Identification of Oil Sands Naphthenic Acid Structures and Their Associated Toxicity to *Pimephales promelas* and *Oryzias latipes*

by

Anthony Bauer

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Abstract

The oil sands, located in north-eastern Alberta, are one of the largest deposits of oil worldwide. Because the Alberta Environmental Protection and Enhancement Act prohibits the release of oil sands process-affected material into the environment, industry is storing vast quantities of tailings on mine lease sites. The oil sands industry is currently accumulating tailings waste at a rate of $>10^5$ m^3/day, for which reclamation strategies are being investigated. Naphthenic acids (NAs) have been identified as the most toxic component of oil sands tailings as they are considered acutely toxic to a variety of biota, and are therefore a target contaminant for tailings pond reclamation strategies. Current literature based on Microtox® assays (marine bacteria *Vibrio fischeri*) suggests that lower molecular weight NAs are more toxic than higher molecular weight NAs. The following thesis involves the utilization of NA fractions and their relative toxicities to determine if NA toxicity is related to NA molecular weight.

A previous study generated an oil sands-derived naphthenic acid extract (NAE), which was fractionated by distillation at stepped temperatures, yielding five fractions with increasing median molecular weights (Daltons). In the present study, the same extract and five fractions were utilized. To expand on the earlier characterization which involved a low resolution electrospray ionization mass spectrometry (ESI-MS), the whole extract and five fractions were analysed using electrospray ionization high-resolution mass spectrometry (ESI-HRMS) and synchronous fluorescence spectroscopy (SFS). Mean molecular weights were generated for each fraction, and an increase in molecular weight with increasing fraction number was confirmed. Respective mean Daltons and relative proportions for each fraction are as follows: 237 and 11.9 % (fraction 1), 240 and 32.3% (fraction 2), 257 and 33.4% (fraction 3), 308 and 16.8% (fraction 4), and 355 and 5.6% (fraction 5). When chemical analyses of fractions were compared, it was

determined that structures contributing to increased molecular weight included increased cyclic structures (up to 7-ring structures), aromaticity (mono- and diaromatics), nitrogen, sulfur, and oxygen heteroatoms, and dihydroxy/dicarboxy compounds. In addition, characterization data suggested the presence of NAs exhibiting estrogenic structures.

Following chemical characterization, NA fractions were subject to embryo/larval bioassays using two fish species: *Oryzias latipes* (Japanese medaka) and *Pimephales promelas* (fathead minnow). Endpoints evaluated were mortality, time to hatch, hatch length, and abnormalities. Results suggest that relative NA fraction toxicity is not related to molecular weight, as no trend relating mean Dalton weight to toxicity was observed for any endpoint in both species. Acute toxicity data indicated differences between fractions as high as 2-fold, although results were species-dependent. Fraction 1 displayed the lowest potency (highest LC_{50}) for both Japanese medaka (0.291 mM) and fathead minnow (0.159 mM). Fractions 3 and 2 for Japanese medaka (0.149 and 0.157 mM, respectively), and fractions 5 and 2 for fathead minnow (0.061 and 0.080 mM, respectively) displayed the greatest potencies for mortality (lowest LC_{50}). When fraction LC_{50}s for Japanese medaka were compared to the whole NAE (0.143 mM), the mid molecular weight fractions (fractions 2 and 3) appeared most similar to the whole NA. . In terms of relative toxicity and proportion, constituents in the mid molecular range fractions (2 and 3) likely represent greater risk compared to other fractions, and further chemical and toxicological characterization of constituents within these fractions is warranted particularly for long-chained, monocarboxylic acids, with low aromaticity.

Japanese medaka and fathead minnow varied in their sensitivity and their relative response to different fractions. In general, fathead minnow were more sensitive than Japanese medaka based on lower estimates of LC_{50} and threshold (growth) values in addition to the

presence of developmental abnormalities (predominately yolk sac edema) associated with a few of the fractions. Compared to differences in toxicity between fractions for a given species (>2-fold for fathead minnow), there was more variability between species for a given fraction (> 3-fold for fraction 5). Also, the relative toxicity of fractions as indicated in the present study is contrary to the results generated using *Vibrio fischeri* for the same fractions. Thus, there is a need for multi- endpoint and species toxicity evaluations to assess the efficacy of remediation and reclamation options for reducing toxicity of oil sands tailings.

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Chapter 1. General Introduction

1.1 Oil Sands Background

1.1.1 *Global Context*

Alberta holds the third largest reserve of oil next to Saudi Arabia and Venezuela and the largest deposit of oil sands worldwide (Alberta Government, 2012). The Canadian oil sands deposits in Alberta are comprised of three regions, the largest being the Athabasca, followed by the Cold Lake and Peace River regions. These three deposits encompass ~140,200 km^2 (Alberta Government, 2012). Moreover, Alberta's oil sands deposits are estimated to contain 1.71 trillion barrels of bitumen of which 10 percent can be economically obtained (Alberta Treasury Board, 2009).

Alberta's oils sands are a major source of revenue for the province and the country. Oil sands royalties in 2008 (not including conventional oil, natural gas, or offshore drilling) totalled $2.97 billion from the production of 1.19 million barrels per day (bbl/d) of marketable crude bitumen (Alberta Energy, 2009b). The oil sands have also made Canada a major political force through the sheer size of the reserves. Roughly 65% of Alberta's crude oil is transported south for consumption in the USA (Alberta Energy, 2009b). Alberta's oil sands are, and will continue to be, a vital source of energy and economic security for Canada and elsewhere.

1.1.2 *What are Oil Sands?*

Oil sands material is a heavy crude oil deposit that require upgrading to a lighter oil for transportation along pipelines (Alberta Energy, 2009a). It is composed of 68% sand, 23% bitumen and 9% water, the bitumen being the sought after resource (Fair, 2010). Bitumen, a

viscous hydrocarbon mixture, occurs naturally in the Athabasca, Peace River and Cold Lake regions of Alberta. Historically it was utilized by aboriginal peoples in this region to waterproof birch bark canoes, and was even presented as an item of trade in 1719 (Alberta Energy, 2009c). Bitumen is an important resource as it can be upgraded to synthetic crude oil. Synthetic crude oil can then be used to produce gasoline, diesel, and jet fuel, as well as industrial chemicals and polymers such as plastics and rubber (Alberta Treasury Board, 2009).

1.2 Oil Sands Processes

To extract and process bitumen requires the withdrawal on average of 3 barrels of water for every barrel of oil produced (Shell, 2009; Suncor Energy, 2009). The oil sands industry currently extracts an average of 0.6% of the Athabasca River's annual flow and 5% at its lowest flow during the winter (Shell, 2009; Suncor Energy, 2009; Syncrude Canada Ltd., 2009). Three major oil sands lease holders: Suncor, Syncrude and Shell, claim that 85-90% of their operations' water requirements are met through recycling water from tailings and settling basins (Shell, 2009; Suncor Energy, 2009; Syncrude Canada Ltd., 2009).

In order for the oil to reach market, it must first be extracted as bitumen and subsequently separated from the associated sand material. Because oil extracted from bitumen is very crude oil it must then go through upgrading to a synthetic crude oil as well as refining processes. The bi-product of extraction, separation, and upgrading of bitumen is the production of $\sim 10^5$ m^3 of tailings/day which is currently stored in large pits on site (FTFC, 1995).

1.2.1 Extraction

There are two main methods being utilized to recover bitumen, "in-situ" and open pit mining. Roughly 80 percent of Alberta's recoverable oil sands are too far below the surface to utilize open-pit mining, but can be extracted by "in-situ" methods (Alberta Energy, 2008). This method is similar to conventional oil extraction in that it uses a stationary pump to bring oil to the surface. But, because bitumen is a much more viscous mixture than conventional light oils, it requires technologies such as steam injection to allow the bitumen to flow to a pump to bring it to the surface (Alberta Energy, 2009a).

Bitumen that is close to the surface is mined and then separated from the sand before it can be processed and upgraded. In order to extract the bitumen, open-pit mining in combination with the Clark Hot Water Extraction process are employed (FTFC, 1995). Open-pit mining involves removing large quantities of earth by shovel where it is transported by truck to a cleaning facility. The Clark Hot Water Extraction process is a separation method that mixes the oil sand with hot water, sodium hydroxide and steam, separating it into three distinct layers of sand, water and bitumen. The bitumen is then skimmed off the top to be refined further while the water and sand are used for land reclamation and elsewhere in the operation, such as recycling water for in-situ steam injection (Alberta Energy, 2009c). After the extraction process, bitumen requires upgrading to a synthetic crude oil. This involves thermally cracking the bitumen using hydrocrackers or fluid cokers. The oil is then hydrotreated with hydrogen and high temperature and pressure to remove impurities like trace metals, nitrogen and sulphur (Harris, 2001).

1.2.2 Tailings and Reclamation

Unrecycled water generated from extraction, upgrading and refining, is a mixture of trace metals, nitrogen, sulphur, and residual hydrocarbons (Allen, 2008). The resulting water is termed process water, and, due to its high concentration of organic acids, is considered toxic to biota (Colavecchia et al., 2004; Farwell et al., 2006; Kamaluddin and Zwiazek, 2002; Nero et al., 2006a). The product of mining, upgrading, and refining is a waste mixture comprised of 70-80% process and/or fresh water, 20-30% solids, and 1-3% unrecovered bitumen (Allen, 2008). The process water contains trace metals and dissolved compounds, while the solid portion can be sand and/or fines, composing a slurry which is collectively termed tailings (Allen, 2008).

The Alberta Environmental Protection and Enhancement Act prohibits the release of tailings waste into the natural environment and requires that lease sites be remediated to their original state or better (Madill et al., 2001). Because of the "zero discharge" policy, which also ensures that no process affected waste can be discharged back into the Athabasca River or its tributaries, industry is adopting methods of storing the process tailings on-site (Allen, 2008). The mine tailings are pumped to specially constructed settling basins and tailings ponds where it is left to settle out. In general, the tailings ponds are constructed by creating large dykes composed of sand. Contained within the dykes, are constructed sand or coarse tailing beaches on to which the tailings are discharged (Fair, 2010). Over time, the tailings slurry forms three distinct phases. The faster settling solids, such as sand, will fall to the bottom of the water column first, forming a sedimentary layer within a few weeks (Scott et al., 1985). These fine tailings will reach 30-35 wt% solid after several years, at which point it is termed mature fine tailings (MFT) (Allen, 2008). Due to different suspended solid settling rates, the tailings pond will also form a middle

layer of suspended silt and/or clay, and finally a surface water layer (Allen, 2008). Tailings are continually discharged into these basins for storage.

In addition to tailings, open-pit mining leaves large pits on the natural landscape rendering the environment unproductive. The oil sands industry is tackling this by investing heavily in extensive reclamation strategies.

The ultimate goal of industry is to combine waste disposal techniques with reclamation strategies to create expansive wetland areas, which has been termed the "Wet Landscape" approach. The wet landscape approach is a strategy employed to deal with MFT while maintaining vital ecosystems such as lakes and ponds for repopulation by wildlife ranging from algae and benthic invertebrates to fish and migrating waterfowl. The method employed requires the capping of MFT with a depth of about 5 meters of water (Harris, 2001). Water capping incorporates the use of mined-out pits which will create lake fetches of no more than 4 km so as to minimize re-suspension of fine tailings by wind to reduce contaminant bioavailability to organisms (Harris, 2001). To this end, research is being conducted on small test ponds on Syncrude and Suncor lease sites.

1.3 Naphthenic Acids

As mentioned previously, there are many components generated in the extraction, processing, upgrading and refining of bitumen as well as the coagulation of tailings that add to the toxic nature of oil sands tailings. Among the most toxic constituents of oil sands process water (OSPW) and tailings are polycyclic aromatic compounds (PAC), high salinity, and naphthenic acids (NAs). But, because the sodium salts of NAs are soluble in water, they can persist in aquatic systems for longer periods of time than particulate-associated PACs which

eventually settle out of suspension. Therefore, this study focuses on the toxicity of oils sands

NAs because of their persistence in aquatic environments and potential to be the most toxic

component of tailings (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986).

1.3.1 Properties of Naphthenic Acids

NAs are a complex group of alkyl-substituted acyclic, monocyclic and polycyclic

carboxylic acids (Figure 1.1) (Frank et al., 2008). They are identified as having the general

formula $C_nH_{2n+z}O_2$ where n is the carbon number and z refers to the hydrogen deficiency due to a

ring formation and is zero or a negative even integer (Clemente and Fedorak, 2005). The Z=-4

series predominates in oil sands material comprising up to 50% of the acids present (Lai et al.,

1996). NAs are chemically stable, non-volatile, surfactants and their sodium salts are soluble in

water (Clemente and Fedorak, 2005; Frank et al., 2008).

Many carboxylic acids found in OSPW do not strictly adhere to the chemical formula of

NAs, because they are inconsistent with the classical definition of NAs described above.

Recently, using more sophisticated mass spectrometry, structures in addition to the commonly

cited acyclic and polycyclic monoacids have been discovered. These structures include

dicarboxylic acids as well as some sulfur and nitrogen-containing species (Frank et al., 2009;

Grewer et al., 2010; Headley et al., 2009a). In addition, some "classical" NAs, have been more

definitively characterised by two dimensional gass chromatography (2D GC) coupled with time-

of-flight mass spectrtometry (ToF-MS) methods and include structures such as tri – to

pentacyclic diamondoid acids, aromatic carboxylic acids, diamantane methyl and dimethyl

diamantine acids, acids with ethanoic acid side chains, and estrogen-like steroid acids (Rowland

et al., 2011a-c). Although there are structural differences between "classical" and oil sands NAs,

the term "naphthenic acid" is used herein to refer to the more complex suite of acid extractable organic compounds found within OSPW.
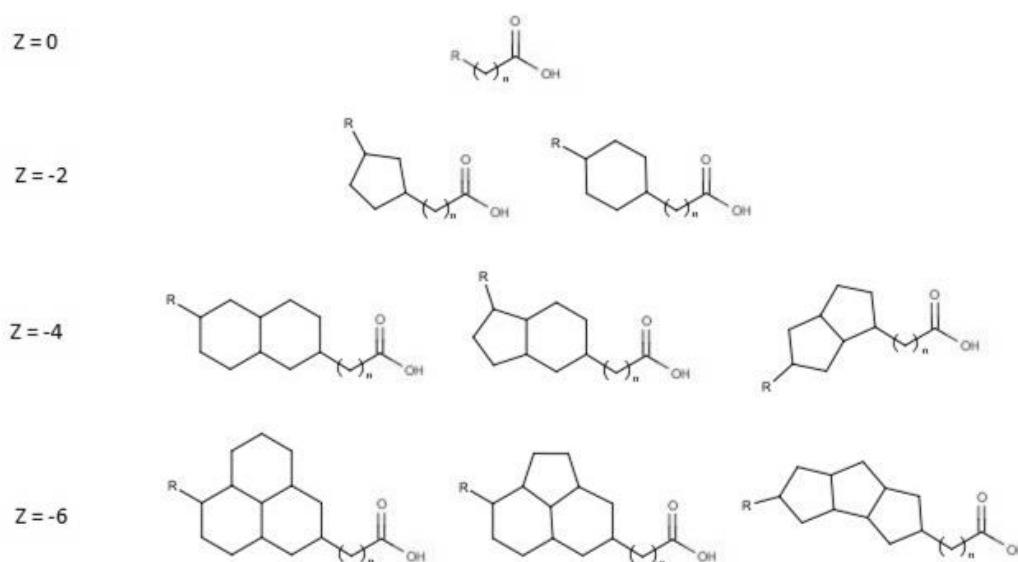
FIGURE 1.1: Examples of various z-series for classical naphthenic acid structures which conform to the general formula $C_nH_{2n-z}O_2$. Alkyl groups are represented by "R".

