The Effects of Oil Sands Process-Affected Waters and their Associated Constituents on Fathead Minnow (*Pimephales promelas*) Reproductive Physiology

by

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ABSTRACT

THE EFFECTS OF OIL SANDS PROCESS AFFECTED WATERS AND THEIR ASSOCIATED CONSTIUENTS ON FATHEAD MINNOW (PIMEPHALES PROMELAS) REPRODUCTIVE PHYSIOLOGY

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As part of their reclamation plan, oil sands operators propose to transfer the mature fine tailings, which are a by-product of the oil sands extraction process, to open-pits and cap them with either a layer of surface water or oil sands process-affected waters (OSPW). These oil sands pit lakes are expected to develop habitats with productive capabilities comparable to natural lakes in the region. The studies presented in this thesis evaluate the potential impact of OSPW and its associated constituents [i.e. acid-extractable organics (e.g. naphthenic acids; NAs) and salts] on the reproductive physiology of adult fathead minnow (*Pimephales promelas*). Through 14-21 day fathead minnow reproduction assays it was demonstrated that aged OSPW can impair spawning, lower plasma sex steroid concentrations, and reduce male secondary sexual characteristics. The acid-extractable organics in OSPW were demonstrated to have an adverse effect on fathead minnow reproductive physiology. Other studies showed that the high salinity which characterizes OSPW also influences toxicity. When fathead minnows were exposed to the OSPW extract and 700 mg/l of $NaHCO_3$, the $NaHCO_3$ reduced the inhibitory effects of the extract on the numbers of reproductive tubercles and plasma

testosterone levels by reducing the uptake of NAE to the fish. Embyro and larval bioassays also revealed that $NaHCO_3$ reduces the acute toxic effects of the OSPW extract. An assessment of a wild population of fathead minnows inhabiting an OSPW pond determined that there were differences in the condition factor (CF), gonadosomatic indices (GSIs), liver somatic indices (LSIs), male secondary sexual characteristics, and 11-ketotestosterone concentrations in the fathead minnows from the OSPW pond relative to fish collected at reference sites. The opercula of fathead minnows from the OSPW pond also differed from those of reference fish and an examination of the gills revealed that were a number of proliferative and degenerative alterations relative to reference fish. Collectively, these studies demonstrate that aged OSPW has the potential to negatively affect the reproductive physiology of fathead minnows and suggest that aquatic habitats with high NAs concentrations (>10 mg/l) will have adverse effects on fish.

Preface

This thesis has been organized into a set of chapters and appendices. This dissertation and the associated manuscripts, with the exception of the manuscript in Appendix 1, were written by Richard Kavanagh. The work in Appendix 1 is the result of a significant collaboration between Richard Frank, a fellow graduate student, and Richard Kavanagh. The naphthenic acid extract method that we developed was used to produce a naphthenic acid extract for my work and Richard Frank's work and this method was published in Chemosphere by Richard Frank (primary author) and me (secondary author). The authorship and contribution of each co-author for each of the publications is described below.

Chapter 2. Kavanagh, R.J., Frank R.A., Oakes, K.D., Servos, M.R., Young, R.F., Fedorak, P.M., MacKinnon, M.D., Solomon, K.R., Dixon, D.G., Van Der Kraak, G., 2011. Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. Aquat. Toxicol. 101, 214-220.

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List of Acronyms and Abbreviations

9-FCA	9-fluorenecarboxylic acid
11KT	11-ketotestosterone
ANOVA	One-Way Analysis of Variance
AR	Androgen Receptor
CF	Condition Factor
CI	Confidence Interval
DEAE	Diethylaminoethyl
E2	17β-estradiol
EDS	Endocrine Disrupting Substances
EIA	Enzyme Immuno Assays
ER	Estrogenic Receptor
ERCB	Energy Resources Conservation Board
ESI-MS	Electrospray Ionization Mass Spectrometry
FTIR	Fourier Transform Infrared
GC-MS	Gas chromatography–mass spectrometry
GCxGC-MS	Multi-dimensional Comprehensive Gas Chromatography-Mass Spectrometry
GL	Gregoire Lake

GSI	Gonadosomatic Index
IC50	Inhibitory Concentration, 50%
LC50	Lethal Concentration, 50%
LSI	Liver Somatic Index
MFO	Mixed-Function Oxygenase
MS-222	Tricaine Methane Sulfonate
NAE	Naphthenic Acid Extract
NAs	Naphthenic Acids
OSPW	Oil Sands Process-affected Water
PAHs	Polycyclic Aromatic Hydrocarbons
PVC	Polyvinyl Chloride
RIA	Radioimmunoassay
RIC	Reconstructed Ion Chromatograms
SC DP	Syncrude Canada Demonstration Pond
SC P5	Syncrude Canada Pond 5
SC P9	Syncrude Canada Pond 9
SD	Standard Deviation
SE	Standard Error
SE N	Suncor Energy MFT-North Pond

SE S	Suncor Energy MFT-South Pond
SIM	Single Ion Monitoring
T	Testosterone
USEPA	United States Environmental Protection Agency

CHAPTER 1. INTRODUCTION

1.1 Thesis overview

Alberta's oil sands are becoming increasingly important as conventional supplies of oil continue to shrink. Surface mining, as well as in-situ methods, are used to recover the bitumen found in oil sands deposits. With surface mining, large quantities of fluid tailings (mixture of bitumen, sand, clay, water, and inorganic and organic oil sands constituents) are produced during the oil sands extraction process. The fluid tailings are transported to settling basins and allowed to settle. The mature fine tailings (MFT) that form on the bottom of basin remain there while the overlying waters [oil sands process-affected waters (OSPW)] is reused in the extraction process. As part of their wet landscape option, oil sands developers eventually plan to transfer the MFT to geotechnically-secure mined-out pits and cap it with a layer of surface water or OSPW. It is expected that the acute toxicity of the OSPW will diminish over time in these oil sands pit lakes and that this will lead to the development of stable and viable lake habitats. These man-made lakes are required by regulators to have similar biological capability as natural lakes in the region.

Short-term lethal responses to OSPW are easy to evaluate, but chronic and sublethal impacts are more difficult to assess in the context of reclamation success. In establishing the effectiveness of the wet landscape option, chronic effects of aged OSPW on aquatic organisms in these reclaimed systems must be understood. This thesis focuses on determining if aged OSPW and its associated constituents have an adverse effect on the reproductive physiology of fathead minnows (*Pimephales promelas*). Fathead minnows were chosen for these studies as this species is native to the oil sands region and these forage fish are expected to be present in oil sands pit lakes. Fathead minnows were exposed to OSPW and its constituents in the laboratory and their reproductive fitness was determined using short term reproductive bioassays in which effects on the numbers of eggs spawned, fertilization success and measures of individual reproductive status including ovary size, plasma sex steroids and secondary sex characteristics were evaluated. In order to understand, the long-term of effects of OSPW exposure on fish, fathead minnows were also collected from a pond containing OSPW and from selected

reference sites within the oil sands region and their plasma sex steroid concentrations, secondary sexual characteristics, and gonad size were compared.

1.2 Alberta Oil Sands

Oil sands ore consists of a mixture of bitumen (heavy, biodegraded crude oil), sand, clay, and formation water. There are three main deposits of oil sands in Alberta: the Athabasca, the Peace River, and the Cold Lake oil sands deposits (Figure 1.1). They occupy a combined area of 140,200 km^2 and contain an estimated 1.7 trillion barrels of oil, however, only approximately 170 billion barrels are accessible using current technologies (Alberta: Department of Energy, 2010a). With the rising price of oil and the decline of conventional oil supplies, industrial development has increased in the Alberta oil sands region. In 2009, there were approximately 5012 oil sands (mineral rights) agreements, which total about 82,542 km^2, with the province of Alberta (Alberta: Department of Energy, 2010a). There are over 91 active oil sands projects in Alberta; only four of these projects use surface mining, the rest use various in situ recovery methods. In 2009, 1.49 million bbl/d of bitumen were produced in Alberta with surface mining accounting for 55% and in situ for 45% (Alberta: Department of Energy, 2010a). It is believed that bitumen production will increase to 3.2 million bbl/d by 2019 (ERCB, 2010a).

The Athabasca oil sands deposit is the largest of the Alberta oil sands deposits (100,000 km^2) and is closest to the surface. A large quantity of the ore in this deposit is “rich” oil sand (bitumen content of 12 – 19 % by weight). It is for these reasons that development has been primarily focused in this area. By 2009, a total of 602 km^2 have been disturbed by oil sands mining (Alberta: Department of Energy, 2010a).

Figure 1.1. Map of the Alberta Oil Sands

1.3 Oil Sands Operations

Oil sands can be recovered using surface mining when the oil sands formations lie within 75 m of the surface. Out of the 170 billion barrels of oil available in the Alberta Oil Sands deposits, only 20 % can be recovered using surface mining (Alberta: Energy, 2010a). The remaining oil sand can only be reached by using in situ methods. These in situ methods involve injecting steam, water, or solvents into the reservoir; the viscosity of

the bitumen is reduced and this allows it to flow to a vertical or horizontal wellbore. Only 35-60 % of the bitumen can be recovered from the deposit when in situ methods are used; oil sands operators prefer to use surface mining, where possible, as the recovery is approximately 90% when this method is employed (Alberta: Department of Energy, 2010b).

With surface mining, the trees and vegetation are cleared and then the top soil is removed and stored for future reclamation. The overburden (muskeg, clay, and sand) is then drained before being removed and stored for later use (e.g. building dykes, reclamation). Enormous shovels and trucks are used to excavate the oil sand. The oil sand is transported to extraction facilities where modifications of the Clark Water Extraction process are used to separate the bitumen from the oil sands. In this process, an aqueous digestion with hot water (>50 ^oC), usually with NaOH as a process aid (<200g/ton of oil sand), will yield recoveries of >90% (FTFC, 1995). In separation vessels, the bitumen (froth) floats to the surface where it is skimmed; it then undergoes further treatment to remove residual water and fine solids. The bitumen is then either upgraded (bitumen is turned into a synthetic crude oil) at the facility or it is diluted and pumped to a refinery elsewhere.

1.4 Wet Landscape Option

With surface mining, 3 m^3 of water are required for each m^3 of oil sand processed (Clemente et al., 2003). Large volumes of fluid tailings, which contain un-recovered bitumen, sand, clay, and water with inorganic and organic constituents associated with oil sands ore, are produced as a by-product of the oil sands extraction. Large settling basins, referred to hereafter as tailings ponds, are required during the operational phase of the surface mine development to store these fluid tailings. Over time, the fluid tailings de-water and eventually produce a soft fines-rich suspension, known as mature fine tailings (MFT). The water (i.e. OSPW) from these tailings ponds is recycled; however, the accumulation of fine clays, inorganic ions (e.g. Na^+, Cl^-), and naphthenic acids (NAs) eventually prevents oil sands operators from using this water for extraction purposes.

The Alberta Environmental Protection and Enhancement Act prohibits the release of the large volumes of OSPW and MFT that are currently stored in large settling basins. As a result of a zero discharge policy, over 840 million m^3 of tailings are currently being stored in tailings ponds in the Athabasca oil sands region (ERCB, 2010a). Oil sands operators must eventually reclaim their leases; as part of their "wet landscape" option, operators plan to transfer these materials to mined-out pits and cap them with a layer of clean water. These artificially constructed end pit lakes are expected to have similar biological capability as natural lakes in the region. Therefore, survival, growth, and reproduction of aquatic organisms are expected to be unimpaired by any of the constituents found in these oil sands pit lakes.

1.5 Endocrine Disrupting Substances (EDS)

It is well known that a wide variety of substances, both natural and anthropogenic, can disrupt the reproductive physiology of both humans and wildlife; exposure to these substances can have indirect (e.g. induce stress) or direct effects (e.g. endocrine disruption) on the reproductive system. In fish, stress has been shown to cause changes in osmotic status, development, immune capacity and learning, shifts in energy stores, energy biosynthesis, and use efficiency (Shreck et al., 2010). These changes, in turn, can alter the reproductive capacity of fish (Fuzzen et al., 2011; Shreck et al., 2010). Substances that are capable of disrupting the endocrine system directly are called endocrine disrupting substances (EDS; Damstra et al., 2002). EDS were once thought to primarily interfere with hormone receptors and thereby impact a wide range of signaling processes. We now know that EDS can interfere with hormone binding, transport, and production; gene expression; and a host of other cell regulatory mechanisms. These disruptions may affect the endocrine, immune and neural systems and may lead to developmental, reproductive, metabolic, neural, and behavioral problems.

A variety of methods are used to assess whether substances are capable of disrupting the endocrine system of fish. These range from short-term *in vitro* tests, through to *in vivo* reproductive tests, and assessments of reproductive fitness in wild populations of fish. Receptor binding assays are used to determine if compounds are capable of binding

to cellular receptors (e.g. androgen receptors (AR), estrogen receptors (ER)) and initiating a biological response or blocking the binding sites, which prevents natural hormones from binding to the receptors. Other *in vitro* studies expose gonadal tissues from fish directly to a compound and then sex steroid production is measured using radioimmunoassays (RIAs) or enzyme immunoassays (EIAs). These types of tests are fast, inexpensive, and may reveal the specific mechanisms of action of a particular compound. Unfortunately, the results from receptor binding assays and *in vitro* gonadal incubations are confined to the cellular and organ level, respectively, and thus they can provide minimal information on the exact biological response of an organism exposed to an EDS. Short-term toxicity tests in which organisms (e.g. fish) are exposed to an EDS for a short-time are capable of evaluating such endocrine endpoints (e.g. vitellogenin induction, plasma sex steroid production). However, even these types of tests are not capable of determining the impact that a substance may have on fecundity, fertility, or development. Full lifecycle and multi-generational toxicity tests can measure these responses in the laboratory or though the assessment of fitness in a wild fish population that has been exposed to chemicals for many years. A limitation of many of these tests is that they are time consuming and resource intensive (Ankley et al., 2001). The fathead minnow reproduction assay developed by Ankley et al. (2001) is a compromise between short-term toxicity tests and full lifecycle tests; fecundity, fertility, and larval development can still be assessed but the results can be acquired in a much shorter time frame. Other reproductive endpoints, such as gonadal development, plasma sex steroid concentrations, and secondary sexual characteristic expressions, can also be assessed by using this bioassay.

Agricultural runoff, domestic sewage treatment plant effluents, and industrial effluents (e.g. pulp and paper mills) are the sources of the majority of estrogenic and androgenic compounds in the environment (Hays et al., 2002; Jobling et al., 1998; Larson et al., 2000). Reductions in plasma sex steroids, gonadosomatic indices (GSIs), fecundity, and secondary sexual characteristics have been reported in fish exposed to these effluents (Jobling and Tyler, 2003; McMaster et al., 1992; McMaster et al., 2005; Munkittrick et al., 1992; Orlando et al., 2004; Porter and Janz, 2003). While fish are expected to be

introduced to oil sands pit lakes, there has been little research conducted on OSPW and the effects that it could have on reproduction.

1.6 Acute and Chronic Toxicity of OSPW

Only a few studies have looked at the effects that oil sands constituents may have on the reproductive physiology of fish and none have determined whether these constituents are capable of impacting the fecundity of fish. It is, however, now widely understood that there are distinct differences in the responses of organisms, including fish, to freshly produced OSPW and aged (<1-2 years old) OSPW.

1.6.1 Fresh OSPW

Freshly produced OSPW is acutely toxic to a variety of aquatic organisms. MacKinnon and Boerger (1986) demonstrated that fresh OSPW is acutely toxic to *Daphnia magna* (LC50 = 2% $v\ v^{-1}$ OSPW after 48 hours), rainbow trout (*Onchorhyncus mykiss* LC50 = 7 % $v\ v^{-1}$ OSPW after 96 hours), and *Vibrio fisheri* (IC50 between 20% and 30% $v\ v^{-1}$ after 15 min incubation). Peters et al. (2007) demonstrated that fresh OSPW can also cause higher incidences of deformities in yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) larvae compared to control fish. These types of deformities have also been seen when fish eggs were exposed directly to oil sands sediment. Fathead minnow and white sucker (*Catostomus commersoni*) larvae exposed to sediment from the oil sands region and from a wastewater pond from Suncor Energy Inc. had significantly higher incidences of mortality and deformities including edemas, hemorrhages, and spinal malformations compared to control fish (Colavecchia et al., 2004; Colavecchia et al., 2006).

1.6.2 Aged OSPW

The acute lethality of freshly produced OSPW has been shown to decline after 1-2 years (MacKinnon and Boerger, 1986) but aged OSPW has still been demonstrated to have some toxic effects. Body mass and skeletal size were found to be significantly lower

in mallard (*Anas platyrhynchos*) ducklings after 2, 5, 9, and 13 days of exposure to wetlands containing aged Suncor OSPW (Gurney et al., 2005). Significantly reduced growth, delayed metamorphosis, and lower survival were reported when *Bufo boreas* and *Rana pipiens* were exposed to aged Suncor OSPW (Pollet and Bendell-Young, 2000).

Rainbow trout exposed to aged OSPW have been shown to have significantly fewer leukocytes, increased fin erosion, and decreased ability to produce antibodies to the inactivated A. salmonicida (McNeil et al., 2012). Histopathological changes in gill and liver tissue have been reported in several fish species exposed to aged OSPW (Nero et al., 2006b; van den Heuvel et al., 2000). Increases in liver size and hepatic mixed-function oxygenase (MFO) activity have also been observed in fish exposed to aged OSPW (van den Heuvel et al., 1999). Fish collected within the oil sands region have been shown to produce lower concentrations of sex steroids. Tetreault et al. (2003) demonstrated with *in vitro* studies that gonadal tissues collected from slimy sculpin (*Cottus cognatus*) within the oil sands region produced lower concentrations of sex steroids (basal and forskolin-stimulated) compared to fish from a reference site and Lister et al. (2008) showed that goldfish (*Carassius auratus*) held in ponds containing aged OSPW had lower plasma steroid levels than reference fish (Lister et al., 2008).

1.7 Constituents of OSPW

If OSPW does negatively affect reproduction, then there is some urgency in identifying the constituent's responsible for these effects. As a result of the extraction and water recycling activities in the surface mine operations, OSPW has elevated concentrations of NAs and inorganic ions (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) relative to surface waters in the region (MacKinnon and Boerger 1986; Schramm et al., 2000). OSPW also has very low concentrations of metals and polycyclic aromatic hydrocarbons (PAHs), present predominately as the alkylated series of the PAHs. These petrogenic PAHs, which are natural constituents of bitumen, have very low solubility in OSPW (Madill et al., 2001).

Unlike PAHs, NAs are highly soluble in OSPW and occur at concentrations as high as 110 mg/L (FTFC, 1995a). They are a mixture of acyclic, monocyclic, and polycylic carboxylic acids that have the general formula $C_nH_{2n+Z}O_2$, where *n* represents the carbon number and *Z* indicates the number of hydrogen atoms removed to accommodate the number of five- or six-carbon rings within the structure (Figure 1.2; Clemente and Fedorak, 2005; Rogers et al., 2002). NAs in their dissociated ionic forms are thought to be primarily responsible for the acute toxicity found in fresh OSPW (Frank et al., 2008; MacKinnon and Boerger, 1986; Madill et al., 2001).

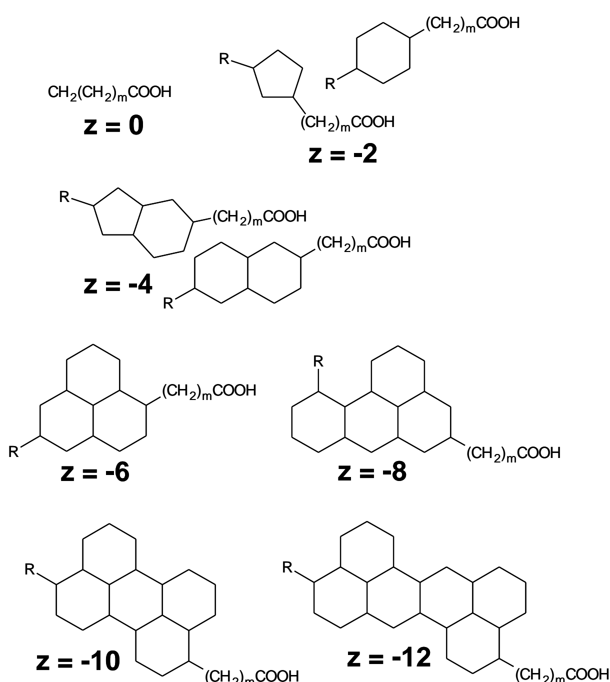

Figure 1.2. Examples of NA structures. Z indicates the hydrogen deficiency, R is an alkyl group, and m is the number of CH_2 units (Frank et al., 2006).

NAs have hydrophobic alkyl groups and a hydrophilic carboxylic moiety; a probable primary mode of action for the acute toxicity of NAs is narcosis (Frank et al., 2008). The acute toxicity of "fresh" OSPW declines over time and this is believed to be due to a decrease in the proportion of lower molecular weight NAs (Holowenko et al., 2002; MacKinnon and Boerger, 1986). Frank et al. (2008) fractionated an extracted NA mixture by molecular weight and to assess each fraction's toxicity and determined using

assays with *Vibrio fischeri* bioluminescence (via Microtox assay) that the lowest molecular weight NAs collected had higher potency than the highest molecular weight NAs collected. The natural biodegradation of NAs in OSPW is slow and yields an increasing proportion of mono-, di- and tri-hyroxylated NAs (Han et al., 2009).

Rogers et al. (2002) demonstrated that only very large doses of NAs can increase liver size and glycogen storage and cause liver damage in rats. NAs are surfactants and it is therefore not surprising that NAs are much more toxic to aquatic organisms than terrestrial mammals. Experiments with a commercial NAs mixture and a NAs mixture extracted from aged OSPW showed proliferative changes in the gill (epithelial, mucous, and chloride cell) of fish and that these changes increased with the addition of salt (Nero et al. 2006a). As a result of these changes, the gill surface area was reduced and it was suggested that this would not only reduce the rate of absorption of NAs but reduce the rate of gas exchange as well, leading to respiratory stress.

Recently, NAs from the produced waters of offshore oil platforms and from commercial sources have been shown to be weak estrogenic receptor agonists and potent androgen receptor antagonists (Thomas et al., 2009). Using the H295R Steroidogenesis Assay, fresh OSPW was demonstrated to significantly decrease testosterone and increase 17β-estradiol concentrations (He at al., 2010). This effect was mainly due to decreased 17β-estradiol metabolism. Analysis of CYP19A (aromatase) mRNA abundance and enzyme activity suggested that induction of this enzyme activity may have also contributed to these effects. Numerous studies have shown the importance of 17β-estradiol and testosterone to normal reproductive development and any constituent that was shown to activate or block these two hormones would be expected to represent a significant hazard to the sustainability of fish populations in oil sands pit lakes.

1.8 Objectives and Hypotheses

The primary objective of this thesis was to determine if aged OSPW adversely affects the reproductive physiology of fathead minnow. To accomplish this, fathead minnows were exposed to OSPW in the laboratory and then various reproductive

endpoints were monitored. Wild fathead minnows were also collected from a demonstration pond containing OSPW and various biological endpoints were compared to fish that had been collected from various reference sites within the region. A second objective was to determine whether NAs, one of the main constituents of OSPW, adversely affect the reproductive physiology of fathead minnows; this was completed by exposing fathead minnows to NAs in the laboratory. Because NAs concentrations tend to increase with increasing salinity, it was also important to understand whether salts modify the acute and chronic toxicity of NAs.

Chapter 2

Ho: OSPW has no effect on the reproductive physiology of fathead minnows.

Description: A series of experiments were conducted in which male and female fathead minnows were exposed to OSPW from Syncrude Canada Ltd. and Suncor Energy Inc. in the laboratory for 14-21 days. Reproductive endpoints including fecundity, fertility, secondary sexual characteristics, and plasma sex steroids, were measured in OSPW exposed fish and compared to control fish.

Chapter 3

Ho: NAs have no effect on the reproductive physiology of fathead minnows.

Ho: Salts do not modify the effects the acute and chronic toxicity of NAs.

Description: Given that NAs are a major constituent of OSPW and is thought to be responsible for the toxicity of these effluents, tests were conducted in which male and female fathead minnows were exposed to a naphthenic acid extract (NAE) in the laboratory for 21 days and the effects on reproduction were quantified. Reproductive endpoints, including fecundity, fertility, secondary sexual characteristics, and plasma sex steroids, were measured in NA exposed fish and compared to control fish. In a second assay, male and female fathead minnows were exposed to a NAE along with $NaHCO_3$ in order to determine if the presence of high concentrations of this salt, which is present in OSPW, modified the effects that NAs have on fathead minnow reproductive physiology. A further series of tests was conducted to determine the role of salts in the acute toxicity

of NAs. In these studies, fathead minnow embryos and larvae were first exposed to a NAE along with NaCl, Na_2SO_4, or $NaHCO_3$ and their effects on survival were determined.

Chapter 4

Ho: The salinities measured in OSPW have no effect on fathead minnow reproduction.

Description: Fathead minnows were held in water with salinities equivalent to Pond 9 in the laboratory for 21 days and reproductive endpoints, such as fecundity, fertility, secondary sexual characteristics, and plasma sex steroids, were measured and compared to control fish. In a second assay, fathead minnows were acclimated to the salinities measured in Pond 9 OSPW before being exposed in the laboratory to Pond 9 OSPW for 21 days. Reproductive endpoints, such as fecundity, fertility, secondary sexual characteristics, and plasma sex steroids, were measured in OSPW exposed fish and compared to control fish.

Chapter 5

Ho: The reproductive physiology of wild fathead minnows collected from a pond containing OSPW (NAs ~13 mg/l) will not differ from reference fish collected within the oil sands region where NA concentrations are much lower (NAs ~1 mg/l).

Description: Fathead minnows, which were exposed to NAs for generations, were collected from Syncrude Canada Ltd.'s Demonstration pond. Gonadosomatic indices, condition, plasma sex steroids, and secondary sexual characteristics were compared to fathead minnows collected from sites within the oil sands region that naturally contain low levels of NAs.

Chapter 6

The major findings reported in this thesis are summarized in this chapter.

Appendix 1

The procedure that was developed to extract NAs from OSPW is described in this section. This method was used to produce the NAE that fathead minnows were exposed to in Chapter 3.

Appendix 2

This section describes how synchronous fluorescence can be used to detect NAs in groundwater and surface waters in the oil sands region. This semi-quantitative method requires no sample preparation or manipulation and can be used to determine if NAs are present in water samples in less than five minutes. It is anticipated that this procedure would be used as a rapid-screening test and is not intended to replace existing analytical methods such as fourier transform ion cyclotron resonance mass spectrometry and HPLC/QTOF-MS which are used when measuring NAs in water samples.

CHAPTER 2. FATHEAD MINNOW (*PIMEPHALES PROMELAS*) REPRODUCTION IS IMPAIRED IN AGED OIL SANDS PROCESS-AFFECTED WATERS

2.1 Abstract

Large volumes of fluid tailings are generated during the extraction of bitumen from oil sands. As part of their reclamation plan, oil sands operators in Alberta propose to transfer these fluid tailings to end pit lakes and, over time, these are expected to develop lake habitats with productive capabilities comparable to natural lakes in the region. This study evaluates the potential impact of various oil sands process-affected waters (OSPW) on the reproduction of adult fathead minnow (*Pimephales promelas*) under laboratory conditions. Two separate assays with aged OPSW (>15 years) from the experimental ponds at Syncrude Canada Ltd. showed that water containing high concentrations of naphthenic acids (NAs; >25 mg/L) and elevated conductivity (>2000 µS/cm) completely inhibited spawning of fathead minnows and reduced male secondary sexual characteristics. Measurement of plasma sex steroid levels showed that male fathead minnows had lower concentrations of testosterone and 11-ketotestosterone whereas females had lower concentrations of 17β-estradiol. In a third assay, fathead minnows were first acclimated to the higher salinity conditions typical of OSPW for several weeks and then exposed to aged OSPW from Suncor Energy Inc (NAs ~ 40 mg/L and conductivity ~2000 µS/cm). Spawning was significantly reduced in fathead minnows held in this effluent and male fathead minnows had lower concentrations of testosterone and 11-ketotestosterone. Collectively, these studies demonstrate that aged OSPW has the potential to negatively affect the reproductive physiology of fathead minnows and suggest that aquatic habitats with high NAs concentrations (> 25 mg/L) and conductivities (>2000 µS/cm) would not be conducive for successful fish reproduction.

2.2 Introduction

Alberta's oil sands developments account for more than 40% of Canada's oil production (CAPP, 2009). Oil sands ore consists of a mixture of bitumen (heavy, biodegraded crude oil), sand, clay, and formation water. In surface mining operations,

modifications of the Clark Water Extraction process are used to separate the bitumen from the oil sands. In this process, an aqueous digestion with hot water, usually with NaOH as a process aid, will yield recoveries of >90% (FTFC, 1995). This results in large volumes of fluid fine tailings that slowly de-water, producing a soft fines-rich suspension, known as mature fine tailings (MFT). The resulting released water is reused in the oil sands extraction process (process water) and accounts for >80% of the water used in a surface oil sands operation. Large settling basins are required during the operational phase of the surface mine development as an essential part of current water management strategies, but eventually they must be reclaimed when no longer actively being used (ERCB, 2009). Current inventories of fluid tailings (process affected water and MFT) within the various settling basins exceed 10^9 m^3 of tailings and are a focus of concern (Del Rio et al., 2006).

As a result of the extraction and water recycling activities in the surface mine operations, the resulting oil sands process-affected waters (OSPW) will have elevated concentrations of naphthenic acids (NAs) and inorganic ions (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) relative to surface waters in the region (MacKinnon and Boerger 1986; Schramm et al., 2000). The un-recovered bitumen in the oil sands process-affected material is comprised of primarily saturated and polar organic compounds, with a minor fraction being polycylic aromatic hydrocarbons (PAHs), present predominately as the alkylated series of the PAHs. These petrogenic PAHs are natural constituents of bitumen that have very low solubility in OSPW (Madill et al., 2001).

Freshly produced OSPW can be acutely toxic to aquatic organisms; NAs in their dissociated ionic form are thought to be primarily responsible (Frank et al., 2008; MacKinnon and Boerger 1986; Madill et al., 2001). These NAs are a mixture of saturated acyclic, monocyclic, and polycyclic carboxylic acids that are highly soluble natural surfactants associated with the oil sands bitumen fraction (Clemente et al., 2005). The acute toxicity of “fresh” OSPW declines over time, likely due to a decrease in the proportion of lower molecular weight NAs (Holowenko et al., 2002; MacKinnon and Boerger 1986). The natural biodegradation of NAs in OSPW is slow and yields an increasing proportion of mono-, di- and tri-oxidized NAs (Han et al., 2009).

The wet landscape reclamation option to deal with the large amounts of MFT and residual OSPW involves their transfer to geotechnically-secure mined-out pits. It is expected that the toxicity of the waters in these end pit lakes will diminish over time leading to the development of stable and viable lake habitats with a biological capability similar to natural lakes in the region. In establishing the effectiveness of this remediation approach, chronic effects of aged OSPW on semi-aquatic and aquatic organisms in these systems must be understood.

Fish studies have been conducted to assess the effects of OSPW exposure. Peters et al. (2007) demonstrated that fresh OSPW can cause deformities in yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*). Histopathological changes in gill and liver tissue have been reported in several fish species exposed to aged OSPW (Nero et al., 2006b; van den Heuvel et al., 1999). Increases in liver size and hepatic mixed-function oxygenase (MFO) activity have also been observed in fish exposed to oil sands constituents (Nero et al., 2006a; van den Heuvel et al., 1999). These constituents may also have an adverse effect on the reproductive physiology of fish. *In vitro* studies on gonadal tissues demonstrated reduced production of sex steroids (basal and forskolin-stimulated) in slimy sculpin (*Cottus cognatus*) collected within the oil sands area relative to fish from a reference site (Tetreault et al., 2003). Goldfish (*Carassius auratus*) held in ponds containing aged OSPW had lower plasma steroid levels than reference fish (Lister et al., 2008).

This study was designed to determine the effects of aged OSPW on the reproductive physiology of fathead minnow (*Pimephales promelas*). Sexually mature fathead minnows were exposed to aged OSPW in the laboratory for 21 days and the numbers of eggs spawned, secondary sexual characteristics, and plasma sex steroid levels were monitored. Fathead minnows are native to the oil sands region and these forage fish are expected to be present in aquatic reclamation options such as end pit lakes. The OSPW was collected from experimental ponds at Syncrude Canada Ltd. and Suncor Energy Inc, within the Alberta oil sands region. In the study using Suncor OSPW, fathead minnows were acclimated to the higher salinities typically associated with OSPW prior to the start of the experiment; in the Syncrude OSPW exposures there was no acclimation. This

research will aid in predicting potential challenges regarding the success of fish in the wet landscape reclamation option and will help to identify the risks that must be addressed as oil sands operators implement end pit lakes.

