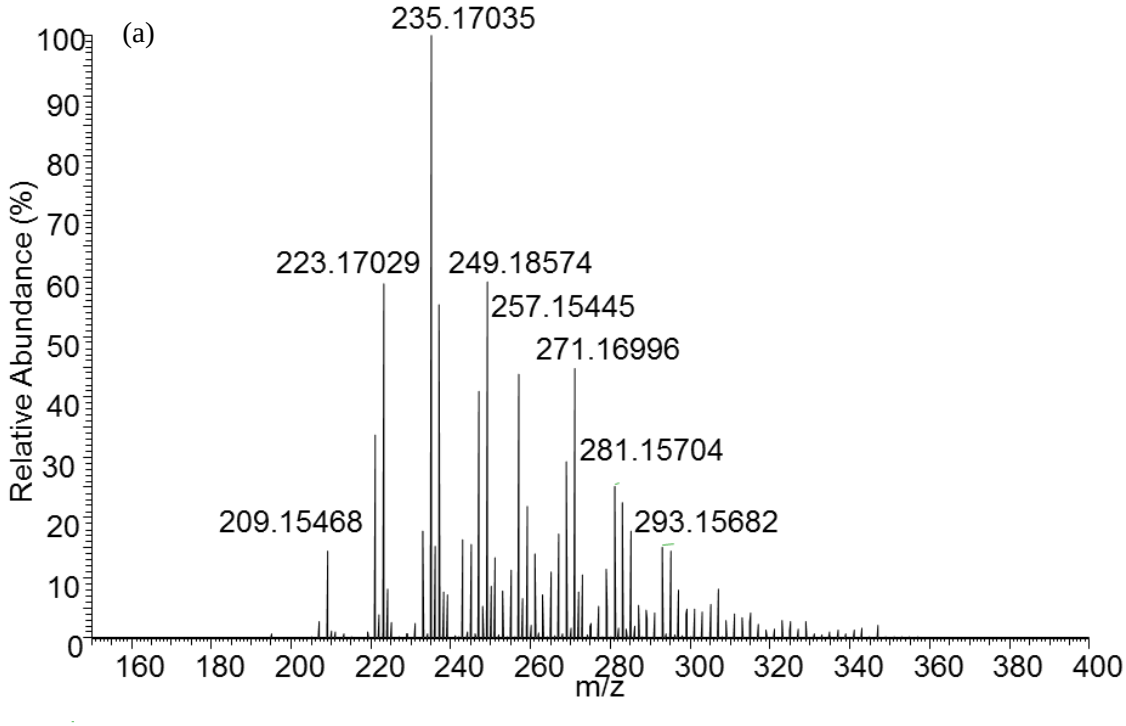
To completely remove NAs in OSPW is a long-term goal in OSPW treatment and detoxification. However a priority list of toxic and/or estrogenic compounds to degrade can be set in the short-term. Since fractionation can isolate the most toxic and estrogenic components from OSPW, those toxic fractions can be used to enrich for specific degrading populations. Bacteria that can degrade those toxic components can be preferentially cultivated and enriched in bioreactor systems like the ISBR to detoxify OSPW more efficiently.