1.3.2 Fate of NAs in the Environment

NAs are naturally occurring compounds in crude oil and constitute about 2% of total bitumen by weight (Headley and McMartin, 2004). Due to the relatively shallow deposits of bitumen in the Athabasca region, NAs can enter surface waters by erosion of riverbanks and mixing with groundwater (Headley and McMartin, 2004). On the Athabasca oil sands leases, these organic acids are released under alkaline conditions (Lai et al., 1996). These alkaline conditions are present in the Clark Hot Water Extraction process employed during the extraction and separation of bitumen, resulting in high concentrations of NAs in tailings.

On oil sands lease sites, reclamation test pond NA concentrations can range from 0.8-67 mg/L depending on the reclamation technique employed (Farwell et al., 2009; Siwik et al., 2000). Some of the highest NA concentrations occur in Mildred Lake Settling Basin (MLSB), the primary tailings basin on the Syncrude lease site, with typical concentrations between 68 mg/L and 120 mg/L (CFRAW, 2007; Holowenko et al., 2002). Adjacent to the lease sites, the Athabasca River contains NA concentrations that are typically below 1 mg/L, while lakes in the region unaffected by oil sands industry contain concentrations between 1 and 2 mg/L which are likely due to natural inputs (Allen, 2008; Golder Associates Ltd., 2002).

1.3.3 Toxicity of Naphthenic Acids

Narcosis is the probable mode of acute toxicity from NAs, and NAs have been proposed endocrine disruptors and potential carcinogens, but not mutagens (Headley and McMartin, 2004; Roberts, 1991; Scarlett et al., 2012). Recent studies have identified carcinogenic potential of NAs using the ADMET predictor™ and found TD_{50} values >50 mg/kg/d, with NAs containing an aromatic ring having greater carcinogenicity (TD_{50}s 19-85 mg/kg/d) than other NAs tested (Scarlett et al., 2012). Conversely, NAs subject to the Ames test for mutagenicity did not display mutagenic potential (Scarlett et al., 2012). With regard to endocrine disruption, estrogen-like NAs have been identified by their structural similarities to estrogen, as well as predicted estrogen and androgen receptor activation using ADMET predictor software (Rowland et al., 2011b). Additionally, OSPW containing NA concentrations >25 mg/L have been shown to inhibit fathead minnow spawning, reduce secondary sex characteristics and testosterone in males, and reduce 17B-estradiol in females (Kavanagh et al., 2011). As detailed below, NAs have been shown to negatively affect reclamation environments in high concentrations by reducing

phytoplankton and aquatic invertebrate community diversity, and displaying acute toxicities to fish and plants (Leung et al., 2001; McCormick, 2000; Nero et al., 2006a). The difficulty in extracting, then separating and analysing NAs in the lab is the greatest issue faced in studying them (Headley and McMartin, 2004).

The following is a synopsis on NA mixtures and OSPW toxicity tests reported to date.

1.3.3.1 Bacteria (*Vibrio fischeri*)

Toxicity studies have employed the Microtox assay as a quick assessment of acute toxicity of NAs to the marine bacterium, *Vibrio fischeri*. In a study by Frank et al. (2008), results suggest that oil sands-derived NA-fraction toxicity displays an inverse relationship with the molecular weight of each fraction. In a follow-up study, Frank et al. (2009) determined that a decrease in acute toxicity to *Vibrio fischeri* observed in NAs of greater molecular weight was possibly due to an increase in carboxylic acid content. Most recently, Jones et al. (2011) discovered when assessing the toxicity of individual monocarboxylic NAs, that an increase in alkyl chain length conferred an increase in toxicity to *Vibrio fischeri*.

1.3.3.2 Phytoplankton

Process water from MFT and consolidated tailings (CT) sources that is less than five years old is acutely toxic to phytoplankton (Leung et al., 2001). In addition, it appears that NAs, with concentrations above 20 mg/L directly affect phytoplankton community structure (Leung et al., 2001; Leung et al., 2003). But, test ponds displaying an effect on phytoplankton community composition (high NAs and salinity) did not promote a reduction in total biomass (Leung et al., 2001). This may be a result of opportunistic niche-filling behaviour by more robust taxa of phytoplankton, specifically species of Chlorophyta, which effectively mitigates NA toxicity in the environment (Leung et al., 2001; Leung et al., 2003).

1.3.3.3 Aquatic Invertebrates

In the past, there have been very few studies identifying NA toxicity to aquatic invertebrates. The only study involving live *Daphnia magna* by CEATAG (1988) utilised OSPW as the test contaminant. With an assumed OSPW NA concentration of 80 mg/L, and a *Daphnia magna* LC_{50} of 76-98% OSPW, the LC_{50} to NAs equalling a range from 61-78 mg/L can be derived (Armstrong, 2008). *D. magna* exposed to Syncrude OSPW displayed an acute 96 h EC_{50} value of 10% OSPW (MacKinnon and Boerger, 1986), although NA concentrations were not reported. It has also been determined that wetlands impacted by effluent containing NAs display a decrease in aquatic invertebrate community diversity, but with an increase in densities of the opportunistic invertebrate *Chironomus* spp. when compared to reference sites (Bendell-Young et al., 2000). *Chironomus tentans* exposed to Suncor OSPW displayed a 14 d LC_{50} value of 71% OSPW (Whelly, 1999).

1.3.3.4 Fish

Most studies regarding NAs and toxicity to fish have performed assays using oil sands process material (OSPM) and/or OSPW. Therefore, associated toxicities cannot be solely attributed to NAs. In general, OSPW has been shown to increase deformities and mortality to larval and juvenile fathead minnow, yellow perch, and Japanese medaka (Kavanagh et al., 2011; Nero et al., 2006a; Peters et al., 2007; Siwik et al., 2000). In adult fathead minnow, yellow perch, and goldfish, a shift in blood cell composition, cytological impacts on the liver and gill arch, and viral tumors have been observed (Farrell et al., 2004; Nero et al., 2006b; van den Heuvel et al., 2000). Reproductive impairment was observed in fathead minnow and goldfish, with fish exhibiting a reduction in spawning, fecundity, secondary sex characteristics, and sex steroids (Kavanagh et al., 2011; Lister et al., 2008). These impairments to reproduction may be due to

chronically acting monoaromatic steroidal NAs recently identified in OSPW (Rowland et al., 2011b). Thus, at high OSPM (NA) concentrations, one would expect to observe high larval/ juvenile fish mortality, while at lower concentrations a population decline may manifest due to reproductive impairment and immunosuppression in adult fishes.

1.3.4 Napthenic Acid Degradation

NAs have been shown to definitively influence microbial community composition (Hadwin et al., 2006). As a result of a natural background level of NAs in this region, indigenous microbial communities have adapted to degrade NAs and effectively reduce the toxicity of OSPW (Clemente and Fedorak, 2005; Frank et al., 2008; Lai et al., 1996). This biodegradation of NAs occurs in two phases: a relatively quick degradation of a labile fraction, followed by a much slower degradation of highly alkyl-branched, more recalcitrant NAs (Del Rio et al., 2006; Han et al., 2008; Scott et al., 2005). When OSPM contains a higher proportion of recalcitrant NAs, the half-life for biodegradation is between 44-240 days. In contrast, commercial NA biodegradation half-life ranges between 1-8 days, with full biodegradation taking only 14 days (Han et al., 2008; Scott et al., 2005). Furthermore, the identification of NA composition in aged OSPW indicates that an extremely resilient fraction may have a half-life of up to 13.6 years (Han et al., 2009).

From a remediation standpoint, it is important to note that aging tailings experience a greater degradation of lower molecular weight (less recalcitrant) NAs by microbial communities (Frank et al., 2008), decreasing the toxicity of the water fraction present, and, over time, changing the composition of NAs present in tailings. In addition, it has been suggested that NAs of higher molecular weight (HMW) are less toxic than lower molecular weight (LMW) NA compounds when evaluated with a *Vibrio fischeri* Microtox assay (Frank et al., 2008). Thus, it is

apparent that as tailings age they become less toxic to *Vibrio fischeri,* and possibly other biota, due to a shift in NA composition to a predominance of HMW NA compounds.

1.4 Research Objectives

The primary objective of this study was to determine if oil sands NA fraction acute toxicity was associated primarily with molecular weight. More specifically, toxicities of five oil sands NA fractions, of increasing mean Dalton (Da) weights, were evaluated to determine if they differed in acute toxicity to two fish species (*Oryzias latipes* and *Pimephales promelas*). Therefore the null hypothesis tested was that all fractions displayed equivalent toxicities. In addition, because of knowledge gaps regarding oil sands derived NAs, this study incorporated the characterization of oil sands NA fractions and their associated structures using sophisticated mass spectrometry, to determine what contributed to NA molecular weight.

To determine structures contributing to NA molecular weight, oil sands NAs previously extracted from the Syncrude lease site in Alberta (Frank et al., 2006), and fractionated by Kugelrohr distillation (Frank et al., 2008), were provided by the primary author of Frank et al (2008). The five NA fractions (with increasing molecular weights) used in this study were previously analysed using electrospray ionization mass spectrometry (ESI-MS), from which molecular weights and ring structures were derived (Frank et al., 2008). This study expanded on this knowledge by using novel electrospray ionization high resolution mass spectrometry (ESI-HRMS) and synchronous fluorescence spectroscopy (SFS) chemical analysis to explore molecular weight, ion distribution, degree of unsaturation, and aromaticity inherent in each fraction. The information provided by this analysis will further understanding about the chemical

structures of oil sands NAs, and provide insight into the reasons for the recalcitrant nature of HMW NAs and reduced toxicity of biodegraded tailings.

Based on studies exploring the degradation of NAs by oil sands microbes (described above), it was predicted that NA fractions of LMWs would be more toxic than those with HMWs. To test the proposed hypothesis, that oil sands NA acute toxicity is associated primarily with molecular weight, *Pimephales promelas* (fathead minnow) and *Oryzias latipes* (Japanese medaka) embryos were exposed to five oil sands NA fractions with increasing molecular weights. Acute toxicity was investigated by evaluating mortality, time to hatch, and hatch length endpoints. In addition, teratogenicity was investigated by observing developmental abnormalities in newly hatched larvae. Endpoints were evaluated to derive LC_{50} and threshold values for each fraction. Subsequently, LC_{50}s and thresholds values were compared for each endpoint to determine if fractions displayed equivalent toxicities, or, alternatively, a molecular weight-toxicity relationship. The results of these analyses have the potential to impact oil sands lease site reclamation strategies and policy regarding potential priority NAs.

Chapter 2. Enhanced Characterizations of Naphthenic Acids Derived from Oil Sands Process Waters Using Fractional Distillation and High Resolution Mass Spectrometry

2.1 Introduction

Due to its vast deposits of bitumen, Alberta alone holds the largest deposit of oil sands and third largest reserve of oil worldwide next to Saudi Arabia and Venezuela (Alberta Government, 2012). These deposits encompass ~140,200 km^2 (Alberta Energy, 2008) and contain an estimated 1.71 trillion barrels of bitumen (Alberta Treasury Board, 2009). Tailings generated from extraction, upgrading and refining of bitumen are a mixture of organic acids, PACs, trace metals, nitrogen, sulphur, and residual hydrocarbons (Allen, 2008), and are considered acutely toxic to biota (Colavecchia et al., 2004; Farwell et al., 2006; Kamaluddin and Zwiazek, 2002; Nero et al., 2006a). The Alberta Environmental Protection and Enhancement Act prohibits the release of tailings waste into the natural environment and requires that lease sites be remediated to the pre-mined state or better (Madill et al., 2001). Because of the "zero discharge" policy, industry is adopting methods of storing process tailings on-site until remediation is thoroughly evaluated (Allen, 2008). Among the most toxic constituents of OSPW and tailings are NAs which possess narcotic and estrogenic potential, and display persistence in aquatic systems (Dokholyan and Magomedov, 1983; Headley and McMartin, 2004; Kavanagh et al., 2011; MacKinnon and Boerger, 1986).

NAs are a complex group of alkyl-substituted acyclic, monocyclic and polycyclic carboxylic acids and are identified as having the general formula $C_nH_{2n+z}O_2$, where *n* is the

carbon number and z refers to the hydrogen deficiency due to a ring formation (Clemente and

Fedorak, 2005). However, many oil sands carboxylic acids do not strictly adhere to this chemical

formula as there are many structures within OSPW naphthenic acid extracts (NAEs) that are

inconsistent with this definition. Recently, the use of more sophisticated mass spectrometry, has

led to the discovery of compounds that do not possess the criteria commonly defined for acyclic

and polycyclic monoacids. This includes structures such as dicarboxylic acids, diamantane acids,

diamondoid acids, monoaromatic and diaromatic carboxylic acids, heteroatoms, and estrogen-

like steroid acids (Frank et al., 2009; Grewer et al., 2010; Headley et al., 2011a; Headley et al.,

2009a; Rowland et al., 2011a; Rowland et al., 2011b; Rowland et al., 2011c).

Because of the difficulty in extracting NAs from OSPW, commercial NAs have widely

been used in toxicity tests relating to oil sands (Apostol et al., 2004; Bendell-Young et al., 2000;

Jones et al., 2011; Kamaluddin and Zwiazek, 2002; Lai et al., 1996). However, the structural

composition of commercial NAs is significantly different from oil sands-derived NAs. When

compared to a Kodak or Merichem commercial NAE, oil sands-derived NAs are more complex

and display a higher percentage of higher molecular weight C22+ (22 carbons) structures

(Clemente et al., 2003). Additionally, many commercial NAs contain "classical" NA structures

(those conforming to the NA formula $C_nH_{2n+z}O_2$) and are devoid of some "non-classical" NA

structures typical of oil sands NAEs. This includes steroidal NA compounds that have recently

been identified in oil sands NAs but which are absent from commercial NAs (Rowland et al.,

2011b).