2.3 Materials and methods

2.3.1 Collection of OSPW

OSPW with various concentrations of NAs for this study were sourced from ponds on the leases of Syncrude Canada Ltd. and Suncor Energy Inc. in Northern Alberta. The waters from these sources were naturally aged OSPW and this water, through a battery of standard bioassays (fish, invertebrates, bacteria), has been demonstrated to be not acutely toxic. To evaluate the wet landscape reclamation option, Syncrude Canada Ltd. constructed a series of test ponds on part of their lease in Northern Alberta (57°05.050'N, 111°41.505'W) in 1989 and 1993. These ponds contained MFT and OSPW collected from Syncrude's Mildred Lake Settling Basin. Pond 5 was constructed in 1989 and filled with 1000 m^3 of MFT and capped with 1000 m^3 of OSPW (depth 2.5 m). Pond 9 was constructed in 1993 and filled with approximately 50 000 m^3 of OSPW with no MFT (maximum depth of 5 m). Demonstration Pond was constructed in 1993 and filled with approximately 70 000 m^3 of MFT (maximum depth of 12 m) and capped with approximately 70 000 m^3 of non-OSPW (muskeg surface water from the area). Aged OSPW was also collected from Suncor Energy Inc.'s North and South MFT ponds (56°59.478'N, 111°32.138'W). These ponds were constructed in 1991 and filled with MFT (~14 500 m^3) and capped with OSPW (approximately 12 000 m^3, 2.5 m depth). Gregoire Lake (56°27′06″N, 111°07′38″W), located approximately 15 km south of Fort McMurray, Alberta, was used to source reference water for each year of study.

Water from all of the locations listed above was collected using a submersible pump positioned approximately 1.0 m below the surface and then stored in 1000 L polypropylene containers before they were used. The water was collected in 2004, 2005, and 2006 for Assays 1, 2, and 3, respectively.Test waters used for fish exposures were characterized (Table 2.1) at Syncrude's Research Facility in Edmonton, AB using

standard aquatic chemical methods. The concentrations of total NAs were measured using Fourier transform infrared (FTIR) spectroscopy (Holowenko et al., 2001). Kodak NAs (Eastman Kodak Company, Rochester, NY) were used as standards in the FTIR analysis.

Table 2.1. Water chemistry of aged OSPW and reference waters used in the fathead minnow reproduction assays.

	GL	SC P5	SC P9	SC DP	SE S	SE N
pH	7.6	9.2	8.8	9.0	8.8	8.9
Conductivity (µS/cm)	142	2780	2240	1420	1960	2050
Naphthenic acids (mg/l)	<0.5	19.2	28.6	10.7	40.4	40.9
Na^{+} (mg/l)	4.9	675	555	319	434	477
K^{+} (mg/l)	<0.5	20.8	11.2	6.9	11.6	11.3
Mg^{2+} (mg/l)	5.5	34.3	10.6	16.5	21.8	15.9
Ca^{2+} (mg/l)	18.3	21.1	14.6	17.1	21.9	17.8
F^{-} (mg/l)	<0.01	<0.01	<0.01	<0.01	2.1	2.3
Cl^{-} (mg/l)	2.1	159	239	96.7	42	52
SO_4^{2-} (mg/l)	5.3	773	210	141	470	370
CO_3^{2-} (mg/l)	<5	60.6	22	17	25	35
HCO_3^{-} (mg/l)	81	539	781	578	610	810

GL = Gregoire Lake, SC P5 = Syncrude Canada Pond 5, SC P9 = Syncrude Canada Pond 9, SC DP = Syncrude Canada Demonstration Pond, SE S = Suncor Energy MFT-South Pond, SE N = Suncor Energy MFT-North Pond.

2.3.2 Fathead Minnow Reproduction Assay

Fathead minnow reproduction assays were conducted in a trailer located on Syncrude's site. Each of the tanks (n=4 per treatment) contained four female and two male fathead minnows and three halved polyvinyl chloride (PVC) pipes which served as the spawning substrate. The tanks were filled with 15 L of Gregoire Lake water and placed inside a reservoir maintained at 25 ±1 ^{o}C, under a photoperiod of 16 h light: 8 h dark. Dissolved oxygen remained above 8 mg/L throughout the exposures. Fathead

minnows were fed frozen adult brine shrimp (*Artemia* sp.) *ad libitum* twice daily. After a 14-21 day pre-exposure period, the fish were transferred to tanks containing either Gregoire Lake water (reference) or one of the treatment waters (OSPW) described below for an additional 21 days. Water renewals occurred every two days.

The endpoints monitored included the number of spawns, fecundity (number of eggs spawned), fertility, hatching success, and survival to swim-up. For the purposes of determining the number of spawns, eggs observed on a single piece of PVC pipe were counted as one spawn while eggs found on two pieces of PVC pipe were counted as two spawns. Fecundity was calculated by counting the number of eggs in each tank and then the total number of eggs per female per day was calculated for each tank. The fertility of all the eggs was determined under a dissecting microscope. A subsample of these eggs was kept to measure hatching success and survival to swim-up (96 h post hatch). This involved transferring 10 eggs into a Petri dish containing water from the corresponding treatment and placing them in an incubator ($25 \pm 2^{o}C$, 16 h light: 8 h dark). There were four Petri dishes for each tank and each tank was evaluated twice. The eggs were checked daily for mortality and hatching and any dead eggs or larvae were recorded and removed. Approximately 70% of the test waters in the Petri dishes were changed daily.

2.3.3 Assay 1: Syncrude aged OSPW Exposure

Fathead minnows (~1-2 years old) were purchased from Silhanek Baitfish Farms (Bobcaygeon, ON, Canada) and transported to Fort McMurray. The pre-exposure phase of the experiment lasted 14 days. During the exposure phase, fish were held for 21 days in either Gregoire Lake water (reference) or OSPW from Demonstration Pond or Pond 9.

2.3.4 Assay 2: Syncrude aged OSPW Exposure

Fathead minnows were purchased from Aquatic Research Organisms (Hampton, NH, USA) and raised at the University of Guelph for several months before being transported to Fort McMurray. The pre-exposure phase lasted 21 days. During the exposure phase, fathead minnows (age: 1 year old) were held for 21 days in OSPW from Pond 5 or Pond 9, or non-OSPW from Gregoire Lake.

2.3.5 Assay 3: Suncor aged OSPW Exposure

Fathead minnows (Aquatic Research Organisms) were raised at the University of Guelph and then transported to Fort McMurray. To minimize the stress on fish moving from low conductivity water (Gregoire Lake) to higher conductivity water (OSPW), salts ($NaHCO_3$ at 378 mg/L, NaCl at 33 mg/L, Na_2CO_3 at 17 mg/L, and Na_2SO_4 at 350 mg/l) were added to increase the conductivity of Gregoire Lake water from 140 µS/cm to about 1200 µS/cm. Fathead minnows (age: 9 months) were held in this higher salinity reference water for 1 month prior to the experiment. They were then held in Gregoire Lake water (salt amended) for 14 days (pre-exposure) before being transferred to either non OSPW from Gregoire Lake (salt amended), or to the Suncor OSPW from North MFT, South MFT, or 50 % South MFT (diluted with Gregoire Lake water) for 21 days.

2.3.6 Fish Sampling

Fathead minnows were anesthetized with tricaine methane sulfonate (MS-222). The mass (g) and fork length (cm) of each fish was measured and the condition factor (CF) was calculated [CF = 100 x total wt (g) / fork length^3 (cm)]. Blood was collected from the caudal vessels with a heparinized microcapillary tube and the plasma was isolated by centrifugation (13000 × g for 6 min). The packed cell volume was then measured (Assays 2 & 3) before storing the plasma at –20 ^{o}C. Fish were euthanized by spinal severance. Gonads and livers were removed and weighed in order to calculate the gonadosomatic index (GSI) [GSI = 100 x gonadal tissue wt (g) / total wt (g)] and liver somatic index (LSI) [LSI = 100 x liver tissue wt (g) / total wt (g)]. Tubercles, a secondary sex characteristic on male fathead minnows, were counted under a dissecting scope. In Assay 2, the spleen was weighed to calculate spleen-somatic indices (SSI) [SSI = 100 x spleen weight (g) / body weight (g)]. Plasma sex steroid concentrations were measured in Assays 2 and 3. After extracting the plasma three times with diethyl ether, concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone were determined using enzyme immuno assays (EIA; Cayman Chemical, Ann Arbor, MI) as per the manufacturer's instructions. Dilutions of the samples were shown to be parallel to the standard curves. Carcasses (excluding head and internal organs) from fathead minnows used in Assay 2

were frozen and stored at –80° C until they were analyzed for NAs content following the protocol of Young et al. (2007).

2.3.7 Statistical analyses

Male and female fish were analyzed separately. For all of the assays, each tank (n=4) was considered a replicate and no outliers were removed. A Levene's test was used to evaluate data for homogeneity of variance; data that did not meet parametric assumptions were log transformed for normalization and/or to reduce the heterogeneity in variance. One-way analysis of variance (ANOVA) was used to test for differences across all treatment groups. A Tukey’s post hoc test was used to determine which treatment(s) differed from the control. Differences were considered significant at p <0.05. All statistical analyses were conducted using SPSS 16.0. The data are presented as mean ± SE unless otherwise noted.

2.4 Results

2.4.1 Assay 1: Syncrude aged OSPW Exposure

A total cessation of spawning was observed in fathead minnows held in aged OSPW from Pond 9, but fish held in Demonstration Pond OSPW did not have significantly lower spawning success relative to Gregoire Lake water (Figure 2.1A). During the exposure, the mean fecundity rates (eggs/female/day) were 9.0 ± 0.6, 7.3 ± 1.7, and 0.0 for fish held in water from Gregoire Lake, Demonstration Pond, and Pond 9, respectively. The mean number of spawns per treatment for Gregoire Lake, Demonstration Pond, and Pond 9 was 5.5 ± 1.0, 5.8 ± 0.9, and 0.0, respectively. There was a significant difference in mean fecundity rate (eggs/f/d) and the number of spawns per treatment between fish held in water from Gregoire Lake and Pond 9 and between fish held in water from Demonstration pond and Pond 9.

Although there were no significant differences in GSIs in males, females held in water from Pond 9 had significantly smaller ovaries compared to Gregoire Lake fish (Table 2.2). The mean number of tubercles on male fathead minnows held in water from

Gregoire Lake, Demonstration Pond, and Pond 9 were 27.0 ± 1.7, 26.0 ± 2.0, 11.5 ± 2.4 tubercles/fish, respectively. Males in Pond 9 water had tubercles that were significantly reduced both in size and number compared to fish held in Gregoire Lake water. There were no significant changes in the packed cell volume (data not shown), the LSIs or CF in fathead minnows from any of the treatments (Table 2.2). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks.

2.4.2 Assay 2: Syncrude aged OSPW Exposure

A significant reduction in the mean fecundity rate and number of spawns was observed in fish held in Pond 9 OSPW compared to the other two waters (Figure 2.1B). There was no significant difference in spawning between fathead minnows held in Pond 5 OSPW and those from the reference Gregoire non-OSPW. During the exposure, the mean fecundity rates (eggs/female/day) were 9.6 ± 1.9, 7.5 ± 1.2, and 2.1 ± 1.3 for fish held in water from Gregoire Lake, Pond 5, and Pond 9, respectively. The mean number of spawns in fish held in water from Gregoire Lake, Pond 5, and Pond 9 was 5.3 ± 0.6, 5.3 ± 0.5, and 1.5 ± 0.5, respectively. No significant differences in the GSIs of male and female fathead minnows were observed (Table 2.2). The number of tubercles on male fathead minnows held in water from Gregoire Lake, Pond 5, and Pond 9 were 23.8 ± 1.8, 21.6 ± 3.4, 10.8 ± 3.5 tubercles/fish, respectively. Tubercles were significantly reduced in size and number in fathead minnows held in Pond 9 water. There were no significant differences in the packed cell volume (data not shown), LSIs (Table 2.2), SSIs (data not shown) or CF in fathead minnows from any of the treatments (Table 2.2). In comparison to fish exposed to Gregoire Lake water, male fathead minnows held in Pond 9 water had significantly lower concentrations of testosterone and 11-ketotestosterone in plasma (Figure 2.2A). Female fish held in Pond 9 water also had significantly lower levels of 17β-estradiol compared to control fish (Figure 2.2B). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks.

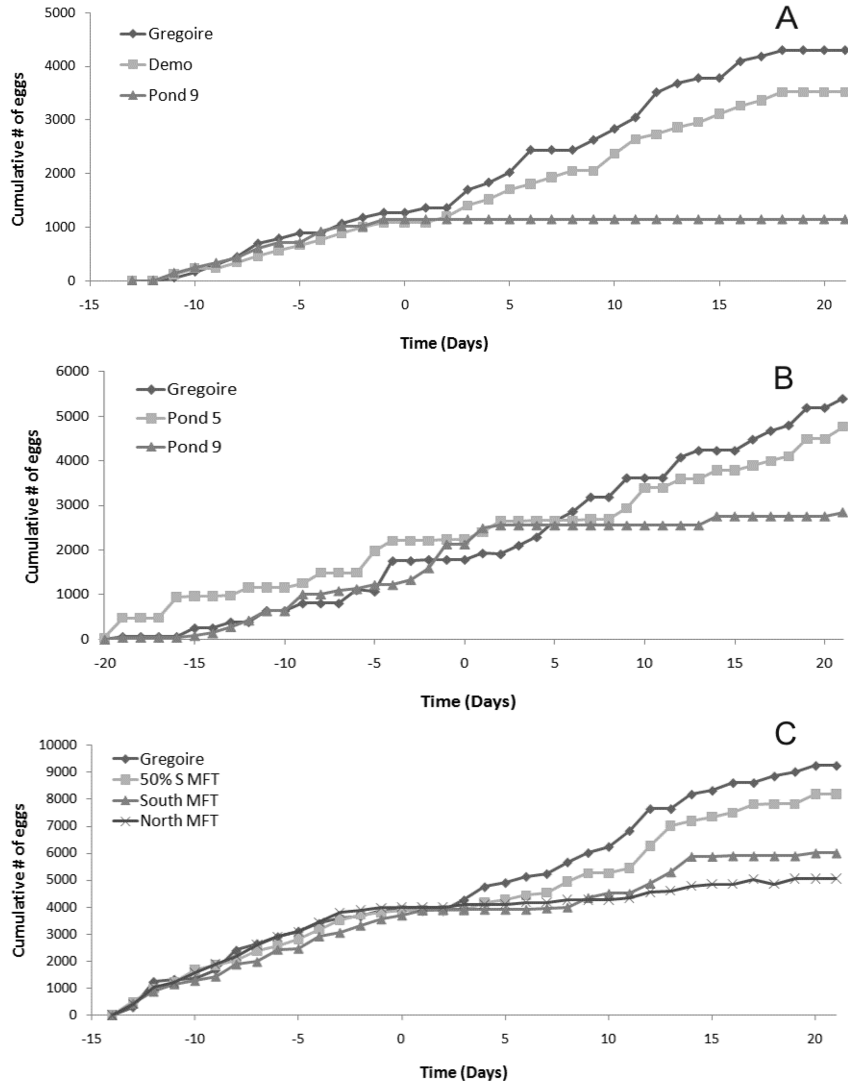

Figure 2.1. Cumulative number of eggs spawned by fathead minnows exposed to different OSPWs in A) Syncrude OSPW Assay 1, B) Syncrude OSPW Assay 2, C) Suncor OSPW Assay 3.

T= testosterone, 11KT= 11-ketotestosterone, E2= 17β-estradiol.

Table 2.2. Condition factor (CF), gonadosomatic indices (GSIs), and liver somatic indices (LSIs) for fathead minnows held in OSPW and Gregoire Lake water (reference site).

Males

Assay	Source	Length (mm)	Mass (g)	CF	GSI (%)	LSI (%)
1	GL	5.63 ±.13	2.37 ±.14	1.35 ±.02	0.96 ±.08	2.11 ±.18
1	SC DP	5.80 ±.14	2.55 ±.22	1.64 ±.32	0.95 ±.16	2.30 ±.16
1	SC P9	5.54 ±.14	2.13 ±.04	1.28 ±.09	1.01 ±.11	1.94 ±.20
2	GL	7.03 ±.18	4.03 ±.26	1.20 ±.05	1.34 ±.10	1.76 ±.07
2	SC P5	6.45 ±.10	2.91 ±.24*	1.12 ±.08	1.34 ±.20	1.91 ±.29
2	SC P9	6.68 ±.20	3.12 ±.38	1.07 ±.05	1.38 ±.23	1.87 ±.20
3	GL	6.25 ±.21	3.65 ±.43	1.47 ±.03	1.46 ±.08	2.03 ±.10
3	SE S-50%	6.56 ±.21	4.25 ±.41	1.46 ±.03	1.58 ±.19	2.11±.10
3	SE S	6.54 ±.06	3.46 ±.23	1.56 ±.03	1.55 ±.16	2.27 ±.12
3	SE N	6.38 ±.13	3.80 ±.14	1.40 ±.02	1.49 ±.05	2.31± .15

Females

Assay	Source	Length (mm)	Mass (g)	CF	GSI (%)	LSI (%)
1	GL	4.75 ±.49	1.34 ±.01	1.17 ±.04	8.98 ±.78	1.89 ±.16
1	SC DP	4.82 ±.83	1.27 ±.02	1.18 ±.06	7.25 ±.44	1.98 ±.24
1	SC P9	4.76 ±.92	1.44 ±.17	1.22 ±.03	5.90 ±.43*	2.22 ±.10
2	GL	5.48 ±.17	1.48 ±.10	1.05 ±.06	10.6 1 ±.91	1.53 ±.22
2	SC P5	5.24 ±.37	1.35 ±.51	1.13 ±.05	13.72 ± 2.90	1.70 ±.16
2	SC P9	5.36 ±.14	1.42 ±.10	1.05 ±.01	9.85 ±1.04	1.80 ±.07
3	GL	4.64 ±.08	1.27 ±.07	1.22 ±.02	11.66 ±.80	2.15 ±.10
3	SE S-50%	4.50 ±.05	1.21 ±.03	1.30 ±.02	16.34 ±3.45	1.95 ±.08
3	SE S	4.71 ±.03	1.24 ±.11	1.30 ±.03	21.61 ±5.10	2.17 ±.10
3	SE N	4.44 ±.07	1.14 ±.05	1.26 ±.10	13.24 ±.98	2.26 ±.18

Note: Values are means ± SE. Values with an * are significantly different ($p < 0.05$) compared with GL fish. GL = Gregoire Lake, SC P5 = Syncrude Canada Pond 5, SC P9 = Syncrude Canada Pond 9, SC DP = Syncrude Canada Demonstration Pond, SE S = Suncor Energy MFT-South Pond, SE N = Suncor Energy MFT-North Pond. For each parameter, n=4.

2.4.3 *Assay 3: Suncor aged OSPW Exposure*

A decrease in spawning was observed in both North and South MFT Pond aged OSPW compared to Gregoire Lake non-OSPW water (Figure 2.1C). During the exposure, the mean fecundity rates (eggs/female/day) were 16.9 ± 1.8, 14.4 ± 1.5, 7.2 ± 0.6 and 3.8 ± 1.0 for Gregoire Lake, 50 % South MFT, South MFT, and North MFT, respectively. The mean number of spawns in Gregoire Lake, 50 % South MFT, South MFT, and North MFT was 9.5 ± 0.5, 7.0 ± 0.4, 4.75 ± 0.9, and 3.0 ± 0.9, respectively. There was a significant difference in the mean fecundity rate and the number of spawns of fathead minnows held in North and South MFT Pond water when compared to reference fish. No

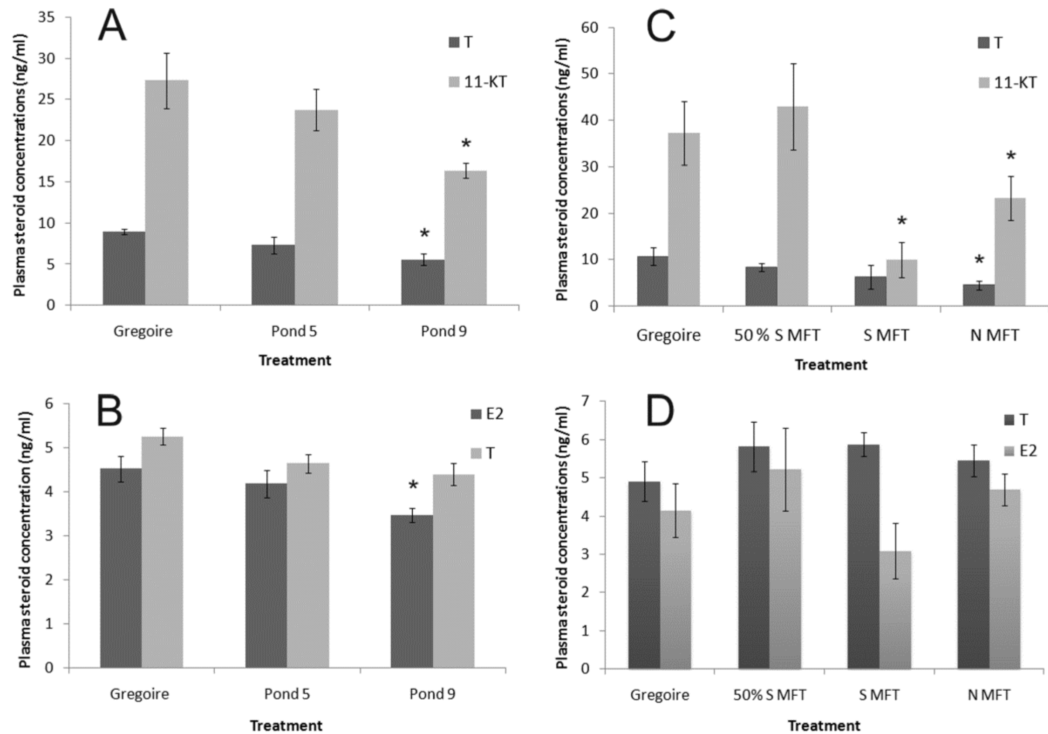

Figure 2.2. Plasma steroid concentrations measured in A) Syncrude OSPW Assay 2 males, B) Syncrude OSPW Assay 2 females, C) Suncor OSPW Assay 3 males, D) Suncor OSPW Assay 3 females.

T= testosterone, 11KT= 11-Ketotestosterone, E2= 17β-estradiol. Values with an * are significantly different ($p < 0.05$) when compared to Gregoire Lake fish.

significant differences in the packed cell volume (data not shown), LSIs, GSIs, or CF of male and female fathead minnows were observed (Table 2.2). The number of tubercles on male fathead minnows held in Gregoire Lake, 50 % South MFT, South MFT, and North MFT water were 19.8 ± 1.2, 18.6 ± .1, 16.9 ± 2.5, and 15.9 ± 1.8 tubercles/fish, respectively and did not differ significantly between sites. In comparison to fish held in Gregoire Lake water, male fathead minnows held in Suncor North MFT Pond water and South MFT Pond water had significantly lower levels of 11-ketotestosterone (Figure 2.2C). Testosterone was also significantly reduced in male fathead minnows exposed to Suncor North MFT Pond water relative to reference water fish. No significant differences in plasma steroid concentrations were observed in female fathead minnows (Figure 2.2D). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks.

2.4.4 Larvae

Fertility, hatching success, and survival to swim-up could not be evaluated in fish held in Pond 9 water because there was no spawning in Assay 1and very limited spawning in Assay 2. Fertility rate, hatching success, and survival to swim-up were not affected by exposure to any other aged OSPW (data not shown). There were no significant differences in mortality in any of the experiments.

2.4.5 Analysis of NAs by GC-MS

The extracts of fathead minnows exposed to OSPW (Pond 5 and Pond 9) from Assay 2 contained a broad, unresolved peak between 15 and 20 min in the reconstructed ion chromatograms for nominal mass $m/z = 267$ (Figure 2.3). The detection of a broad, unresolved peak at this retention time indicates the presence of NAs (Young et al., 2007). One of the Gregoire Lake fish extracts showed a very small peak between 15 and 20 min which suggests the presence of NAs (Figure 2.3A).

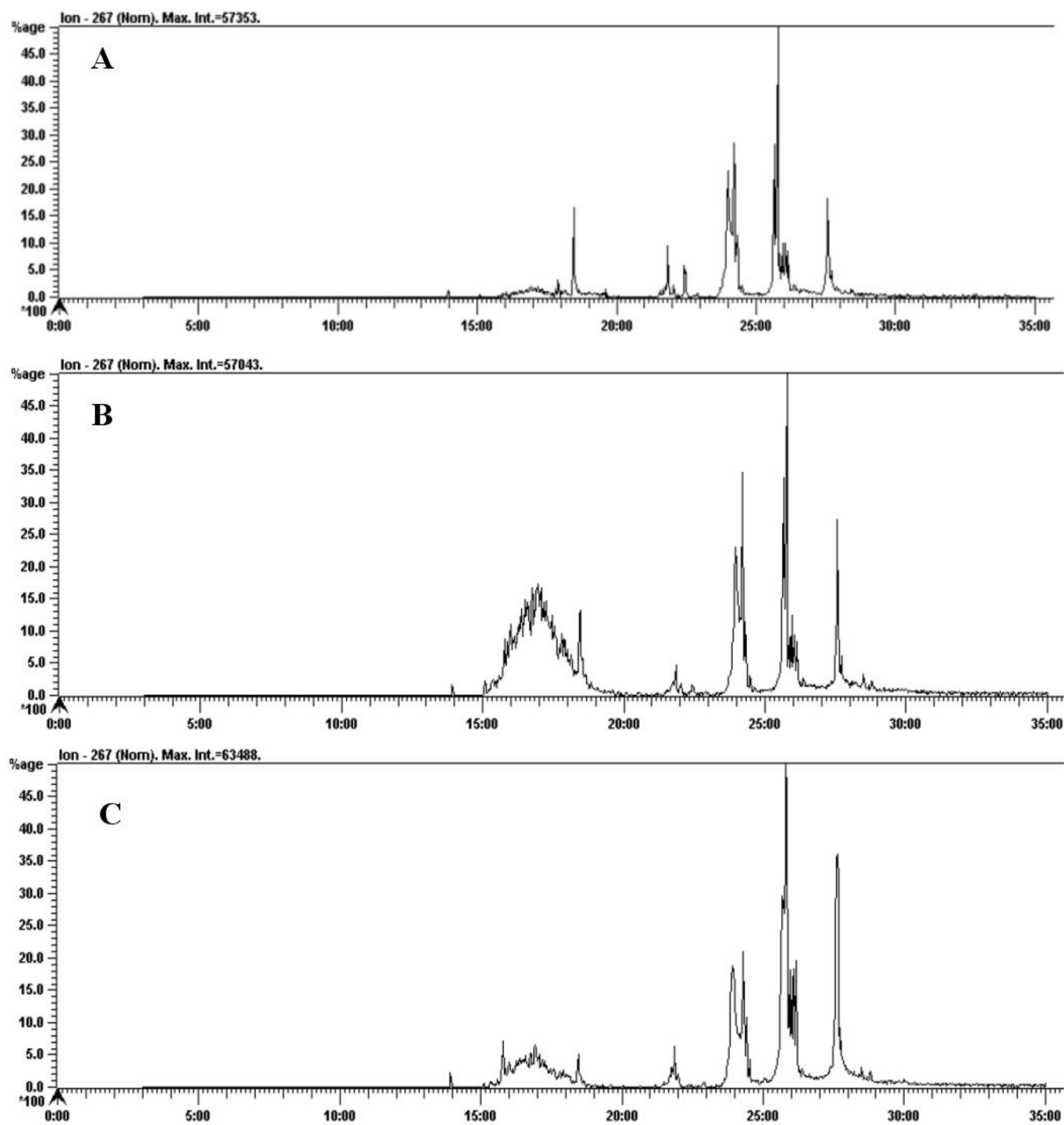

Figure 2.3.The reconstructed ion chromatograms for nominal m/z = 267 from the GC-MS analyses of the extracts of a 5 g samples of minnows held in A) Gregoire Lake water, B) Syncrude Pond 5 water, C) Syncrude Pond 9 water.

Broad, unresolved peak between 15 and 20 min indicates the presence of naphthenic acids. Peaks with retention times greater than 20 min are due to the natural fatty acids in fish.

2.6 Discussion

Using the fathead minnow reproduction assay described by Ankley et al. (2001), aged OSPW from Syncrude's Pond 9 and Suncor's North and South MFT ponds were shown to impair fathead minnow reproductive physiology in terms of a marked reduction in fecundity and the number of spawns. Nuptial tubercles were also significantly reduced in male fish held in water from Pond 9. Plasma sex steroids were significantly reduced in fathead minnows exposed to aged OSPW. Reductions in the production of sex steroids have been reported in slimy sculpin collected within the oil sands deposit (Tetreault et al., 2003) and in goldfish caged in ponds containing aged OSPW (Lister et al., 2008). Reduced sex steroid levels could explain the lower fecundity and the reduction in male secondary sexual characteristics. The expression of nuptial tubercles and other male secondary sexual characteristics is controlled by androgens (Smith et al., 1978). A reduction in tubercles could be due to either a decrease in plasma androgens, a down-regulation of the androgen receptors, or exposure to androgen receptor antagonists. Miles-Richardson et al. (1999) found that when fathead minnows were exposed to estrogen receptor agonists, the number of tubercles was observed to decrease.

OSPW has higher concentrations of inorganic ions (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-), and dissolved organics (NAs), compared to the non-OSPW from Gregoire Lake (Table 2.1). The concentrations of NAs tend to increase with increasing salinity and this makes it more difficult to determine conclusively which constituent, the salts or the NAs, is most responsible for the reproductive disruptions observed in fathead minnows held in aged OSPW. In the first two assays, fathead minnows were transferred from a low conductivity (142 μS/cm) water (Gregoire Lake) to a higher conductivity (1420-2780 μS/cm) water (OSPW) so salinity could have been a contributor to the reproductive impacts observed in Assays 1 and 2. Pistole et al. (2008) showed that metabolic rate increased in fathead minnows exposed to higher salinity and this was attributed to the increased energetic costs of osmoregulation. Oxygen consumption was also observed to increase when southern redbelly dace (*Phoxinus erythrogaster*) and northern studfish (*Fundulus catenatus*) were exposed to higher salinities (Toepfer at al., 1994).

A significant amount of energy is devoted to reproduction; male fathead minnows must allocate their reproductive resources among territorial defense, mating, and egg care (Unger, 1983). Consequently, the increased energetic costs of osmoregulation could impair fathead minnow reproduction. It is important to note, however, that in the second experiment with Syncrude OSPW, reproduction was not impaired in fish held in Pond 5 water, whose conductivity (2780 µS/cm) was greater than that seen in Pond 9 waters (2240 µS/cm). If the cause of the reproductive impairment was simply the change from water with low conductivity to water with high conductivity, reproduction should have been impaired more in fish held in Pond 5 water since its conductivity was higher than that from Pond 9. In Assay 3, the fathead minnows were salt-acclimated prior to OSPW exposure using the aged Suncor OSPW, so any effects that salinity might have on fish reproduction should have been mitigated. Since there was no salinity acclimation prior to the Syncrude OSPW experiments (Assays 1 and 2), the exposure to bio-degraded NAs coupled with the change in salinity may have had a greater impact on fathead minnow reproduction. This difference could explain why there was a greater reduction in tubercles, a reduction in plasma estradiol in females, and a complete cessation of spawning in fish held in Pond 9 aged OSPW.

Freshly produced OPSW can be acutely toxic to aquatic organisms and NAs are believed to be the constituent primarily responsible (MacKinnon and Boerger 1986). Over relatively short times (6-12 months) of ageing under natural conditions, the OSPW loses its acute toxic response and this has been attributed to the biodegradation of NAs (MacKinnon and Boerger 1986). Han et al. (2009) have shown that over time there is a loss of the more labile fraction of the NAs in OSPW and a relative increase in the more refractory constituents in the NAs group. In addition to the relative higher content of NAs with higher c-numbers (molecular weight) and z-values (ring structures), there is also evidence which suggests that a major fraction of the remaining NAs in aged OSPW are hydroxylated.

The OSPW used in this study had been aged for >15 years under natural conditions in ponds isolated from fresh input of OSPW as well as any recharge (aside from precipitation/ surface water runoff) or discharge with surface or ground waters. No acute

toxicity to either larval or adult fathead minnows was observed and these results confirm previous work; Siwik et al. (2000) determined that Syncrude OSPW would not significantly impact the survival or growth of fathead minnows in their early life stages. There were NAs detected in the tissues of fathead minnows exposed to waters from Pond 5 and Pond 9 (Figure 2.3). This is consistent with the results of Young et al. (2007) who found these acids in fish that were kept in a solution of commercial NAs (3 mg/L) or Pond 9 water. The method of Young et al. (2007) is not quantitative, but its minimum detection limit is ~1 μg NAs/g of fish. The barely discernable peak in the reconstructed ion chromatograms (RIC) between 15 and 20 min suggests that fish held in the Gregoire Lake water were exposed to very low levels of NAs (Figure 2.3A). This is not unexpected as NAs occur naturally in the Athabasca oil sands region and have been detected at concentrations ranging between 0.1 and 0.9 mg/L (Schramm et al., 2000).