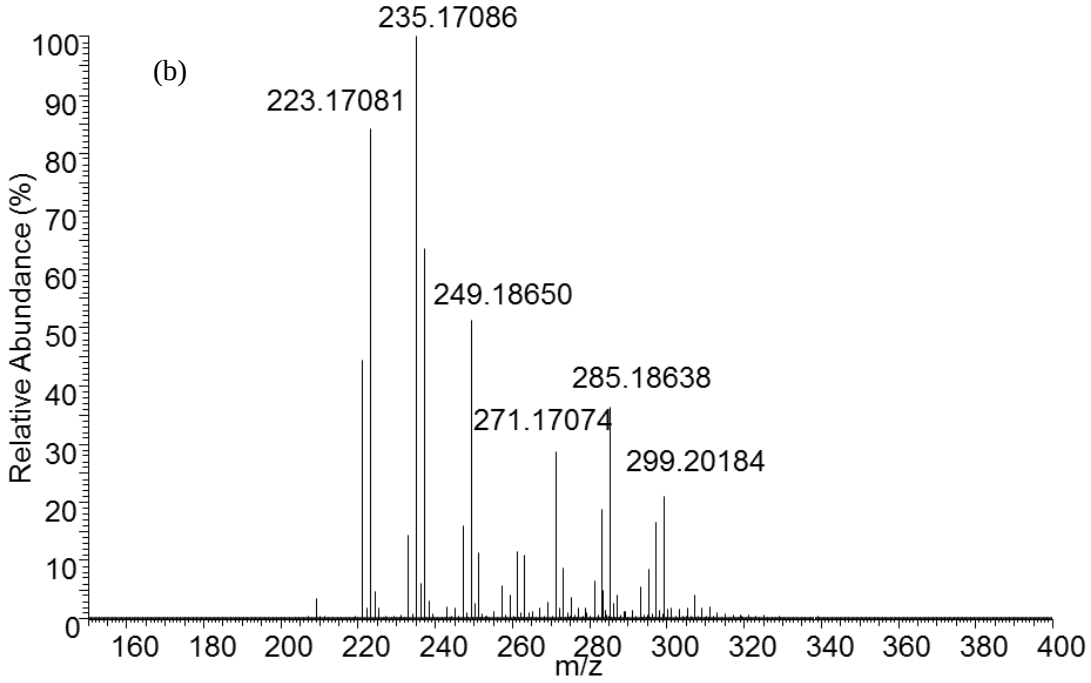
7.3 References

Headley, J. V., K. M. Peru, M. H. Mohamed, R. A. Frank, J. W. Martin, R. R. Hazewinkel, D. Humphries, N. P. Gurprasad, L. M. Hewitt, D. C. Muir, D. Lindeman, R. Strub, R. F. Young, D. M. Grewer, R. M. Whittal, P. M. Fedorak, D. A. Birkholz, R. Hindle, R. Reisdorph, X. Wang, K. L. Kasperski, C. Hamilton, M. Woudneh, G. Wang, B. Loescher, A. Farwell, D. G. Dixon, M. Ross, S. Pereira Ados, E. King, M. P. Barrow, B. Fahlman, J. Bailey, D. W. McMartin, C. H. Borchers, C. H. Ryan, N. S. Toor, H. M. Gillis, L. Zuin, G. Bickerton, M. McMaster, E. Sverko, D. Shang, L. D. Wilson and F. J. Wrona (2013). "Chemical fingerprinting of naphthenic acids and oil sands process waters-a review of analytical methods for environmental samples." Journal of Environmental Science and Health, Part A **48**(10): 1145-1163.

Appendix A

Chapter 4 supplementary material





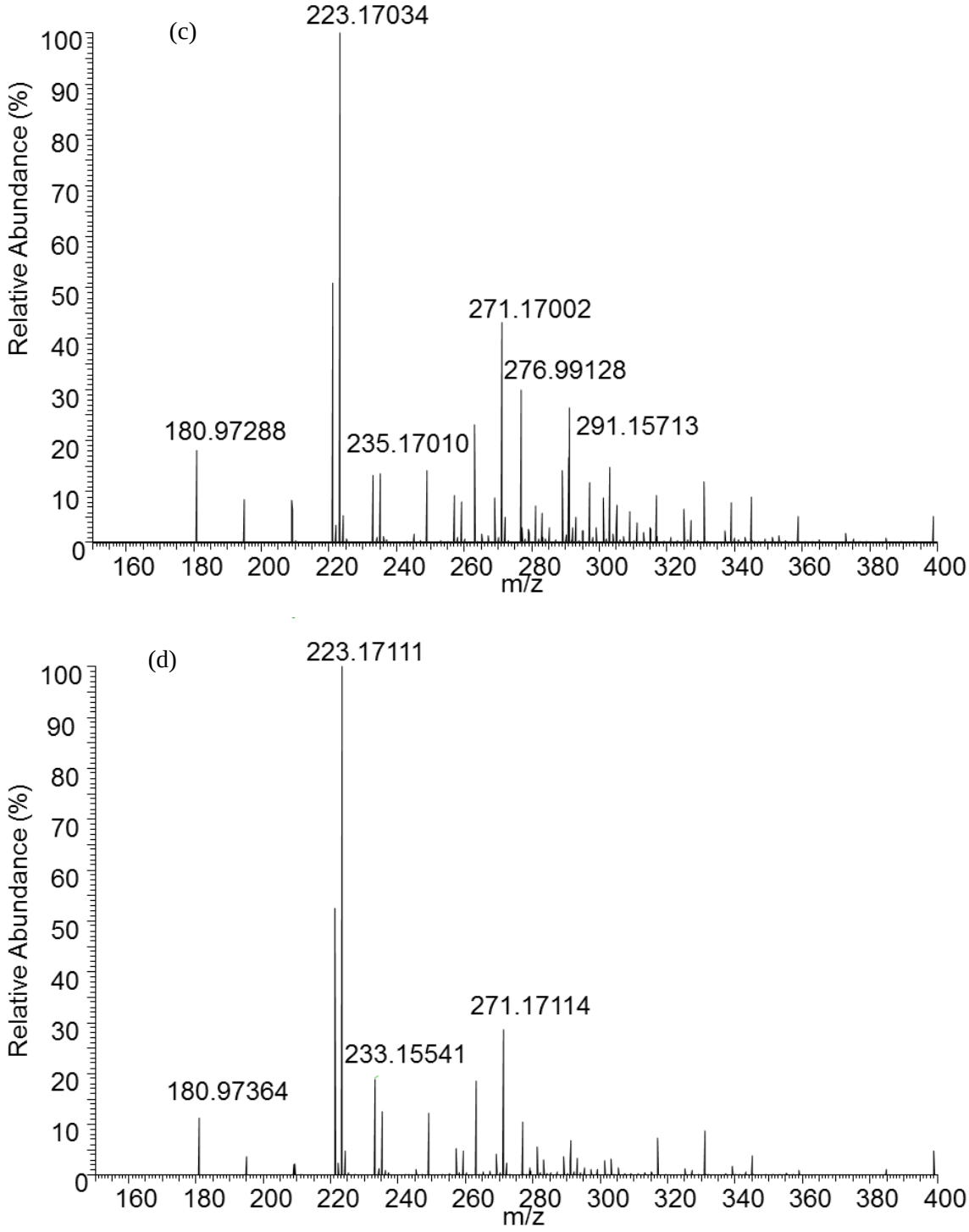


Figure S1. The MS spectra of SPE fractions (a) F2 (untreated), and (b) F2T (biologically treated), and HPLC subfractions (c) F2.31 (untreated), and (d) F2.31T (biologically treated).