A strategy being investigated for the remediation of oil sands tailings ponds includes the

use of indigenous microbial communities to reduce the toxicity of tailings. As a result of a

natural background level of NAs in the Athabasca region, indigenous microbial communities

have adapted to degrade NAs and effectively reduce the toxicity of OSPW (Clemente and Fedorak, 2005; Frank et al., 2008; Lai et al., 1996). This is thought to result from the preferential degradation of lower molecular weight (less recalcitrant) NAs by microbial communities, which over time change the composition of NAs present in tailings (Clemente et al., 2004; Lai et al., 1996). This is exemplified in a study by Frank et al. (2008), in which it was determined that oil sands- derived NA fractions of LMW were more toxic than HMW NA fractions when evaluated with *Vibrio fischeri* (Microtox assays). In contrast, a study by Jones et al. (2011) determined that individual carboxylic acids increased in toxicity with increasing molecular weight when subject to Microtox assays. Another study by Frank et al. (2009) clarifies this discrepancy by revealing that toxicities of NA surrogates (carboxylic acids structurally similar to NAs) increased with increasing molecular weight, but dramatically decreased with increasing carboxylic acid content. As toxic potential of individual NAs appears to be modified by their molecular structure (Frank et al., 2009; Jones et al., 2011; Stanford et al., 2007), it becomes increasingly important to identify non-classical NA structures in order to determine toxic potential of oil sands-derived NAs and aid in oil sands tailings pond remediation efforts.

In order to determine what structures are contributing to oil sands NA molecular weight, five oil sands NA fractions of increasing molecular weight (fraction source. Frank et al., 2008), were utilized herein. The fractions represent NAs of increasing mean molecular weight, which contain "non-classical" oil sands acid structures not present in commercial NAEs. Previous analysis of these fractions was conducted using unit-resolution electrospray ionization mass spectrometry (ESI-MS) with limited structural elucidation (Frank et al., 2008; Frank et al., 2006). By applying electrospray ionization high resolution mass spectrometry (ESI-HRMS) and synchronous fluorescence spectroscopy (SFS) I was able to significantly extend previous work

and determine ion classes present, degree of cyclic structures, degree of oxygenation, and aromaticity of NA fractions of different molecular weights.

2.2 Materials and Methods

Oil sands naphthenic acid extract (NAE) and five NA fractions utilized in this study were provided by Dr. Richard Frank. The collection of tailings pond water and the extraction, purification, methylation, and fractionation of NAs were performed previously as described in Frank et al. (2006 and 2008). Demethylation and solid phase extraction cleanup were performed by myself at the University of Waterloo in Waterloo, Ontario. Chemical analysis of NAE and fractions using ESI-HRMS was conducted at the Aquatic Ecosystem Protection Research Division of Environment Canada in Saskatoon, Saskatchewan. Synchronous fluorescence spectroscopy was conducted by myself at Environment Canada in Burlington, Ontario. The methods and materials described hereafter are in brief.

2.2.1 Extraction

2.2.1.1 Tailings Pond Water Collection and Extraction of Organic Acids

On the Syncrude lease site, north of Fort McMurray, Alberta, 3000 L of tailings pond water was collected from a pump house at West End pit settling basin in 2005. Using 18 M sulphuric acid (H_2SO_4), collected samples were acidified to pH between 2 and 3 to allow organic acids to precipitate out of solution. After settling for 72 h, organics were transferred into 1-L high-density polypropylene centrifuge bottles (Nalgene, Fisher Scientific, Whitby, ON) and centrifuged at 3000 rpm for 15 min. The supernatant was siphoned off and discarded. In order to

solubilise NAs present, 200 mL of 0.1 M sodium hydroxide (NaOH) was then added to each 1-L

bottle, which were then shaken for 1 min. Bottles were then centrifuged at 10000 rpm for 10 min

after which the aqueous layer was collected in 25-L polyethylene carboys.

2.2.1.2 Purification of Organic Acid Solution

Diethylaminoethyl (DEAE) cellulose (75 g; Sigma Chemical Co., St. Louis, MO) mixed

with distilled water (300 mL) was used to filter out humic-like material from the organic acid

fraction of the tailings pond water. DEAE cellulose was pre-treated according to methods

outlined by Miles et al. (1983). DEAE cellulose was added to a negative flow filtration unit with

a 90 mm diameter 1-µm glass fibre filter paper. The organic acid solution was then poured into

the pre-wetted column and a vacuum was maintained at a pressure of 330 mm Hg so that the

filtration rate did not exceed ~1 L per 15 min. The final filtrate, now free of humic acids, was

collected in 1-L amber glass bottles (Fisher Scientific).

To remove base/neutral organic compounds, 300 mL of the organic acid cellulose filtrate

was poured into 2-L separatory funnel with 60 mL dichloromethane (DCM; Optima grade,

Fisher Scientific) and mixed. The funnel was left to settle for 10 minutes, resulting in two layers:

a DCM layer at the bottom and NaOH layer on top. The DCM layer was discarded and DCM

solvent wash was repeated 2 more times. The NaOH layer that now contained NAs free of

base/neutral organics, was collected in 25-L polyethylene carboys, and henceforth considered a

NA solution.

To precipitate out NAs, 800 mL of NA solution were transferred to a 1-L beaker, to

which 18 M sulphuric acid (H_2SO_4) was added dropwise to solution until pH 2 was obtained.

Precipitated NAs were then transferred to centrifuge bottles and centrifuged for 10 min at 3000

rpm. Supernatant was poured off and the bottles now contained the pellet from 1 L of acidified

18

NA solution. The remaining pellet was scraped off the bottom of the bottle, transferred to a 1 L

beaker and 250 mL of 0.05 M NaOH (solvent) was added. The bottle was shaken for 1 min and

sonicated for 10 min. The concentrated yellow-brown NA solutions were then transferred to 1 L

amber glass bottles and stored at 4 ^{o}C until further use.

2.2.2 Fractionation

2.2.2.1 Preparation and Methylation of Naphthenic Acids

In preparation for distillation, the NA stock solution was filtered and the remaining NA

residue was methylated to produce volatile methyl-naphthenate compounds. This was achieved

firstly by acidifying the NA stock solution to pH 2 with 12 M hydrochloric acid (HCl).

Precipitated NAs were then filtered using 0.2-µM PTFE filter paper (Sartorius, Fisher Scientific)

on a negative flow filtration unit (90 mm glass holder, Advantec MFS Inc., Dublin, CA). Filter

paper was then sonicated in 100 mL of methanol (HPLC grade, Fisher Scientific). NAs dissolved

in methanol were then transferred to a pre-weighed, 250 mL round-bottom flask. Methanol was

then rotovapped off until constant weight was achieved, leaving a NA residue. Each round-

bottom flask was then stored in a dessicator for at least 48 hrs.

The methylation of the NA residue required the use of ethereal diazomethane (DZM),

which was generated according to methods outlined in Frank et al. (2008). The DZM was added

dropwise to the NA residue in 250 mL round-bottom flask until bubbling (release of N_2 gas)

stopped. DZM-NAs were then filtered through a 1-mL column containing Fisher HyFlo supercel

(Fisher Scientific) and collected in a 100 mL round-bottom flask. DZM+NA+ether solution was

then rotovapped to evaporate off excess DZM and ether, leaving a methylated NA residue in an

oily state.

2.2.2.2   Fractional Distillation and De-methylation of Naphthenic Acids

Fractional distillation was achieved using a Kugelrohr distillation apparatus. Methylated NAs were evaporated off at 130 ^{o}C, 160 ^{o}C, 190 ^{o}C, 220 ^{o}C, with the remaining NAs representing a >220 ^{o}C fraction. The vacuum pressure was set at 28 mbar, while voltage was initially set to 120 V. Each fraction was held at $\pm$5 ^{o}C of set temperature for 20 min. Each fraction was collected in clean 25-mL round-bottom flask and subsequently transferred to 20-mL sample vials by rinsing with 10 mL of DCM. The final result was the generation of five NA fractions: fraction 1 (130 ^{o}C), fraction 2 (160 ^{o}C), fraction 3 (190 ^{o}C), fraction 4 (220 ^{o}C), fraction 5 (>220 ^{o}C).

In order to analyse the fractions by ESI-HRMS and SFS, demethylation was required. From each of the five fractions, 1 mL was transferred to separate sample vials and subject to a N_2 gas bath until constant weight was obtained indicating the complete evaporation of DCM. This was followed by the addition of 9 mL of methanol and 3 mL of 1.67 M NaOH to each vial containing dried NAs. The vials were placed in an oven at 55-60 ^{o}C for 7 days, after which the NA+NaOH+methanol solution was transferred to a 250 mL round-bottom flask and the methanol and water was rotovapped off. Dried NAs+NaOH were then reconstituted in HPLC-grade water, transferred to 10-mL glass vials, and vortexed for 3-5 min. Demethylated NA fractions in NaOH solution were stored in the dark at 4 ^{o}C until further use.

2.2.3   Analysis

2.2.3.1 Solid Phase Extraction Cleanup of Naphthenic Acids

In order to obtain concentration estimates for NAs, the mixture was diluted with Milli-Q water to below an estimated value of 10 mg/L NAs so that the tested concentration was within

the range of a previously established concentration curve (concentrations of 0-150 mg/L).

Actual concentrations were extrapolated thereafter. An aliquot of 10 mL of diluted NA solution

was acidified to a pH of ~2 using 12 M HCl to allow NAs to precipitate. Acidified NA mixtures

were filtered at a flow rate of ~1 mL/min through a preconditioned (with HPLC-grade

acetonitrile and Milli-Q water) 6 mL solid phase extraction (SPE) cartridge connected to a SPE

manifold and vacuum pump. All eluent was collected and discarded. Organic acids associated

with the SPE cartridge were then eluted using 7 mL of acetonitrile at a flow rate of ~ 1 mL/min.

Resulting eluent was collected in a 12 mL scintillation vial. Vials were then placed under a N_2

bath to remove acetonitrile, resulting in a NA residue. The NA residue was then reconstituted

with 950 µL of a solution of 50:50 acetonitrile:milli-Q water with 0.1% NH_4OH (ammonium

hydroxide) and vortexed. The 950 µL sample was then transferred to a 2-mL amber GC vial and

stored at 4^oC. 50 µL of an internal standard solution, consisting of 25 mg of 5β-cholanic acid

dissolved in 500 mL of HPLC-grade methanol, was added to each of the 950 µL NA samples.

2.2.3.2 Electrospray Ionization High Resolution Mass Spectrometry and Synchronous

Fluorescence Spectroscopy

Mass spectral analysis of collected samples was performed at the Aquatic Ecosystem

Protection Research Division of Environment Canada in Saskatoon, Saskatchewan. Analysis was

conducted using electrospray ionization high resolution mass spectrometry (ESI-HRMS) in

negative ion mode. The equipment used was an LTQ Orbitrap Velos (Thermo Fisher Scientific,

Hampton, NH). ESI source conditions were as follows: heater temperature 50 ^{o}C, sheath gas

flow rate 25 (arbitrary units), auxiliary gas flow rate 5 (arbitrary units), spray voltage 2.90 kV,

capillary temperature 275 ^{o}C and the S lens RF level 67%. Samples were analysed in full scan

with an m/z range of 100-600, resolution was set to 100,000. For quality control assurance,

distilled water blanks were also analyzed. Resulting NA concentrations were determined by comparison to a pre-defined 5-point regression of NAs at known concentrations, collected from OSPW in the Athabasca region.

The five fractions and a whole NAE were subject to high resolution mass spectrometry enabling the derivation and analysis of class distribution data, double-bond equivalents and the generation of van Krevelen plots. Composer v 1.0.2 (Sierra Analytics, Inc.) and Xcalibur v 2.1 (Thermo Fisher Scientific) software were used to derive the class distribution data, double bond equivalents and van Krevelen plots.

Each of the five fractions and NAE were analysed with synchronous fluorescence spectroscopy to determine the potential presence of aromatic structures. Spectrofluorometry was performed at Environment Canada in Burlington, Ontario. The instrument used was a Perkin-Elmer Luminescence spectrometer LS50B, and data were interpreted using FL Winlab 3® software (Perkin-Elmer). Briefly, 3 mL quartz cuvettes with PTFE stopper (Hellman, Concord, ON, Canada) were cleaned with DI water and methanol. Blanks were run using HPLC-grade water (Caledon Laboratory Chemicals, Georgetown, ON, Canada), after which some water was removed (1 µL to 10 µL) and replaced with NA fractions. Blanks were considered adequate when their intensity signal was below 50 nm. The emission spectra were collected with an excitation wavelength of 250-400 nm and a Δλ of 18 nm. Excitation and emission monochromator slit widths were 5 nm, while scan speed was set at 50 nm/min with a resolution of 0.5 nm. Fraction data were normalized according to their dilution factor and blank corrected using Microsoft Excel®, after which generated graphs were smoothed and normalized using Origin Pro 8.1® software (OriginLab Corp.).