Fathead minnow reproduction was impacted the most in OSPW from Syncrude's Pond 9 and Suncor's North and South MFT ponds. These three ponds were found to have the highest concentrations of NAs of all the OSPWs tested (Table 2.1). There is evidence which suggests NAs cause respiratory stress in fathead minnows (Nero et al., 2006a). It has been demonstrated that stress can have an adverse effect on the reproductive physiology of fish, reducing plasma sex steroids (Castranova et al., 2005; Pickering et al., 1987). Experiments with a commercial NAs mixture and a NAs mixture extracted from aged OSPW showed proliferative changes in the gill (epithelial, mucous, and chloride cell) of fish and that these changes increased with the addition of salt (Nero et al., 2006ab). As a result of these changes, the gill surface area was reduced and it was suggested that this would not only reduce the rate of absorption of NAs but reduce the rate of gas exchange as well, leading to respiratory stress.

While NAs have the potential to impair the reproductive system indirectly, these compounds may also affect the endocrine system directly. The NAs from the produced waters of offshore oil platforms and from commercial sources have been shown to be weak estrogenic receptor agonists and potent androgen receptor antagonists (Thomas et al., 2009). The reduction in androgens and male secondary sexual characteristics in fathead minnows exposed to OSPW would be consistent with these observations.

While the present studies have shown that aged OSPW negatively affect reproduction in the fathead minnow, identifying the constituents responsible for these effects will require further investigation. The current work suggests that oil sands operators should strive to reduce the NAs content and salinities in aquatic reclamation systems in order to achieve the water quality conditions that are conducive to the successful reproduction of fish. We feel that further studies testing the effects of NAs alone or in combination with salts on reproduction in fish are warranted.

CHAPTER 3. FATHEAD MINNOW (*PIMEPHALES PROMELAS*) REPRODUCTION IS IMPAIRED WHEN EXPOSED TO A NAPHTHENIC ACID EXTRACT

3.1 Abstract

Previous studies have demonstrated that oil sands process-affected water (OSPW) impair the reproduction of fish and that naphthenic acids (NAs), a natural constituent of oil sands, are suspected of being responsible. This study evaluates the potential impact of NAs on the reproduction of adult fathead minnows (*Pimephales promelas*) under laboratory conditions. Fathead minnows exposed to a 10 mg/l naphthenic acid extract (NAE) for 21 days spawned fewer eggs and males had reduced expression of secondary sexual characteristics. Male fathead minnows exposed to a 5 mg/l NAE had lower plasma levels of 11-ketotestosterone whereas those exposed to a 10 mg/l NAE had lower concentrations of both testosterone and 11-ketotestosterone. Since OSPW also contains high concentrations of salts, this study also investigated whether they modify the toxicity of NAEs. Spawning was significantly reduced in fathead minnows exposed to a 10 mg/l NAE alone and in combination with $NaHCO_3$ (700 mg/l), typical of concentrations in OSPW. Interestingly, the addition of $NaHCO_3$ reduced the inhibitory effects of the NAE on the numbers of reproductive tubercles and plasma testosterone levels. Further studies showed that $NaHCO_3$ acted by reducing the uptake of the NAE to the fish. $NaHCO_3$ but not NaCl or Na_2SO_4 reduced the acute toxic effects of the NAE on fathead minnow embryo and larvae mortality. Collectively, these studies show that the NAs in OSPW have the potential to negatively affect reproduction in fathead minnows and that HCO_3^- reduces the acute and chronic toxicity of NAs.

3.2 Introduction

There is an increasing reliance on unconventional sources of oil as conventional supplies diminish. Alberta's oil sands developments represent a significant source of unconventional oil and currently account for an average of 1.35 million barrels per day of marketable bitumen (heavy, biodegraded crude oil) and upgraded synthetic crude oil (Ministry of Energy, 2010). In surface mining operations, modifications of the Clark

Water Extraction process are used to separate the bitumen from the oil sands. As a result of this process, large volumes of fluid tailings are produced and stored in large settling basins, commonly referred to as tailings ponds. Even though a large percentage of water is recycled from these ponds for the extraction process, there are approximately 720 million m^3 of tailings being stored in tailings ponds (ERCB, 2009).

The oil sands process-affected water (OSPW) stored on oil sands leases will eventually be transferred to mined-out pits and there are plans for these to be capped with a layer of natural surface water. Despite the higher concentrations of naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs) and inorganic ions (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) that are present in OSPW (MacKinnon and Boerger, 1986; Schramm et al., 2000), it is expected that viable ecosystems will develop in these end pit lakes with a biological capability similar to natural lakes in the region. However, in studies to date, OSPW has been demonstrated to be acutely and chronically toxic to aquatic organisms. For example, yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) larvae exposed to freshly produced OSPW exhibit higher incidences of deformities compared to control fish (Peters et al., 2007). Histopathological changes in gill and liver tissue and increases in liver size and hepatic mixed-function oxygenase (MFO) activity have also been observed when fish were exposed to aged OSPW (Nero et al., 2006a; van den Heuvel et al., 1999; van den Heuvel et al., 2000). OSPW also has an adverse effect on the reproductive physiology of fish; goldfish (*Carassius auratus*) held in ponds containing aged OSPW had lower plasma steroid levels than reference fish (Lister et al., 2008) while fathead minnows exposed to aged OSPW in the laboratory were shown to have lower fecundity, lower plasma sex steroid concentrations, and a reduction in male secondary sexual characteristics (Kavanagh et al., 2011). Recently, it has been demonstrated *in vitro* that OSPW can decrease testosterone production and increase 17β-estradiol production by cultured H295R cells (He et al., 2010).

Currently, the oil sands constituents that are responsible for the reproductive effects seen following exposure to OSPW are not known. The acid-extractable organics in OSPW, which includes NAs, are prime candidates. NAs are a mixture of acyclic, monocyclic, and polycyclic carboxylic acids (chemical formula $C_nH_{2n+Z}O_2$) that are

highly soluble natural surfactants associated with the oil sands bitumen fraction (Clemente et al., 2005). In their dissociated ionic form, they are thought to be primarily responsible for the lethal toxicity of "fresh" OSPW (Frank et al., 2008; MacKinnon and Boerger, 1986; Madill et al., 2001). It has been demonstrated that NAs are weak estrogenic receptor agonists and potent androgen receptor antagonists (Thomas et al., 2009) making them candidates for the chemicals that modulate reproductive function. OSPW also contains high concentrations of Na^+, Cl^-, SO_4^{2-}, and HCO_3^- and it is possible that these ions acting alone or in combination with other constituents may contribute to the toxicity of OSPW. Nero et al. (2006b), for example, demonstrated that there were proliferative changes in gill epithelial, mucous, and chloride cells when yellow perch were exposed to a naphthenic acid extract (NAE) produced from OSPW, and these were increased with the addition of 1 g/l of Na_2SO_4.

The primary objective of this study was to determine the effect of a NAE on the reproductive physiology of fathead minnows (*Pimephales promelas*). We studied the fathead minnow because it is native to the oil sands region and would be expected to be present in end pit lakes. Sexually mature fathead minnows were exposed to a NAE in the laboratory for 21 days and the number of eggs spawned, expression of secondary sexual characteristics, and plasma sex steroid levels were monitored. A secondary objective was to determine if salts modify the toxicity of NAs. This involved tests in which sexually mature fathead minnows were exposed to a NAE alone or in combination with $NaHCO_3$ and the number of eggs spawned, expression of secondary sexual characteristics, and plasma sex steroid levels were monitored for 14 days. A further series of tests was conducted to determine the role of salts in the acute toxicity of NAs. In these studies, fathead minnow embryos and larvae were first exposed to a NAE along with NaCl, Na_2SO_4, or $NaHCO_3$ and their effects on survival were determined.

3.3 Materials and methods

3.3.1 NAE preparation

The method described by Frank et al. (2006) was used to extract NAs (and other organic acids) from OSPW. Briefly, OSPW was collected from Syncrude Canada Ltd.'s (Syncrude) West In-pit settling basin in 2004 and 2005. This was acidified to pH 2, the aqueous layer was decanted, and the precipitate (slurry) was centrifuged. The pellet was then dissolved in a 0.1 M NaOH solution before being passed through a bed of the weak anion exchanger diethylaminoethyl-cellulose to remove humic-like substances. The filtered NAE was washed with dichloromethane to remove neutral organics such as PAHs. The extract was again acidified and the precipitated acids were collected on a 0.2 μm Teflon filter. The acid precipitate was then re-dissolved in 0.05 M NaOH. The concentration of the resulting NAE stock solution (2408 mg/l of NAs in a 0.05 M NaOH) used in Assay 1 and the acute toxicity tests was determined using electrospray ionization mass spectrometry (ESI-MS; Headley et al., 2002) at the National Water Research Institute in Saskatoon, Saskatchewan, Canada. For Assay 2, the concentration of the resulting NAE stock solution (3375 mg/l of NAs in a 0.05 M NaOH) was measured using Fourier transform infrared (FTIR) spectroscopy (Holowenko et al., 2001) at Syncrude's Research Facility in Edmonton, AB. Kodak NAs (Eastman Kodak Company, Rochester, NY) were used as standards in the FTIR analysis. The two analytical methods produce similar results when determining NA concentrations. A lower detection method can be achieved with ESI-MS (0.01 mg/l) compared to FTIR (1 mg/l) but at the concentrations used in this study it would not have had an impact. Nominal concentrations of NAs are presented throughout this paper The NAs found in OSPW are recalcitrant to biodegradation (Scott et al., 2005) and half-lives of about 13 years have been reported (Han et al., 2009). Thus, no measurable degradation of the NAs would be expected during these experiments.

The NAE stock solution was analyzed for the concentrations of 16 priority PAHs as defined by the United States Environmental Protection Agency (USEPA), alkylated PAHs, and dibenzothiophenes by the ALS Laboratory Group, Edmonton, AB, Canada

using gas chromatography and mass spectrometry. Naphthalene, quinoline, C4 substituted phenanthrene/anthracene, and C3 substituted fluorene were measured at concentrations of less than 0.3 µg/l in the NAE. For the NAE concentrations used in the fathead minnow reproduction assays, the concentrations of PAHs would have been less than 1.3 ng/l. At these concentrations, the PAHs would be expected to have no measurable effects on fathead minnow reproductive physiology (Hall and Oris, 1991; Hoffman and Oris, 2006).

A low recovery of lower molecular weight NAs was observed with this extraction method; the loss of some of the lower molecular weight NAs was attributed to the initial acidification of the OSPW (Frank et al., 2006). The NAE was also aerated at room temperature for ~1.5 months and this may have helped to further deplete the lower molecular weight NAs. This was considered to be advantageous because the NAE more closely resembled the NA profile of aged OSPW (Figure 3.1). Holowenko et al. (2002) demonstrated that as OSPW ages, there is a decrease in the lower molecular weight NAs and this is accompanied by a decrease in the acute toxicity of OSPW.

3.3.2 Assay 1: NAE exposure

Adult fathead minnows were purchased from Aquatic Research Organisms (Hampton, NH, USA) and raised at the University of Guelph for several months before being used in a fathead minnow reproduction assay described by Ankley et al. 2001. Due to the hardness of the well water in Guelph, Ontario, Canada, reconstituted water was used in this assay. The reconstituted water was prepared by adding the following reagent-grade salts (Fisher-Scientific) to deionized water (ASTM, 1980): 48 mg/l of $NaHCO_3$, 30 mg/l of $CaSO_4 \cdot 2H_2O$, 30 mg/l of $MgSO_4$, and 2 mg/l KCl. Fathead minnows were acclimated to the reconstituted water one month prior to the start of the experiment.

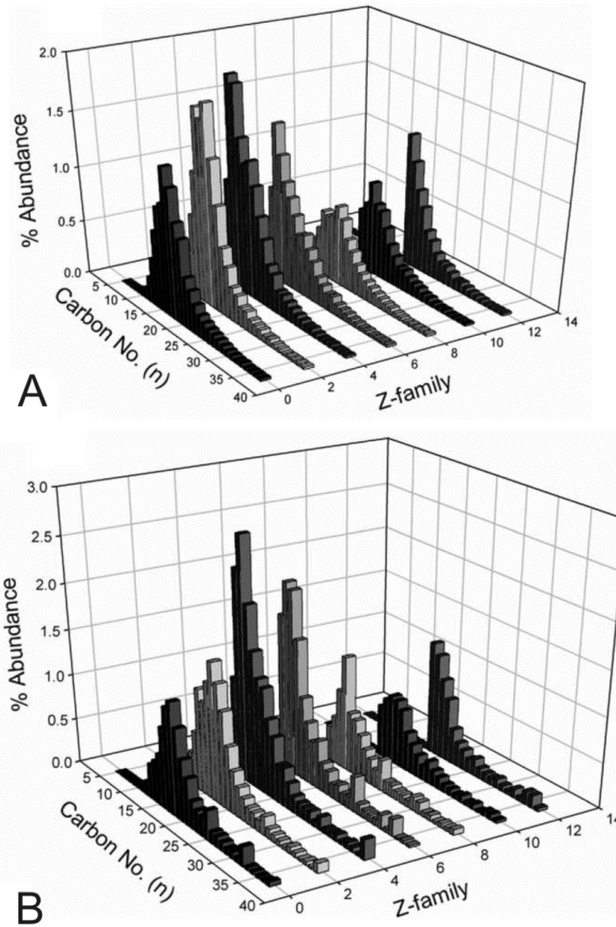

Figure 3.1. Three-dimensional plots of NA z-series (Frank et al., 2006). A) NA component of initial OSPW, B) NA component of extract following DEAE-cellulose treatment.

During the pre-exposure phase, fathead minnows (age: 9 mo) were held in the reconstituted water for 21 days and fecundity, fertility, and hatching success were monitored to ensure they had similar egg production rates and that fertility, hatching success, and survival to swim-up were greater than 95%, 90%, and 90%, respectively. During the exposure phase, fathead minnows were held for 21 days in the following treatments: control (reconstituted water), NaOH control, 5 mg/l NAE, and 10 mg/l NAE. The NaOH control had a NaOH concentration (8.3 mg/l) equivalent to the highest NAE treatment. The pH of the reconstituted water was 7.8 ± 0.1. In the NaOH control and

NAE treatments, the pH was adjusted to a pH of 8.6 ± 0.2 with 18M H_2SO_4 (Sigma-Aldrich, Milwaukee, WI).

3.3.3 Assay 2: NAE + $NaHCO_3$ exposure

A second test examined the interactive effects of a NAE and $NaHCO_3$ on reproduction in the fathead minnow. Fathead minnows were purchased from Aquatic Research Organisms (Hampton, NH, USA) and raised at the University of Guelph for several months before being transported to Fort McMurray, AB. This assay was conducted in a laboratory trailer located on Syncrude's site using water (carbon filtered, UV treated) collected from Gregoire Lake (56°27'06"N, 111°07'38"W), which is located approximately 15 km south of Fort McMurray, AB. Fathead minnows were held in water from Gregoire Lake for one month at Syncrude while fish that were to be treated with $NaHCO_3$ were acclimated in Gregoire Lake water containing 700 mg/l of $NaHCO_3$. This concentration was selected to be comparable to the concentrations of HCO_3^- measured in the aged OSPW from tailings ponds. Fresh and age OSPW have similar concentrations of HCO_3^-. However, unless there are fresh water inputs to end pit lakes, salt concentrations will increase over time. Fathead minnows (age: 1 yr) were held in the same waters during the 14 day pre-exposure phase and fecundity, fertility, and hatching were monitored to ensure they had similar egg production rates and that fertility, hatching success, and survival to swim-up were greater than 95%, 90%, and 90%, respectively. During the exposure phase, fathead minnows were held for 14 days in the following treatments: control (Gregoire Lake water), NaOH control (5.9 mg/l), $NaHCO_3$ control (700 mg/l $NaHCO_3$), 10 mg/l NAE, and 10 mg/l NAE plus $NaHCO_3$ (700 mg/l $NaHCO_3$). The pH of Gregoire Lake water was 7.6 while the pH for all other treatments was 8.6 ± 0.2 (adjusted with 18M H_2SO_4). Due to the amount of NAE available, fathead minnows were only exposed to the NAE for 14 days as opposed to standard 21 days.

3.3.4 Assay methodology

Each of the tanks (n=4 per treatment) contained four female and two male fathead minnows and three halved polyvinyl chloride (PVC) pipes for spawning substrate. The tanks were filled with 15 L of control or treatment water and placed inside a reservoir

maintained at 25 ±1°C, under a photoperiod of 16 h light: 8 h dark. Tanks were cleaned and 100 % of the water renewed every two days. Dissolved oxygen was maintained above 8 mg/l throughout the exposures and ammonia was <0.05 mg/l. Fathead minnows were fed frozen adult brine shrimp (*Artemia* sp.) *ad libitum* twice daily. The endpoints monitored included the number of spawns, fecundity (number of eggs spawned), fertility, hatching success, and survival to swim-up. Each morning spawning and fecundity were determined by counting the numbers of eggs laid on the PVC pipe. For the purposes of determining the number of spawns, eggs observed on a single piece of PVC pipe were counted as one spawn while eggs found on two pieces of PVC pipe were counted as two spawns. Fecundity was calculated by counting the number of eggs in each tank and then the total number of eggs per female per day was calculated for each tank. The fertility of all the eggs was determined under a dissecting microscope. A subsample of these eggs was kept to measure hatching success and survival to swim-up (96 h post hatch). This involved transferring 10 eggs into a Petri dish containing water from the corresponding treatment and placing them in an incubator (25 ± 2°C, 16 h light: 8 h dark). There were four Petri dishes for each tank and each tank was evaluated twice. The eggs were checked daily for mortality and hatching, and any dead eggs or larvae were recorded and removed. Approximately 70% of the exposure solutions in the Petri dishes were changed daily.

3.3.5 Fish sampling

Fathead minnows were anesthetized with tricaine methanesulfonate (MS-222). The weight (g) and fork length (cm) of each fish was measured and the condition factor (CF) was calculated [CF = 100 x total wt (g) / fork length^3 (cm)]. Blood was collected from the caudal vessels with heparinized microcapillary tubes and the plasma was isolated by centrifugation (13000 × *g* for 6 min) and stored at –20°C. Fish were euthanized by spinal severance. Gonads and livers were removed and weighed in order to calculate the gonadosomatic index (GSI) [GSI = 100 x gonadal tissue wt (g) / total body wt (g)] and liver somatic index (LSI) [LSI = 100 x liver tissue wt (g) / total body wt (g)]. Tubercles, a secondary sex characteristic on male fathead minnows, were counted under a dissecting scope. In Assay 2, the spleen was weighed to calculate the spleen-somatic index (SSI)

[SSI = 100 x spleen weight (g) / body weight (g)]. Concentrations of sex steroids were measured in both experiments. After extracting the plasma three times with diethyl ether, concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone were determined using enzyme immunoassays (EIA; Cayman Chemical, Ann Arbor, MI) as per the manufacturer's instructions. Dilutions of a set of subsamples were shown to be parallel to the standard curves.

3.3.6 Analysis of Tissue for NAs

Carcasses (excluding head and internal organs) of fathead minnows from Assay 2 were frozen and stored at –20° C until they were analyzed for NAs. Approximately 1-g samples of eviscerated fish per tank from the NAE and NAE + $NaHCO_3$ treatments were analyzed for NAs. Females and males were analyzed separately. Fish samples corresponding to the same treatment were analyzed as a batch. For every batch of analyzed minnows, a 1-g sample of purchased rainbow trout was spiked with 10 µg of Merichem NAs (Merichem Chemicals and Refinery Services LLC, Houston, TX) and carried through the same extraction procedure and GC-MS method. The mean NAs recovered (±SD) from these samples was 9.7 ± 1.0 µg/g.

The method used to extract and analyze the fish samples was that of Young et al. (2008). For each fish sample, 5 µg of 9-fluorenecarboxylic acid (9-FCA, Sigma-Aldrich, Milwaukee, WI) were added as a surrogate standard prior to extraction. Sample extracts were derivatized with N-methyl-N-(*t*-butyldimethylsilyl)triflouroacetamide, without 1% *t*-butyldimethylsilylchoride (Lots LA140128A and LH141909, Sigma-Aldrich, Milwaukee, WI; Young et al., 2010) and analyzed by GC-MS in SIM mode for *m/z* 267 (Young et al., 2008) at high resolution (peak width ~0.5 amu). The MS signal integration parameters were set to integrate the NAs hump and the 9-FCA peak, and ratios were compared to a calibration curve to estimate NAs concentrations. To prepare a calibration curve, five 5-g samples of purchased rainbow trout were spiked with Merichem NAs at concentrations ranging from 0 to 50 µg/g with 5 µg of 9-FCA added before extraction.

3.3.7 Acute toxicity tests with embryos and larvae

Embryos

Using reconstituted water (described in Assay 1), embryos were held for 9 days (from fertilization until 96 h post hatch) in one of the following treatments: control, NaOH control (84 mg/l), and various concentrations of NAE (10, 14.7, 21.5, 31.6, 46.4 , 68.1, and 100 mg/l). The NaOH control had a NaOH concentration equivalent to the NaOH concentration found in the highest NAE treatment. NaOH was added to the NAE treatments so that all of the treatments contained the same amount of NaOH. The pH was adjusted with 18M H_2SO_4 to a pH of 8.6 ± 0.2. In a second and third set of experiments, embryos were exposed to the NAE (at the same concentrations used previously) and NaCl, Na_2SO_4, or $NaHCO_3$ at concentrations of 350 mg/l and 700 mg/l, respectively. A concentration of 350 mg/l was selected as it is comparable to the concentrations of Cl^- and SO_4^{2-} measured in OSPW. HCO_3^- occurs at higher concentrations (>500 mg/l) in OSPW and therefore, $NaHCO_3$ as well as NaCl and Na_2SO_4 were also evaluated at a concentration of 700 mg/l. In each of the experiments, each treatment was evaluated using 3 replicates of 10 larvae each in 100 mm x 15 mm Petri dishes containing 55 ml of test solutions. Embryos were raised in an incubator at 25 ± 2°C and a photoperiod of 16 h light: 8 h dark. The eggs were checked daily for mortality and hatching and any dead eggs or larvae were recorded and removed. Approximately 70% of the test waters in the Petri dishes were changed daily.

Larvae

Fathead minnow larvae (5 days old) were held for 96 h in one of the following treatments: control, salt control, NaOH control (84 mg/l), and various NAE concentrations (listed above) plus 700 mg/l of NaCl, $NaSO_4$, or $NaHCO_3$. The NaOH control had a NaOH concentration equivalent to the NaOH concentration found in the highest NAE treatment. NaOH was added to the NAE treatments so that all of the treatments contained the same amount of NaOH. The pH was adjusted with 18M H_2SO_4 to a pH of 8.6 ± 0.2. Each treatment was evaluated using 3 replicates of 10 larvae each in 500 ml glass pyrex dishes containing 250 ml of test solutions. Fish were fed *Artemia*

nauplii 4 h prior to daily sample renewal. Tests were conducted at 25 ± 2°C with a 16 h light: 8 h dark photoperiod. Mortality was recorded daily.

3.3.8 Statistical analysis

Male and female fish were analyzed separately. For all of the assays, each tank (n=4) was considered a replicate and no outliers were removed. A Levene's test was used to test data for homogeneity of variance. Data that did not meet parametric assumptions were log transformed for normalization and/or to reduce the heterogeneity in variance. One-way analysis of variance (ANOVA) was used to test for differences across all treatment groups. A Tukey's post-hoc test was used to determine which treatment(s) differed from the control. A t-test was used in the fish tissue analysis. Differences were considered significant at $p < 0.05$. With the exception of the acute toxicity tests, all statistical analyses were conducted using SPSS 19.0; the data are presented as mean ± SE unless otherwise noted. The Log-probit analysis for the embryo and larvae acute toxicity test data was conducted using the USEPA Probit Analysis Program Version 1.5, which generated LC50 values with 95% Confidence Intervals (CIs). Significant differences between the LC50 values that were generated for the NAE and NAE + salt exposures were identified by a lack of overlap between 95% CIs, which is a conservative comparison with $\alpha < 0.005$ (Payton et al., 2003).

3.4 Results

3.4.1 Assay 1: NAE exposure

During the exposure, fecundity was reduced in the fathead minnows exposed to the 10 mg/l NAE treatment (Figure 3.2A). The mean fecundity rates (eggs/female/day) were 12.2 ± 1.2, 10.6 ± 1.0, 6.9 ± 1.7, and 3.9 ± 0.7 for fathead minnows held in the control, NaOH control, 5 mg/l NAE, and 10 mg/l NAE, respectively. The mean number of spawns for fathead minnows held in the control, NaOH control, 5 mg/l NAE, and 10 mg/l NAE treatments were 8.8 ± 0.8, 7.0 ± 0.8, 5.5 ± 0.9, and 2.8 ± 0.5, respectively. There was no significant difference in mean fecundity rate (eggs/female/day) and number of spawns between fathead minnows exposed to the 5 mg/l NAE and the control and the

NaOH control. A significant reduction in the mean fecundity rate (eggs/female/day) and number of spawns was observed in fathead minnows exposed to the 10 mg/l NAE

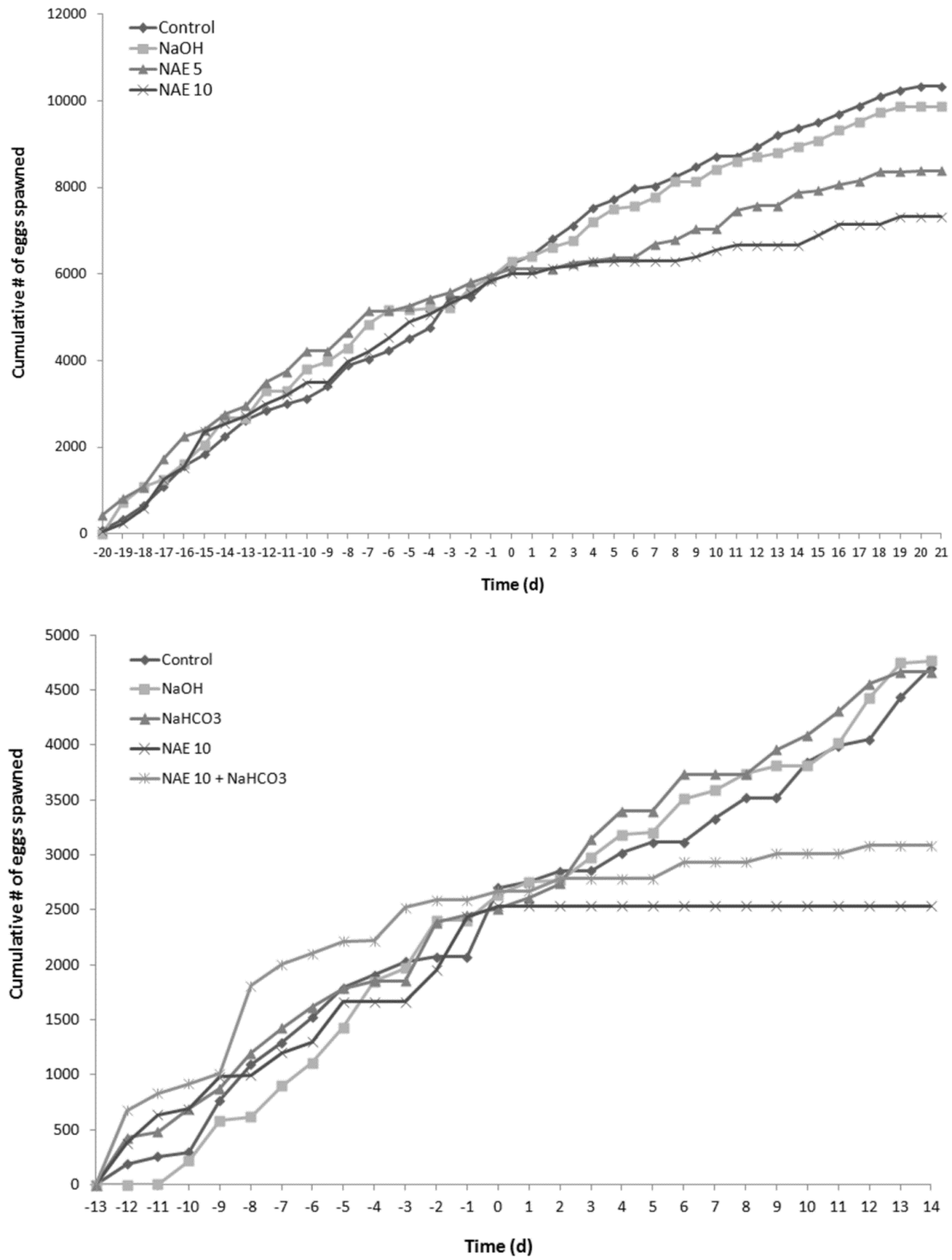

Figure 3.2. Cumulative number of eggs spawned by fathead minnows during NAE exposures A) Graph shows fecundity during a 21 day pre-exposure period prior to exposure to control, NaOH control, 5 mg/l of NAE, and 10 mg/l of NAE for 21 days. B) Graph shows fecundity during a 14 day

pre-exposure period prior to exposure to control, NaOH control, $NaHCO_3$ control, 10 mg/l of NAE, and 10 mg/l of NAE + 700 mg/l $NaHCO_3$ for 14 days.

compared to the control and NaOH control. The mean number of tubercles on male fathead minnows held in the control, NaOH control, 5 mg/l NAE, and 10 mg/l NAE were 18.4 ± 1.0, 18.5 ± 1.6, 11.8 ± 3.1, and 8.0 ± 2.1 tubercles/fish, respectively. Male fathead minnows from the 10 mg/l NAE treatment had tubercles that were significantly reduced both in size and number compared to fathead minnows in the control groups. Male fathead minnows in the 5 mg/l and 10 mg/l NAE treatments had significantly larger livers (LSI, Table 3.1) compared to fathead minnows in the control groups. There were no significant changes in the GSIs or CF in fathead minnows with any of the treatments (Table 3.1). Male fathead minnows exposed to the 5 mg/l NAE had significantly lower concentrations of 11-ketotestosterone in plasma while those exposed to the 10 mg/l NAE had significantly lower concentrations of both testosterone and 11-ketotestosterone in plasma (Figure 3.3). The fertility rate, hatching success, and survival to swim-up were not affected by any of the treatments (data not shown). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks.

3.4.2 Assay 2: NAE + $NaHCO_3$ Exposure

During the exposure, fecundity was reduced in the fathead minnows exposed to NAE and NAE + $NaHCO_3$ (Figure 3.2B). The mean fecundity rates (eggs/female/day) were 8.9 ± 0.6, 9.5 ± 1.6, 9.6 ± 1.2, 0.0 ± 0.0 and 1.39 ± 0.4 for fathead minnows in the control, NaOH control, $NaHCO_3$ control, 10 mg/l NAE, and 10 mg/l NAE + $NaHCO_3$ treatments, respectively. The mean number of spawns for fathead minnows held in the control, NaOH control, $NaHCO_3$ control, 10 mg/l NAE , and 10 mg/l NAE + $NaHCO_3$ treatment was 3.8 ± 0.5, 5.3 ± 0.6, 4.0 ± 0.4, 0.0 ± 0.0 and 1.3 ± 0.4, respectively. A significant reduction in the mean fecundity rate (eggs/females/day) and number of spawns was observed in fathead minnows exposed to NAE and NAE+ $NaHCO_3$ when compared to the control groups. There were no significant differences in fecundity or in

Table 3.1. Condition factor (CF), gonadosomatic indices (GSIs), liver somatic indices (LSIs), and spleen-somatic indices (SSIs) for fathead minnows used in Assays 1 and 2.

Males

Assay	Treatment	Length (cm)	Mass (g)	CF	GSI (%)	LSI (%)	SSI (%)
1	Control	6.90±0.08	4.33±0.08	1.29±0.02	1.19±0.12	1.63±0.13	-
	NaOH control	7.03±0.25	4.46±0.38	1.28±0.06	1.19±0.16	2.30±0.15	-
	NAE 5 mg/l	7.06±0.12	4.77±0.24	1.33±0.03	1.09±0.16	2.50±0.31*	-
	NAE 10 mg/l	7.09±0.37	4.54±0.59	1.26±0.09	1.20±0.07	2.37±0.13*	-
2	Control	5.41±0.07	2.22±0.10	1.40±0.04	1.30±0.09	2.21±0.24	0.38±0.10
	NaOH	6.10±0.30	3.12±0.52	1.34±0.09	1.62±0.19	2.59±0.13	0.43±0.18
	HCO_3^-	5.76±0.11	2.72±0.15	1.40±0.12	1.10±0.08	2.22±0.07	0.33±0.07
	NAE 10 mg/l	5.90±0.11	2.58±0.31	1.24±0.09	1.25±0.14	2.80±0.26	0.25±0.07
	NAE 10 mg/l + HCO_3^-	5.63±0.11	2.25±0.15	1.30±0.12	1.32±0.08	2.25±0.07	0.27±0.07

Females

Assay	Treatment	Length (cm)	Mass (g)	CF	GSI (%)	LSI (%)	SSI (%)
1	Control	5.23±0.08	1.60±0.07	1.10±0.02	12.1 ±1.5	2.33±0.11	-
	NaOH control	5.43±0.05	1.79±0.09	1.11±0.03	12.5 ±1.4	2.61±0.26	-
	NAE 5 mg/l	5.44±0.05	1.79±0.08	1.11±0.05	11.4 ±2.2	2.14±0.23	-
	NAE 10 mg/l	5.25±0.08	1.73±0.05	1.17±0.04	14.6±0.99	2.62±0.17	-
2	Control	4.36±0.13	1.14±0.08	1.36±0.04	10.6±1.4	2.57±0.20	0.41±0.13
	NaOH	4.27±0.02	1.09±0.04	1.37±0.03	11.4±0.81	2.44±0.07	0.38±0.10
	HCO_3^-	4.29±0.13	1.05±0.10	1.30±0.02	9.30±0.69	2.19±0.10	0.51±0.12
	NAE 10 mg/l	4.38±0.11	1.13±0.10	1.32±0.03	8.65±1.69	2.81±0.16	0.29±0.04
	NAE 10 mg/l + HCO_3^-	4.35±0.13	1.12±0.09	1.33±0.07	8.75±1.35	2.38±0.19	0.29±0.05

Note: Values are means ± SE. Values with an * are significantly different ($p < 0.05$) when compared with control and "-" indicates that the parameter was not measured. For each parameter, n=4.

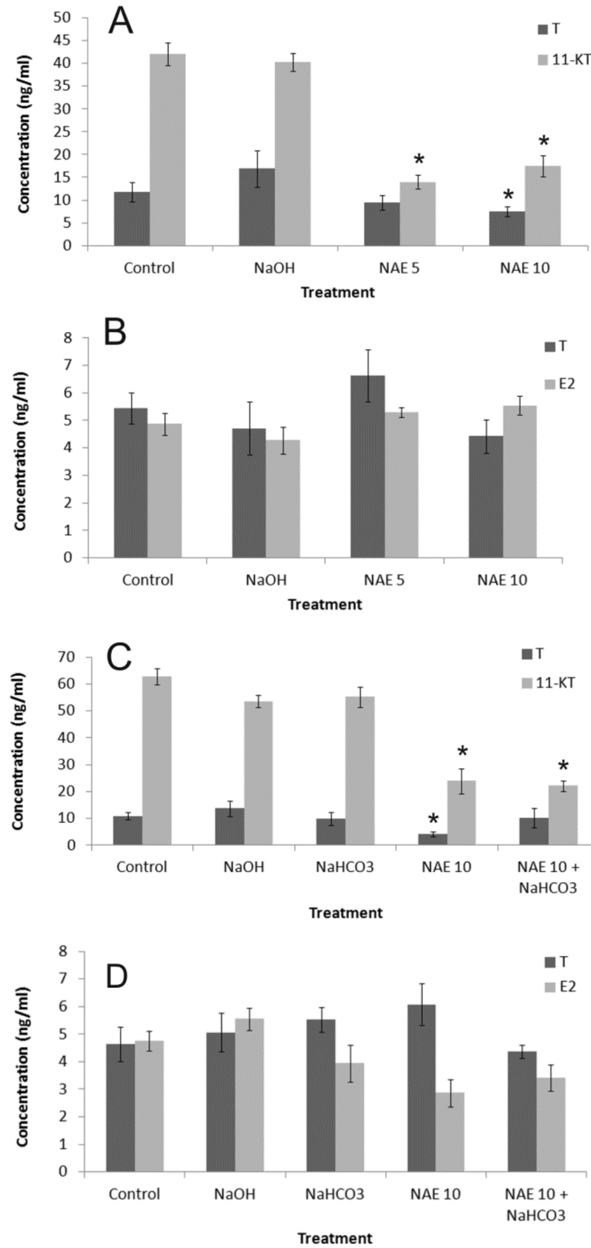

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Figure 3.3. Plasma steroid concentrations measured in A) Male fathead minnows exposed to 5 mg/l and 10 mg/l NAE for 21 days, B) Female fathead minnows exposed to 5 mg/l and 10 mg/l NAE for 21 days C) Male fathead minnows exposed to 10 mg/l of NAE and 10 mg/l of NAE + 700 mg/l of NaHCO3 for 14 days, D) Female fathead minnows exposed to 10 mg/l of NAE and 10 mg/l of NAE + 700 mg/l of NaHCO3 for 14 days.

T= testosterone, 11KT= 11-ketotestosterone, E2= 17β-estradiol. Values (means ± SE) with an * are significantly different ($p < 0.05$) when compared to controls.

the number of spawns between fathead minnows in the control group and fathead minnows in NaOH and $NaHCO_3$ control groups. The mean number of tubercles on male fathead minnows held in the control, NaOH control, $NaHCO_3$ control, 10 mg/l NAE , and 10 mg/l NAE + $NaHCO_3$ treatment were 14.9 ± 2.2, 18.1 ± 1.5, 15.4 ± 2.5, 1.0 ± 0.5, and 12.9 ± 2.0 tubercles/fish, respectively. Tubercles were significantly reduced in size and number in fathead minnows held in the 10 mg/l NAE treatment compared to all other groups. There were no significant differences in GSIs, LSIs, SSI or CF of fathead minnows from any of the treatments (Table 3.1). In comparison to the control groups, fathead minnows from the 10 mg/l NAE treatment had significantly lower concentrations of testosterone and 11-ketotestosterone in plasma while fathead minnows from the 10 mg/l NAE + $NaHCO_3$ treatment had lower concentrations of 11-ketotestosterone (Figure 3.3). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks. The fertility rate, hatching success, and survival to swim-up were not affected by exposure to any of the treatments (data not shown).

3.4.3 Analysis of tissue for NAs

The mean NAs content (±SD) measured in the fathead minnows from the NAE and NAE/$NaHCO_3$ treatments were 8.9 ± 3.3 µg/g wet weight and 5.4 ± 2.1 µg/g wet weight, respectively. There was a statistically significant difference between the two treatments ($p<0.05$; t-test). No statistically significant differences ($p<0.05$) were observed between females and males.

3.4.4 Acute toxicity tests with embryos and larvae

When fathead minnow embryos were exposed to the NAE, a LC50 of 32.8 mg/l was calculated (Figure 3.4; Table 3.2). The LC50 for the NAE did not change when NaCl or Na_2SO_4 were added. The LC50 did not significantly change when 350 mg/l of $NaHCO_3$ was added, however, in the presence of 700 mg/l of $NaHCO_3$, the LC50 for the NAE was increased to 59.9 mg/l. For the acute toxicity tests with fathead minnow larvae, the LC50 was 51.8 mg/l for NAE (Figure 3.4; Table 3.2). The LC50 for NAE did not change when

700 mg/l of NaCl or Na_2SO_4 were added. However, the LC50 value for the NAE was significantly increased to 121 mg/l when 700 mg/l of $NaHCO_3$ was added.

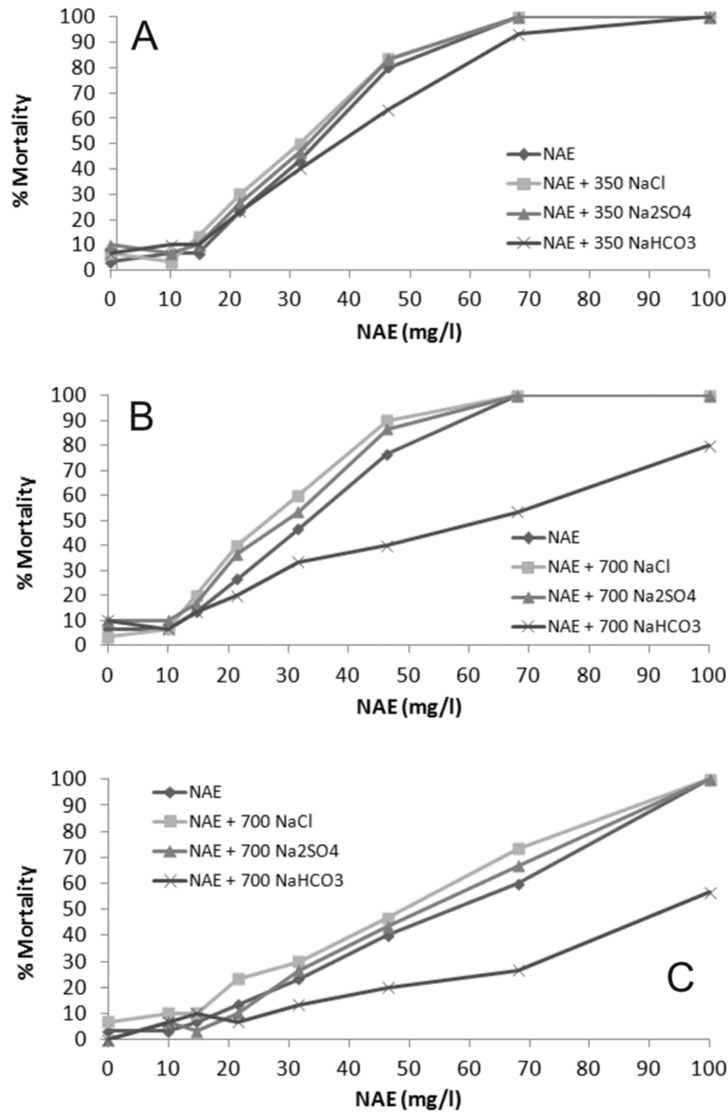

Figure 3.4. Acute toxicity tests for A) Embryos exposed to various concentrations of NAE and 350 mg/l of NaCl, Na_2SO_4, or $NaHCO_3$ for 9 days, B) Embryos exposed to various concentrations of NAE and 700 mg/l of NaCl, Na_2SO_4, or $NaHCO_3$ for 9 days, C) Larval fathead minnow exposed to a NAE (0-100 mg/l) and 700 mg/l of NaCl, Na_2SO_4, or $NaHCO_3$ for 96 h.

Table 3.2. Estimated LC50 Values and Confidence Limits.

Experiment	Treatment	LC50 (mg/l)	95% Confidence Limits Lower	Upper
Embryo Exposure (NAE + 350 mg/l of salts)	NAE	32.8	28.4	36.9
	NAE + NaCl	30.3	25.7	34.4
	NAE + Na_2SO_4	32.4	27.5	36.6
	NAE + $NaHCO_3$	36.6	30.6	42.0
Embryo Exposure (NAE + 700 mg/l of salts)	NAE	32.6	27.6	37.2
	NAE + NaCl	27.0	22.3	31.0
	NAE + Na_2SO_4	29.5	24.4	33.9
	NAE + $NaHCO_3$	59.9*	45.4	77.9
Larvae Exposure (NAE + 700 mg/l of salts)	NAE	51.8	44.0	59.7
	NAE + NaCl	47.4	38.9	55.1
	NAE + Na_2SO_4	46.8	33.4	72.8
	NAE + $NaHCO_3$	121*	81.6	277

Note: Values with an * are significantly different ($p < 0.05$) when compared with NAE only groups. n=3.

3.5 Discussion

The current studies provide evidence that the NAE impairs the reproductive physiology of fathead minnows. The NAE had negative effects on the fecundity rate and the number of spawns, the size and number of nuptial tubercles in males, and on the concentration of sex steroids in plasma of males. These results were similar to the responses seen in fish exposed to OSPW, which contain elevated concentrations of NAs. For example, male and female goldfish caged in ponds containing OSPW had lower concentrations of 17β-estradiol and testosterone in plasma (Lister et al., 2008) and fathead minnows held in OSPW in the laboratory were shown to have lower fecundity and male fathead minnows had lower numbers of tubercles and lower concentrations of testosterone and 11-ketotestosterone in plasma (Kavanagh et al., 2011). Androgens, particularly 11-ketotestosterone, have control over the expression of secondary sexual characteristics, reproductive behavior, and spermatogenesis (Borg et al., 1994). The lower fecundity and the reduction in the number of tubercles on male fathead minnows

induced by the NAE in this study may, therefore, be a result of the lower testosterone and 11-ketotestosterone concentrations observed in plasma of male fathead minnows.

The NAs could be impacting steroid production by acting either indirectly or directly on the endocrine system of the exposed fish. Stress, for example, can indirectly affect the reproductive physiology of fish, reducing plasma sex steroids (Castranova et al., 2005; Pickering et al., 1987). During the stress response, energy is directed towards processes that will restore homeostasis and away from other functions, such as reproduction (Fuzzen et al., 2011). If, for example, there is damage from exposure to a substance, the fish may reallocate energy expenditures in order to repair the affected system (Beyers et al., 1999). Experiments with a commercial NAs mixture and a NAs mixture extracted from OSPW showed proliferative changes in the gill (epithelial, mucous, and chloride cell) of yellow perch (Nero et al., 2006a). As a result of these changes, the gill surface area was reduced and it was suggested that this would not only reduce the rate of absorption of NAs but reduce the rate of gas exchange as well, leading to respiratory stress. The physiological changes that might be required to compensate for the lower rate of gas exchange could, therefore, affect the reproductive physiology of these fish. Indeed hypoxia is known to impair the reproductive capacity of fish (Thomas et al., 2007; Thomas and Rahman, 2009). It is important to note, however, that the NAE used in our study may not have caused respiratory stress as there was a significant difference between the acute toxicity of NAE (LC50=32 mg/l) produced for this study and the NAE (LC 100= 6.8 mg/l) used by Nero et al. (2006a). The artificial aging (aeration) of the extract in our study could account, at least in part, for the differences observed in the toxicity of the two extracts.

It is well known that a variety of compounds detected in the aquatic environment can directly affect the endocrine system of fish. Estrogenic substances such as 17α-ethinylestradiol have been demonstrated to alter reproductive behaviors, plasma sex steroids, and secondary sexual characteristics in male fathead minnows (Martinović et al., 2007; Salierno and Kane, 2009; Dammann et al., 2011). Similar results have been obtained in studies with fathead minnows exposed to fungicides with anti-androgenic properties; fathead minnows exposed to vinclozolin had lower fecundity and reduced

male secondary sexual characteristics (Martinović et al., 2008) and male fathead minnows exposed to prochloraz had significantly lower plasma concentrations of testosterone and 11-ketotestosterone (Ankley et al., 2005). Recently, it has been demonstrated that OSPW and NAs have both estrogenic and anti-androgenic properties. He et al. (2010) determined that after 48 h of exposure to OSPW, H295R cells produced significantly less testosterone and significantly more 17β-estradiol. The NAs found in OSPW are most likely responsible for these effects; Thomas et al. (2009) extracted NAs from the produced waters (mixture of formation water and injected water of the oil reservoir) of offshore oil platforms and demonstrated that the extract and commercial NAs were weak estrogenic receptor agonists and potent androgen receptor antagonists. The results from these studies would suggest that NAs are capable of directly impairing the reproductive physiology of fathead minnows.

OSPW contains high concentrations of NAs as well as salts so it was important to determine whether salts modify the toxicity of NAEs. In the second assay, fathead minnows were exposed to 10 mg/l of NAE and a 10 mg/l of NAE + 700 mg/l of $NaHCO_3$. Unlike the fathead minnows held in the 10 mg/l NAE treatment, tubercle numbers and testosterone concentrations in male fathead minnows from the 10 mg/l NAE + $NaHCO_3$ treatment did not differ significantly from control fish. The $NaHCO_3$ appeared to reduce the chronic reproductive toxicity of the NAE. GC-MS analyses of fathead minnow tissues for NAs showed that they were significantly higher in fathead minnows from the 10 mg/l NAE treatment than they were in the 10 mg/l NAE+ $NaHCO_3$ treatment. This indicates that less NAs are accumulated by fathead minnows when $NaHCO_3$ was present and suggests that HCO_3^- interferes with NAs and their movement through the cell membrane. Ions such as HCO_3^- are known to influence the uptake and toxicity of a number of metals usually through the formation of less toxic metal-base complexes (Wurts and Perschbacher, 1994). NAs would not be expected to form complexes with HCO_3^-, however, HCO_3^- could reduce their uptake in other ways. For example, it has been suggested that calcium could influence the uptake of zinc by biological mechanisms (e.g. decrease membrane permeability, fewer uptake sites, etc.) or

some other chemical mechanism such as competition for absorption sites (Barron and Albeke, 2000).

At higher concentrations, the NAE was shown to be acutely toxic to fathead minnow embryos and larvae. $NaHCO_3$ was shown to significantly reduce the acute toxicity of NAs but this reductive effect was not observed with NaCl or Na_2SO_4. It is not understood why HCO_3^- had this effect and not Cl^- or SO_4^{2-}. The acute toxicity of NAs was not unexpected as NAs are believed to be primarily responsible for the lethal toxicity of "fresh" OSPW (Frank et al., 2008; MacKinnon and Boerger, 1986; Madill et al., 2001). Peters et al. (2007) demonstrated that fresh OSPW and commercial NAs can also cause higher incidences of mortalities and deformities in yellow perch and Japanese medaka larvae compared to control fish. Similar results have also been observed when fathead minnow and white sucker (*Catostomus commersoni*) larvae were exposed to sediment from the oil sands region and from a wastewater pond from Suncor Energy Inc. (Colavecchia et al., 2004; Colavecchia et al., 2006). In the present study, higher incidences of mortalities or deformities were not observed in the fathead minnow reproduction assays. The frequency of deformities that occurred during the acute toxicity tests was not examined in this study.

The lethal toxicity of “fresh” OSPW declines over time and this is believed to be due to a decrease in the proportion of lower molecular weight NAs (Holowenko et al., 2002; MacKinnon and Boerger, 1986). Frank et al. (2008) fractionated a NAE and demonstrated that the lowest molecular weight NAs had higher potency than the highest molecular weight NAs. Despite the loss of lower molecular weight NAs during the OSPW extraction method and the aeration of the extract afterwards, the chronic toxicity of NAs in this study differ from what has been observed in studies with OSPW. Kavanagh et al. (2011) showed that fathead minnow reproductive physiology was not impacted unless NAs concentrations were >30 mg/l. Although there may have been differences in the NAs used in this study compared to what has been observed in aged OSPW, other environmental factors could also contribute. Colloidal clays are known to bind with carboxylic acids and other organic acids (Kang and Xing, 2007). A certain percentage of the organic acids found in OSPW could, therefore, be expected to be bound

to these clay particles making them unavailable for uptake into aquatic organisms such as fish. Humic acids have also been shown to reduce the bioconcentration of dehydroabietic acid and benzo(a)pyrene into *Daphnia magna* (Kukkonen and Oikari, 1987). They have also been demonstrated to reduce the uptake of other hydrophobic compounds into rainbow trout (Qiao and Farrel, 2002) and are also known to bind organic solutes primarily by hydrophobic interactions (Rav-Acha and Rhebun, 1992). It is possible that the hydrophobic alkyl groups of NAs could associate with the hydrophobic sites in humic acids in OSPW and thereby make them less bioavailable. It is also possible that there could be other compounds in the extract that might do this. Throughout the literature and in this study also, the term “naphthenic acids” has been used to describe the acid-extractable organics in OSPW. However, using ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, Grewer et al. (2010) have recently demonstrated that this terminology is misleading because <50% of the total abundance could be assigned to the classical (chemical formula $C_nH_{2n+Z}O_2$) and oxy-NAs. Most of the acid-extractable compounds in OSPW have yet to be identified and no one has isolated these compounds from classical NAs so it is, therefore, not known if unidentified compounds or classical NAs are responsible for the effect that the NAE had on the reproductive physiology of fathead minnows. However, using multidimensional comprehensive gas chromatography-mass spectrometry (GCxGC-MS) Rowland et al. (2011) has recently determined that some of the acid-extractable organics in OSPW have structural similarities with estrone and estradiol and this would suggest that these compounds could account for some of the endocrine disrupting properties reported in OSPW and OSPW extracts.

The results of this present study demonstrate that NAE impairs the reproductive physiology of fathead minnows. Due to the recycling of the OSPW in tailings ponds, both NAs and salts occur at high concentrations relative to surface waters in the region. Surprisingly HCO_3^-, which is also present in high concentrations in OSPW, reduces the toxicity of NAs. If HCO_3^- did not occur at high concentrations (>500 mg/l) in these ponds, the OSPW, even after aging, would likely be more acutely and chronically toxic. In order to promote the long term sustainability of fish in reclaimed areas with the oil

sands region, oil sands operators should endeavor to reduce the concentration of NAs in OSPW prior to the construction of end pit lakes.

CHAPTER 4. THE EFFECTS OF SALINITY AND OIL SANDS PROCESS-AFFECTED WATERS ON FATHEAD MINNOW REPRODUCTION

4.1 Abstract

Previous studies have shown that oil sands process-affected waters (OSPW) produced as a by-product in oil sands mining operations have adverse effects on the reproductive physiology of fish. Salts and acid-extractable organics are the two main constituents of OSPW. The acid-extractable organics in OSPW have been determined to have adverse effects on fathead minnow reproduction but the effects that inorganic ions may have on reproduction have not been examined. In this study, the interactive effects of salts and OSPW on the reproduction of fathead minnows were examined. These studies showed that exposure of fish for one month to a moderate concentration of salt (940 mg/L; 50% of OSPW) had no effect on the numbers of eggs spawned or male secondary sexual characteristics (tubercles). However, fish previously held in low salt conditions of a reference lake (93 mg/L) and then exposed for 3 weeks to the high salt concentrations found in OSPW (1890 mg/l) led to reduced egg production and tubercle numbers in males. The high salt concentrations found in OSPW did not affect the reproduction of fish previously acclimated to 50% salts. In a second experiment, exposure of fish for 6 weeks to moderate salt concentrations had no effect on reproduction. Subsequent exposure of these acclimated fish to the high salt concentrations equivalent to OSPW had no effect on reproduction. In contrast, salt acclimated fish exposed to OSPW had a 46% reduction in egg production although this was not statistically significant. Collectively, these results suggest that if fathead minnows are not acclimated to the higher salinities found in OSPW, then salts alone can impair reproduction. This study demonstrates the importance of taking into account differences in water quality characteristics (e.g. salinity) when conducting bioassays with complex industrial effluents.

4.2 Introduction

Large volumes of oil sands process-affected waters (OSPW) are produced as a by-product of oil sands mining extraction technologies used in the Athabasca oil sands region of Alberta. Naphthenic acids (NAs) and inorganic ions (such as Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) are concentrated in OSPW reaching levels of 110 mg/l and over 2000 mg/l (total), respectively (FTFC, 1995; Zubot et al., 2012). Fathead minnow reproduction bioassays have shown that OSPW and NAs can impair reproduction (numbers of eggs laid) and reduce secondary sexual characteristics and plasma sex steroids in male fathead minnows (Kavanagh et al., 2011; Kavanagh et al., 2012). When conducting these tests with OSPW from Syncrude Canada Ltd. (Syncrude), fathead minnows were transferred from low salinity water (reference condition found in Gregoire Lake) to high salinity water (OSPW) without any prior acclimation to the higher salinities (Kavanagh et al., 2011). Given this experimental paradigm it is not possible to determine if NAs, salt concentration or other constituents in OSPW contributed to the reproductive effects that were observed. It is possible that the abrupt change in salinity alone could affect fathead minnow reproduction. A sudden increase in conductivity (almost 20 fold) is not something that fathead minnows would typically encounter naturally in the environment and could induce a stress response. Stress can have an adverse effect on the reproductive physiology of fish including a reduction in plasma sex steroid levels (Campbell et al., 1994; Castranova et al., 2005; Fuzzen et al., 2011; Pickering et al., 1987). High concentrations of inorganic ions have been shown to adversely affect fish reproduction and recruitment. McCarraher and Thomas (1968) observed that fathead minnow reproduction and abundance was adversely effected in lakes that had total alkalinities of 1800 mg/l or more. An examination of 27 small watersheds in Eastern Wisconsin, USA determined that fathead minnows from streams with higher conductivities produced fewer eggs (Corsi et al., 2011).

In this study, an initial experiment was conducted to determine whether a sudden change in salinity could adversely affect fathead minnow reproduction. For one month, two groups of fathead minnows were held in a 1000 L tank containing Gregoire Lake water (control and non-acclimated treatment group) which had a salt concentration of 93

mg/l and a third group was held in a 500 l tank containing Gregorie Lake water with salts added at concentrations equivalent to 50% of the salts observed in Pond 9 OSPW (940 mg/l; acclimated). Fathead minnows were then transferred to 38 l tanks containing the same waters and polyvinyl chloride (PVC) pipe as a spawning substrate and the numbers of eggs spawned were then monitored over a 14 day period. The next phase of the experiment involved continued holding of the control group in Gregoire Lake water and transferring the non-acclimated and acclimated fish to 100% of the salt concentration observed in Pond 9 (1789 mg/l). Various reproductive endpoints (egg production, secondary sexual characteristic expression, fertility, hatching success, and survival to swim-up) were monitored over a 21 day period.

A second experiment was conducted to determine whether acclimation of fathead minnows to high salinities for a period of one month would modify the subsequent response of the fish to OSPW. For one month, one group of fathead minnows (control) were held in a 500 l tank containing Gregoire Lake water and two groups of fathead minnows (salt control and Pond 9 OSPW treatment) were held in Gregorie Lake water with salts added at concentrations equivalent to 50% of the salts observed in Pond 9 OSPW (940 mg/l). Fathead minnows were then transferred to 38 l tanks containing the same waters and PVC pipe for spawning substrate and the numbers of eggs spawned were then monitored over a 14 day period. The next phase of the experiment involved continued holding of the control group in Gregoire Lake water and transferring the acclimated fish to either Gregoire Lake water with salts added at concentrations equivalent to Pond 9 OSPW (1789 mg/l; salt control) or Pond 9 OSPW (Pond 9 treatment). Reproductive endpoints as listed above were then monitored over a 21 day period.

4.3 Materials and Methods

4.3.1 Collection of Water

Two sources of water were used in these experiments. In 2006, reference water was collected from Gregoire Lake (56°27′06″N, 111°07′38″W), a clean lake located

approximately 15 km south of Fort McMurray, Alberta. OSPW was collected from Pond 9 (57°05.020′N, 111°41.50′W) on Syncrude's Mildred Lake lease site, which was a man-made pond of approximately 0.4 hectares in size that was constructed in 1993 to evaluate the wet landscape option for handling oil sands fluid tailings. Pond 9 was filled with 50, 000 m^3 of tailings pond water. Water was collected from both sites using a submersible pump positioned approximately 1.0 m below the surface and then stored in 1000 L polypropylene containers before they were used. Water from both sites was characterized at Syncrude's Research Facility in Edmonton, AB using standard aquatic chemical methods (Table 4.1). The concentrations of total NAs were measured using Fourier transform infrared (FTIR) spectroscopy (Holowenko et al., 2001). Kodak NAs (Eastman Kodak Company, Rochester, NY) were used as standards in the FTIR analysis.

Table 4.1 Water chemistry of Syncrude's Pond 9 and Gregoire Lake waters that were used in the fathead minnow reproduction assays. The characteristics of salt amended Gregoire Lake waters are also reported. This includes Gregoire Lake with salts added to achieve approximately 50% and 100% of the concentrations previously measured in Pond 9.

	GL	GL-50 % Salts	GL-100 % salts	SC P9
pH	7.6	8.4	8.5	8.6
Conductivity (μS/cm)	142	1165	2425	2180
Naphthenic acids (mg/l)	<0.5	<0.5	<0.5	21
Na^+ (mg/l)	5	271	537	470
Cl^- (mg/l)	2	120	239	230
CO_3^{2-} (mg/l)	<5	11	22	33
HCO_3^- (mg/l)	81	431	781	674
SO_4^{2-} (mg/l)	5	107	210	145

GL = Gregoire Lake, SC P9 = Syncrude Canada Pond 9, GL-50% Salts= Gregoire Lake water with salts added at concentrations equivalent to 50% Pond 9 OSPW, GL-100% Salts= Gregoire Lake water with salts added at concentrations equivalent to Pond 9 OSPW.

4.3.2 *Experiment 1: Salt Exposure*

Fathead minnows (age: 6 months) were purchased from Aquatic Research Organisms (Hampton, NH, USA) and raised at the University of Guelph for several months before being transported to Fort McMurray, AB, Canada. For one month prior to the start of the experiment, fathead minnows for the control and non-acclimated treatments groups were held in 1000 l tanks in water collected from Gregoire Lake while fathead minnows for the acclimated treatment group were held in Gregoire Lake water with the following salts added to it: NaCl at 195 mg/l, Na_2CO_3 at 19 mg/l, $NaHCO_3$ at 482 mg/l, and Na_2SO_4 at 152 mg/l (Table 4.2). The salts were added at concentrations equivalent to 50% of what has been observed in Pond 9 OSPW. A 50 % concentration was used because conductivities at 1200-1400 µS/cm were shown to have no effect on fathead minnow reproduction (Kavanagh et al., 2011). These salts were added to minimize the stress on fish that would otherwise occur if fish were moved from a low salinity water (Gregoire Lake) to a high salinity water (salts equivalent to Pond 9 OSPW) during the exposure phase of the experiment.

Fathead minnows were then transferred to 38 l tanks that had PVC pipe for spawning and contained the same water that they had been held in during the 1 month acclimation period. Over the next 14 days, their fecundity, fertility, and hatching were monitored. Subsequently, fathead minnows in the control group were held for 21 days in Gregoire Lake water while fathead minnows in the acclimated and non-acclimated treatment groups were held in Gregoire Lake water with salts added at concentrations equivalent to Pond 9 OSPW (NaCl at 391 mg/l, Na_2CO_3 at 39 mg/l, $NaHCO_3$ at 963 mg/l, and Na_2SO_4 at 303 mg/l).

Table 4.2 Description of waters used during the Experiment 1 reproduction assay.

	Holding water used in the experimental groups		
	Control	**Non-acclimated**	**Acclimated**
1 Month Acclimation	Gregoire	Gregoire	Gregoire plus 50 % salts
Initial 14 Day reproduction trial	Gregoire	Gregoire	Gregoire plus 50 % salts
Secondary 21 Day reproduction trial	Gregoire	100 % salts (Equivalent to Pond 9)	100 % salts (Equivalent to Pond 9)

4.3.3 Experiment 2: Aged OSPW Exposure

Fathead minnows (age: 6 months) were purchased from Aquatic Research Organisms (Hampton, NH, USA) and raised at the University of Guelph for several months before being transported to Fort McMurray, AB, Canada. For one month prior to the start of the experiment, one group of fathead minnows for the control group were held in 1000 l tanks with water collected from Gregoire Lake while two groups of fathead minnows were held in Gregoire Lake water with salts added at concentrations equivalent to 50% of what has been observed in Pond 9 OSPW (see above and Table 4.3).

Fathead minnows were then transferred to 38 l tanks that had PVC pipe for spawning and contained the same water that they had been held in during the 1 month acclimation period. Over the next 14 days, their fecundity, fertility, and hatching were then monitored. The next phase of the experiment involved continued holding of the control group in Gregoire Lake water and transferring one group of the acclimated fish to Gregoire Lake water with salts added at concentrations equivalent to Pond 9 OSPW as in experiment 1 (salt control) or Pond 9 OSPW (Pond 9 treatment).

Table 4.3 Description of waters used during the Experiment 2 reproduction assay.

	Holding water used in the experimental groups		
	Control	**Salt Control**	**Pond 9 OSPW**
1 Month Acclimation	Gregoire	Gregoire plus 50 % salts	Gregoire plus 50 % salts
Initial 14 Day reproduction trial	Gregoire	Gregoire plus 50 % salts	Gregoire plus 50 % salts
Secondary 21 Day reproduction trial	Gregoire	Gregoire plus 100 % salts (Equivalent to Pond 9 OSPW)	Pond 9 OSPW

4.3.4 Assay methodology

Each of the tanks (n=4 per treatment) contained four female and two male fathead minnows and three halved PVC pipes for spawning substrate. The tanks were filled with 15 L of control or treatment water and placed inside a reservoir maintained at 25 ±1°C, under a photoperiod of 16 h light: 8 h dark. Tanks were cleaned and 100 % of the water was renewed every two days. Dissolved oxygen was maintained above 8 mg/l throughout the exposures and ammonia was <0.05 mg/l. Fathead minnows were fed frozen adult brine shrimp (*Artemia* sp.) *ad libitum* twice daily. The endpoints monitored included the number of spawns, fecundity (number of eggs spawned), fertility, hatching success, and survival to swim-up. Each morning spawning and fecundity were determined by counting the numbers of eggs laid on the PVC pipe. For the purposes of determining the number of spawns, eggs observed on a single piece of PVC pipe were counted as one spawn while eggs found on two pieces of PVC pipe were counted as two spawns. Fecundity was calculated by counting the number of eggs in each tank and then the total number of eggs per female per day was calculated for each tank. The fertility of all the eggs was determined under a dissecting microscope. A subsample of these eggs was kept to measure hatching success and survival to swim-up (96 h post hatch). This involved transferring 10 eggs into a Petri dish containing water from the corresponding treatment and placing them in an incubator (25 ± 2°C, 16 h light: 8 h dark). There were four Petri dishes for each tank and, where possible, each tank was evaluated twice. The eggs were checked daily for mortality and hatching, and any dead eggs or larvae were recorded and removed. Approximately 70% of the exposure solutions in the Petri dishes were changed daily.