2.3   Results

2.3.1 Electrospray Ionization High Resolution Mass Spectrometry

The generated chromatograms from ESI-HRMS (Figure 2.1) of the five fractions displayed peaks that increased in carbon number with increasing distillation temperature (and fraction number). Relative ion abundance was calculated by multiplying each m/z value (ion) by its peak intensity. The sum of the relative ion abundances was divided by the total peak intensity for each fraction to determine mean Dalton weights. Table 2.1 describes the characteristics of each fraction and includes each fraction's proportional contribution to the whole NA mixture. Although, molecular weight ranges indicated varying degrees of overlap between fractions, mean Daltons increased with increasing fraction number. Fractions 4 and 5 both displayed a bimodal distribution (Appendix A), with the first distribution at an m/z range of 140-250. It was indicated by Xcalibur v 2.1 software that this range displayed double charges, therefore, masses in this range were doubled. The doubled masses were included in mass calculations resulting in higher mean Daltons and Dalton weight ranges of fractions 4 and 5. Using total mass intensity for each fraction, its relative proportion to the whole NAE was identified. Fractions 2 and 3 contributed the most to the NAE (65.7% total) while fractions 1 and 5 contributed the least (17.5% total). Overall, increases in distillation temperature (fraction number) yielded fractions of increasing molecular weight.
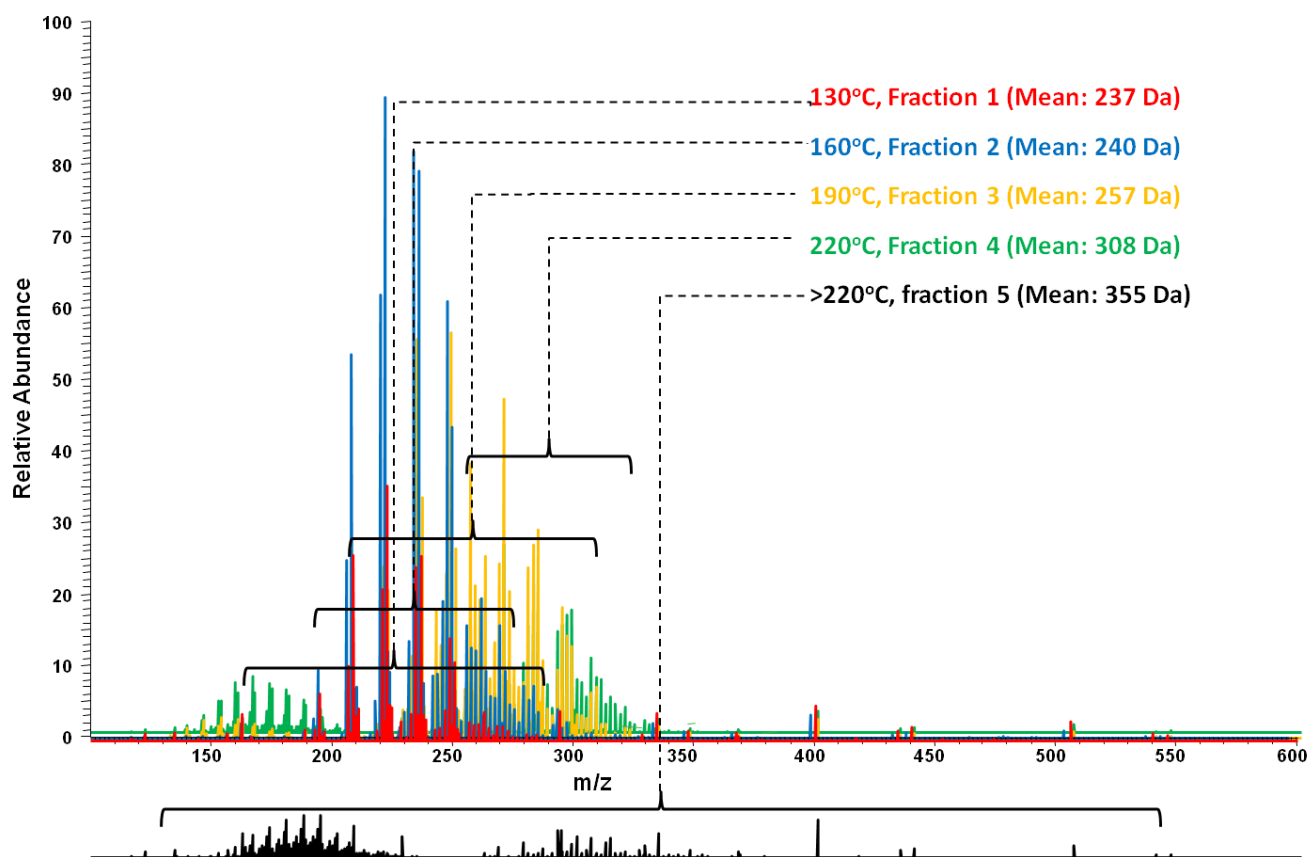
FIGURE 2.1: ESI Mass spectral analysis of five NA fractions. Daltons (Da) are considered equivalent to m/z values. Brackets encompass Da (m/z) ranges while the bracket apex indicates the mean value for each fraction.

TABLE 2.1: Naphthenic acid fraction and their corresponding Kugelrohr distillation temperatures, mean Dalton weights, Dalton weight ranges, and proportional contribution to whole NAE. Mean Dalton weights and ranges were derived from ESI-MS spectrum intensities. Fraction proportions were derived from the sum of all fraction intensities, representing the whole extract.

Fraction #	Distillation Temp (°C)	Mean Daltons	Range (Da)	Proportion of NAE
1	130	237	162-294	11.9%
2	160	240	195-274	32.3%
3	190	257	209-310	33.4%
4	220	308	255-322	16.8%
5	>220	355	122-547	5.6%

2.3.2 Double-Bond Equivalents

Figures 2.2a-e display double-bond equivalents (DBE) data of O_2 for each fraction. DBE data reports the degree of unsaturation of a compound, and can, therefore, be interpreted as the number of rings present (DBE minus 1 equals number of rings), as well account for the aromaticity present in the NA compounds tested.

DBE of 3 and 4 were most abundant in fractions 1 and 2, with DBE values values ranging from 2-7. Fraction 3 displayed a bimodal distribution of DBE values with high percent abundance of DBE values of 4 and 7 (range 2-8). The greatest increase in DBE values occurred in fraction 4, where values of 7 and 8 were most predominant and the highest DBE was 10. Finally, fraction 5 displayed a DBE distribution most similar to fractions 1 and 2, with a DBE of 3 predominating. Overall, there was a marked increase in DBE values as fraction number increased, with the exception of fraction 5.
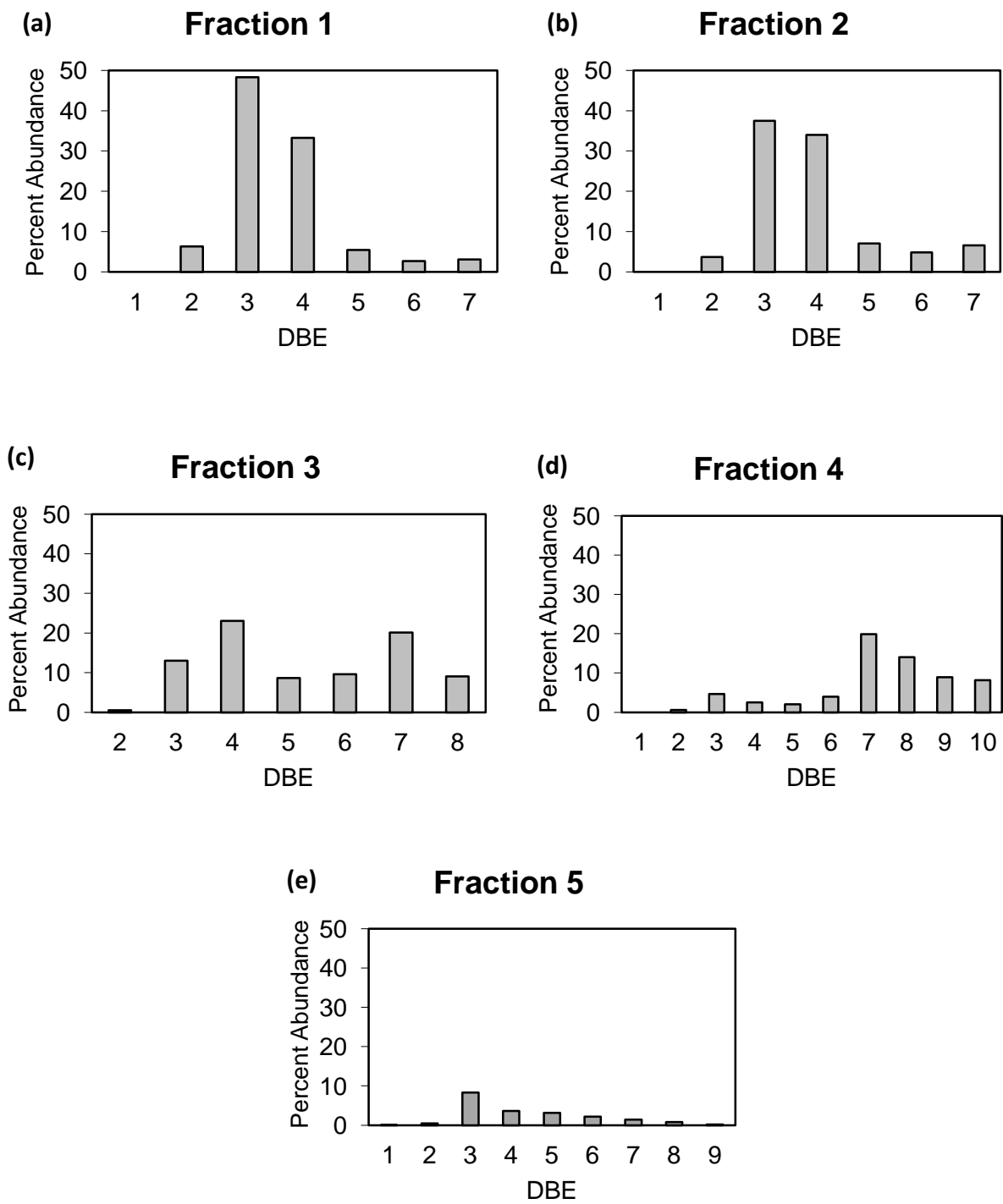
FIGURE 2.2: Double-bond equivalents of O_2 for fractions 1-5 (a-e respectively).

2.3.3 Ion Class Distribution

With respect to ion class distribution data obtained, O_2-containing species were the most abundant ion in all fractions (Figure 2.3a-e). However, with increasing fraction number was a corresponding increase in nitrogen, oxygen and sulfur-containing (NSO) ions. Fraction 1 displayed the lowest abundance of ions, with the O_2 ion comprising nearly 100% of the fraction. Fraction 2 displayed a slight increase in the number of ions present in comparison to fraction 1, as well as the appearance of sulfur-containing ions. Fraction 3 displayed an increase in ions in comparison to fractions 1 and 2, with both nitrogen and sulfur-containing ions almost tripling. Fraction 3 also displayed the presence of oxygen ions with greater than 2 oxygen atoms (O_3). Oxygen ions increased again in fraction 4, which showed the presence of O_2, O_3, O_4, and O_7 ions, while fraction 5 displayed a range of oxygen ions from O-O_8. In fractions 4 and 5, nitrogen-containing ions dramatically increased, with the greatest contributions by N_2, N_3O, and NO_3. The proportion of compounds with sulfur-containing ions increased most dramatically in the highest molecular weight fraction (fraction 5). Overall, the number of ions present, and the percent contribution of nitrogen and sulfur-containing ions increased with increasing fraction number, while the percent contribution of the O_2 ion decreased.

(a) Fraction 1 — bar chart of Percent Contribution (y-axis 0–120) vs Ion Class (N2O4 [H], O2 [H]), with O2 [H] near 100.

(b) Fraction 2 — bar chart of Percent Contribution (y-axis 0–100) vs Ion Class (N2O4 [H], N2O4S [H], O2 [H], O2S [H]), with O2 [H] near 95.

(c) Fraction 3 — bar chart of Percent Contribution (y-axis 0–100) vs Ion Class (HC [H], N2O4 [H], N2O4S [H], N2O4S2 [H], N2OS2 [H], N3O [H], NO2S2 [H], O2 [H], O2S [H], O3 [H], S [H]), with O2 [H] near 84 and O2S [H] near 14.

(d)

Fraction 4

Percent Contribution — Ion Class

Bars shown for ion classes: N2 [H], N2O2S [H], N2O2S2 [H], N2O4S [H], N2O4S2 [H], N2O5S [H], N2O6 [H], N2O7 [H], N2OS2 [H], N3O [H], N3OS [H], NO2S2 [H], NO3 [H], NO3S [H], O2 [H], O2S [H], O3 [H], O3S [H], O4 [H], O7 [H], S [H]

(e)

Fraction 5

Percent Contribution — Ion Class

Bars shown for ion classes: HC [H], N [H], N2 [H], N2O [H], N2O2 [H], N2O3 [H], N2O3S [H], N2O3S2 [H], N2O4 [H], N2O4S [H], N2O6 [H], N2O7 [H], N2O7S [H], N2OS [H], N2OS2 [H], N2S [H], N2S2 [H], N2S3 [H], N3O [H], N3O2 [H], N3O2S [H], N3O4 [H], N3O5 [H], N3O6 [H], N3OS [H], N3OS2 [H], N3S [H], NO2S [H], NO3 [H], NO3S [H], NO3S2 [H], NO4 [H], NO4S [H], NO5 [H], NO6 [H], NO8 [H], NO9 [H], NOS [H], NS2 [H], O [H], O10 [H], O2 [H], O2S [H], O2S2 [H], O2S3 [H], O3 [H], O3S [H], O3S2 [H], O4 [H], O4S [H], O5 [H], O6 [H], O7 [H], O7S [H], O8 [H], O9S [H], OS [H], OS2 [H], OS3 [H], S [H]

FIGURE 2.3: Class distribution data from ESI-HRMS of five oil sands-derived NA fractions: fractions 1-5 (a-e respectively). Bars represent the respective ion's percent contribution to the whole fraction.

2.3.4 Van Krevelen Plots

NA mass spectrometry data was compiled into van Krevelen plots (Figure 2.4a-e) Xcalibur v 2.1 (Thermo Fisher Scientific) software. Van Krevelen plots display hydrogen/carbon ratios on the y-axis against oxygen/carbon ratios on the x-axis, and simply represents a snapshot of oxidation processes. In this plot, when one moves right along the x-axis, compounds become more oxygenated, and when one moves up along the y-axis compounds contain carbons which are more hydrogen saturated. With this in mind, when looking at van Krevelen plots from fractions 1-3 respectively (Figure 2.4a-c), it was noted that the compounds present became slightly less hydrogen saturated as well as less oxygenated. Fraction 3 displayed two distinct regions, with the lower region showing less hydrogen saturated carbons than the upper region. The upper region, present in fractions 1-3, was absent in fraction 4 and replaced by a new region that displayed a higher oxygen-to-carbon ratio. Fraction 5 only contained the new, more oxygenated, region that first appeared in fraction 4.

Generally, there appeared to be two major shifts in saturation with increasing fraction number, creating 3 main regions. The first small shift occurred in fraction 3, in which a new, less hydrogen saturated region appeared. The second larger shift occurred in fraction 4, in which a new, completely separate region with greater oxygen saturation appeared. Thus, it appears that as molecular weight of oil sands NAs increases, NA compounds become more hydrogen deficient (fractions 1-3), reach a threshold, and then become more hydrogen and oxygen saturated (fractions 4-5).
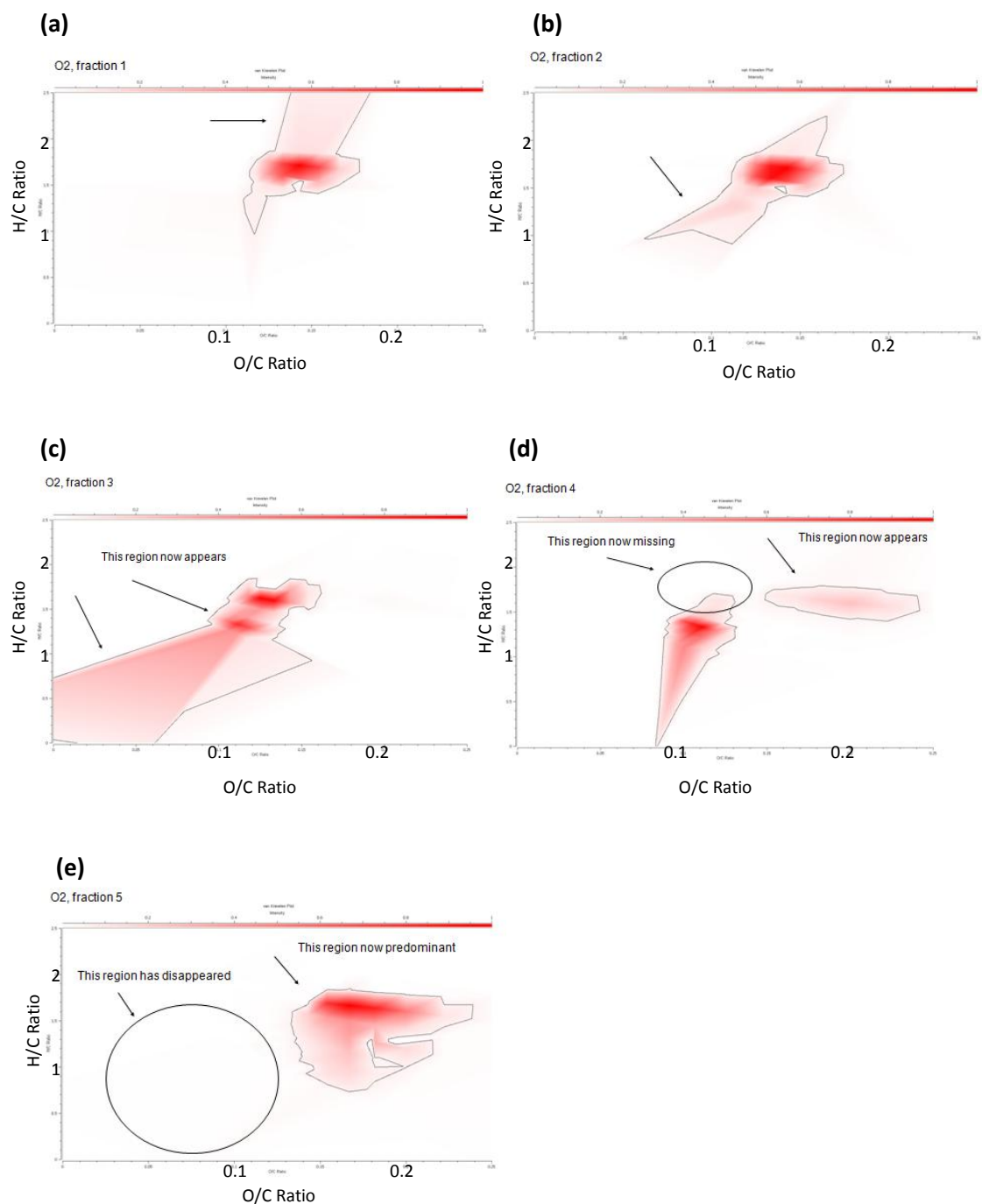
FIGURE 2.4: Van Krevelen plots of fractions 1-5 (a-e respectively) displaying intensity of Hydrogen/Carbon (y-axis) vs Oxygen/Carbon (x-axis) ratios. Intensity is noted by colour saturation with darker saturation indicating higher intensities. High H/C or O/C ratios indicate more hydrogenated or oxygenated compounds, respectively. Arrows and/or circles indicate notable features such as the appearance of new regions or absence of regions.