4.3.5 Fish Sampling

At the end of the experiment, fathead minnows were anesthetized with tricaine methane sulfonate (MS-222; Sigma-Aldrich, Milwaukee, WI). The mass (g) and fork length (cm) of each fish was measured and the condition factor (CF) was calculated [CF

= 100 x total wt (g) / fork length^3 (cm)]. Fish were euthanized by spinal severance. Gonads were removed and weighed in order to calculate the gonadosomatic index (GSI) [GSI = 100 x gonadal tissue wt (g) / total wt (g)]. Tubercles, a secondary sex characteristic on male fathead minnows, were counted under a dissecting scope. Blood was collected from the caudal vessels, but, due to storage problems, the concentrations of sex steroids in the plasma could not be determined.

4.3.6 Statistical analysis

Male and female fish were analyzed separately. For all of the assays, each tank (n=4) was considered a replicate and no outliers were removed. A Levene's test was used to test data for homogeneity of variance. Data that did not meet parametric assumptions were log transformed for normalization and/or to reduce the heterogeneity in variance. One-way analysis of variance (ANOVA) was used to test for differences across all treatment groups. A Tukey’s post-hoc test was used to determine which treatment(s) differed from the control. Differences were considered significant at $p < 0.05$. All statistical analyses were conducted using SPSS 20.0; the data are presented as mean ± SE unless otherwise noted.

4.4. Results

4.4.1 Experiment 1: Salt Exposure

During the initial 14 day breeding trial, no significant differences were observed amongst the three groups. The mean fecundity rates (eggs/female/day) were 17.2 ± 2.1, 18.8 ± 5.4, and 16.6 ± 2.4 for the control fish (Gregoire Lake water), salt-acclimated fish (50 % salt), and non-acclimated fish (Gregoire Lake water), respectively. The mean number of spawns were 7.8 ± 0.5, 5.5 ± 0.6, and 6.3 ± 0.8 for the control fish, salt-acclimated fish, and non-acclimated fish, respectively. During the 21 day breeding trial, a significant reduction ($p<0.05$) in the mean fecundity rate (eggs/female/day) and number of spawns was observed in the non-acclimated fathead minnows when they were exposed to high salt conditions (Figure 4.1). The mean fecundity rates (eggs/female/day) were

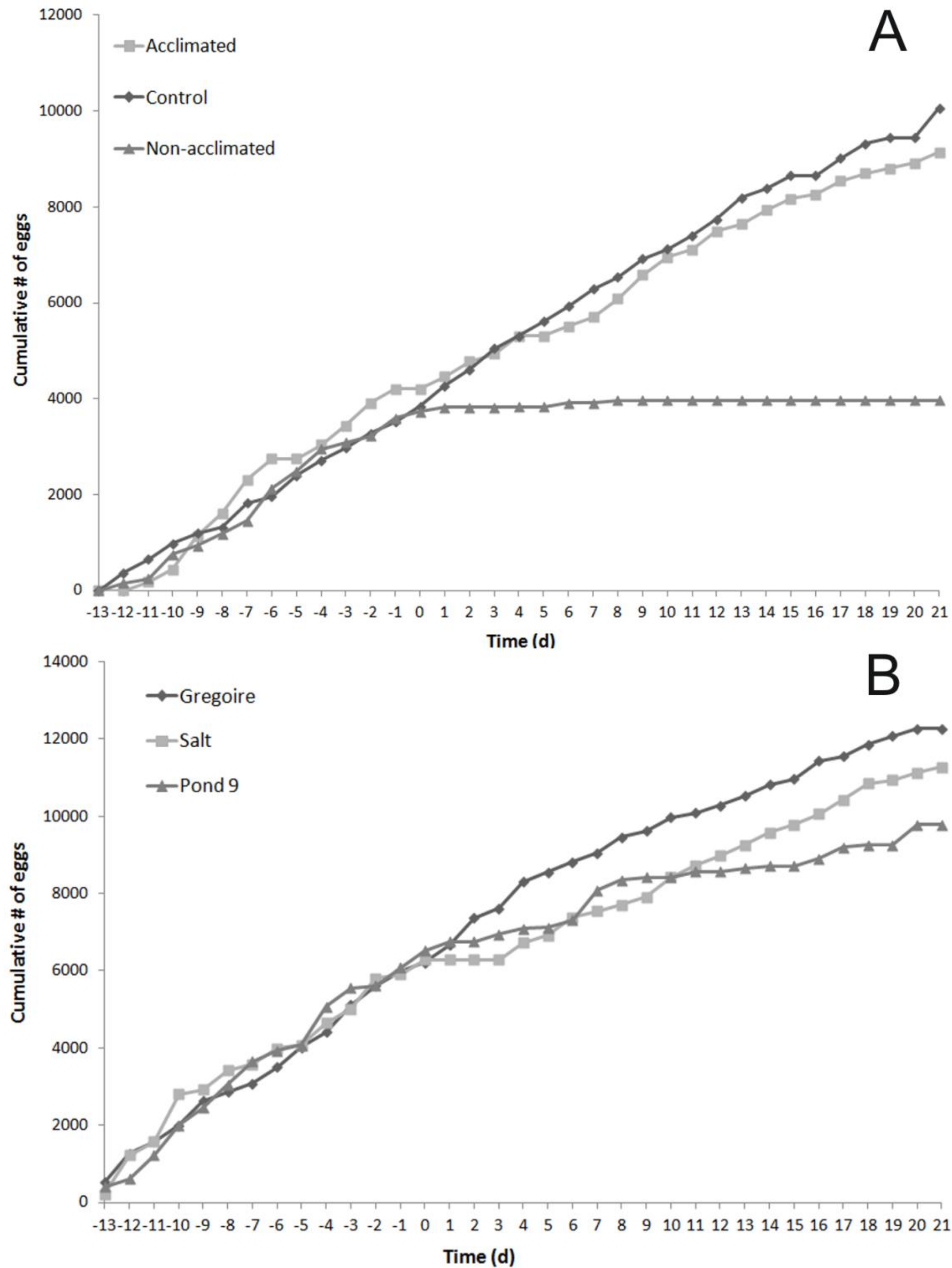

Figure 4.1. Cumulative number of eggs spawned by fathead minnows during the salt and Pond 9 OSPW exposures A) Graph shows fecundity during a 14 day breeding trial period prior to exposure to control, acclimated, and non-acclimated for 21 days. B) Graph shows fecundity during a 14 day breeding trial period prior to exposure to Gregoire Lake water (control), salt (salt control), and Pond 9 (Pond OSPW) for 21 days

18.5 ± 0.91, 14.7 ± 1.4, 0.7 ±0.19 for the control fish, salt-acclimated fish, and non-acclimated fish, respectively. The mean number of spawns were 10.0 ± 0.6, 8.5 ± 0.5, and 1.25 ± 0.3 for the control fish, salt-acclimated fish, and non-acclimated fish, respectively. Non-acclimated male fathead minnows had significantly lower numbers of tubercles relative to control fish. There were no significant differences in the length, mass, CF, and GSIs in the fathead minnows from any of the treatments (Table 4.4). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks. The fertility rate, hatching success, and survival to swim-up were not affected by any of the treatments (data not shown).

4.4.2 Experiment 2: Aged OSPW Exposure

During the initial 14 day breeding trial, no significant differences were observed amongst the three groups. The mean fecundity rates (eggs/female/day) were 27.7 ± 4.8, 28.1 ± 4.5, and 29.1 ± 4.2 for the control fish (Gregoire Lake water), salt control fish (50 % salt), and Pond 9 OSPW fish (50 % salt), respectively. The mean number of spawns 10.8 ± 1.7, 7.8 ± 0.63, and 9.3 ± 0.85 for the control fish (Gregoire Lake water), salt control fish (50 % salt), and Pond 9 OSPW fish (50 % salt), respectively. During the 21 day breeding trial, no significant reduction in the mean fecundity rate (eggs/female/day) and number of spawns was observed in any of the treatments (Figure 4.1). The mean fecundity rates (eggs/female/day) were, 18.0. ± 4.5, 15.1 ± 1.3, and 9.7 ± 2.8 for fathead minnows held in the control, salt control, and Pond 9 OSPW, respectively. The mean number of spawns for fathead minnows held in the control, salt control, and Pond 9 OSPW, were 8.75±0.75, 8.00±1.08, and 6.25±1.03, respectively. There were no significant differences in the length, mass, CF, GSIs, or numbers of tubercles in fathead minnows from any of the treatments (Table 4.4). There was less than 10 % mortality in the control tanks and no significant differences in the mortality for treatment tanks. The fertility rate, hatching success, and survival to swim-up were not affected by any of the treatments (data not shown).

Table 4.4. Length, mass, condition factor (CF), gonadosomatic indices (GSIs), and number of tubercles of the fathead minnows used in Experiments 1 and 2.

Experiment 1

Sex	Treatment	Length (cm)	Mass (g)	CF	GSI	Tubercles
M	Control	6.14±0.09	3.48±0.17	1.51±0.04	1.05±0.05	22.25±0.60
M	Salt (acclimated)	6.31±0.08	3.69±0.04	1.47±0.07	1.05±0.03	21.50±0.94
M	Salt (non-acclimated)	6.30±0.16	3.74±0.20	1.50±0.08	1.06±0.07	12.38±1.28*
F	Control	4.85±0.06	1.64±0.08	1.44±0.05	14.35±0.90	
F	Salt (acclimated)	4.89±0.11	1.66±0.10	1.41±0.06	11.93±1.78	
F	Salt (non-acclimated)	4.92±0.16	1.65±0.14	1.37±0.03	9.73±1.21	

Experiment 2

Sex	Treatment	Length (cm)	Mass (g)	CF	GSI	Tubercles
M	Control	6.15±0.25	3.45±0.27	1.48±0.08	1.10±0.15	18.63±1.34
M	Salt	6.10±0.16	3.27±0.26	1.44±0.09	1.09±0.16	19.88±2.59
M	Pond 9	6.09±0.12	3.13±0.09	1.39±0.05	1.20±0.11	20.63±1.28
F	Control	4.64±0.09	1.38±0.06	1.38±0.09	10.76±0.92	
F	Salt	4.46±0.26	1.34±0.08	1.65±0.43	10.21±0.63	
F	Pond 9	4.83±0.07	1.50±0.04	1.34±0.06	13.04±1.89	

Note: Values are means ± SE. Values with an * are significantly different ($p < 0.05$) compared with control fish. For each parameter, n=4.

4.5 Discussion

The present studies have shown that the high salts concentrations found in OSPW can have adverse effects on the reproductive physiology of fathead minnows. Specifically, transfer of fish from the low salt conditions in Gregoire Lake to the high salt concentrations found in OSPW led to a marked reduction in the numbers of eggs spawned and male secondary sex characteristics. The reproductive physiology of fathead minnows was not affected when fish were acclimated to moderate levels of salts at concentrations equivalent to 50 % of the levels found in OSPW. Moreover, these studies demonstrated that this acclimation can reduce the inhibitory actions that were associated with exposure to OSPW. Collectively, these results demonstrate that the high salt concentrations found in OSPW can impact the results of reproductive bioassays and illustrate need for alternate approaches when conducting these bioassays with OSPW.

Freshwater fish species exhibit a wide range of tolerances to salts and salinity has been known to affect the structure of stream-fish assemblages (Higgins and Wilde, 2005). Fathead minnows, for example, have been found in lakes with salt concentrations other species could not tolerate (Held and Peterka, 1974). It has been known for some time that high concentrations of salts may affect reproduction in fish (McCarraher and Thomas, 1968). An increase in salinity, for example, has been demonstrated to promote gonadal regression in the glass knife fish (*Eigenmannia virescens*; Kirschbaum, 1979). It has also been reported that at concentrations higher than those used in this study certain salts (e.g. CO_3^{2-}, SO_4^{2-}) may adversely affect the reproduction of even salt tolerant species such as the fathead minnow (Burnham and Peterka, 1975; McCarraher and Thomas, 1968).

Fathead minnows that were transferred from the low salinity water (Gregoire Lake) to the higher salinity water (salts equivalent to 100% OSPW) likely experienced stress due to the sudden change in salinity. The reproductive physiology of fish can be altered by exposure to stress (Campbell et al., 1994; Castranova et al., 2005; Pickering et al., 1987). The metabolic rate of fathead minnows has been observed to increase when exposed to higher salinity levels and this increase was attributed to the increased energetic costs of osmoregulation (Pistole et al., 2008). During the breeding season, male fathead minnows must maintain their sexually dimorphic appearance, defend their territory, mate, and care for their eggs (Unger, 1983). When fathead minnows were exposed to the higher salinities, energy may have been diverted from these activities and reallocated towards processes (e.g. osmoregulation) that would restore homeostasis (Beyers et al., 1999; Fuzzen et al., 2011).

While the concentrations of inorganic ions examined in this study did not affect the reproductive physiology of fathead minnows when fish were acclimated, salts as well as acid-extractable organics can be expected to increase in concentration in OSPW as operators become more efficient in their water recycling activities. Directive 74, which was recently issued to oil sands mining operators by the Energy Resources Conservation Board (ERCB), now requires operators to reduce the amount of fluid tailings that are being produced and stored in tailings ponds by 50% (ERCB, 2010b). This method of disposal will free significant amounts of water, which would otherwise trapped be in

MFT, andcan be used again in their operations; this will result in higher concentrations of acid-extractable organics and salts in OSPW.

A previous study demonstrated that OSPW from Syncrude Canada Ltd. and Suncor Energy Inc. (Suncor) can adversely affect the reproductive physiology of fathead minnows (Kavanagh et al., 2011). The acid-extractable organics in OSPW were suspected of being responsible and direct tests with an acid-extractable organics extract showed that these substances adversely affect fathead minnow reproduction in another study (Kavanagh et al., 2012). Fathead minnows were acclimated to higher salinities prior to exposure to Suncor OSPW and, thus, salts were not suspected of being involved in the reproductive impairment. However, in the case of the experiments with Pond 9 OSPW, it is possible that the salts adversely affected reproduction as there was no salinity acclimation prior to exposure to the Pond 9 OSPW (Kavanagh et al., 2011).

In the current study, a small number of tanks were used per treatment (n=4) and there was a high degree of variability in egg production (CV of up to 58 %). Melvin et al. (2009) reported that short-term reproductive bioassays with high variability and low sample size number do not have the power to detect changes less than 40 to 50 % in egg production. A review of 62 published adult fish reproductive papers showed that with current reproductive bioassay protocols only differences in egg production above 50 % were detected with confidence (Bosker et al., 2009). The present study egg production was 46 % lower in fathead minnows held in Pond 9 OSPW but this difference was not found to be significant. This 46% decline in egg production is most likely due to the acid-extractable organics (e.g. NAs) in OSPW as these constituents have been previously shown to adversely affect the reproductive physiology of fathead minnows (Kavanagh et al., 2012) and both OSPW and NAs have been determined to have estrogenic and anti-androgenic properties (He et al., 2010; Thomas et al., 2009). If a larger number of tanks per treatment were used and there was less variability amongst the control tanks, it is likely that the 46 % reduction in egg production would have been determined to be significant. Melvin et al. (2009) reports that to conclusively determine whether a change of less than 40 to 50% in egg production was significant approximately 10 replicates (i.e., aquaria) would be required for each treatment concentration.

Fertility, hatching success, and survival to swim-up were not affected by any of the treatments; however, on six separate occasions the eggs of fathead minnows held in Pond 9 OSPW ruptured during their handling so hatching data could not always be obtained. Despite being fertile, these eggs did not water harden. Soft eggs were also observed on two occasions with eggs collected from fish in the salt controls. Stressors have been known to affect gamete quality (Campbell et al., 1994; Schreck et a., 2001). It is not known why the eggs did not water harden. Higher total dissolved solids of 1420 mg/l and 964 mg/l have been observed to affect embryo water absorption of Arctic Grayling (*Thymallus arcticus*) and Dolly Varden (*Salvelinus malma*), respectively (Brix et al., 2010). Metals, such as cadmium and zinc, have also been observed to delay the processes of water hardening and water uptake in embyros thereby making them more vulnerable to breakage (Brungs, 1969, Alderdice et al., 1979). Male fathead minnows rub their dorsal fatpads against their eggs purportedly for the purpose of aerating and cleaning the eggs and assisting the hatching of the young (Smith and Murphy, 1974); eggs that did not water harden, therefore, could easily rupture during this type of activity.

This study has determined that the inorganic ions in OSPW can adversely affect the reproduction of fathead minnows if they are not acclimated to these salts prior to exposure. Importantly, these studies show that the prior exposure and acclimation to high salinity conditions can modify the response to OSPW. This needs to be considered when conducting fish reproduction bioassays with complex industrial effluents. It is important to consider that species such as fathead minnows exhibit a wide range of tolerance to high salinities. The operators of tailings ponds should consider that many of the species that are endemic to the oil sands region may not have the tolerance to survive let alone reproduce in the high salinity waters that will likely characterize tailings ponds. This represents a significant unknown in predicting the feasibility of the wet landscape option for oil sands operators.

CHAPTER 5. REPRODUCTIVE AND HEALTH ASSESSMENT OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) INHABITING A POND CONTAINING OIL SANDS PROCESS-AFFECTED WATERS

5.1 Abstract

Previous laboratory based studies have shown that oil sands process-affected waters (OSPWs) containing high concentrations of naphthenic acids (> 25 mg/l) have adverse effects on the reproductive physiology of fish. The purpose of this study was to assess the reproductive development and health of a wild population of fathead minnows (*Pimephales promelas*) inhabiting an OSPW pond that has moderate concentrations of naphthenic acids (~10 mg/l). Fathead minnows were collected at various times during the period of 2006 through 2008 from Demonstration Pond (OSPW) located at Syncrude Canada Ltd., and two reference sites, Beaver Creek reservoir and Poplar Creek reservoir, which are all north of Fort McMurray, AB, Canada. Condition factor, gill histopathology, gonadosomatic indices, liver somatic indices, male secondary sexual characteristics, and plasma sex steroids were examined. Depending on the time of year that fathead minnows were collected, there were differences in the condition factor, gonadosomatic indices, liver somatic indices, and secondary sexual characteristics of fathead minnows (in males) from Demonstration Pond when compared to the fathead minnows from the reference sites. In comparison to reference fish, lower concentrations of 11-ketotestosterone were measured in the plasma of male fathead minnows collected from Demonstration Pond in June 2006 and July 2007. Black spot disease and *Ligula intestinalis* were prevalent in fathead minnows from the reference sites but were not observed in fathead minnows from Demonstration Pond. The opercula of fathead minnows from Demonstration Pond also differed from those of reference fish. An examination of the gills of fathead minnows from Demonstration Pond revealed that were a number of proliferative and degenerative alterations relative to reference fish. Even though the fathead minnow population has been maintained in this OSPW pond since 1993, the results of this study demonstrate that the OSPW continues to affect the reproductive development and health of the fathead minnows compared to fish collected at reference sites.

5.2 Introduction

The Athabasca oil sands deposit, located in northern Alberta, Canada, is an important unconventional oil source. Oil sands can be recovered using surface mining when the oil sands formations lie within 75 m of the surface (Alberta Energy, 2012). With surface mining operations, large volumes of fluid tailings (residual bitumen, sand, clay, and water) are produced when the bitumen is separated from the sand. This material is transported by pipelines to settling basins where, over time, some dewatering of the fluid tailings occurs. The water that is released (oil sands process-affected waters; OSPW) is reused in the oil sands extraction process, however, a significant amount of water remains trapped in the tailings and this has resulted in approximately 840 million m^3 of tailings currently being stored in settling basins (ERCB, 2010a). As part of their wet landscape reclamation option, developers of oil sands intend to transfer these mature fine tailings (MFT, ~60 % water) to mined-out pits where they will be capped with either a layer of natural surface water or OSPW. After 40 years of oil sands development, Syncrude Canada Ltd. is creating the first end pit lake in the oil sands region. This end pit lake and the others that follow will be expected to develop viable ecosystems with similar biological capability to natural lakes in the region. This may be difficult to achieve because OSPW contains elevated concentrations of acid-extractable organic acids (e.g., naphthenic acids) and inorganic ions (e.g., Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) and low concentrations of polycyclic aromatic hydrocarbons (PAHs), all of which may be acutely and chronically toxic to aquatic organisms.

Numerous studies have examined the toxicological effects of OSPW. Yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) larvae exposed to freshly produced OSPW exhibit higher incidences of deformities (e.g., optic-cephalic abnormalities, spinal deformities, and edemas) compared to control fish (Peters et al., 2007). Similar deformities were observed in fathead minnow (*Pimephales promelas*) and white sucker (*Catostomus commersoni*) larvae that were exposed to sediment from the oil sands region and from a wastewater pond from Suncor Energy Inc. (Colavecchia et al., 2004; Colavecchia et al., 2006). The lethal toxicity associated with fresh OSPW has been

demonstrated to disappear after 1-2 years (MacKinnon and Boerger, 1986); however, chronic toxicity is still associated with aged OSPW. Body mass and skeletal size were found to be significantly lower in mallard (*Anna platyrhynchos*) ducklings after 2, 5, 9, and 13 days of exposure to wetlands containing aged Suncor OSPW (Gurney et al., 2005). Significantly reduced growth, delayed metamorphosis, and lower survival were reported for northern Canadian toads (*Bufo boreas*) and wood frogs (*Rana pipiens*) exposed to aged Suncor OSPW (Pollet and Bendell-Young, 2000). Histopathological changes in gill and liver tissue and increases in liver size and hepatic mixed-function oxygenase (MFO) activity have also been observed when yellow perch were exposed to aged OSPW (Nero et al., 2006b; van den Heuvel et al., 1999; van den Heuvel et al., 2000). *In vitro* studies have shown that OSPW can decrease production of testosterone and increase that of 17β-estradiol by cultured H295R cells (He et al., 2010). Goldfish (*Carassius auratus*) and yellow perch held in ponds containing aged OSPW had lower concentrations of plasma steroids than reference fish (Lister et al., 2008; van den Heuvel et al., 2012) while fathead minnows exposed to aged OSPW and an extract of naphthenic acids and other acid-extractable organics (produced from OSPW) in the laboratory were shown to have lower fecundity, lower concentrations of plasma sex steroids, and a reduction in male secondary sexual characteristics (Kavanagh et al., 2011, 2012).

To evaluate the wet landscape reclamation option, Syncrude Canada Ltd. constructed a series of test ponds on part of their lease in northern Alberta and filled them with various amounts of MFT, OSPW, and surface water. When Demonstration Pond was capped with surface water, fathead minnows were unintentionally introduced to the test pond and populations of these fish have persisted through the time of this study. The primary objective of this study was to assess the reproductive physiology and health of the fathead minnows inhabiting this OSPW pond. In 2006-2008, fathead minnows were collected from Demonstration Pond and compared to fathead minnows collected from two reference sites within the oil sands region. Condition factor, gill histopathology, gonadosomatic indices, liver somatic indices, male secondary sexual characteristics, and plasma sex steroids were examined.

5.3 Materials and Methods

5.3.1 Collection of Fish

In June 2006, July 2007, August 2007, May 2008, and June 2008, fathead minnows were collected from Demonstration Pond (57°4'57.23" N, 111°41'23.06" W) on Syncrude Canada's Mildred Lake lease site. In June 2006, fathead minnows were collected from Poplar Creek reservoir, a reference site (56°55'9.09" N, 111°29'49.86" W), and in July 2007, August 2007, May 2008, and June 2008, fathead minnows were collected from two reference sites, Poplar Creek reservoir and Beaver Creek reservoir (56°59'16.81" N, 111°36'22.02" W). Demonstration Pond is a 40 000 m^2 pond that was filled with 70 000 m^3 of MFT and capped with 80 000 m^3 of unprocessed water. Since 1993, a viable population of fathead minnows has been present in this pond. The fathead minnows were collected by setting minnow traps overnight for approximately 16 h. The fathead minnows were transferred to aerated buckets and transported to an on-lease laboratory trailer for processing.

5.3.2 Fish Sampling

At each sampling period, 20 male and 20 female fathead minnows from Demonstration Pond and the reference sites were randomly selected and anesthetized with tricaine methane sulfonate (MS-222; Sigma-Aldrich, Milwaukee, WI). The mass (g) and fork length (cm) of each fish was measured and the condition factor (CF) was calculated [CF = 100 x total wt (g) / standard length^3 (cm)]. The presence of any parasites, such as metacercariae of trematodes (black spot disease) and tapeworms was recorded for each fish and were included in the analyses. For fish sampled in June 2006 and July 2007, blood was collected from the caudal artery or vein with a heparinized microcapillary tube and the plasma was separated by centrifugation (13000 X g for 6 min) and stored at –20 °C. After extracting the plasma three times with diethyl ether, concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone in plasma were determined using enzyme immuno assays (EIA; Cayman Chemical) as per the manufacturer's instructions. Dilutions of the samples were shown to be parallel to the

standard curves. Fish were euthanized by spinal severance. Gonads, livers, and spleens were weighed in order to calculate the gonadosomatic index [GSI = 100 x gonadal tissue wt (g) / total wt (g)], liver somatic index [LSI = 100 x liver tissue wt (g) / total wt (g)], and spleen-somatic index [SSI = 100 x spleen weight (g) / body weight (g)]. Tubercles, a secondary sexual characteristic on male fathead minnows, were counted under a dissecting microscope at 10X. In June 2006, fish were fixed in 10% neutral buffered formalin for ~1 week (Fisher-Scientific, Whitby, ON) and then stored in 70% ethanol. The second gill arch of 6 fish per site were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin at the University of Guelph Animal Health Laboratory. All slides were examined under a light microscope without prior knowledge of the sample identity.

In May and June 2008, fathead minnows were fixed in a 10% formalin buffered solution, and then stored in a 70% ethanol solution until the mass, fork length, CF, GSI, LSI, and number of tubercles on these fish could be measured as described previously.

5.3.3 Water Quality

Syncrude Canada Ltd. provided the water quality characteristics (depth = 0 - 1 m) for all of these sites using standard aquatic chemical methods at Syncrude's Research facility in Edmonton, AB (Table 5.1). The naphthenic acid concentrations were measured using Fourier transform infrared (FTIR) spectroscopy (Jivraj et al., 1995; Holowenko et al., 2001). Kodak naphthenic acids (Eastman Kodak Company, Rochester, NY) were used as standards in the FTIR analysis.

5.3.4 Statistical Analysis

Male and female fish data were analyzed separately. A Levene's test was used to test data for homogeneity of variance. Data that did not meet parametric assumptions were log transformed for normalization and/or to reduce the heterogeneity in variance. A t-test was used to test for difference amongst the 2006 data. One-way analysis of variance (ANOVA) was used to test for differences across the treatment groups for 2007 and 2008 data. A Tukey's post hoc test was used to determine which treatment(s) differed from the

control. Differences were considered significant at $p < 0.05$. In cases when data did not meet the parametric assumptions of the ANOVA even after log transformation, Kruskall-Wallis and Mann-Whitney U tests were used and a Bonferroni correction was applied. All statistical analyses were conducted using SPSS 19.0. The data are presented as mean ±SE unless otherwise noted.

Table 5.1. Mean (± SE, n) water chemistry concentrations for Beaver Creek reservoir (2001-2006), Demonstration Pond (2001-2008), and Poplar Creek reservoir (2001-2006).

Site	pH	Conductivity (uS/cm)	Naphthenic Acids (mg/l)	Na^+ (mg/l)	Cl^- (mg/l)	SO_4^{2-} (mg/l)	HCO_3^- (mg/l)
Demo	8.88 (0.1, 40)	1433 (24, 40)	10.8 (0.5, 37)	331 (6, 40)	93.8 (2, 40)	150 (3, 40)	554 (9, 40)
Beaver	7.95 (0.3, 6)	279 (19, 7)	0.86 (0.2, 7)	30.4 (2, 9)	1.92 (0.4, 9)	16.9 (1, 9)	163 (15, 9)
Poplar	8.15 (0.2, 7)	299 (21, 8)	1.25 (0.7, 4)	34.7 (2, 9)	2.70 (0.4, 9)	16.1 (2, 9)	196 (13, 9)

Demo = Demonstration Pond, Poplar = Poplar Creek reservoir, Beaver = Beaver Creek reservoir.

5.4 Results

The length of male and female fathead minnows varied markedly across the different sampling times (Figure 5.1). Fathead minnows from Demonstration pond were either shorter, longer or did not change relative to the fathead minnows from reference sites. Similarly, the mass of male and female fathead minnows varied during the different sampling periods with no consistent trends being observed (Figure 5.1). The CF of male and female fathead minnows from Demonstration Pond was generally significantly higher when compared to fathead minnows from reference sites (Figure 5.1).

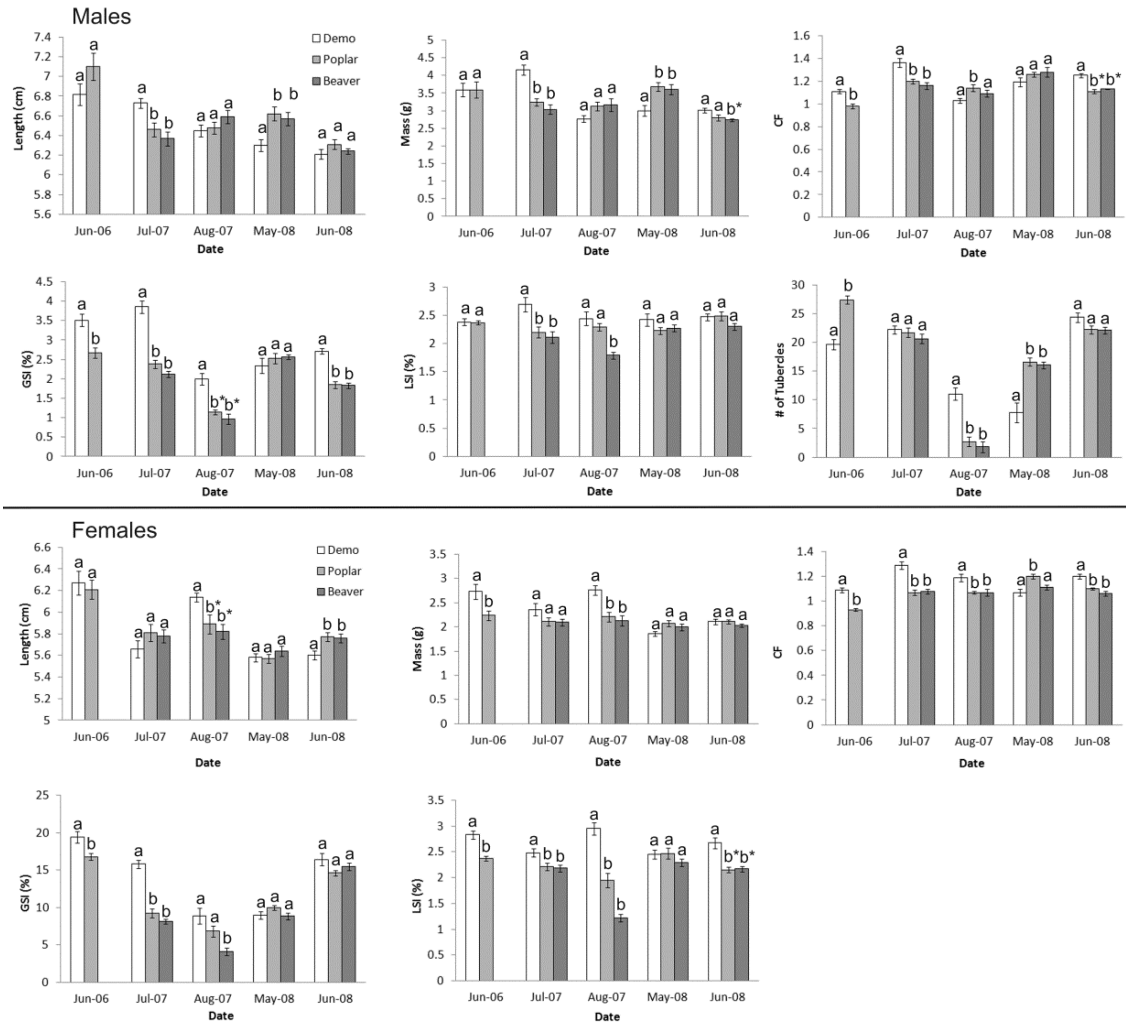

Figure 5.1. Length, mass, condition factor (CF), gonadosomatic indices (GSIs), liver somatic indices (LSIs), and number of tubercles in fathead minnows collected from Demonstration Pond and two reference sites.

Values that are not significantly different share common superscripts; $p<0.05$ unless otherwise indicated. Demo = Demonstration Pond, Poplar = Poplar Creek reservoir, Beaver = Beaver Creek reservoir, n= 20 for each group, "*"= $p<0.0167$.

With the exception of the fathead minnows collected in May 2008, male fathead minnows from Demonstration Pond had significantly larger GSIs in comparison to fathead minnows from reference sites (Figure 5.1). Female fathead minnows from Demonstration Pond had significantly larger GSIs in June 2006, July 2007, and August

2007 when compared to fathead minnows from reference sites (Figure 5.1). Male fathead minnows from Demonstration Pond had significantly larger LSIs in July 2007 and August 2007 in comparison to fathead minnows from reference sites. With the exception of the female fathead minnows collected in May 2008, female fathead minnows from Demonstration Pond had significantly larger LSIs when compared to fathead minnows from reference sites (Figure 5.1). Male fathead minnows from Demonstration Pond had significantly lower numbers of tubercles in June 2006 and May 2008 when compared to fathead minnows from reference sites. The reverse was observed in August 2007 with significantly larger numbers of tubercles being observed in fathead minnows from Demonstration Pond in comparison to fathead minnows from reference sites (Figure 5.1). In June 2006 and July 2007, significantly lower concentrations of 11-ketotestosterone were measured in the plasma of male fathead minnows from Demonstration Pond relative to fathead minnows from reference sites (Figure 5.2; $p \leq 0.05$).

For all sampling periods, male and female fathead minnows from Poplar Creek and Beaver Creek reservoir had significantly larger SSIs compared to fathead minnows from Demonstration pond (Table 5.2; $p \leq 0.05$). A number of white nodules were observed throughout the enlarged spleens. At the time of sexual maturity, male fathead minnows develop dark heads and dark vertical bands. All of the male fathead minnows from Demonstration Pond were observed to be significantly lighter in color in comparison to reference fish (Figure 5.3).

Every fathead minnow collected from Demonstration pond also appeared to have abnormal opercula. The opercula of these fish were rounded, extending outwards to varying degrees (Figure 5.3). This deformity was not observed in any of the fathead minnows collected from Poplar Creek reservoir and Beaver Creek reservoir. A histological examination of the fathead minnows collected in 2006 revealed that were a number of proliferative and degenerative alterations in the gills of fathead minnows from Demonstration Pond and Poplar Creek (Figure 5.4). Epithelial cell hypertrophy was observed along with a proliferation of epithelial cells at the base and tips of some of the secondary lamellae. A large number of mucous cells were also observed at the base of some of the secondary lamellae. There was also mild epithelial necrosis noted and it was

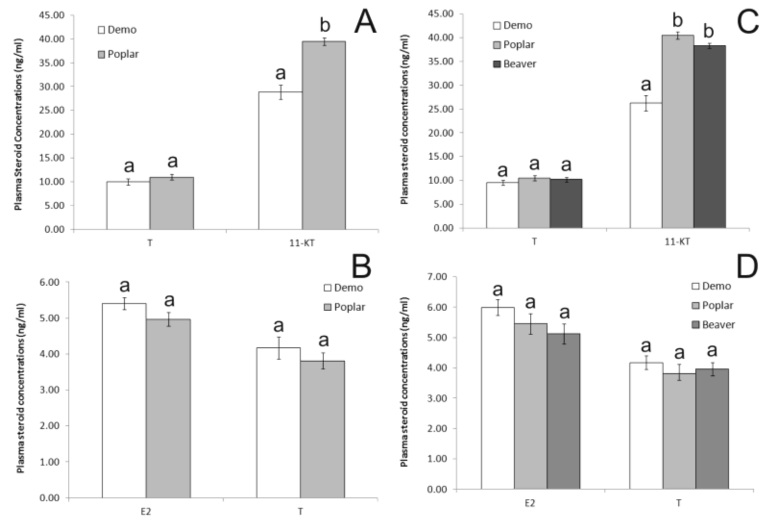

Figure 5.2. Plasma steroid concentrations measured in A) Male fathead minnows collected in June 2006, B) Female fathead minnows collected in July 2006, C) Male fathead minnows collected in July 2007, D) Female fathead minnows collected in July 2007.

Demo = Demonstration Pond, Poplar = Poplar Creek reservoir, Beaver = Beaver Creek reservoir, n= 20 for each group, T= testosterone, 11-KT= 11-ketotestosterone, E2= 17β-estradiol. Values that are not significantly different share common superscripts; $p<0.05$ unless otherwise indicated.

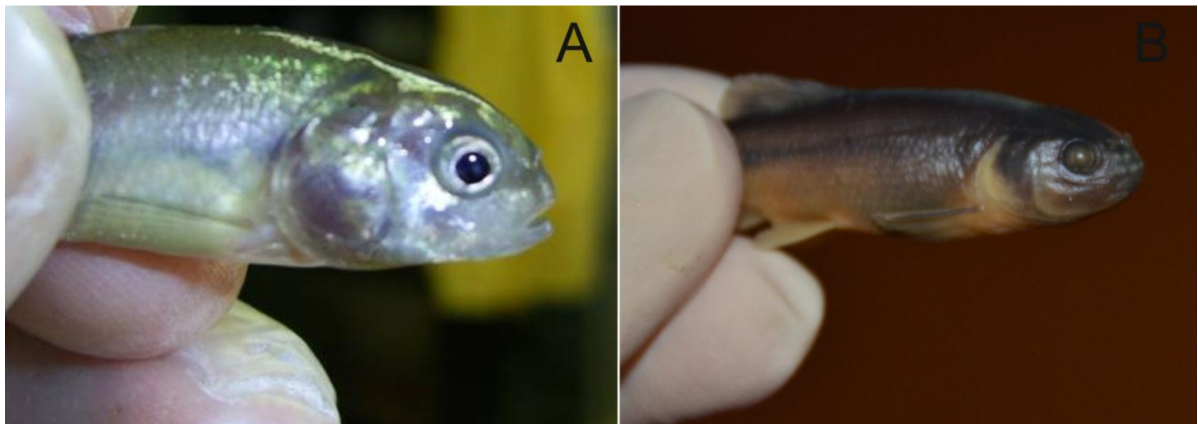

Figure 5.3. A) Fathead minnow from Demonstration Pond displaying abnormal operculum and the light body coloration, B) Fathead minnow from Popular Creek with normal operculum and the darker coloration typical of males.

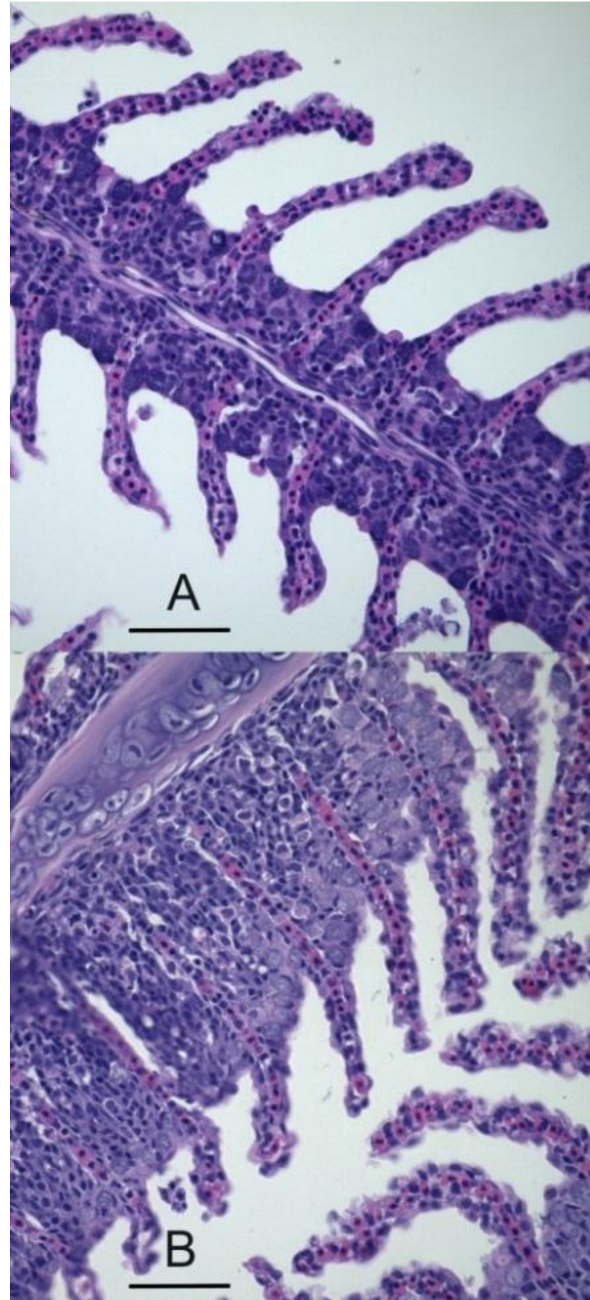

Figure 5.4. Gill histopathology of fathead minnows collected from Poplar Creek reservoir (A) and Demonstration Pond (B). A) Gill section displaying a slightly increased number of mucous cells, B) Proliferation of mucous cells and epithelial cells at base of secondary lamellae.

Bar = 100 μm

Table 5.2. Mean (± SE) spleen-somatic indices (SSIs), black spot prevalence, and tapeworm prevalence of fathead minnows collected from Demonstration Pond and two reference sites.

Year	Month	Sex	Site	SSI	Black Spot Prevalence %	Tapeworm Prevalence %
2006	June	Male	Demo	$0.27 \pm .01^{a}$	0.0	0.0
			Poplar	$0.65 \pm .01^{b}$	5.0	50.0
		Female	Demo	$0.26 \pm .01^{a}$	0.0	0.0
			Poplar	$0.57 \pm .02^{b}$	5.0	55.0
2007	July	Male	Demo	$0.44 \pm .03^{a}$	0.0	0.0
			Poplar	$0.58 \pm .05^{b}$	5.0	65.0
			Beaver	$0.65 \pm .04^{b}$	60.0	25.0
		Female	Demo	$0.25 \pm .02^{a}$	0.0	0.0
			Poplar	$0.63 \pm .04^{a}$	10.0	60.0
			Beaver	$0.58 \pm .03^{b}$	60.0	25.0
2007	August	Male	Demo	$0.28 \pm .03^{a}$	0.0	0.0
			Poplar	$0.61 \pm .04^{b}$	5.0	65.0
			Beaver	$0.62 \pm .01^{b}$	55.0	25.0
		Female	Demo	$0.30 \pm .03^{a}$	0.0	0.0
			Poplar	$0.52 \pm .05^{b*}$	5.0	55.0
			Beaver	$0.52 \pm .03^{b*}$	50.0	35.0
2008	May	Male	Demo	---	0.0	0.0
			Poplar	---	20.0	40.0
			Beaver	---	30.0	50.0
		Female	Demo	---	0.0	0.0
			Poplar	---	50.0	20.0
			Beaver	---	80.0	50.0
2008	June	Male	Demo	---	0.0	0.0
			Poplar	---	35.0	20.0
			Beaver	---	30.0	45.0
		Female	Demo	---	0.0	0.0
			Poplar	---	50.0	25.0
			Beaver	---	70.0	45.0

Values that are not significantly different share common superscripts; $p<0.05$ unless otherwise indicated. Demo = Demonstration Pond, Poplar = Poplar Creek reservoir, Beaver = Beaver Creek reservoir, n =20 for each group, “*”= $p<0.0167$.

accompanied by a moderate number of inflammatory cells such as lymphocytes and macrophages. These morphological changes were expressed to a greater degree in fathead minnows from Demonstration Pond (Figure 5.4).

Between and surrounding the gill filaments, dozens of protozoans were observed. These round but dorsoventrally-flattened organisms had a basophilic nucleus, were ~100 μm in diameter, and had a circular ring with hook-like denticles. The morphology of the parasite is characteristic of *Trichodina* spp. These parasites were also observed in fathead minnows from Poplar Creek reservoir and Beaver Creek reservoir. Black spot (trematode) and tapeworms (*Ligula intestinalis*) were also observed in fathead minnows collected from Beaver Creek reservoir and Poplar Creek reservoir but these parasites were not observed in the fathead minnows from Demonstration pond (Table 5.2).

5.5 Discussion

The current study has demonstrated significant differences in the general condition and reproductive physiology of the fathead minnows from Demonstration Pond compared to the fathead minnows from Beaver Creek and Poplar Creek reservoir. In particular, there were marked differences in the expression of male secondary sexual characteristics in the fathead minnows collected from Demonstration Pond. In Alberta, the fathead minnow breeding season typically begins in late May and ends in July or early August (McMillan and Smith, 1974; Price et al., 1991). Fathead minnows begin to display their sexual dimorphism approximately 30 days prior to spawning, with females developing an ovipositor, a fleshy protuberance used for laying eggs (Flickinger, 1969). As male fathead minnows mature sexually, they develop nuptial tubercles, a dorsal fatpad, a darker colored head, and dark vertical bands (Smith 1978). The GSIs of male and female fathead minnows have been observed to be the largest in June and have been observed to decrease in size by August (Smith, 1978). The number of tubercles on male fathead minnows has also been reported to decrease at the end of the breeding season (Tetreault et al., 2012). This pattern was observed in fathead minnows collected from Poplar Creek reservoir and Beaver Creek reservoir. Fathead minnows from the reference sites had

significantly larger numbers of tubercles in May (2008) compared to fathead minnows from Demonstration Pond while the reverse was observed in August (2007). Male and female fathead minnows from Demonstration Pond also had significantly larger GSIs than reference fish in August. These variations could suggest that the sexual maturity of male fathead minnows from Demonstration Pond was delayed and that spawning may have been offset to a later date. Delays in the sexual maturation of fish have been previously observed with industrial effluents such as those from pulp and paper mills and sewage treatment plant effluents (McMaster et al., 1992; Tetreault et al., 2012) and in laboratory exposures with anti-androgens (Bayley et al., 2002). It is possible that one of the constituents in OSPW disrupted the endocrine system of the fathead minnows and delayed their sexual maturity; a previous study demonstrated that OSPW can reduce steroid concentrations in plasma, male secondary sexual characteristics, and fecundity in fathead minnows (Kavanagh et al., 2011). Similar results were later achieved with an OSPW extract containing naphthenic acids and other acid-extractable organic acids which suggests that these organic acids are responsible for the adverse effects observed when fish are exposed to OSPW (Kavanagh et al., 2012).

While the constituents (e.g., organic acids) of OSPW may have delayed the sexual maturation of fathead minnows in Demonstration Pond, it is possible that the environmental conditions in the pond itself may have had an effect as abiotic and biotic factors can also influence reproductive timing. Temperature, for example, can affect the initiation of reproduction (Gillet and Dubois, 2007). Water temperature was not continually recorded during the study so differences in temperature cannot be ruled out. However, the ponds are located within ~10 km of each other so significantly lower temperatures in Demonstration Pond would not be expected. In fact, if there is a difference, temperatures would be expected to be higher in Demonstration Pond as this pond becomes free of ice before the two reference sites.

The energetic condition of fish after winter can also affect the timing of reproduction (Divino and Tonn, 2007). However, it is unlikely that the energetic condition of the fathead minnows in Demonstration Pond differed significantly from those of the reference sites in May 2008 because there was no significant difference in the CFs of the

fathead minnows from all three sites; CF has been shown to be correlated with the energetic condition of fish (Neff and Cargnelli, 2004). Male fathead minnows in May 2008 were, however, observed to be significantly shorter and weighed less than fathead minnows from reference sites. At other times of the year, fathead minnows from Demonstration Pond had a significantly greater CF than fathead minnows from the reference sites. Increases and decreases in the CF of fish have been observed when fish were collected downstream of pulp and paper mills and domestic sewage treatment plants (Baer et al., 2009; Iwanowicz et al., 2009; McMaster et al., 2005; Porter and Janz, 2003). Siwik et al. (2000), however, did not observe any significant changes in growth after fathead minnows had been held in OSPW for 56 days. Typically, there is also a reduction in the gonad size of fish that have been exposed to domestic and industrial waste effluents (Hewitt et al., 2008; McMaster et al., 2005). In this study, the GSIs of both male and female fathead minnows from Demonstration Pond were usually significantly larger than those of reference fish.

Male fathead minnows develop a darker head and dark vertical bands along the sides of their body during the breeding season (Smith 1978). During all of the months that fathead minnows from Demonstration Pond were collected, their coloration was always lighter than those of reference fish. This could have been due to differences in the water quality characteristics of Demonstration Pond; the water from Demonstration Pond was grey in color and more turbid (due to the presence of colloidal clays) while the water from Beaver Creek reservoir and Poplar Creek reservoir was more yellow in appearance (probably due to more humic acids and/or fulvic acids). It is also possible that this was the result of exposure to endocrine disrupting substances. For example, the orange display coloration in male guppies (*Poecilia reticulate*) was reduced after exposure to anti-androgens (Bayley et al., 2002). Testosterone and 11-ketotesterone have been shown to induce thickening and color changes in the skin of salmonids (Idler et al., 1961; McBride and van Overbeeke, 1971) and structural changes in the skin of brown trout during sexual maturation (Pottinger and Pickering, 1985). The expression of male secondary sexual characteristics is also controlled by androgens in fathead minnows (Smith et al., 1978). In comparison to reference fish, lower concentrations of 11-

ketotestosterone were measured in the plasma of fathead minnows collected from Demonstration Pond in June 2006 and July 2007; these data provide further support to the prospect that a constituent in OSPW is disrupting the endocrine system of fathead minnows, particularly males, from Demonstration Pond. Alterations to the reproductive physiology of male fathead minnows has been previously observed in laboratory studies with OSPW and an extract of acid-extractable organics produced from OSPW (Kavanagh et al., 2011; Kavanagh et al., 2012). Thomas et al. (2009) demonstrated that naphthenic acids are weak estrogenic receptor agonists and potent androgen receptor antagonists and could, therefore, reduce the expression of male secondary sexual characteristics in the fathead minnows from Demonstration Pond. Recently, Rowland et al. (2011) determined that there were compounds with structural similarities to estrone and estradiol in OSPW and suggested that these compounds could account for some of the environmental estrogenic activity reported in OSPW acid-extractable organic acids.

It is possible that the duration of exposure to OSPW constituents may have had an effect on reproductive endpoints. In the Kavanagh et al. (2011) study, the OSPW from Demonstration Pond was not shown to have any impact on the reproductive physiology of the fathead minnows. It is important to note, however, that the fathead minnows were only held in OSPW for 21 days while the fathead minnows in the present study have been exposed to OSPW for generations. Multi-generational experiments with sheepshead minnows (*Cyprinodon variegatus*) have demonstrated that endocrine disrupting substances, such as 17β–estradiol and 17β-trenbolone, can impact F1 and F2 generations at lower concentrations than those that would have had an effect on the F0 generation (Cripe et al., 2009; Cripe et al., 2010).

All of the fathead minnows from Demonstration Pond had unusual opercula. The cause of this deformity is not known. Opercular deformities have been observed previously in yellow perch collected from areas that received discharges from pulp and paper mills (Lindesjöö et al., 1994). Colavecchia et al. (2004; 2006) observed cranial-facial abnormalities in fathead minnow and white sucker larvae that were exposed to sediment from the oil sands region and from a wastewater pond from Suncor Energy Inc. The authors suspected that PAHs or some other constituent of oil sands is responsible for

these effects. PAHs are likely to be present in Demonstration Pond as bitumen has been observed in the pond and low concentrations of PAHs have been previously detected in the porewater of MFT (Madill et al., 1999). The majority of the PAHs should be associated with the sediment in Demonstration Pond but fathead minnows could still be exposed to them through the food chain and thus they could still have some impact on fathead minnow larval development.

OSPW and naphthenic acids have been shown to cause proliferative changes (epithelial, mucous, and chloride cell) in the gills of yellow perch (Nero et al., 2006a; Nero et al., 2006b; van den Heuvel et al., 2000) and fathead minnow (Farrell et al., 2004). These same changes were observed in fathead minnows from Demonstration Pond. It is well known that gills can undergo histopathological changes when exposed to a variety of contaminants (Mallat, 1985). Naphthenic acids are a surfactant and may be responsible for these histopathological changes. Perhaps, as a result of a lifetime of exposure to naphthenic acids, a larger gill cavity is required to accommodate these proliferative changes and this has the caused the morphological changes in the opercula of Demonstration Pond fathead minnows.

There may be another reason for the histopathological alterations observed in the gills of fathead minnows collected from Demonstration Pond. An examination of the gills revealed that there were a number of *Trichodina* spp. between and surrounding the gill filaments. Gill parasites such as *Trichodina* spp. are known to cause proliferation of mucous cells and hyperplasia in the secondary lamellae of infected fish (Raissy and Anasari, 2011). Interestingly, Khan (1990) observed an increase in the prevalence and intensity of the *Trichodina* spp. on marine fish that had been exposed to petroleum hydrocarbons in the laboratory. Trichodina were also observed on the gills of fathead minnows from the reference sites; as no comparison was made between the number of Trichodina on fathead minnows from Demonstration Pond and the number of Trichodina on fathead minnows from the reference sites, it not known if the prevalence and number of Trichodina was significantly greater in fathead minnows from Demonstration Pond.

Black spot disease and tapeworms were observed in fathead minnows from Beaver Creek and Poplar Creek but no trematodes or tapeworms were observed in fathead minnows from Demonstration Pond. Fish-eating birds, the final host of these parasites, have been observed to frequent Demonstration Pond on occasion so these two parasites have the potential to be introduced into the pond. This would suggest that either the conditions (e.g. higher salt or NA concentrations) in Demonstration Pond are unfavorable to the parasites or to the initial host of these organisms. Usually *Neascus* spp. use snails as the first intermediate hosts, fish as the second intermediate hosts, and fish-eating birds as the final hosts (Blouin et al., 1984). *Ligula intestinalis*, on the other hand, use a copepod for its first intermediate host, fish as the second intermediate host, and a fish-eating bird for its final host (Arme and Owen, 1968). It is possible that the higher concentrations of ions (e.g., Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) or naphthenic acids found in OSPW could make the pond uninhabitable for either snails or the trematode itself. In the case of *Ligula intestinalis*, copepods are known to be present in Demonstration Pond so the conditions are likely unfavorable to the tapeworm itself. Fathead minnows from Beaver Creek reservoir and Poplar Creek reservoir also had enlarged spleens with white nodules throughout them. The source of these nodules was not determined; white nodules have been observed in the enlarged spleens of fish afflicted with cyprinid herpesviruses (Jeffrey et al., 2007), bacterial infections such as *Mycobacteria* spp. (Heidel et al., 2002), and myxosporean parasites (Fernandez-de-Luco et al., 1997).

Our previous research has shown that OSPW containing high concentrations of naphthenic acids adversely affects the reproductive physiology of fathead minnows (Kavanagh et al., 2011). The results of this study indicate that OSPW that has lower concentrations of naphthenic acids may still have an effect on the reproductive health and development of fathead minnows that have been exposed throughout their entire lifecycle. The underpinnings of the wet landscape reclamation option is that the toxicity of the OSPW in the end pit lakes will diminish over time and will have the same biological productivity as those of natural lakes in the region. At the time of this study, the OSPW in Demonstration Pond was 15 years old and yet still appeared to be chronically toxic to fish. This suggests that some treatment to remove the organic acids in

the OSPW will most likely be required in order for these end pit lakes to successfully sustain fish populations. As inflows and outflows of these lakes will need to be integrated with adjacent watersheds the removal of salts will most likely also be required in order to meet Alberta Environment's water release criteria.

CHAPTER 6. GENERAL DISCUSSION

Oil sands process-affected waters (OSPW) and its associated acid-extractable organics [e.g. naphthenic acids (NAs)] were demonstrated to have adverse effects on the reproductive physiology of fathead minnows. This is a cause for concern as the creation of oil sands pit lakes is part of every operator's mine reclamation plan. Oil sands operators plan to use these lakes to reclaim the large volume of fluid tailings that are currently being stored in tailings ponds in the Athabasca oil sands region. Since these water bodies must be integrated with adjacent undisturbed areas, they must meet water quality release criteria. They must also be capable of developing viable ecosystems that have a biological capability similar to natural lakes in the region but based on the results obtained in this thesis it is unlikely that this will be achievable without some type of water treatment.

Although the lethal toxicity of fresh OSPW has been demonstrated to disappear after 1-2 years (MacKinnon and Boerger, 1986), several studies have shown that aged OSPW have some acute and chronic toxic effects (Gurney et al., 2005; Nero et al., 2006a; Pollet and Bendell-Young, 2000; van den Heuvel et al., 1999; van den Heuvel et al., 2000). My thesis considered the effects of aged OSPW on reproduction in fish as this would be a key feature in establishing whether oil sands pit lakes would be capable of supporting fish communities that are self-sustaining.

Fathead minnow reproduction assays, embryo bioassays, and larval bioassays were used to achieve these objectives in order to determine what effect OSPW and its associated constituents would have on reproductive success. The two main constituents of OSPW are acid-extractable organics and salts so it was important to look at the effects that these constituents might have on fathead minnow reproductive physiology both separately and together (Chapter 3 & 4). A wild fathead minnow population in an OSPW pond also provided the opportunity to examine the long-term effects of OSPW exposure (Chapter 5). It was thought that this approach of multiple endpoints would cover all aspects of adult reproductive success including the viability of the next generation and

that the results of these studies could ultimately be used to comment on population sustainability.

Laboratory studies with aged OSPW (>28 mg/ of NAs) was demonstrated to reduce fecundity, male secondary sexual characteristics, and plasma sex steroids of fathead minnows (Chapter 2). An assessment of a wild population of fathead minnows inhabiting Syncrude Canada Ltd.'s Demonstration Pond revealed that OSPW with more moderate concentrations of NAs (~10 mg/l of NAs) still have an adverse effect on the reproductive physiology of fathead minnows (Chapter 5). Differences in the gonadosomatic indices and male secondary sexual characteristics of fathead minnows from Demonstration Pond suggested that the sexual maturity of male fathead minnows from Demonstration Pond was delayed and that spawning was offset to a later time. As with the laboratory studies with OSPW, the concentrations of 11-ketotestosterone measured in the plasma of fathead minnows from Demonstration Pond was significantly lower when compared to reference fish. The results of both the laboratory studies and the field studies suggest that a constituent in OSPW disrupted the endocrine system of fathead minnows.

Since the acid-extractable organics in OSPW were thought to be the prime candidates, an acid-extractable organics extract (i.e. NA extract) was produced from OSPW (Appendix 1) and then fathead minnows were exposed to this extract in the laboratory (Chapter 3). Fathead minnows exposed to a 10 mg/l NA extract for 21 days spawned fewer eggs and males had reduced expression of secondary sexual characteristics and lower concentrations of testosterone and 11-ketotestosterone. The specific compounds responsible for disrupting the endocrine system of these fish remain an unknown. NAs were once thought to make up the majority of the organic acids detected in OSPW, however, using ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, Grewer et al. (2010) have recently determined that <50% of the total abundance of the acid-extractable organics could be assigned to the classical (chemical formula $C_nH_{2n+Z}O_2$) and oxy-NAs. This suggests that over 50 % of the organics present in OSPW are unknown compounds. There is no information on their toxicity or rates of biodegradation; to date, no one has been able to

isolate these compounds from classical NAs so it is not known if these compounds or the classical NAs are responsible for the effects on fathead minnow reproductive physiology. Future research should, therefore, focus on identifying these compounds and determining their no observed effect concentrations.

Male fish appear to be particularly affected by the acid-extractable organics in OSPW. Many studies of EDCs have identified that compounds that are estrogenic and/or anti-androgenic may negatively affect reproduction in males. Anti-androgens, such as prochloraz, flutamide, and bicalutamide, have been shown to reduce fecundity, sex steroid concentrations, and male secondary sexual characteristics of fathead minnows (Ankley et al., 2005; Jensen et al., 2004; Panter et al., 2012), Similar results have been observed when fathead minnows were exposed to estrogenic substances (Harries et al., 2003; Kramer et al., 1998).

Recent research provides support for the premise that OSPW contain estrogenic and/or anti-androgenic compounds. He et al. (2010), for example, demonstrated that after 48 h of exposure to OSPW, H295R cells produced significantly less testosterone and significantly more 17β-estradiol which suggests that anti-androgenic compounds and estrogenic compounds are both present in OSPW. Thomas et al. (2009) determined that the NAs from the produced waters of offshore oil platforms and from commercial sources are weak estrogenic receptor (ER) agonists and potent androgen receptor (AR) antagonists. Additional tests with Fluka and ACROS naphthenic acid mixtures showed that there were compounds in these commercial mixtures that had greater AR antagonist potency than flutamide, a known anti-androgen (Thomas et al., 2009). These results and the positive response that was achieved with the yeast estrogen screening assay indicate that estrogenic and anti-androgenic substances are present in OSPW (Thomas et al., 2009). Using multidimensional comprehensive gas chromatography-mass spectrometry (GCxGC-MS), Rowland et al. (2011) also showed that some of the acid-extractable organics in OSPW have structural similarities with estrone and estradiol.

Inorganic ion (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) concentrations have been observed to increase with increasing NAs concentrations. Embryo and larval bioassays

demonstrated that HCO_3^-, an ion that occurs in high concentrations (>500 mg/l) in OSPW, can reduce the acute toxicity of NAs. When adult fathead minnows were exposed to an acid-extractable organics extract and $NaHCO_3$, spawning and 11-ketotestosterone concentrations were still reduced but there no significant differences observed in secondary sexual characteristics or testosterone concentrations. The $NaHCO_3$ appears to reduce the effects that the extract of acid-extractable organics had on the reproductive physiology of fathead minnows by reducing the uptake of these compounds. Calcium has been suggested to influence the uptake of zinc by biological mechanisms (e.g. decrease membrane permeability, fewer uptake sites, etc.) or some other chemical mechanism such as competition for absorption sites (Barron and Albeke, 2000); it is possible that HCO_3^- could have a similar influence on the uptake of the acid-extractable organics in fish. If HCO_3^- did not occur at high concentrations (>500 mg/l), the OSPW, even after aging, would likely be more acutely and chronically toxic.

The concentration of the ions that will be present in OSPW is dependent on the salts that are present in the oil sands deposits. Thus, ion concentrations can differ amongst the different mines. Suncor Energy Inc., for example, has higher concentrations of SO_4^{2-} and lower concentrations of Cl^- in their OPSW compared to Syncrude Canada Ltd. (Kavanagh et al., 2011). While there is obviously an advantage to having high concentrations of HCO_3^- in OSPW, the higher concentrations of inorganic ions can be problematic for the oil sands developers that must eventually integrate these aquatic systems with their surrounding watersheds. Spawning was impaired when fathead minnows were directly exposed (naïve exposure) to the salt concentrations found in Pond 9 OSPW (Chapter 4). However, these ion concentrations did not affect the reproductive physiology of fathead minnows that had been acclimated to higher salinities prior to exposure (Chapter 4). Higher concentrations of ions as well as acid-extractable organics can be expected to increase as operators become more efficient in their water recycling activities (e.g. dewatering of fluid tailings using dedicated disposal areas). Higher concentrations of inorganic ions have been observed to have adverse effects on freshwater fish. McCarraher and Thomas (1968) reported that total alkalinities above 1800 mg/l impair the reproduction and abundance of fathead minnows. Burnham and

Peterka et al. (1975) also reported that Na^{+}/ SO_4^{2-} dominated lakes with conductivities above 7000-8000 µcm/s could impair fathead minnow reproduction. An examination of 27 small watersheds in Eastern Wisconsin, USA determined that an increase in conductivity (possibly due to Na^{+} and Cl^{-}) was related to a decrease in spawning success of fathead minnows (Corsi et al., 2011). As the inorganic ions concentrations increase in OSPW, these constituents can, therefore, be expected to adversely affect fish reproduction.

Zubot et al. (2012) reports that the conductivities typically observed with Syncrude Canada Ltd.'s OSPW are at present ten times higher than the Athabasca River watershed. The amount of Cl^{-} in this OSPW is twice the amount measured in Pond 9 and is almost five times higher than the limit (120 mg/l) set by Canadian Water Quality Guidelines for the long-term protection of aquatic life. Desalinization of the OSPW will likely be necessary before water from these oil sands pit lakes can be released into the environment. Oil sands operators should take into consideration the fact that the removal of HCO_3^{-} could increase the toxicity of the acid-extractable organics in OSPW. It would, therefore, be advisable for future researchers to conduct acute and chronic toxicity bioassays with desalinized OSPW.

The acid-extractable organics in OSPW are expected to degrade over time but the process is slow and could take several decades. After twenty years, the NAs measured in Syncrude Canada Ltd.'s test ponds are still at high enough concentrations to have adverse effects on fish. Given the results presented in this thesis, some type of water treatment to remove the NAs and other acid-extractable organics in OSPW will likely be required. Considering that the MFT stored at the bottom of these oil sands pit lakes will slowly release pore water (and acid-extractable organics) over a period of several decades, the acid-extractable organics should be removed from MFT prior to being deposited in the pits, otherwise, continual water treatment may be required. The higher concentrations of inorganic ions in the OSPW and MFT will also need to be removed before these lakes can be integrated into the surrounding watersheds. The large volumes of OSPW and MFT and the colloidal clays that are present in these materials will present oil sands operators with both technical and economic challenges. Nonetheless, these

challenges must be surmounted if operators are to succeed in creating pit lakes that have ecosystems with biological production similar to other natural lakes in the Athabasca region.