2.3.5 Synchronous Fluorescence Spectroscopy

The spectrofluorometric analysis performed on the whole NAE and fractions 1-5, displayed in Figure 2.5, revealed the presence of aromatic structures within the extracts. The NAE displayed three aromatic peaks at ~282, 320, and 333 nm. Previous reports have determined that the first peak indicates the presence of monoaromatic NA structures and the second indicates diaromatic structures, while the third 333 nm peak remains unknown (Kavanagh et al., 2009; Rowland et al., 2011b).

When comparing the fractions (Figure 2.5), one can see that fraction 1 displays very little aromaticity with the few present being solely monoaromatics (282 nm). Fraction 2 almost solely contains monoaromatic structures and is virtually devoid of diaromatics (320 nm). Fraction 3 contains a greater amount of aromatic compounds, with the majority being monoaromatic (282 nm), as well as an increase in diaromatics (320 nm), in comparison to LMW fractions 1 and 2. The greatest proportion of diaromatics and unknown aromatics (333 nm) are present in fractions 4 and 5. Fraction 4 contains a high proportion of monoaromatics, which are low in fraction 5. Overall, fraction 3 contains most of the monoaromatic structures present, while fraction 4 contains most of the diaromatic structures present in the whole NAE.

In general, there was an increase in aromatic structures and number of aromatic rings per structure with increasing fraction number and boiling point. Thus, it can be noted that with increasing molecular weight, an increase in aromaticity follows.
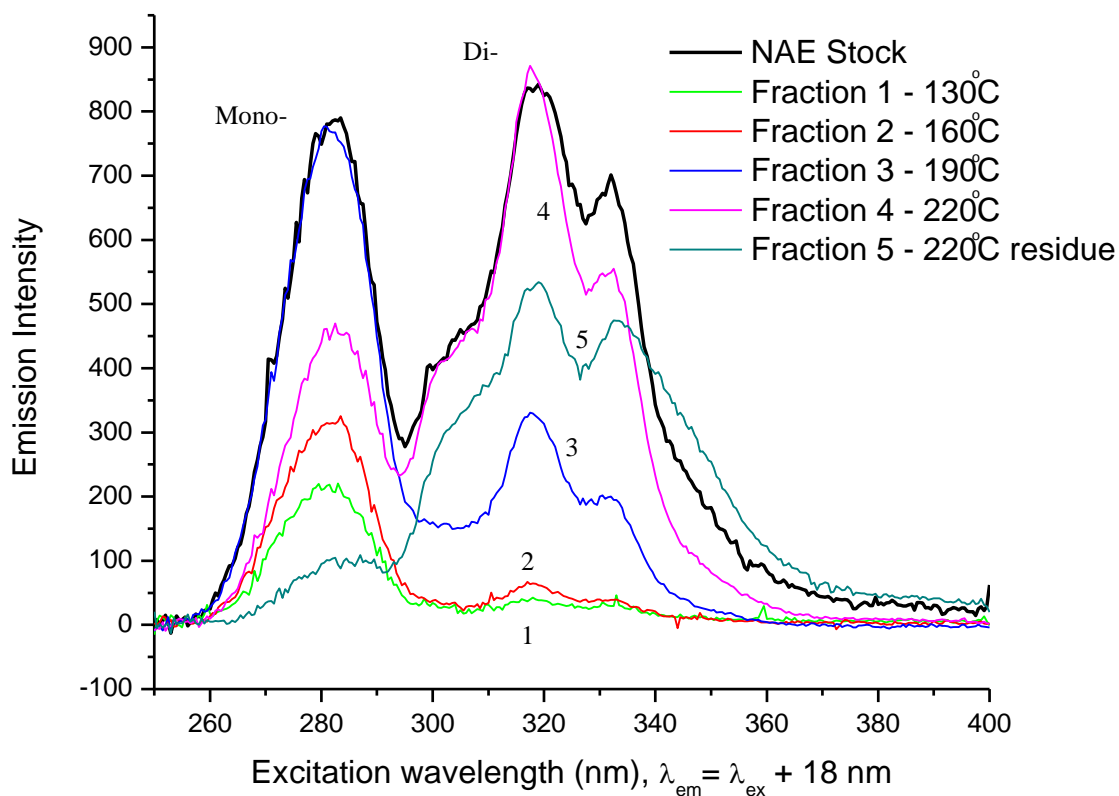
FIGURE 2.5: Synchronous fluorescence spectra of five NA fractions and a whole NAE. Spectra depicted were blank corrected and smoothed with a 5-point averaging adjacent method and then normalized. Peaks represent degrees of aromaticity, with excitation wavelengths of ~282 nm representing monoaromatics and ~320 nm representing diaromatics.

2.4 Discussion

2.4.1 Molecular Weight

The observed increase in molecular weight with an increase in fraction number (Table 2.1) is consistent with low resolution ESI-MS analysis performed previously on these same fractions which reported Dalton values of 223, 243, 261, 287, 346 with increasing distillation temperature (Frank et al., 2008).

Ion class distribution data indicated a predominance of O_2-containing species in all fractions tested. This suggests an abundance of carboxylic acid content in oil sands NAs, which has been well documented in literature (Headley and Peru, 2007; Headley et al., 2012; Stanford et al., 2007). The increase in nitrogen, oxygen and sulfur-containing ions with increasing fraction number (Figure 2.3a-e), suggests an increase in side-chains and "non-classical" NA structures, which has been observed in previous studies (Headley and Peru, 2007; Headley et al., 2011b; Rowland et al., 2011a; Rowland et al., 2011c; Rowland et al., 2011d). Non-classical ion abundance also suggests the potential presence of heteroatoms that appear to increase with increasing molecular weight. The presence of heteroatoms in OSPW has been previously documented with nitrogen, sulfur and oxygen species showing the greatest prevalence (Grewer et al., 2010; Headley et al., 2009a; Stanford et al., 2007), and these new data suggests that these species contribute to oil sands-derived NA higher molecular weight.

2.4.2 Cyclic Structures

Double-bond equivalents data can be interpreted such that DBE values minus 1, are equivalent to the number of rings present in a structure (DBE 1 = 0 rings, DBE 2 = 1 ring, etc.). Fractions 1-3 clearly display an increase in the number of rings present in a given structure as MOLECULAR WEIGHT increases (Figure 2.2a-c). This appears to be supported by the generated van Krevelen plots (Figure 2.4a-c), in which it was noted that fractions 1-3 compounds respectively become less hydrogen-saturated. In the fraction 3 DBE data (Figure 2.2c) there is a "jump" in cyclic structures, in which 3- and 6-ring structures predominate, with 4 and 5-ring structures having a lesser contribution. This noted "jump" in cyclic structures is verified by the fraction 3 van Krevelen plot (Figure 2.4c) in which two "hotspots" are present. The lower, less

hydrogen saturated region is almost entirely separate from the higher region, indicating a "jump" in the degree of saturation of hydrogen. Because the formation of a ring structure necessitates the liberation of hydrogen in an alkyl chain, the parallels between van Krevelen plots and DBE data are to be expected. Fraction 4 DBE data (Figure 2.2d) displays the presence of 6- and 7-ring structures, with 6-ring compounds being predominant, which has been noted by work performed by Rowland et al. (2011b) that involved analysis on this same 220°C fraction. Lastly, fraction 5 displays the lowest degree of cyclic structures (Figure 2.2e) yet a high mean Dalton weight and ion abundance, likely indicative of the presence of highly branched NA compounds.

When the relative contribution to the NAE (Table 2.1) was combined with the relative ring abundance for each fraction (Figure 2.2), it was noted that 2 and 3-ring structures predominate. Together, 2- and 3-ring structures comprise ~47% of the total ring structures present in the NAE. The overall abundance of 2- and 3-ring structures in oil sands NAs has been noted in recent literature (Frank et al., 2008; Headley et al., 2009b; Lo et al., 2006; Martin et al., 2008; Rowland et al., 2011a). Additionally, the low abundance of monocyclic compounds appearing in the NA fractions, as seen in the DBE data (Figure 2.4), has been acknowledged in literature regarding OSPW (Rowland et al., 2011b). Monocyclic NAs represent ~2% of the total ring structures present, which is comparable to the 4% stated in a previous study conducting ESI-HRMS analysis on oil sands NAs (Martin et al., 2008).

In general, the DBE and van Krevelen data indicates that, for fractions 1-4, the degree of cyclic NA structures increases with higher boiling temperatures and increased NA molecular weight. The highest molecular weight fraction (5) appeared to contain more highly branched molecules, with a low degree of cyclic structures.

2.4.3 Dicarboxy/Dihydroxy Components

Dicarboxylic acids and dihydroxy groups are well documented in literature and are commonly a product of the biodegradation of petroleum products (Bataineh et al., 2006; Gibson and Subramanian, 1994) including oil sands process material (Clemente et al., 2004; Headley et al., 2009a; Quagraine et al., 2005; Rowland et al., 2011a). Furthermore, the toxicity of NAs has been shown to decrease in potency with the presence of dicarboxylic acid groups (Frank et al., 2009; Frank et al., 2010).

ESI-HRMS data indicated a slightly greater increase in mean molecular masses in fractions 4 and 5 (Figure 2.1), in comparison to previously published results using low resolution ESI-MS on the same fractions (Frank et al., 2008). This is due to an initial "hump" in the spectra between m/z values 140-250 (Appendix A). Although the "humps" in this range displayed lower molecular weights, on high resolution analysis double charges were indicated. This suggests that there were dicarboxylic and/or dihydroxy components present, effectively doubling the mass of the compounds in this range. Furthermore, the HMW fractions 4 and 5 displayed the presence of 0_4 species (Figure 2.3d,e), which oil sands acid extracts have previously been shown to contain (Barrow et al., 2010; Headley et al., 2009a; Headley et al., 2011b), which is also indicative of dicarboxylic and/or dihydroxy components.

Double bond equivalents data may also suggest the presence of dihydroxy components. If O_2 NA structures included dihydroxy species with a degree of unsaturation that replaced dicarboxy species, it would result in an increase in reported hydrogen deficiencies and appear as an increase in DBEs. This was evident in fraction 4 DBE data but not in fraction 5 (Figure 2.2). If in fact dihydroxy components were present, DBE data combined with mass spectra (double

charged hump in fraction 4 and 5) suggests they likely occur mainly in fraction 4. Thus, the double charge present in fraction 5 would likely be predominantly dicarboxy groups.

The fraction 4 van Krevelen plot (Figure 2.4d) displayed the addition of a completely new region containing a higher proportion of hydrogen and oxygen species. This may indicate the presence and higher proportion of dihydroxy and/or dicarboxylic compounds. Fraction 5 displays only the new region (first appearing in fraction 4), indicating a predominance of more oxygenated compounds (Figure 2.4e), which is likely also due to dihydroxy and/or dicarboxy groups.

Generally, ESI-HRMS spectra, class distribution data, DBEs, and van Krevelen plots suggest the presence of dicarboxy/dihydroxy components in higher molecular weight NA fractions (4 and 5). This is important because the toxicity of NAs can be greatly affected by carboxylic acid content. Although classical NAs are reported to increase in toxicity with increasing carbon number (Frank et al., 2009; Jones et al., 2011), Frank et al. (2009) has previously shown that the presence of dicarboxylic acids can drastically reduce the toxicity of these compounds. This is because carboxylic acids can effectively decrease NA hydrophobicity making it harder for NAs to enter the lipid bilayer of cells and exert their narcotic potential (Schultz, 1989), essentially decreasing NA toxicity. The present study suggests that the dicarboxy/dihydroxy acids occurring in oils sands NAEs can be found primarily in the highest molecular weight fractions and contribute to an overall increase in molecular weight of individual NA compounds.

2.4.4 Aromaticity

The spectrofluorometric analysis (Figure 2.5) displayed the presence of three aromatic peaks at ~282, 320, and 333 nm indicative of monoaromatics, diaromatics, and an unknown aromaticity, respectively. These findings are consistent with studies that have performed synchronous fluorescence spectroscopy on OSPW (Kavanagh et al., 2009). The presence of monoaromatic NA structures is especially warranted as they have also recently been identified in commercial NAs and oil sands-derived NAEs (Rowland et al., 2011b; Rowland et al., 2011c).

With an increase in aromaticity follows an expected decrease in the hydrogen saturation of a compound. With this in mind, when looking at van Krevelen plots from fractions 1-3 respectively (Figure 2.4a-c), it was noted that the compounds present become slightly less hydrocarbon-like. In other words, the van Krevelen data appears to confirm the increase in aromaticity from fraction 1-3. Double-bond equivalents data also seem to confirm aromaticity trends observed in SFS analysis and van Krevelen plots. An increase in double bonds would be expected from the formation of aromatic structures, resulting in an observed increase in DBEs. In this study, concurrent with an increase in aromaticity, is an increase in DBEs (Figure 2.2), with the appearance of diaromatics coinciding with DBE values of 6 and 7 observed in fraction 3. The aromaticity and DBE values continue to increase in fraction 4, while fraction 5 maintains relatively high diaromatic content but relatively low DBE values. This suggests that most cyclic NA compounds in fraction 5 are aromatic in nature.