In conclusion, my thesis has shown that reproductive bioassays are important tools that should be used by oil sands producers to assess the successfulness of the wet landscape option reclamation plan. My work points to the fact that significant concerns still exist with respect to the strategy to allow the acid-extractable organics in OSPW to degrade naturally over time. Due to the fact that some of the ponds examined in these studies were approaching 20 years of age, the expectation that oil sands pit lakes, without water treatment, will develop habitats suitable for fish is questionable. Given the uncertainty regarding the compounds responsible for reproductive effects and the complex interactions between NAs and salts, many questions remain unanswered and these should be a priority for the Canadian industry.

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APPENDIX A. DIETHYLAMINOETHYL-CELLULOSE CLEAN-UP OF A LARGE VOLUME NAPHTHENIC ACID EXTRACT

1. Abstract

The Athabasca oil sands of Alberta, Canada contain an estimated 174 billion barrels of bitumen. During oil sands refining processes, an extraction tailings mixture is produced that has been reported as toxic to aquatic organisms and is therefore collected in settling ponds on site. Investigation into the toxicity of these tailings pond waters has identified naphthenic acids (NAs) and their sodium salts as the major toxic components, and a multi-year study has been initiated to identify the principal toxic components within NA mixtures. Future toxicity studies require a large volume of a NA mixture, however, a well-defined bulk extraction technique is not available. This study investigated the use of a weak anion exchanger, diethylaminoethyl-cellulose (DEAE-cellulose), to remove humic-like material present after collecting the organic acid fraction of oil sands tailings pond water. The NA extraction and clean-up procedure proved to be a fast and efficient method to process large volumes of tailings pond water, providing an extraction efficiency of 41.2%. The resulting concentrated NA solution had a composition that differed somewhat from oil sands fresh tailings, with a reduction in the abundance of lower molecular weight NAs being the most significant difference. This reduction was mainly due to the initial acidification of tailings pond water. The DEAE-cellulose treatment had only a minor effect on the NA concentration, no noticeable effect on the NA fingerprint, and no significant effect on the mixture toxicity towards *Vibrio fischeri*.

2. Introduction

The Athabasca Basin of Fort McMurray, Alberta, Canada was recognized in 2003 as the second largest proven oil reserve on Earth with an estimated 174 billion barrels of

bitumen (AEUB, 2004). Current marketable oil sands production averages 854,000 barrels per day of synthetic crude oil; however, estimates predict an increase in production to 3,000,000 barrels per day by 2020 due to declines in conventional oil reserves (AEUB, 2004). The refineries of the Athabasca Basin utilize the Clark hot water extraction, using hot water (80 ºC) and NaOH to extract bitumen from the oil sand. The result of this extraction is marketable bitumen along with a tailings water waste that has been found to be toxic to aquatic organisms (Warith and Yong, 1994; Herman et al., 1994). Naphthenic acids (NAs), a diverse group of acyclic, monocyclic, and polycyclic carboxylic acids (Figure 2.1), have been identified as the primary toxic components (MacKinnon and Boerger, 1986; Madill et al., 2001; Dokholyan and Magomdeov, 1983; Leung et al., 2003). NAs have the general formula $C_nH_{2n+z}O_2$, where n represents the carbon number and z is the homologous group series number related to the number of 5- or 6-carbon rings within the structure (Rogers et al., 2002).

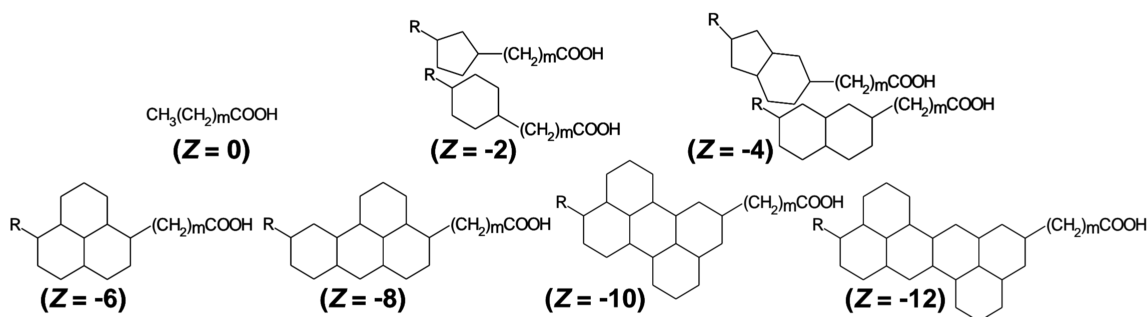

Figure 2.1. Examples of naphthenic acid structures for various z-families. R represents an alkyl group and *m* represents the length of the alkyl chain.

NAs enter surface water systems primarily through effluent discharge, but also through groundwater mixing and erosion of riverbank oil deposits (Headley and McMartin, 2004). Their occurrence and fate in aquatic environments is the topic of a recent review, in which attention was drawn to the need for improved methods for fractionation and isolation of the principal toxic components. Prior to toxicity testing, NAs are extracted from tailings pond water (TPW) using existing procedures (Headley et

al., 2002; Rogers et al., 2002. Within the acidic fraction extracted from TPW, NAs are present along with higher molecular weight (MW) humic-like materials. NAs have a MW less than 1000 Daltons. In the published extraction protocols, one of the final steps removes the higher MW materials by ultrafiltration using a 1,000 MW cut-off membrane (Rogers et al., 2002). Although efficient when extracting from smaller volumes of TPW for analytical purposes, this ultrafiltration step is time-intensive when processing larger volumes of TPW for toxicity testing. Following successful fractionation of the NA mixture, a range of bioassays (including, but not limited to, acute toxicity tests using *Daphnia* sp., the Basic Microtox test using *Vibrio fischeri*, and *in vitro* gonadal incubations) will be conducted to determine if different groups of NAs exhibit varying toxicities between different species. Due to the uncertainties regarding the success of the fractionation experiments (i.e. the number of fractions it is possible to generate) as well as the number and repetition of bioassays, the required volume of the NA stock solution is not known, suffice to say that a large volume is required.

To rapidly extract NAs from a large volume of TPW, a method was developed using diethylaminoethyl-cellulose (DEAE-cellulose) to remove humic-like material from the organic acid fraction of TPW, eliminating the need for ultrafiltration. DEAE-cellulose is a weak anion exchanger that efficiently reacts with carboxyl and hydroxyl functional groups on humic molecules (Burnison et al., 1996; Miles et al., 1983). This paper reports the development of a bulk procedure for extracting NAs from oil sands TPW, followed by using a DEAE-cellulose column to remove humic-like material from the organic acid fraction.

3. Materials and Methods

3.1 Sample collection and extraction of organic pellet

Approximately 2,000 L of oil sands TPW were collected from Syncrude Canada Ltd. West Endpit settling basin in Fort McMurray, Alberta, Canada in June 2004. The water was stored in 60 L polyethylene containers and acidified to a pH of 2 using 18 M sulphuric acid (H_2SO_4) (Figure 2.2). By acidifying the TPW, NAs and other organic acids

would precipitate. The acid-insoluble material was allowed to settle for 24 h. The overlaying acidic water was then siphoned off and discarded. The precipitated residue was transferred to 5-L containers and allowed to settle for 72 h. Following this, the supernatant was siphoned off and the precipitate was transferred to clear 1-L high-density polyethylene containers (Nalgene, Fisher Scientific, Whitby, ON) and centrifuged at 3,300 *g* for 15 min, at which time the mixture separated. The supernatant was carefully siphoned off and discarded. The acid precipitate was collected in eight 500-mL amber glass bottles (Fisher Scientific) and stored at 4 °C.

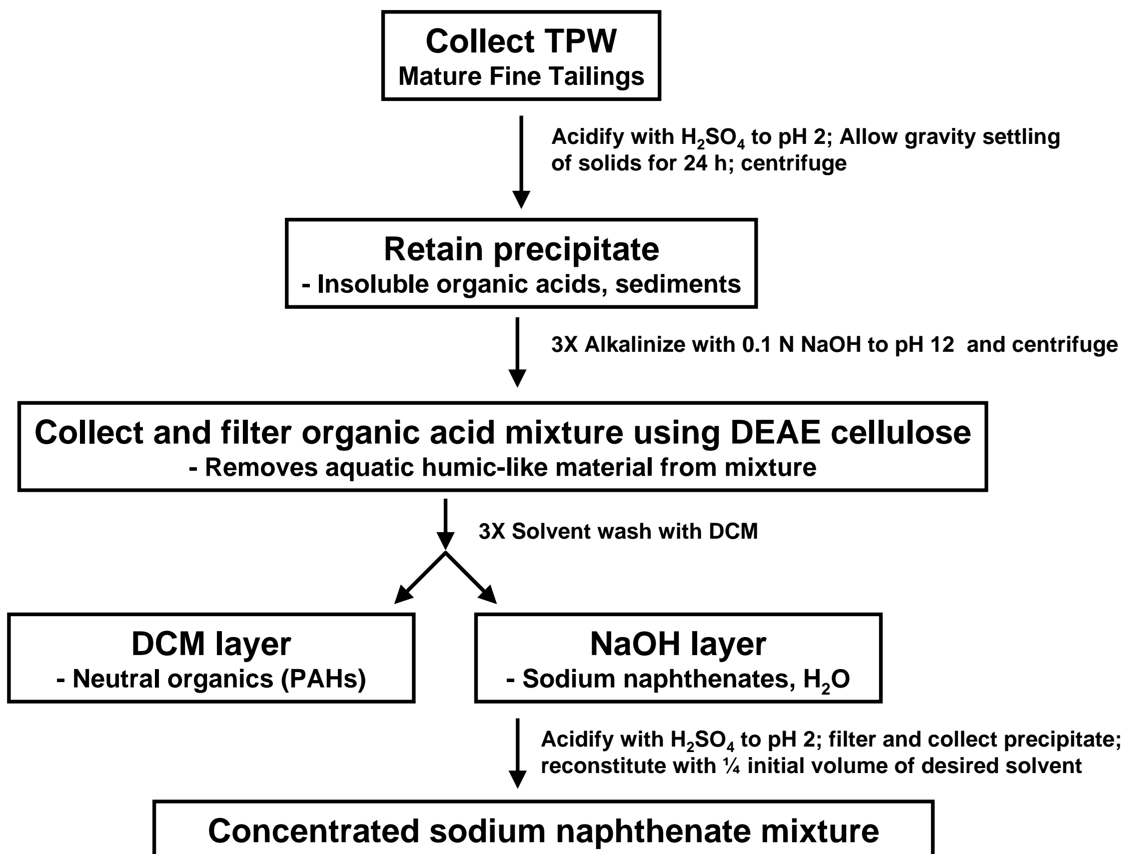

Figure 2.2. Procedure for bulk extraction and clean-up of naphthenic acids extracted from oil sands tailings pond water.

Twenty grams of the wet, organic precipitate were weighed into each of six 250-mL polyethylene centrifuge bottles (Fisher Scientific). In order to re-dissolve the acids, 200

mL of 0.1 N NaOH were added to each centrifuge bottle and shaken for one minute. The bottles were centrifuged at 17,000 *g* for 10 min, and the mixture separated into a transparent, dark liquid over a two-layered black and grey pellet. The supernatant contained NAs and organic acids (i.e. humic acid-like material), which are soluble at higher pH, while the pellet consisted of materials that were insoluble (e.g. sand, clay, humins). The supernatant was collected in 1-L amber glass bottles (Fisher Scientific) and another 40 g (20 g x 2) of organic precipitate were added to the existing pellet in the centrifuge bottles. The alkali solubility and centrifugation steps were repeated as before and the resulting supernatant was collected in 1-L amber glass bottles. To ensure that the majority of the organic acids were dissolved by the 0.1 N NaOH, the pellets in each of the centrifuge bottles were washed three times with 200 mL, 150 mL, and 100 mL of NaOH per centrifuge bottle, respectively. Thus, each polyethylene centrifuge bottle contained 60 g of the organic precipitate and yielded 1050 mL of supernatant. Following each wash and centrifugation, the resulting supernatant was collected in 1-L amber glass bottles. All of the collected supernatant was then combined in 20 L polyethylene carboys (Fisher Scientific). The carboys were transported at 4 ºC to the National Water Research Institute, Burlington, Ontario, for further processing. The supernatant is hereafter referred to as the reconstituted organic solution.

3.2 Purification of NAs

DEAE-cellulose (Sigma Chemical Co., St. Louis, MO) was cleaned using hydrochloric acid (HCl) and NaOH (Miles et al., 1983) prior to column formation. A slurry of DEAE-cellulose (75 g, dry wt.) and 300 mL distilled water was added to a vacuum filtration unit (90 mm glass holder, Advantec MFS, Inc., Dublin, CA, USA) fitted with a glass fibre filter (Whatman GF/F, 1 µm pore size). The cellulose was allowed to settle before a vacuum was applied (330 mm Hg). When the water level reached the top of the settled cellulose, the water filtrate collected was discarded. Five hundred mL of the reconstituted organic solution (0.1 N NaOH solution containing NAs and other organic acids) were added to the cellulose column. The vacuum pump was briefly turned on to start filtration and the reconstituted organic solution was continually

added to prevent drying of the column. The collected filtrate was yellow in color and the DEAE-cellulose column retained the dark brown humic-like material. The column could be used to filter approximately 6 L of the reconstituted organic solution before the humic-like material band migrated close to the bottom of the column. After 6 L of the reconstituted organic solution had been filtered, or there had been noticeable migration of the humic band, the dark brown cellulose was discarded and the white cellulose from the bottom of the column was collected for use in future columns. Another DEAE-cellulose column was created using the recycled cellulose from the previous column along with clean, unused DEAE-cellulose. DEAE-cellulose filtration was continued until the majority of the humic-like material was removed from the reconstituted organic solution. This removal was visible through the change in color of the NA solution from dark-brown to golden-yellow following passage through the DEAE-cellulose column.

Neutral organic compounds, including polycyclic aromatic hydrocarbons (PAHs), were removed from the column eluate by a dichloromethane (DCM) wash. Three hundred mL of the column eluate were added to a 2,000-mL separatory funnel, followed by the addition of 60 mL of DCM (Optima grade, Fisher Scientific). The separatory funnel was shaken for several minutes, regularly releasing any gas build-up. The separatory funnel was allowed to stand for 10 min, allowing the DCM and aqueous fractions to separate. The lower DCM layer containing PAHs and other neutral organic compounds was then removed and discarded while the upper aqueous layer containing polar organic compounds, primarily sodium naphthenates, was retained for further DCM washing. Two more washes were performed in the same manner, each using 60 mL of DCM. Following the third wash, the lower DCM fraction was completely removed and the aqueous NaOH-sodium naphthenate fraction was collected in 20-L polyethylene carboys. There was minimal loss of the aqueous fraction during these washes. This procedure was repeated for all of the DEAE-cellulose column eluate.

3.3 Recovery and concentration of NAs

One litre of the yellow NaOH-sodium naphthenate solution was transferred from the carboy into a beaker. Concentrated H_2SO_4 was slowly added to the solution to obtain a

pH of 2, resulting in the visible formation of a light yellow NA precipitate. The acidified NA suspension was then transferred to 250-mL centrifuge bottles and centrifuged at 3,300 *g* for 10 min. The supernatant was filtered through a 0.2 μm pore size PTFE filter (Sartorius) to collect any residual precipitate, and the pale yellow filtrate was discarded. Following filtration, the filter paper was submerged in 300 mL of 0.05 N NaOH and was sonicated for 10 min (Ultrasonic cleaner model FS30, 130 W, Fisher Scientific). Additional acidified NA suspension was then added to the 250-mL centrifuge bottles still containing the initial NA pellet at the bottom. The filtration procedure described above was repeated, discarding the filtrate and reconstituting the NA precipitate in the same 300 mL of 0.05 N NaOH as before. The final pellet in each of the 250-mL centrifuge bottles represented 1 L of the acidified NA suspension. The precipitate was washed by resuspension in 250 mL of pH 2 distilled H_2O followed by centrifugation at 3,300 *g* for 10 min. The addition of pH 2 distilled H_2O diluted the remaining more concentrated acid that was present in the centrifuge bottles, minimizing the amount of NaOH required to obtain a pH of 8. The supernatant was discarded and the pellet was dissolved with the aid of vigorous shaking in 250 mL of 0.05 N NaOH for 1 min, followed by sonication for 10 min. The resulting solution was a golden-yellow concentrated solution of NAs. This procedure was repeated for all of the NaOH-sodium naphthenate solution. The concentrated NA solution was stored in 1-L amber glass bottles at 4°C.

3.4 Ultrafiltration of the organic solution

For the purpose of assessing the throughput of the new method, an aliquot of the acid precipitate was purified using a previously published method (Rogers et al., 2002). The humic-like material in the organic solution was removed through a 1,000 MW cut-off ultrafiltration system in place of DEAE-cellulose. The reconstituted organic solution underwent sequential filtration using a 1 μm pore size glass fibre filter (Whatman GF/F), followed by a 0.45 μm pore size cellulose acetate filter (Sartorius), and finally a 0.2 μm pore size PTFE filter (Sartorius). It was then filtered through a 50,000 MW cut-off ultrafiltration cell (XM50, Amicon Inc., Beverly, MA) before being filtered through a 1,000 MW cut-off ultrafiltration cell (YM1, Amicon Inc., Beverly, MA).

3.5 Sample analysis using ESI-MS

Analyses of samples collected throughout the extraction procedure were conducted using electrospray ionization mass spectrometry (ESI-MS; Headley et al., 2002) at the National Water Research Institute in Saskatoon, Saskatchewan, Canada. In brief, mass spectrometric analysis was conducted using a Quattro Ultima mass spectrometer (Micromass, UK) equipped with an electrospray interface operating in the negative ion mode. MS conditions for analysis of the mixtures were set as follows: source temperature 90 ºC, desolvation temperature 220 ºC, cone voltage setting 62 V, capillary voltage setting 2.63 kV, cone gas N_2 158 L h^{-1}, desolvation gas N_2 489 L h^{-1}. The multiplier was set at 650 V. Full scan MS was employed in the m/z range 50 – 550. Samples (5 µL) were loop injected using a Waters 2695 (Waters Corp) Separations Module using 50:50 acetonitrile:water containing 0.4% ammonium hydroxide as the eluent at 200 µL min^{-1}. Distilled water blanks were analyzed revealing no external contribution of NAs to the mixture. All of the analyses were performed using ESI-MS and the results were reported as total NA concentrations, as determined by comparison to an aliquot of NAs previously extracted from OSPW (Rogers et al., 2002). Following serial dilution of the aliquot, a five-point regression curve was created for quantification of unknown NA extracts. The quantification of NAs from OSPW was further verified by comparing area counts of the OSPW NA extract with the area counts produced by the same concentration of a Fluka commercial NA extract.

3.6 Microtox toxicity assay

Microtox analysis was performed on a Microtox Model 500 Analyzer using the Basic test provided by Azur Environmental (Azur Environmental, 1995). Samples for Microtox analysis were diluted from the initial concentrated NA solution (2,500 mg L^{-1}) to 577.0 mg L^{-1}. A portion of this diluted NA solution was cleaned using a DEAE-cellulose column and the resulting solution had a concentration of 499.0 mg L^{-1}. The toxicity of the two NA mixtures (prior to, and following, DEAE-cellulose clean-up) was investigated following exposure at 15 minutes, assessing the impact on the

bioluminescence of the marine bacterium, *Vibrio fischeri*. Serial dilutions were set up to investigate the toxicity at 20%, 10%, 5%, and 2.5% of each solution. Concentrations tested for the pre-cellulose solution were 0 mg L^{-1} (0%), 14.4 mg L^{-1} (2.5%), 28.9 mg L^{-1} (5%), 57.7 mg L^{-1} (10%), and 115.4 mg L^{-1} (20%). Concentrations tested for the post-cellulose solution were 0 mg L^{-1} (0%), 12.5 mg L^{-1} (2.5%), 25.0 mg L^{-1} (5%), 49.9 mg L^{-1} (10%), and 99.8 mg L^{-1} (20%). The EC_{50} values expressed are defined as the NA concentration required to reduce bioluminescence by 50%. A 4-parameter logistic model was used to fit regression lines to the data.

3.7 Statistical analysis

All statistical analyses were performed using the proc glm function of SAS (V8.02, SAS Institute, Cary, NC). Significant differences in *Vibrio fischeri* bioluminescence were analyzed at $\alpha = 0.05$ using Student's t-test in which NA solution treatment was the variable.

4. Results and Discussion

Using DEAE-cellulose to remove humic-like material from the organic extract of oil sands TPW significantly reduced the clean-up time when compared to ultrafiltration techniques. For example, a 50,000 MW cut-off filter required approximately 2 ½ hours per litre to process the raw NaOH-sodium naphthenate solution, and visually, not all of the dark brown humic-like material was removed. A second 1-hour filtration through a 1,000 MW cut-off filter was completed to remove the remaining humic-like material. Therefore, the total clean-up time required by the Rogers et al. (2002) method was 3 ½ hours. However, the DEAE-cellulose column removed the humic-like material at a rate of approximately 15 minutes per litre.

To determine the extraction efficiency of this method, NA concentrations were measured by ESI-MS for the original TPW sample and the final NA stock solution. The overall extraction efficiency for this bulk-extraction method was 41.2%, and a total of 14 L of concentrated NA solution (~2,500 mg L^{-1}) was extracted from approximately 2,000

L of TPW. Although the extraction efficiency is much lower than the 85% efficiency reported by Rogers et al. (2002) the time required to produce a large volume of a concentrated purified NA solution is much less, making this method beneficial for toxicity testing protocols requiring a large volume of NA solution.

As illustrated in Figures 2.3 a, b and Figures 2.4 a, b, there are clear differences in the electrospray ionization fingerprints obtained for the same sample between pre- and post-DEAE cellulose treatment. There appears to be a somewhat lower recovery of the lower molecular weight NAs in the $z = 0$ and $z = -2$ NA families for the acid precipitate extract recovered from the procedure described in this paper, compared to the initial TPW NAs recovered by the solid phase extraction procedure described in Headley et al. (2002). The loss in recovery was attributable to the two acidification steps, during which some NAs did not precipitate out at a pH of 2. However, as illustrated in Figures 2.3 b, c and Figures 2.4 b, c, the DEAE-cellulose treatment caused minor changes, if any, in the NA fingerprint. The initial acidification of the TPW contributed to approximately 40% of initial NAs lost, while the final acidification step added approximately 10% more NA loss. The first acidification step allowed for concentration of the NAs at the oil sands to limit the amount of material being transported off-site. Following the acidification and centrifugation of 2,000 L of TPW, only eight 500-mL amber glass bottles were required to contain all of the solidified organic acids. Acidification and filtration of the resulting organic acid precipitate was required following the DCM to wash to remove final traces of DCM.

To determine the impact of DEAE-cellulose clean-up on NA mixture toxicity, the marine bacterium *Vibrio fischeri* was exposed to varying concentrations of NA solutions prior to, and following, DEAE-cellulose clean-up. As reported above, there was a slight loss of NAs following the DEAE-cellulose clean-up, reducing the NA concentration from 577.0 mg L^{-1} to 499.0 mg L^{-1}. However, this loss was not NA fraction-specific and the toxicity of the solutions was investigated by setting up serial dilutions of 20%, 10%, 5%, 2.5%, and 0% of the pre- and post-DEAE-cellulose NA solutions. The Basic test of the

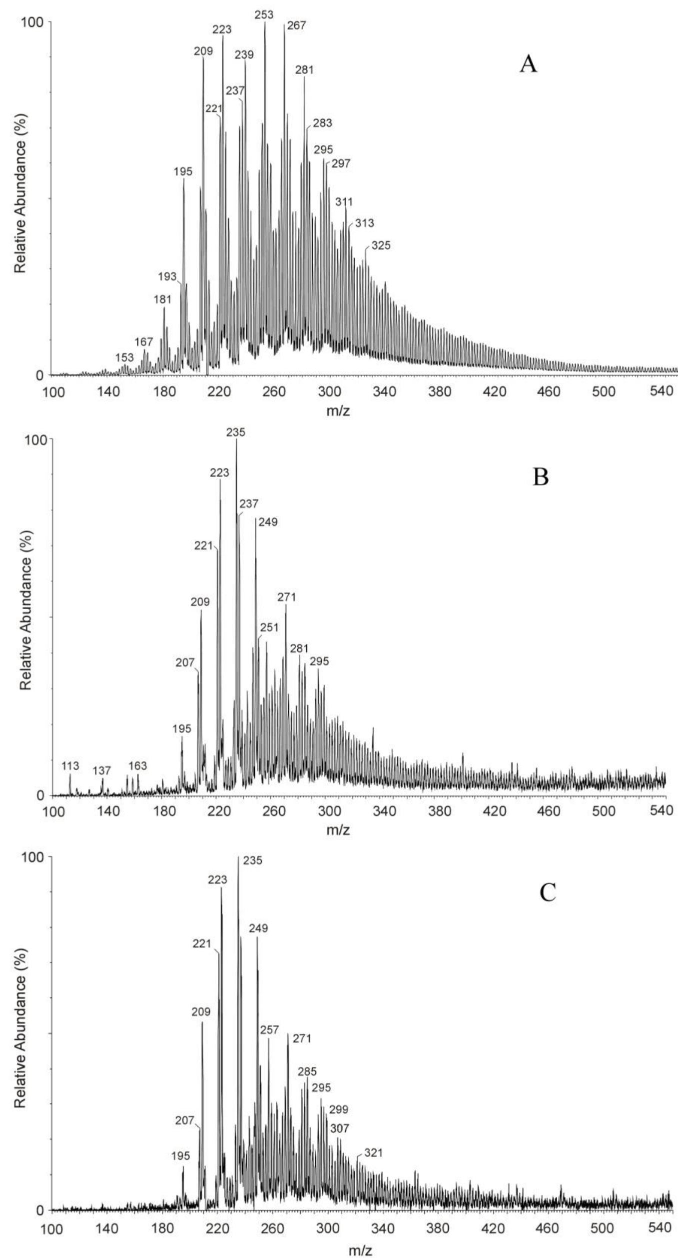

Figure 2.3. Mass spectral analyses of naphthenic acids. A) Naphthenic acid component of initial oil sands tailings pond water, B) Naphthenic acid component of organic acid solution prior to DEAE-cellulose treatment, and C) Naphthenic acid component of organic acid solution following DEAE-cellulose treatment.

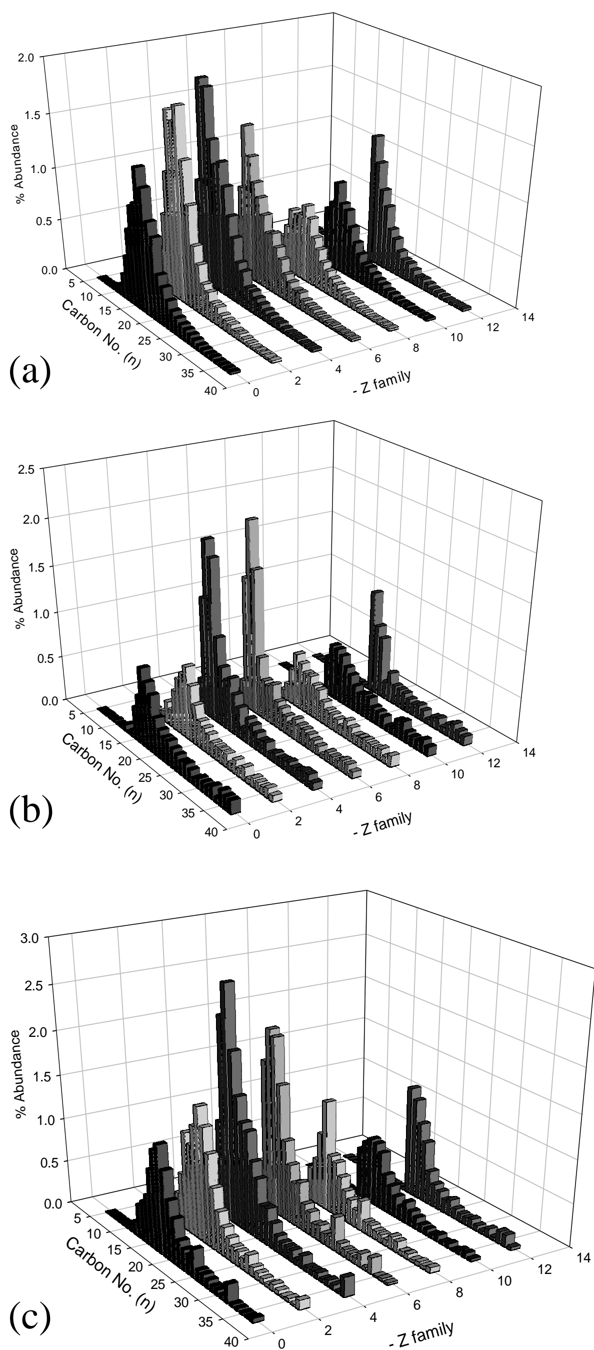

Figure 2.4. Three-dimensional plots of naphthenic acid z-series. A) Naphthenic acid component of initial oil sands tailings pond water, B) Naphthenic acid component of organic acid solution prior to DEAE-cellulose treatment, and C) Naphthenic acid component of organic acid solution following DEAE-cellulose treatment.

Microtox toxicity assay (Azur Environmental, 1995) was used to determine the EC_{50} values for the pre-cellulose and post-cellulose solutions at 15 minutes (Figure 2.5). The calculated EC_{50} value for the pre-DEAE-cellulose NA solution was 31.4 mg L^{-1} (SE ± 1.8) and for the post-DEAE-cellulose NA solution, 30.5 mg L^{-1} (SE ± 2.2). Student's t-test was used to detect a potential difference in toxicity at $\alpha = 0.05$, however, no significant difference was detected between the NA solution toxicities.

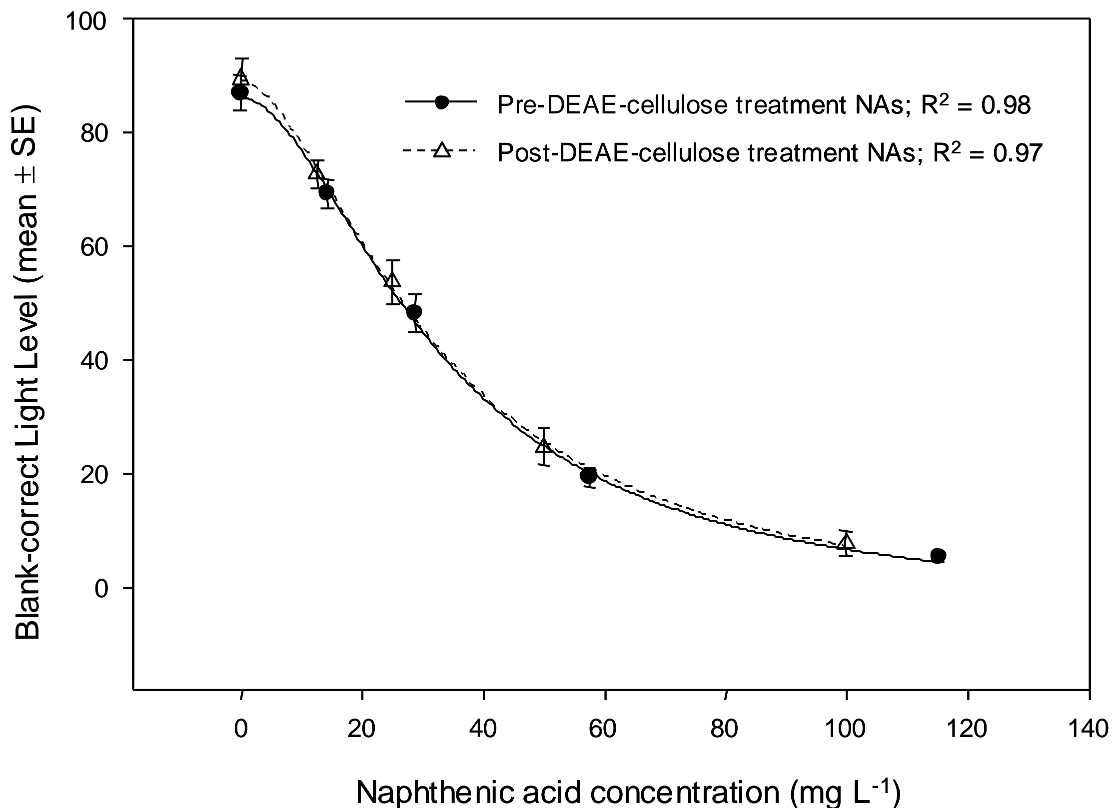

Figure 2.5. Effect of naphthenic acid solutions, prior to and following, DEAE-cellulose clean-up on *Vibrio fischeri* bioluminescence following exposure at 15 minutes. A 4-parameter logistic model was used to fit regression lines to the data.

The goal of the bulk NA extraction and rapid clean-up was to generate a large volume of NAs present in oil sands TPW. Although the acidification steps of the extraction contributed to a slight loss of the lower MW NAs, there was not complete removal of any of the compounds. Therefore, successful fractionation of the mixture would allow for separation of all existing classes of NAs, even if there is a slightly reduced concentration of the lower MW NAs.

This improved method was a fast and effective way of extracting NAs from a large volume of oil sand TPW, removing unwanted organic compounds from the solution, and concentrating the NAs in the final extract. The time required for DEAE-cellulose to remove the unwanted organic compounds was approximately 1/14 the time required using ultrafiltration techniques, and the toxicity of the mixture was not compromised during this cleaning step. Ongoing research is attempting to fractionate a NA mixture with the intention of conducting toxicity identification evaluation driven bioassays. The goal of this research is to provide a better understanding of the toxicity of individual NAs contained within TPW mixtures.

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APPENDIX B. DETECTING OIL SANDS PROCESS-AFFECTED WATERS IN THE ALBERTA OIL SANDS REGION USING SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

1. Abstract

Large volumes of oil sands process-affected waters (OSPW) are produced during the extraction of bitumen from oil sand. There are approximately 10^9 m^3 of OSPW currently being stored in settling basins on oil sands mining sites in Northern Alberta. Developers plan to create artificial lakes with OSPW and it is expected that this water may eventually enter the environment. This study was conducted in order to determine if synchronous fluorescence spectroscopy (SFS) could detect OSPW contamination in water systems. Water samples collected from ponds containing OSPW and selected sites in the Alberta oil sands region were evaluated using SFS with an offset value of 18 nm. OSPW ponds consistently displayed a minor peak at 282.5 nm and a broad major peak ranging between 325-340 nm. Water from reference sites within the oil sands region had little fluorescence at 282.5 nm but greater fluorescence beyond 345 nm. Naphthenic acids are the major toxic component of OSPW. Both a commercial naphthenic acid and a naphthenic acid extract prepared from OSPW had similar fluorescent spectra with peaks at 280 nm and 320 nm and minor shoulders at approximately 303 and 331 nm. The presence of aromatic acids closely associated with the naphthenic acids may be responsible for unique fluorescence at 320-340 nm. SFS is proposed to be a simple and fast method to monitor the release of OSPW into ground and surface waters in the oil sands region.

2. Introduction

Alberta's oil sands industry currently produces an average of 1,184,000 barrels (188241 m^3) of marketable bitumen and sweet crude oil per day (Alberta Energy &

Utilities Board, 2007-2008). Using the Clark Water Extraction process, the bitumen (a heavy form of crude oil) is separated using hot water (79-93 ^{o}C) and NaOH (Rogers et al., 2002). The large volume of fluid tailings (the remaining bitumen, residual sand, clay, water, inorganic and organic contaminants) produced by this process are not permitted to be released into the environment (FTFC, 1995). These tailings, currently estimated to exceed 10^9 m^3, are transferred to on-site settling basins (Del Rio et al., 2006). Fluid tailings, commonly referred to as oil sands process-affected water (OSPW), have been found to have high concentrations of naphthenic acids. Naphthenic acids are a mixture of acyclic, monocyclic, and polycyclic carboxylic acids that have the general formula $C_nH_{2n+z}O_2$, where n represents the carbon number and z indicates the number of hydrogens removed to accommodate the number of five- or six-carbon rings within the structure (Rogers et al., 2002; Clemente and Fedorak, 2005). High concentrations of inorganic ions such as Na^+, Cl^-, SO_4^{-2}, and HCO_3^- and very low concentrations of polycyclic aromatic hydrocarbons (PAHs), the majority of which are alkylated, have been measured in OSPW (MacKinnon and Boerger, 1986; Schramm et al., 2000; Madill et al., 2001).

OSPW has been found to be acutely toxic to fish, however, this toxicity has been found to disappear after 1-2 years (MacKinnon and Boerger, 1986). As part of their “wet landscape reclamation option”, oils sands developers plan to transfer OSPW to mined-out pits and then cap these with a layer of natural surface water. An aquatic ecosystem, which is expected to have the same biological productivity as those of natural lakes in the region, will then be established in these “end pit lakes”. Gradually, OSPW will be released from these lakes into the environment, and thus, the monitoring of OSPW release into ground and surface waters is important.

Fluorescence spectroscopy is a fast and sensitive method that enables the analysis of complex mixtures without the need for pretreatment. This method can be used to detect aromatic compounds such as proteins, humic and fulvic acids, steroids, phenols, oils, surfactants, and PAHs all of which exhibit characteristic fluorescent emissions after excitation by visible or near-UV light (Wu et al. 2006; Baker et al. 2008). Synchronous fluorescence spectroscopy (SFS) has been used for a variety of purposes including

characterizing natural organic matter in rivers (Ahmad et al. 2002), biomonitoring of PAHs (Eickoff et al., 2003), and identifying the source of petroleum contaminants (Pharr et al., 1992; Han et al., 2006). It has also been used to determine if various water bodies are contaminated by pulp and paper wastewater (Santos et al., 2001; Carvalho et al., 2008), agriculture runoff (Baker, 2002), untreated domestic sewage (Baker et al., 2003), and landfill leachate (Baker et al., 2005; Lu et al., 2009). SFS offers some advantages over other forms of fluorescence spectroscopy, such as excitation-emission matrix (EEM), as the spectral profile is simpler, there is less background because of a reduction of scattered light, and there are sharper peaks (Pharr et al., 1992). The objective of this study was to determine if SFS could be used to detect OSPW in the Alberta Oil Sands region. We first set out to determine if OSPW had a consistent fluorescent signature and subsequently, attempted to determine what constituents were responsible for that signature.

3. Materials and Methods

3.1 Sites

To evaluate the wet landscape reclamation option, Syncrude Canada Ltd. constructed a series of ponds on part of their lease (57°05.050'N, 111°41.505'W) in northern Alberta. These ponds were constructed in 1993 and filled with various amounts of OSPW. In this study, water was collected from Pond 1, Pond 3, Demonstration Pond, and Pond 9 of Syncrude Canada Ltd. Pond 1 is a 1600 m^2 pond that was filled with 2000 m^3 of unprocessed water (clean water from a diversion channel of lower Beaver Creek). Pond 3 is a 1600 m^2pond that was filled with 1000 m^3 of a mixture of mature fine tailings (MFT) and 1000 m^3 of unprocessed water. Demonstration Pond is a 40 000 m^2pond that was filled with 70 000 m^3 of MFT and capped with 80 000 m^3 of unprocessed water. Pond 9 is a 40 000 m^2 pond that was only filled with 50 000 m^3 of tailings pond water. Another sample was collected from a 5000 m^2 pond constructed in 1992 which contained a mixture of fine tailings, recycled process water, and area runoff (MFT South pond, 56°59.478'N 111°32.138'W) at Suncor Energy Inc. Water from all of these sites was

collected using a submersible pump positioned approximately 0.5- 1.0 m below the surface and was stored at 4° C until SFS analysis. Using a rope and bailer, ground water samples were also collected at Suncor Energy Inc. from wells that had been drilled into surficial sand. One sample was collected from a well beside the dyke of a tailings pond and a second sample was collected from a well in an area where no tailings pond water was present (reference site). Dr. Mike MacKinnon from Syncrude Canada Ltd. provided the water quality characteristics of the surface waters while Charity Zayonc from Suncor Energy Inc. provided the water quality characteristics of the ground water samples (Table 1). The naphthenic acid concentrations were measured using Fourier transform infrared (FTIR) spectroscopy (Jivraj et al., 1995; Holowenko et al., 2001). Kodak naphthenic acids (Eastman Kodak Company, Rochester, NY) were used as standards in the FTIR analysis of the surface waters while Aldrich naphthenic acids (Sigma-Aldrich, Oakville, ON, Canada) were used for the analysis of the ground waters. The other water quality data for the surface and ground water samples were collected using standard aquatic chemical methodologies at Syncrude's Research facility in Edmonton, Ab and ALS Laboratory Group, Edmonton, AB, respectively.

Grab sampling was also used to collect water from several reference sites within the oil sands region. Gregoire Lake (56°27′06″N 111°07′38″W), located approximately 15 km south of Fort McMurray, Alberta, was used as a reference site. Other locations for collection were Poplar Creek Reservoir and Beaver Creek Reservoir (~20 km and ~25 km north of Fort McMurray, respectively) in addition to several rivers in the area (Figure 1). These samples were stored at 4° C until SFS analysis.

3.2 Reagents

A naphthenic acid extract (NAE) was produced using the method described by Frank et al. (2006). Briefly, OSPW was collected from Syncrude Canada Ltd.'s West In-pit settling basin. It was acidified to pH 2, the aqueous layer was decanted, and the precipitate (slurry) was centrifuged. The pellet was then dissolved in a 0.1 M NaOH solution before being passed through a bed of the weak anion exchange diethylaminoethyl-cellulose to remove humic-like substances. The filtered NAE was

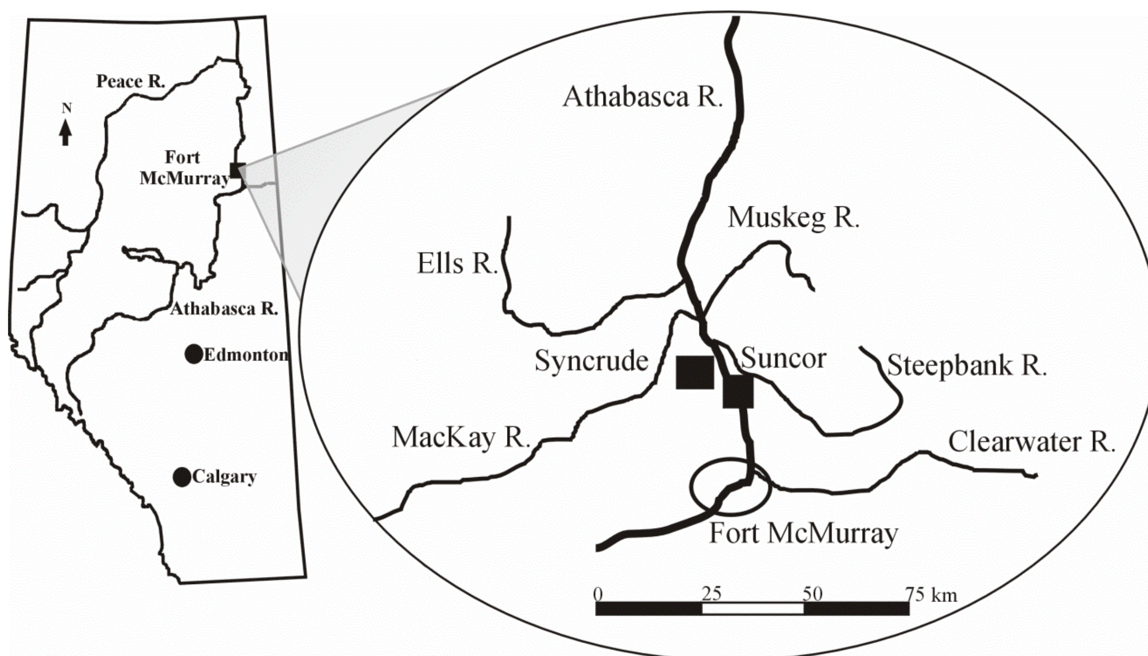

Figure 1. Map of the region showing the locations of the study sites.

washed with dichloromethane (DCM) to remove neutral organics such as PAHs. The extract was again acidified and then filtered through a 0.2 μm Teflon filter. The precipitate was then re-dissolved in 0.05 M NaOH. The resulting NAE was analyzed for the concentrations of 16 priority PAHs as defined by the EPA, alkylated PAHs, and dibenzothiophenes by the ALS Laboratory Group, Edmonton, AB, Canada using gas chromatography and mass spectrometry.

Several selected compounds were used for comparative purposes: Fluka naphthenic acid (FNA), naphthalene, phenanthrene, anthracene, fluorene, quinoline, and humic acid were obtained from Sigma-Aldrich (Oakville, ON, Canada) and used without further purification. Toluene (HPLC grade) was obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). The Savannah River fulvic acid was provided by Dr. Dale Van Stempvoort, Environment Canada, Burlington, ON, Canada.

3.3 Synchronous fluorescence spectroscopy

The SFS procedure outlined by Peuravuori et al. (2002) for analyzing aquatic humic matter was followed with minor modifications. Synchronous fluorescence spectra were recorded with a Perkin-Elmer Luminescence Spectrometer LS50B and the data collected using FL WinLab 3 software (Perkin-Elmer, Norwalk, CT). All samples were scanned in a 1 cm quartz cuvette with PTFE stopper (Hellman, Concord, ON, Canada) at 20°C. The wavelength difference between the excitation and emission monochromators ($\Delta\lambda$) was optimized by measuring the spectra of dilute NAE at various offset values (10 nm to 60 nm). The recommended $\Delta\lambda$ of 18 nm (Miano et al., 1988, Peuravuori et al., 2002) was chosen and synchronous fluorescence spectra were collected in the 250 – 400 nm excitation wavelength range. Excitation and emission monochromator slit widths were set at 5 nm, scan speed at 50 nm min^{-1} and resolution at 0.5 nm. The spectra were blank corrected with either Milli-Q water (reference sites) or 0.05 M $NaHCO_3$ (pH 8.3 for OSPW ponds and selected known compounds) and then smoothed with a 5-point averaging adjacent method using Origin software ver. 7.5 (OriginLab Corp., Northampton, MA). The effect of pH on the spectra of the commercial naphthenic acid was measured in various phosphate buffers and compared to NAE at pH 8.3. No further corrections for instrument response were made to the fluorescence spectra. Environmental samples were filtered through a washed 0.2 µm filter (Millipore) before fluorescent analysis to remove particulates. Washing was important to remove interfering fluorescent contaminants from the filter.

4. Results and Discussion

Initial SFS analysis of OSPW from Syncrude's Demonstration Pond revealed a peak at 282 nm and a second larger peak at 320 nm and emission intensities beyond 330 nm (Figure 2A). This OPSW spectra differed from the reference sites which exhibited broad peaks of fluorescence starting at 310 nm and these continued past the 400 nm wavelengths (Figure 2B). This pattern is likely due to the presence of fulvic and humic acids in these natural water as spectra at these wavelengths have been observed

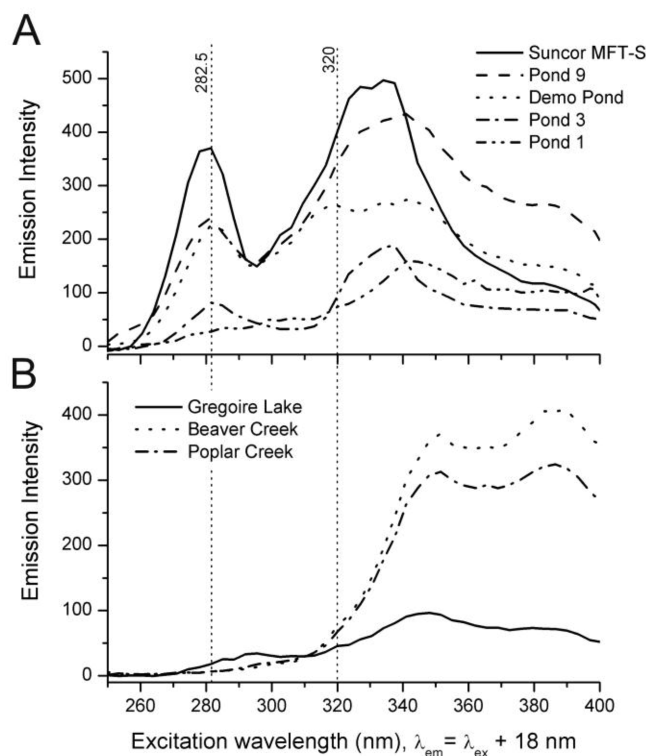

Figure 2. (A) SFS spectra for Syncrude Canada Ltd.'s Pond 1, Pond 3, Pond 9, and Demonstration Pond and Suncor Energy Inc.'s Mature Fine Tailings South pond and (B) reference sites near the Alberta oil sands region.

previously with these substances (Miano and Senesi, 1992; Peuravuori et al., 2002). Extending the excitation range to 600 nm in our SFS procedure, the fulvic acid showed a peak intensity at 390 nm while the peak intensity for humic acid occurred beyond 400 nm (Figure 3A). The emissions at 282 nm and 320 nm suggested that some substances in the Demonstration Pond were responsible for the difference from the reference sites. However, some natural aquatic waters also have a fluorescent peak at ≈280 nm (Peuravuori et al., 2002) using a similar SFS procedure. These authors attributed this fluorescence to aromatic amino acids or volatile acids containing highly conjugated aliphatic structures. Other monoaromatics are also known to fluoresce in this region, including substances detected in aged algal cultures (Ferrari and Mingazzini, 1995) and marine oil spills (Gordon and Keizer, 1974; Gordon et al., 1976).

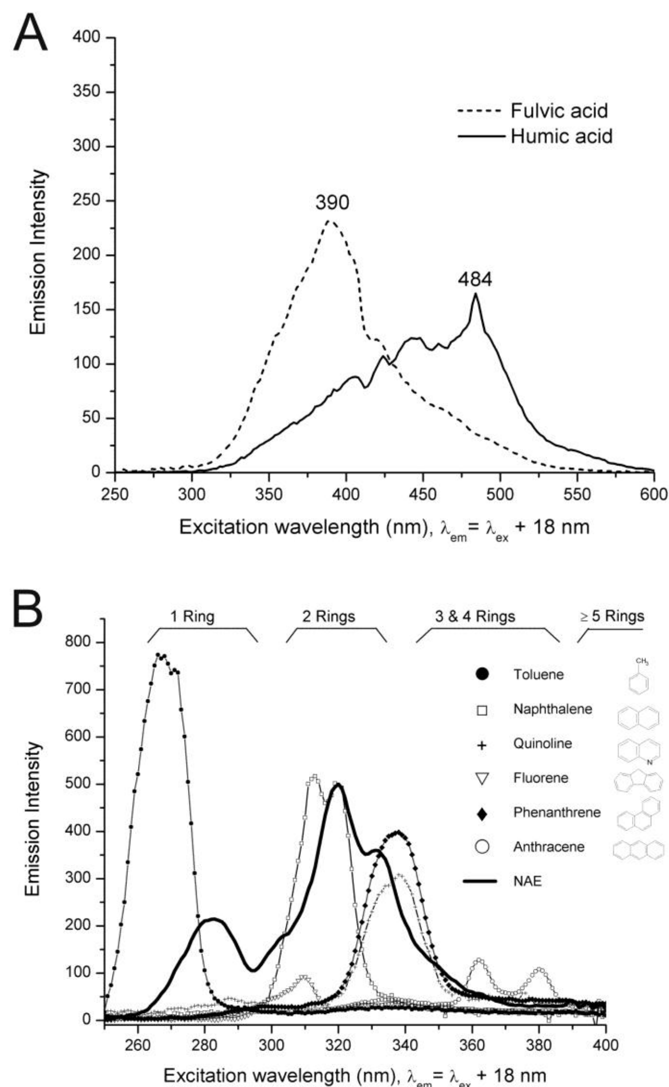

Figure 3. (A) SFS spectra of fulvic acid and humic acid. (B) SFS spectra of various PAHs detected in the NAE preparation and toluene as an example of a monoaromatic compound

Naphthenic acids are natural constituents of oil sands and become concentrated in OSPW during the bitumen extraction process. Concentrations ranging from 80 to 110 mg L^{-1} are found in the settling basins where tailings are presently being stored (FTFC, 1995). An analysis of the oil sands NAE (pH 8.3) and the commercial preparation of naphthenic acid (FNA, pH 7.5) showed similar fluorescence profiles as that of OSPW, with peaks at 280 nm and 320 nm. Previous studies have shown that pH can alter both the

fluorescent intensities as well as spectral shape in some environmental samples (Cabaniss and Shuman, 1987; Pullin and Cabaniss, 1997). Over a pH range from 4.3 to 10.5, there was a decrease in the SFS emission intensity but no significant shift in the various fluorescent peaks for the FNA (data not shown).

FNA is reported to be a mixture of alkylated cyclopentane carboxylic acids (Sigma-Aldrich). Negative ion electrospray-mass spectrometry has shown that this petrochemical-based Fluka product has a very different congener distribution from the naphthenic acids isolated from the Alberta oil sands (Lo et al., 2006), yet, using SFS the same fluorescent spectra was observed. Compounds such as the "classical" naphthenic acids should not be fluorescent because they lack an aromatic ring and are usually described as acyclic, monocyclic and polycyclic carboxylic acids (Brient et al., 1995). A possible explanation is that compounds with similar chemical properties such as aromatic acids may "co-isolate" with naphthenic acids and therefore be present as contaminants in NAE and FNA samples and could account for the fluorescent spectra observed with these compounds.

Of course, PAHs are well-known fluorescent compounds and low concentrations have been detected in OSPW (Madill et al., 2001; Rogers et al., 2002). However, the extraction method called for solvent washes using dichloromethane to remove neutral organics, including PAHs, from the final mixture. Despite these steps, there was no reduction in the characteristic fluorescence signature or its intensity, suggesting that the compounds responsible for the fluorescence may not be PAHs. The NAE stock solution (~5000 mg L^{-1} of naphthenic acids) was analyzed for PAHs and extremely low concentrations were detected. Naphthalene, quinoline, C4 substituted phenanthrene/ anthracene, and C3 substituted fluorene were measured at concentrations of less than 0.3 μg L^{-1} in the naphthenic acid extract. Given the volume of NAE being used in the SFS procedure, PAH concentrations would have been less than 1 ng L^{-1}. Individual SFS of these compounds determined that they were not detectable at these low concentrations (data not shown). When they were scanned at higher concentrations (μg L^{-1}), these PAHs had fluorescent signatures that differed from those observed in OSPW and the naphthenic acid extract (Figure 3B). None of the detected compounds had a peak at 282.5 nm and

even the monoaromatic compound, toluene, had a peak at 267 nm. However, naphthalene did exhibit a double peak (313 nm and 320 nm). Other naphthalene-like compounds may explain the spectra in the 320 nm to 340 nm area in the OSPW (Figure 2A). The concentrations of quinoline and phenanthrene (λ_{max} = 338 nm) were not measured in the OSPW, but it is unlikely they would be in sufficient quantities to account for the emission intensities in this spectral region.

Along with naphthenic acids, fatty acids and aromatic acids have been detected in certain crude oils (Qian et al., 2008). An analysis showed that the acids found in South American heavy crude oil had structures ranging from C15-C55 with cyclic (1-6 rings) and aromatic (1-3 rings) structures and that "classical" naphthenic acids made up less than half of the total acids observed (Qian et al., 2001a,2000b). ^{13}C-Nuclear magnetic resonance spectroscopy has shown that commercial Fluka naphthenic acid contains 7.1% aromatic carbons (Rudzinski et al., 2002). Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy analysis of fractions collected from the NAE indicated an increased presence of aromatic compounds in association with naphthenic acids of higher molecular weight (Frank et al., 2008). It is, therefore, possible that aromatic acids are responsible for the fluorescent signature that has been observed in OSPW, NAE, and FNA.

Analyses of additional OSPW samples from Syncrude and Suncor Energy Inc. revealed spectra with the highest peak intensities were found in water samples containing the highest concentrations of naphthenic acids (Table 1; Figure 2). When these two variables are plotted, a positive correlation was observed with increasing naphthenic acid concentration (Figure 4). Environmental samples in several rivers in the Alberta oil sands region were tested with SFS (Figure 5) and there were no peaks at 320 nm and low intensities at 282.5 nm. This low intensity at 282.5 nm would concur with the natural background levels of naphthenic acids of 1 – 2 mg L^{-1} in surface waters in this region (Leung et al., 2003). Using FTIR, Scott et al. (2008) demonstrated that naphthenic acid concentrations were less than 0.6 mg L^{-1} in the Athabasca River, Ells River, Mackay River, and the Muskeg River. The spectral intensities at higher wavelengths (> 340 nm) were comparable to known fluorescence signals for fulvic and humic acids. Fluorescence

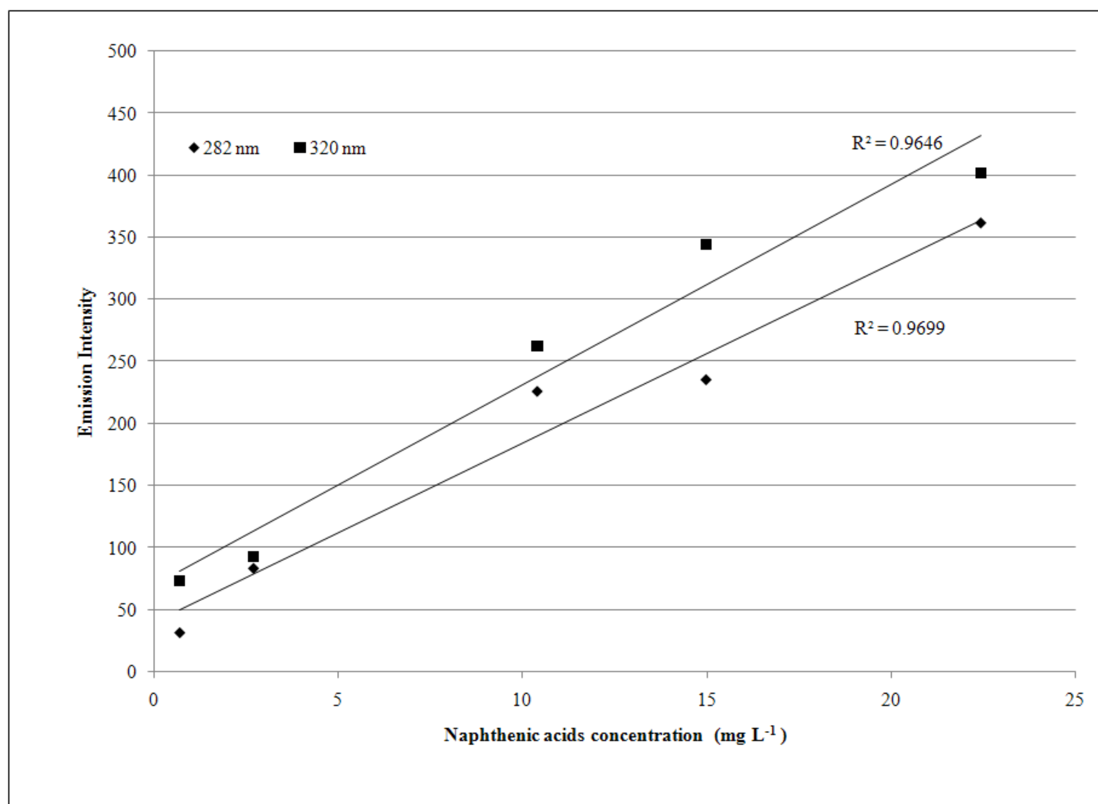

Figure 4. Correlation of the SFS emission intensities and the naphthenic acid concentrations (determined using Fourier transform infrared (FTIR) spectroscopy) in Syncrude Canada Ltd.'s Pond 1, Pond 3, Pond 9, and Demonstration Pond and Suncor Energy Inc.'s Mature Fine Tailings South pond. Note: Due to the high intensities observed with the Pond 9 and the MFT-S samples, they were diluted by 1/3 with 0.05 M $NaHCO_3$.

in this spectral region was also noted in the reference sites (Beaver Creek and Poplar Creek), however, the concentration of naphthenic acids in these creeks was measured at less than 1 mg L^{-1} and the characteristic spectra of OSPW were not observed (Figure 2B).

The groundwater sample collected near the tailings pond water dyke showed an almost identical fluorescence signature to the NAE, with peaks at 282.5 nm and 320 nm but this signature was not observed in the groundwater reference sample (Figure 6). The naphthenic acid concentration of the sample near the tailings pond was 26 mg L^{-1} while the naphthenic acid concentration in the reference sample was not detectable (less than 1 mg L^{-1}). Considering the concentration of the naphthenic acids in the ground water

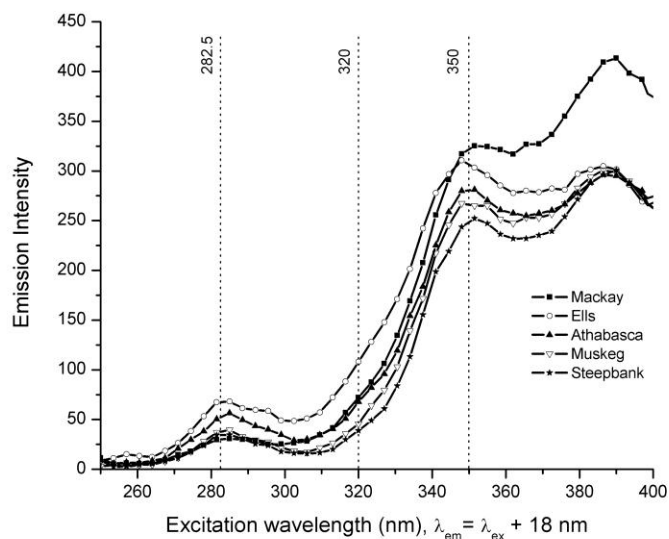

Figure 5. SFS spectra for various rivers near the Alberta oil sands region.

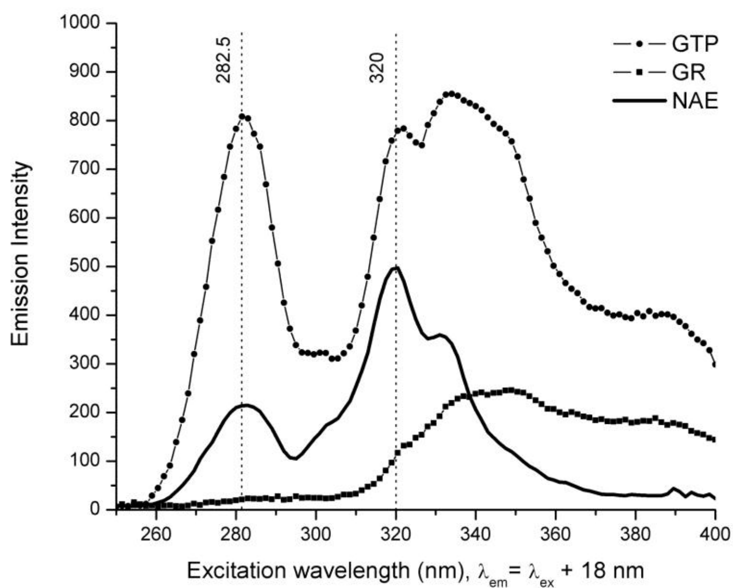

Figure 6. SFS spectra for groundwater collected at Suncor Energy Inc. GTP = groundwater near tailings pond; GR = groundwater reference site; NAE = naphthenic acid extract.

sample near the tailings pond, the intensity of the peaks at 280 nm and 320 nm was higher than was expected. This is likely due to the difference in the age of the OSPW at this location. The OSPW stored in the tailings pond was much “fresher” compared to the OSPW in the ponds used in this study as these ponds were filled with OSPW over 13 years ago and have had no new inputs of OSPW since the time of their construction. It would not be unreasonable to assume that over time there has been some degradation of the aromatic acids believed to be responsible for the fluorescent signature observed with OSPW.

A number of quantitative methods have been used to measure the concentration of naphthenic acids in water samples and these include Fourier transform infrared (FTIR) spectroscopy (Jivraj et al., 1995; Holowenko et al., 2001), negative-ion electrospray mass spectrometry (Headley et al., 2002), high performance liquid chromatography (Yen et al., 2004), and gas chromatography-mass spectrometry (Scott et al., 2008). All of these methods are laborious and time consuming. SFS is a rapid screening method that will detect the presence of aromatic acids closely associated with the naphthenic acids and do so in less than 5 minutes without the need for pre-treatment. If semi-quantitative data were required for a particular site, it would be feasible to measure water samples by SFS and one of the methods listed above and then to generate a dilution calibration curve for estimating the naphthenic acid content in environmental samples when using SFS analysis.

OSPW is currently being stored in large settling basins on oil sands sites and there are increasing concerns over OSPW entering the environment due to its demonstrated adverse effects on semi-aquatic and aquatic organisms (van den Heuvel et al., 1999, 2000; Pollet and Bendell-Young 2000; Gurney et al., 2005; Nero et al., 2006; Lister et al. 2008). Thus, developing SFS as a method to monitor the movement of OSPW into the environment would provide a useful and time-efficient screening tool.

5. Conclusions

The OSPW from Syncrude Canada Ltd. and Suncor Energy Inc. show a characteristic fluorescence signature. This fluorescent signature, which was also observed in both Fluka naphthenic acid and naphthenic acid extract, may be caused by the presence of aromatic compounds closely associated with “classical” naphthenic acids. Although not a quantitative measurement of naphthenic acids, the peak intensities at 282.5 nm and 320 nm in OSPW correlate with the determined naphthenic acid concentrations. Consequently, SFS appears to be a fast and effective way to monitor the presence of OSPW in ground and surface waters within the oil sands region.

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