The presence of aromatic structures in oil sands-derived NAs indicates the potential presence of estrogenic NA structures. Additionally, the existence of estrogenic compounds is further substantiated by possible presence of dihydroxy O_2 species. In fact, OSPW has been deemed more estrogenic than commercial NAs (Rowland et al., 2011b), and has demonstrated a

negative effect on plasma sex steroid levels, secondary sex characteristics, and spawning in fathead minnows (Kavanagh et al., 2011). The present study suggests that mid to high molecular weight NAs contain the majority of the aromatic structures and may be responsible for the reproductive toxicity of fish observed in OSPW (Kavanagh et al., 2011). Fraction 4 appears to be most likely to contain estrogen-like compounds, as it fulfills the structural requirements of an estrogen; 4-ring structures, monoaromatics, and dihydroxy groups.

2.5 Conclusions

Advanced chemical analyses using ESI-HRMS and SFS, has improved our understanding of the structural complexity of the five NA fractions. Structures not elucidated using low resolution ESI-MS (Frank et al., 2008), were identified herein. These structures include aromatic rings and novel oil sands NA ions, as well as the suggestion of dihydroxy/dicarboxy groups, heteroatoms, and estrogenic NAs. In addition, the fractional characterization herein, provided insight into where in the molecular weight spectrum of oil sands NAs, these structures are likely to occur.

In general, the method of extracting and fractionating NAs from OSPW outlined in a previous study by Frank et al. (2008) produced fractions which increase in molecular weight, but with some degree of overlap. Mean Dalton weights and ranges outlined in Figure 2.1, coincide quite well with the weights and ranges produced previously. Concurrent with greater molecular weight and increasing distillation temperature, is also an increase in cyclic structures. This is verified by DBE data (Figure 2.2), which display increasing DBE values with increasing fraction number with the exception of fraction 5. As fraction number and cyclic structures increase, so does aromaticity, as seen in the fluorometric analysis (Figure 2.5). Lastly, the presence of

dicarboxylic acids in HMW fractions (Figure 2.1, fractions 4 and 5) has been suggested by use of the mass spectra, van Krevelen plots (Figure 2.4), and confirms findings of a study by Frank et al. (2009).

The low abundance of monocyclic compounds and predominance of 2- and 3-ring compounds appearing in OSPW and verified in literature (Frank et al., 2008; Headley et al., 2009b; Lo et al., 2006; Martin et al., 2008), was confirmed in this study by the DBE data. The general structural characteristics of each fraction can be observed in Tables 2.1 and 2. 2. The structures present in the highest molecular weight fractions (4 and 5) suggest the presence of heteroatoms and estrogenic compounds. In addition, this study has shown that the majority (two thirds by mass contained in fractions 2 and 3) of the NAs present in OSPW are 2-6 rings, mostly monoaromatic with some diaromatics and contains some monosulfur content.

TABLE 2.2: Physical and chemical properties of NA Fractions as determined by ESI-MS and SFS analysis. *Total ions* presents the total number of ions detected in class distribution data. *Class Distribution* represents those ions that exhibit > 10% contribution to the given fraction. The *O_2 DBE* numbers represent the double bond equivalents present which comprise > 50% of each fraction. *Cyclic structures* refers to the proposed number of ring structures contributing > 50% of that fraction, derived from DBE data. *Aromaticity* is subdivided into monoaromatics (mono-) and diaromatics (di-), with "+" indicating the presence and relative abundance and "-" the absence within each fraction.

Fraction #	Total Ions	Class Distribution	O_2 DBE	Cyclicity (rings)	Aromaticity Mono-	Di-
1	2	O2	3,4	2,3	+	-
2	4	O2,	3,4	2,3	+	-
3	11	O2, O2S	4,7	3,6	+++	+
4	21	O2	7,8	6,7	++	+++
5	60	O2, N2, N03, N3O	3,4	2,3	+	++

Overall, this study suggests that with increasing molecular weight of oil sands-derived NAs, is an increase in cyclic structures, aromaticity, and possibly molecular branching due to increasing N, S, and O species (Table 2.2), as well as the presence of dihydroxy and dicarboxy structures. As the toxicity of NAs may not simply be a function of molecular weight, but may be more closely associated with structure and solubility, the elucidation of some of these structures and how they relate to molecular weight becomes increasingly important. Many of the structures identified in this study deviate from the definition of classical NAs, which may change the toxic potential of some of these oil sands-derived NAs. Identifying individual NA structural components will aid in determining toxic potential of these compounds, vastly benefitting toxicological assessments and remediation efforts.

Chapter 3. Naphthenic Acid Toxicity to *Pimephales promelas* and *Oryzias latipes*

3.1 Introduction

Bitumen, a viscous hydrocarbon mixture extracted from oil sands and upgraded to synthetic crude oil, occurs naturally covering a total area of 140,200 km^2 in the Athabasca River, Peace River and Cold Lake regions of northern Alberta, Canada (Alberta Energy, 2008). Because the oil sands industry is currently producing over 1 million barrels of bitumen a day, vast quantities of tailings waste are stored on lease sites in large tailings ponds (Alberta Energy, 2008; Allen, 2008). Under provincial regulations (Alberta Environmental Protection and Enhancement Act) no tailings water can be discharged into the natural environment and all leased land must be remediated to pre-lease conditions or better (Madill et al., 2001). Since tailings constituents can cause acute toxicity to biota (Colavecchia et al., 2004; Farrell et al., 2004; Siwik et al., 2000), industry is responsible for environmental remediation which involves the detoxification of tailings. Among the most toxic constituents of tailings water (referred to as oil sands process water, OSPW) are water soluble NAs. Although NAs are present in the Athabasca region at background levels of ~2 mg/L, their high concentration in tailings ponds and aquatic reclamation (~ 40-130 mg/L) are cause for concern (Allen, 2008; Golder Associates Ltd., 2002; Holowenko et al., 2002; Schramm et al., 2000).

In general, the classical definition of NAs refers to compounds that adhere to the formula $C_nH_{2n+z}O_2$, where n is the carbon number and z refers to the hydrogen deficiency associated with ring formation (Clemente and Fedorak, 2005). Alkyl-substituted acyclic, monocyclic and polycyclic carboxylic acids are classical NA structures however these types of compounds only

represent a proportion of the chemicals present within the acid-extractable fraction of OSPW. As such, OSPW-derived acids including dicarboxylic acids, diamondoid acids, aromatic carboxylic acids, heteroatoms and others (Frank et al., 2009; Headley et al., 2011b; Kavanagh et al., 2009; Rowland et al., 2011a) as well as classical NAs are collectively referred to as oil sands NAs. Within this complex mixture of oil sands NAs, sublethal concentrations of identified estrogen-like steroid acids are thought to act as endocrine disruptors (Headley and McMartin, 2004; Roberts, 1991; Rowland et al., 2011b). However at lethal concentrations of oil sands NAs, narcosis is the probable mode of toxicity. The toxic potency of narcotics is a function of solubility and molecular size (Schultz, 1989). In this case, oil sands NAs with lower water solubility (more hydrophobic) could more easily penetrate a cell's lipid bilayer, and of those, higher molecular weight NAs likely cause more disruption within the membrane.

There have been a number of studies that have evaluated the toxic potential of whole OSPW, comprised of a complex mixture of organic and inorganic constituents, on aquatic organisms. Most notably, OSPW caused alterations in phytoplankton community composition (Leung et al., 2003), reduced benthic invertebrate abundance (Gould, 2000), and reduced growth and impaired reproduction of fish (Farrell et al., 2004; Lister et al., 2008; Nero et al., 2006b; Peters et al., 2007; Siwik et al., 2000). More specifically, laboratory and field studies involving fathead minnow, yellow perch, and/or Japanese medaka exposures to commercial or oil sands NAE or OSPW, showed similar results with respect to embryo-larval endpoints. Common responses were increased larval mortality (Lai et al., 1996; Peters et al., 2007; Siwik et al., 2000) and decreased hatch length (Farwell et al., 2006; Peters et al., 2007). Additionally, NAs have been shown to cause abnormalities in developing fish embryos, degenerative effects in gills of

juvenile fish (Nero et al., 2006a; Peters et al., 2007), and reproductive impairment in adult fish (Kavanagh et al., 2011).

Although numerous studies have evaluated the effects of whole OSPW on aquatic organisms, there are major information gaps with respect to toxicity of specific groups of constituents such as oil sands NAs. Few studies have evaluated the toxicity of oil sands NAs extracted from OSPW. Of these studies, two have evaluated NAEs on *Vibrio fischeri* (Frank et al., 2008; Lo et al., 2006), a marine bacterium, and one has evaluated NAEs on embryo-larval Japanese medaka (Farwell et al., 2006). Only one has evaluated toxicity to a geographically relevant organism, *Perca flavascens* (Nero et al., 2006a). Furthermore, although commercial NAs have widely been used for the tests on fish and bacteria (Biryukova et al., 2007; Clemente et al., 2004; Nero et al., 2006a; Peters et al., 2007; Scott et al., 2005; Young et al., 2007), the composition of commercial NAs is significantly different than oil sands NAs. In a study conducted by Nero et al. (2006a), commercial NAs elicited greater toxicity to young-of-the-year yellow perch than an oil sands NAE. When compared to Kodak and Merichem commercial NAs, oil sands NAs were more complex, displayed a higher percentage of C22+ (greater than 22 carbons) structures, and the overall composition showed a majority of NAs in the mid mass (150-350 amu) range (Clemente et al., 2003).

To advance the understanding of the toxic effects of oil sands NAs, this study evaluated the toxicity of whole oil sands NAs and five previously generated oil sands NA fractions on embryo-larval fish (Frank, 2008; Frank et al., 2008; Frank et al., 2006). These oil sands NA fractions, that increased in molecular weight at increasing distillation temperatures, were individually analysed to determine the structures contributing to increased molecular weight. As discussed in Chapter 2, HMW fractions (with mean Daltons of 308 and 355) possessed increased

cyclic structures, aromaticity, heteroatomic branching, and carboxylic acid content (Frank, 2008). Additionally, a *Vibrio fischeri* (Microtox®) toxicity study was previously performed on these same fractions revealing a general decrease in toxicity with increasing molecular weight (Frank et al., 2009). Thus, the purpose of this study was to investigate the toxicity of each fraction to two species of fish; *Pimephales promelas* (fathead minnow), and *Oryzias latipes* (Japanese medaka), to determine validity of these findings to organisms of a higher trophic level.

Japanese medaka and fathead minnow were used in this study because they are among the most commonly used small-bodied, freshwater fishes used in aquatic toxicology, and have been extensively studied by institutions such as the Organisation for Economic Co-operation and Development (OECD), Environment Canada, and the United States Environmental Protection Agency (USEPA). Thus, standard operating protocols for husbandry and testing on these fishes is widely available. Both are ideal laboratory test species because of their short generation time, rapid egg production, and small size. Lastly, the fathead minnow is indigenous to the oil sands region, and is present in test ponds and un-affected lakes within oil sands lease sites and therefore provides an opportunity to compare lab results to those observed in the field.

The major objective in the present chapter was to test the null hypothesis that NA fractions of increasing molecular weight displayed equivalent toxicities, against an alternate hypothesis that NA toxicity is simply a function of molecular weight. Fulfilling this objective included comparing the relative toxicity of each fraction and correlating the observed toxicities with the molecular weight present in respective fractions. In addition fraction toxicities were compared between both fish species, and findings related to the narcotic properties, and structures present within each NA fraction.

3.2 Materials and Methods

3.2.1 Source of Oil Sands Naphthenic Acid Fractions

Oil sands whole NAE and fractions were provided by Dr. Richard Frank (Frank et al., 2008), and analysed using electrospray ionization high resolution mass spectrometry (ESI-HRMS) and synchronous fluorescence spectrometry (SFS) (Chapter 2). The source, extraction, and fractionation of oil sands NAs are provided in Frank et al. (2006, 2008), and a brief description of the methods are described in Chapter 2. Fraction molecular weight and structural characteristics are provided in Tables 2.1 and 2.2.

3.2.2 Breeding Cultures

Breeding cultures of fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*) were established to provide the embryos necessary for toxicity testing. Fathead minnow and Japanese medaka were purchased from Aquatic Research Organisms (Hampton, New Hampshire). All fish were kept at the wetlab facility, in the Department of Biology at the University of Waterloo (Waterloo, Ontario, Canada). Culturing conditions maintained for fathead minnow followed Environment Canada fathead toxicity test protocols (Environment Canada, 2011), while culturing conditions for Japanese medaka were based on USEPA protocols (USEPA, 1991). Upon arrival, fish were separated by sex and placed into 10 gallon (~38 L) tanks for a week to acclimatize to water and temperature conditions. Water temperature in the tanks was maintained at close to room temperature, 18-22 $^\circ$C, with a photoperiod of 12 h light : 12 h dark. Culture water consisted of 50% well water and 50% milli-Q water on a re-circulating system, and water was renewed weekly. Well water was diluted in order to maintain water hardness below 150 mg/L, pH below 8, and conductivity below 1000 µS/cm, to provide an

optimal culturing environment, as University of Waterloo well water tended to be very high for these parameters (300+ mg/L, ~8.3, and 2000+ µS/cm, respectively). Water pH, due to dilution of well water, was in the range of 7.7 ± 2. Aeration of water was performed by air stones which maintained dissolved oxygen above 75% saturation. All fish were fed at least once a day with frozen brine shrimp and/or flaked food at a rate not exceeding what could be consumed in 10 minutes.

Following the acclimation period, fish were transitioned into breeding conditions. To encourage breeding, water temperature was initially increased by ~1 ^{o}C every two days until a temperature of 26 ± 1 ^{o}C was reached for fathead minnow, and 28 ± 1 ^{o}C for Japanese medaka, where it was then kept for the breeding period. In addition, light duration was increased from 12 h light : 12 h dark to a 16 h light : 8 h dark photoperiod, at a rate of 30 min per day for 8 days with gradual transitions from light to dark.

For breeding, fathead minnows were separated into breeding pairs consisting of 2 females and 1 male and placed into breeding tanks. Breeding tanks contained breeding tiles (3-inch PVC pipe section cut in half), which provided territory for males and breeding substrate for females. Adult fathead minnow females lay their eggs on the underside of the breeding tiles after which males will fertilize and guard the embryos. In order to collect embryos for testing, breeding tiles were removed and embryos were carefully scraped off the tile with blunt forceps. Embryos close to late blastula stage and free of fungal growth were selected and set aside for testing.

Japanese medaka were separated into tanks with a female:male ratio of 1:2 and housed at a density of no more than 20 fish per tank at a minimum ~2 L per fish. All tanks contained floating breeding mops (7-inch long disinfected yarn mops suspended on water surface by plastic bobber), which provided territory for males. Fertilized Japanese medaka egg clutches could be

found attached to the female oviduct pore. Embryo clutches were collected 1-3 hours after the beginning of the light cycle, by placing females on a damp paper towel and removing eggs from oviduct pore with blunt forceps. Embryos were then placed in test water (75% Milli-Q, 25% well water) and teased apart by rolling the clutches with one's finger over a screen. Once separated, embryos were then carefully examined under a dissecting microscope, and those free of fungal contamination, and close to late blastula stage were selected and set aside for testing.

Immediately following selection of fish embryos, each embryo was placed in individual wells of a 48-well tissue culture plate (BD Falcon™, Franklin Lakes, NJ), which were then filled with the appropriate test concentration.

3.2.3 Test Conditions

Oil sands NA fractions were diluted with 75% Milli-Q water and 25% well water to emulate breeding culture conditions, to a concentration of 100 mg/L and stored in 250 mL amber glass bottles with Teflon-lined cap (VWR™, Millville, NJ), and kept as stock solutions. The pH of each stock solution was then adjusted to 7.7 ± 2 and stored at 4 °C. The NA stock solutions were then further diluted using the same dilution water, to appropriate concentrations and stored in 20 mL scintillation vials with Teflon caps (Wheaton™, Millville, NJ). Test conditions were maintained such that there were 9 concentrations (including a control and solvent control), with 30 embryos per treatment. NA concentrations were as follows: Control (0), 3, 5, 10, 15, 30, 50, and 100 mg/L. The solvent control test, which consisted of a sodium hydroxide (NaOH) concentration equal to that present in the 100 mg/L NA test concentration, was performed to determine the possible effects of NaOH on embryo toxicity. Control water used in testing was the same as dilution water (75% Milli-Q: 25% well water). Water quality parameters,

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temperature, dissolved oxygen, pH, and conductivity, were measured for controls, 3 mg/L, 15 mg/L, and 100 mg/L concentrations every 24 h using an Orion model 1230 multi-meter (Orion Research Inc., Beverly, MA). Each embryo was subject to a volume of 0.5 mL of test solution in static conditions and renewed daily. Tissue culture plates containing embryos were kept in a temperature and light controlled Conviron® model E7H plant growth chamber (Conviron®, Winnipeg, MB), where temperature was maintained at 25 ± 1^oC, and photoperiod was held at 16 h light: 8 h dark.

Test concentrations and procedures for Japanese medaka were identical to those used for fathead minnow with the following exceptions. First, the lowest concentration used in toxicity testing was 1.5 mg/L instead of 3 mg/L. Second, once embryos were placed in Falcon™ 48-well tissue culture plates, the plates were placed on a Lab-Line model 3520 orbital shaker (Lab-Line Instruments Inc., Melrose Park, ILL) set to 100 rpm. Studies have found that gentle agitation of Japanese medaka eggs causes earlier hatch times (8 days instead of 12 days) without affecting test results (Farwell et al., 2006). The shaker was then placed into the growth chamber where temperature was held at 25 ± 1^oC, and photperiod was held at 16 h light: 8 h dark.

3.2.4 Endpoint Measurements

Endpoints observed and analysed for both species included: mortality, time to hatch, hatch length, and abnormalities. Observations for hatch time were recorded twice daily and larvae were regarded as successfully hatched only if they had fully emerged from their egg. Following hatch, larval hatch length was recorded to 0.1 mm using a dissecting microscope. Hatch length was recorded only for larvae with no significant spinal deformities to eliminate developmental abnormalities as a factor affecting estimates of larval growth. Mortality was

recorded for embryos displaying lack of heart valve and opercular movement, after which they were removed. Larval abnormalities were investigated under a dissecting microscope and based on a blue sac disease (BSD) score developed by Rhodes et al. (2005). Three abnormality types were identified and included; cranial and skeletal deformities (jaw, mouth, cranial size or structure deformities, and spine curvature), yolk sac abnormalities (yolk sac and pericardial edemas), and heart abnormalities (malformation of the heart, poor circulation, hemorrhaging, and clotting). Each of the three abnormality types were given a score from 1-3 based on severity, where 1 = slight, 2 = moderate, and 3 = severe. Individual larvae were considered abnormal if they exhibited any of the three abnormality types regardless of severity, while normal larvae were those exhibiting no abnormalities. The percentage of larvae in each concentration which exhibited an incidence of abnormality was calculated and compared to percentage abnormal larvae in controls (0 mg/L). Those treatments that showed significantly greater percentage abnormalities ($p \leq 0.05$) than controls were considered significant abnormalities. Tests were complete when all larvae reached 2 days post hatch or died.

Exposure concentrations were prepared based on mg/L concentrations and then converted to mM concentrations to express threshold and LC_{50} values for comparison with characterization data (Chapter 2). Conversions to mM concentrations were based on mean fraction molar masses previously calculated in Chapter 2.

3.2.5 Statistical Analysis

Quantitative data (time to hatch and hatch length endpoints) for each fraction was subject to hypothesis testing to identify test concentration that were significantly different from controls. This allowed for the derivation of no-observed-effects concentrations (NOEC) and lowest-

observed-effects (LOEC) concentrations. Statistical analyses for all quantitative endpoints, including: descriptive statistics (mean, standard error, standard deviation), normal probability plots, Levene's tests for homogeneity of variances, parametric tests (ANOVA and Tukey's post-hoc tests) and non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were performed using SPSS® (v 20). Variables were first tested for normal distribution using normal probability (or P-P) plots, and for homogeneity of variances using Levene's tests. Variables that were normally distributed and significantly homogeneous ($p \geq 0.05$) were analysed using one-way ANOVA to detect differences among treatments (concentrations). Tukey post-hoc tests were then performed for all ANOVA tests that resulted in significant p-values ($p \leq 0.05$) to determine treatment exposure which were significantly different from controls. Variables that did not pass Levene's tests for homogeneity of variances ($p < 0.05$), were subject to nonparametric analysis. Nonparametric analysis consisted of Kruskal-Wallis tests followed by a series of pairwise Mann-Whitney U tests to identify treatment exposures that were significantly different from controls. For both parametric (Tukey's post-hoc tests) and non-parametric (Mann-Whitney U tests), concentrations were considered significantly different from controls if tests resulted in a significant p-value ≤ 0.05.

Abnormality data (percent normal endpoints) were analysed in SPSS® by the same methods described above for quantitative endpoints with the exception that ANOVA tests were substituted with the Student's t-test to determine significant differences from controls. The Student's t-test was used because of the small sample sizes present in abnormality data due to low incidences of abnormalities. Concentrations for which Student's t-tests or Mann-Whitney U tests resulted in a p-value that was significant ($p \leq 0.05$), were considered significantly different from controls.

To compare quantitative endpoints between fractions and test the null hypothesis, that toxicity did not differ significantly between fractions, threshold values were calculated and compared for each fraction. Thresholds were calculated as the geometric mean of the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC). The NOEC was determined as the highest concentration at which an endpoint was not significantly different from controls ($p \geq 0.05$), while LOEC was the lowest concentration at which a significant difference from controls was observed ($p \leq 0.05$).

Quantal data (mortality) was statistically analysed using Microsoft Excel®, where simple functions (means, standard error, percentage conversions) were calculated. The Trimmed Spearman-Karber method (Excel® worksheet acquired from Environment Canada) was used to calculate median lethal concentrations (LC_{50}) and confidence intervals for each fraction ($\alpha = 0.2$, unless stated otherwise) (Hamilton et al., 1977). Fractions with LC_{50} confidence intervals that did not overlap were considered significantly different.

3.3 Results

Both *Oryzias latipes* (Japanese medaka) and *Pimephales promelas* (fathead minnow) were evaluated for each fraction on the following endpoints; mortality, time to hatch, abnormalities, and hatch length. Endpoints were then compared between fractions and whole NAE.

3.3.1 Mortality

In order to provide some comparison between fractions and species' mortality, all LC_{50}s were analysed using the Trimmed Spearman-Karber method at $\alpha = 0.2$. A trimmed Spearman-

Karber LC_{50} table expressed in mg/L is in Appendix C. Fractions were considered significantly different if there was no overlap in confidence intervals between fraction LC_{50}s.

The LC_{50}s values for embryo/larval Japanese medaka occurred in the range of 0.143-0.291 mM of NAs (Table 3.1). Fractions 2 and 3 were most toxic with LC_{50} values of 0.157 mM and 0.149 mM, respectively. Fractions 4 and 5 displayed intermediate toxicities with LC_{50} values of 0.209 mM and 0.192 mM, respectively, while fraction 1 displayed the lowest toxicity at 0.291 mM. Because confidence intervals did not overlap, it was determined that fractions 2 and 3 were significantly different from fractions 1, 4, and 5. When NA fraction toxicities were compared to the whole oil sands NAE toxicity, the LC_{50} of the extract (0.143 mM, Table 3.1) was significantly different from all but fraction 3, while being very close to fraction 2.

Fathead minnow LC_{50} values were between 0.061-0.159 mM ($\alpha = 0.2$, Table 3.1). Fraction 5 appeared most toxic with an LC_{50} of 0.061 mM, while fractions 2, 3, and 4 displayed intermediate toxicity with LC_{50}s of 0.08 mM, 0.104 mM, and 0.092 mM, respectively. Consistent with Japanese medaka bioassay results, fraction 1 showed the lowest toxicity with an LC_{50} of 0.159 mM. Due to high variability for fathead minnow mortality data, confidence intervals could not be derived at $\alpha = 0.2$, thus, comparisons between fractions could not be made for fathead minnow.

TABLE 3.1: Spearman-Karber mortality $LC_{50}s$ ($\alpha = 0.2$) for five NA fractions and a whole NAE for fathead minnow and Japanese medaka. The "-" indicates values that could not be derived at $\alpha = 0.2$. Tests that were not conducted are indicated by "nc". Significantly different $LC_{50}s$ are denoted by letters (a-d).

Fraction	Japanese Medaka (mM)	confidence interval		Fathead Minnow	confidence interval	
		lower	upper		lower	upper
Whole NAE	0.143^a	0.139	0.147	nc		
1	0.291^d	0.280	0.302	0.159	-	-
2	0.157^b	0.151	0.163	0.080	-	-
3	0.149^{ab}	0.147	0.152	0.104	0.084	0.127
4	0.209^c	0.173	0.251	0.092	0.077	0.111
5	0.192^c	0.183	0.201	0.061	-	-

3.3.2 Time to Hatch

In general, mean time to hatch for Japanese medaka varied from 7.6 to 9.3 days, while fathead minnow varied from 4.2 to 5.9 days (Appendix B). Japanese medaka displayed a concentration-response relationship for each fraction, with an increase in concentration conferring a significantly delayed hatch time ($p \leq 0.05$) at higher concentrations, when compared with controls (Figure 3.1a). For fathead minnow tests no concentration-response trends were observed for fractions 1, 2, 3, or 5 (Figure 3.1b), because no concentrations displayed hatch times that were significantly different from controls ($p \leq 0.05$) in any of these fractions. Therefore, fractions 1, 2, 3, and 5 were deemed to have no effect on fathead minnow time to hatch. Fraction 4 was the only fraction to display a significantly shorter hatch time ($p = 0.001$) at

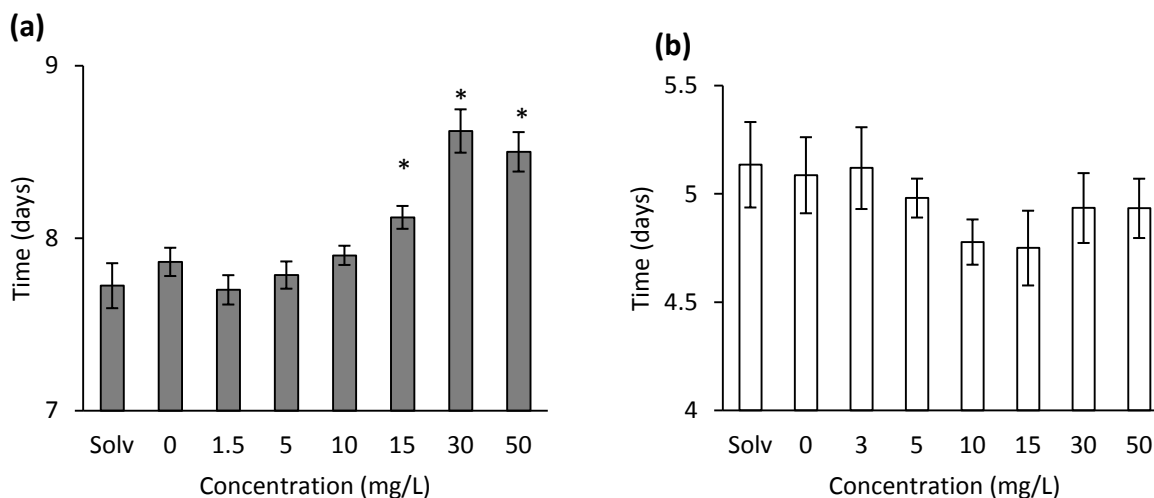the 50 mg/L concentration (Appendix B), and the threshold effect concentration calculated was 0.126 mM.

FIGURE 3.1: Examples of the effects of fraction 2 on Japanese medaka (a) and fathead minnow (b) time to hatch (mean $\pm$SEM). All points significantly different from controls are indicated by an asterisk ($p \leq 0.05$).

Because Japanese medaka displayed concentration-response relationships for all fractions, toxicity threshold values could be derived, allowing for comparison between fractions. Threshold concentrations were calculated as the geometric mean of NOEC and LOEC values, and represent theoretical concentrations above which an effect would be observed. Threshold concentrations for all fractions ranged between 0.048-0.199 mM (Figure 3.2). Fractions 2 and 3 displayed the lowest toxicity thresholds of 0.051 mM and 0.048 mM, respectively. Fractions 1 and 4 displayed toxicity thresholds of 0.163 mM and 0.126 mM, respectively, while the highest threshold was 0.199 mM for fraction 5. It is important to note that fraction 5 is the only fraction

to be observed for time to hatch at 100 mg/L as fractions 1-4 showed complete mortality at this concentration. It can thus be deduced that fraction 2 and 3 displayed the greatest potency and significantly delayed hatch time, followed by fractions 1 and 4, while fraction 5 required much higher concentrations to delay hatch time significantly.
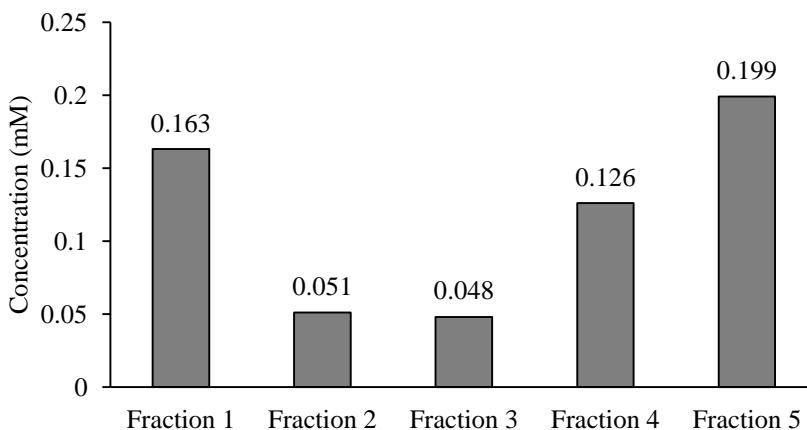

FIGURE 3.2: Naphthenic acid fraction thresholds for Japanese medaka time to hatch endpoint. Thresholds represent concentrations above which significant effects occurred.

3.3.3 Abnormalities

Percent normal Japanese medaka and fathead minnow were compared for all concentrations, and analysed for deviations from water and solvent controls (Figure 3.3a,b). Abnormalities in development included cranial and skeletal deformities, yolk sac/pericardial edema, and heart abnormalities. The most common abnormalities in larvae of both fish species were edema and blood hemorrhaging (Appendix D).

At 100 mg/L, fraction 5 displayed significantly more abnormalities than controls ($p =$ 0.001) in Japanese medaka larvae (Figure 3.3a). No other fractions displayed any abnormalities which were significantly different from controls ($p \leq 0.05$) at any concentration. For fathead minnow, fraction 3 displayed a significant difference from controls at 50 mg/L ($p < 0.001$) and fraction 4 was significantly different at 30 mg/L ($p = 0.001$) (Figure 3.3b). Fathead minnow abnormalities generally appeared to increase in all fractions at higher concentrations (≥ 30 mg/L), although only fractions 3 and 4 displayed significant differences ($p \leq 0.001$).
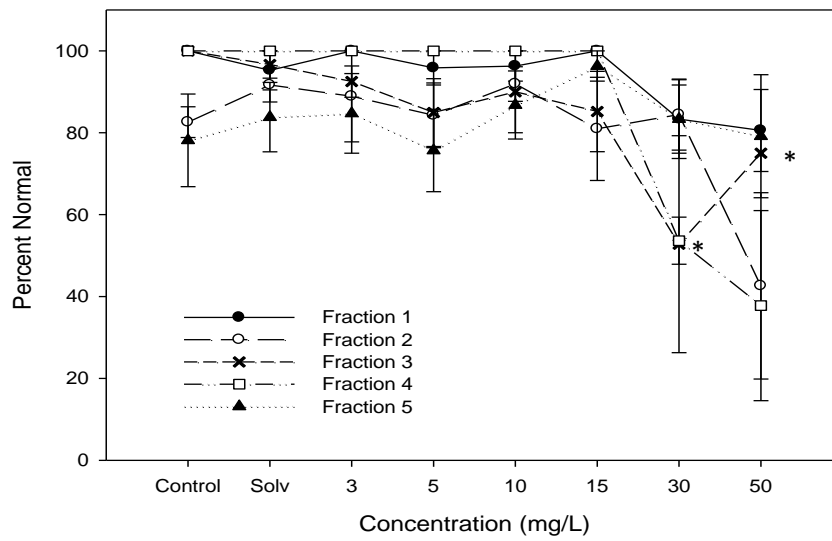
(a)
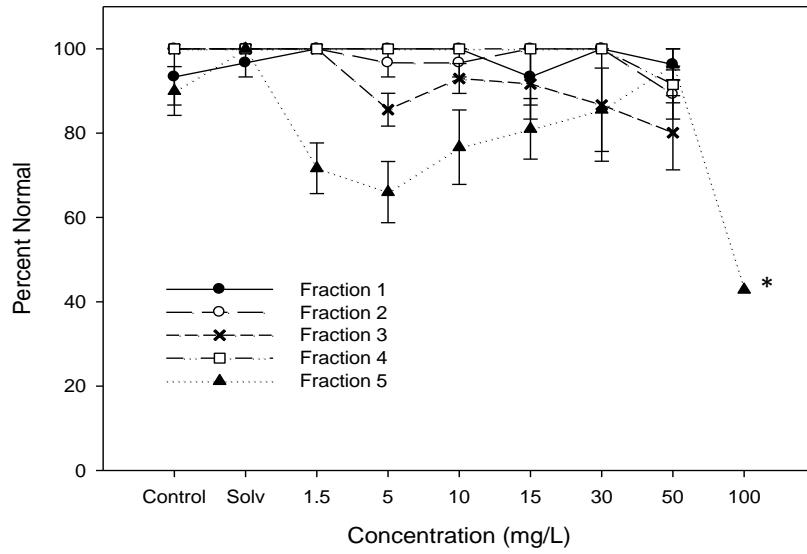
(b)

FIGURE 3.3: Effects of NA fractions on percent normal larvae (mean $\pm$ SEM): (a) percent normal Japanese medaka; (b) percent normal fathead minnow. Asterisks indicate concentrations at which means were significantly different from controls $p \le 0.05$).

3.3.4 Hatch Length

Hatch length for Japanese medaka ranged from 4.16 to 4.93 mm, while fathead minnow hatch length ranged from 3.84 to 5.24 mm (Appendix B).

Larval growth of Japanese medaka was negatively affected by 4 out of the 5 fractions. Specifically, fractions 2-5 showed an inverse concentration-response relationship, where an increase in concentration related to a significant decrease in hatch length ($p \leq 0.05$). Fraction 1 had no effect on hatch length. Toxicity thresholds for hatch length were determined by calculating the geometric mean of NOEC and LOEC values (Figure 3.4). Fractions 2, 3 and 4 threshold concentrations were 0.161 mM, 0.151 mM and 0.126 mM, respectively, while fraction 5 displayed a threshold at 0.199 mM. Fraction 5, therefore, showed the overall lowest toxicity with the highest threshold concentration for hatch length.

For fathead minnow, fractions 1 and 2 displayed no concentration-response relationship for hatch length, whereas fractions 3-5 displayed a slight inverse relationship (Figure 3.4; Appendix B). Fraction 4 displayed the lowest threshold value of 0.04 mM, while fractions 3 and 5 displayed slightly higher thresholds of 0.083 mM and 0.06 mM, respectively. Therefore, NAs within fractions 1 and 2 cause no effect on larval fathead minnow growth, however constituents in fractions 3-5 have a negative effect.
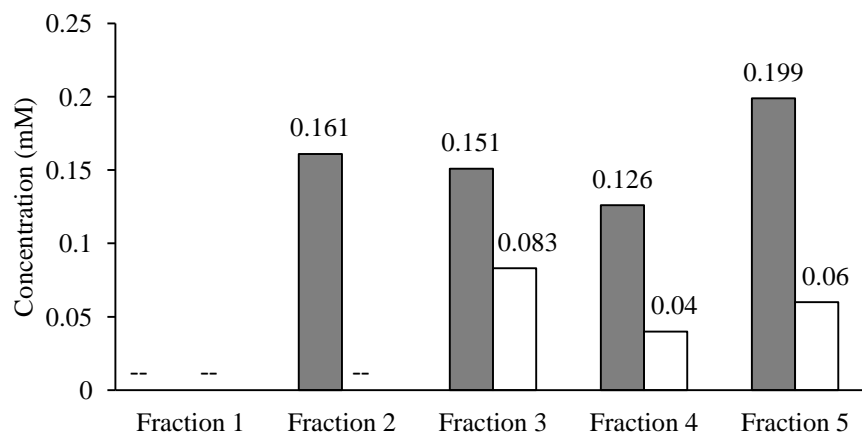
FIGURE 3.4: Naphthenic acid fraction thresholds for Japanese medaka (grey bars) and fathead minnow (white bars) hatch length. Threshold values represent the concentration below which no effects occurred. Tests which had no effect on hatch length are indicated by "--".

3.4 Discussion

3.4.1 Molecular Weight and Toxicity

A whole oil sands NAE, distilled to produce oil sands NA fractions along an increasing temperature/molecular weight gradient (Frank et al., 2008), varied in both their structural properties (Chapter 2) and toxicity. Acute toxicity data, expressed as mM, showed species-dependent differences between fractions (as high as 2-fold) and indicated that toxicity is likely driven by structural properties within fractions and not strictly by broad range molecular weight differences between fractions for this complex mixture of organic acids.

Oil sands studies have suggested that greater toxicity is associated with highly degradable, LMW NAs. Biodegradation of LMW NAs within the oil sands acid-extractable fraction was found to parallel a reduction in toxicity based on bacterial assays (Microtox®) (Clemente and Fedorak, 2005; Frank et al., 2008; Lai et al., 1996). EC_{50} values, expressed as mg/L, generated using bacterial assays for the same 5 oil sands NA distillation fractions used in

the current study, indicated greater toxicity in the lowest molecular range (130 $^{\circ}$C, fraction 1) compared to the highest molecular range (>220 $^{\circ}$C, fraction 5) (Clemente and Fedorak, 2005; Frank et al., 2008; Lai et al., 1996). Interestingly, the most toxic fraction (1), identified using Microtox® (EC$_{50}$ 41.9 mg/L; Frank et al., 2008) was the least toxic fraction identified based on embryo-larval fish mortality data in the current study (LC$_{50}$ values: 68.9 and 37.7 mg/L for Japanese medaka and fathead minnow, respectively; Appendix C). Other studies have also found drastically different results when comparing the toxicity data for oil sands tailings pond water generated using Microtox assays vs. fathead minnow bioassays (Lai et al., 1996). Contrary to Microtox data that proposed that toxicity of this complex mixture of oil sands NAs is simply a function of molecular weight of NA constituents, embryo-larval fish mortality data of NA fractions of increasing mean molecular weight suggests toxicity is likely associated with NA structure and not strictly molecular weight.

There was no molecular weight-associated toxicity trends for Japanese medaka endpoints. Fraction LC$_{50}$ values and their confidence intervals (Table 3.1) showed that fractions 2 and 3 were the most toxic, fractions 4 and 5 were intermediate and fraction 1 was the least toxic. The most potent inhibitors of hatch time and hatch length were the mid molecular weight NA fractions, fractions 2 and 3, and fractions 3 and 4, respectively. For both endpoints, the lowest and highest molecular weight NAs (fractions 1 and 5) displayed the lowest toxic potencies.

When the whole oil sands NAE was compared to NA fraction toxicities for Japanese medaka, the LC$_{50}$ of the whole NAE (Table 3.1) was most similar to fractions 2 and 3. This suggests that for Japanese medaka mortality, fractions 2 and 3 drive the majority of the toxicity associated with the NAE. This is to be expected since NA compounds in fractions 2 and 3

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contribute 32.3 % and 33.4 %, respectively, to the whole NAE composition (Table 2.1). Based on the high toxicity and proportion of fractions 2 and 3, further study of NA constituents within these fractions is required.

Fathead minnow endpoints also displayed no simple relationship between molecular weight and toxicity, and although mortality was highly variable, there were different toxicity ranking to that of Japanese medaka. Estimated LC_{50} values suggested that fraction 5 was most toxic, closely followed by fraction 2, fractions 3 and 4 displayed intermediate toxicities and fraction 1 displayed the lowest toxicity. The fathead minnow hatch endpoints were not greatly affected by NA toxicity. The lowest molecular weight fractions (1 and 2) displayed no significant reduction in larval length at hatch ($p \leq 0.05$), while fractions 1, 2, 3, and 5 had no significant effect on fathead minnow time to hatch ($p \leq 0.05$). Fraction 4 displayed the greatest potency with a significantly pre-mature hatch time ($p = 0.001$) and reduced hatch length ($p = 0.07$). There was high variability observed in fathead minnow mortality, time to hatch, and abnormality endpoints.

The lack of a toxicity-molecular weight relationship, in either fish species tested, indicates that NA toxicity to larval fish is not simply a function of molecular weight. Instead, NA toxicity may be confounded by other factors such as non-classical oil sands NA molecular structures, and/or organismal biological factors, such as metabolism and species sensitivity.

3.4.2 Abnormalities

The most common abnormality observed in both species was yolk sac/pericardial edema. In both fathead minnow and Japanese medaka, there were very few incidences of abnormalities which significantly deviated from controls ($p \leq 0.05$). Japanese medaka displayed only one

significant deviation ($p = 0.001$) in fraction 5 at the highest concentration (100 mg/L; Figure 3.4a). Fathead minnow displayed deviations from controls (Figure 3.4b) in only two treatments: fraction 4 at 30 mg/L (p = 0.001) and fraction 3 at 50 mg/L (p < 0.001). Previous studies have reported high incidences of abnormalities in fathead minnow and white sucker exposed to OSPW and sediments (Colavecchia et al., 2004; Colavecchia et al., 2007). However, these studies have used oil sands material and sediments, which include a multitude of other contaminants such as metals and polycyclic aromatic hydrocarbons (PAH), rather than solely NAs. Moreover, other studies have shown abnormalities in Japanese medaka due to oil sands PAH exposure (Farwell et al., 2006; Rhodes et al., 2005). Therefore, although NAs may be responsible for some abnormalities observed herein, because the majority of them were at very high concentrations, it is likely that abnormalities observed in reclamation scenarios are due to other contaminants like PAHs. Overall, NA fractions did not elicit very high abnormalities and few fractions displayed abnormalities which were significantly different from controls. This indicates that observed abnormalities were within the natural predisposition of both species tested.

3.4.3 Fraction Potencies

There was approximately a 2-fold difference between the toxicity of the least toxic fraction (fraction 1) and the most toxic fraction (varied depending on the species), based on LC_{50s} values. Fraction 1, representing the lowest molecular weight range, elicited the least toxic response for survival and growth, with no significant developmental effects for both species. Exposure to the more toxic fractions (2-5; higher molecular weight ranges) resulted in responses that were highly variable between species for lethal and sublethal endpoints (Table 3.2).

Oil sands NAs are thought to have a non-specific mode of action, narcosis, for acute toxicity (Frank *et al*., 2010). Solubility and molecular size are known to affect the potency of narcotics (Schultz, 1989). NAs are surfactants that have both hydrophobic (alkyl chains) and hydrophilic (carboxylic moiety) surfaces. The aqueous solubility of NAs is inversely related to its hydrophobic surface area (Schultz, 1989; Stanford et al., 2007). Structures such as alkyl branching, polar functional groups, and aromatization effectively reduce hydrophobic surface area, which increases overall solubility therefore reduces toxicity (Stanford et al., 2007). Contrarily, increasing alkyl chain length would have the opposite effect, thus enhancing toxic potency (Stanford et al., 2007). The idea that the structures present in oil sands NAs (Chapter 2) can modify the solubility, and therefore, toxicity of NAs, helps to explain some of the toxicity observed in the present study.

Fraction 1, the lowest molecular weight range, was the least toxic of all the fractions. Constituents of Fraction 1 (mean, 237 Da) had few ions (2) dominated by O2 species, high abundance of 2-3 ring cyclic structures and low abundance of monoaromatics (no diaromatics) (Table 2.2, Chapter 2). The presence of smaller molecules, perhaps with shorter alkyl chains (low hydrophobic surface area) likely influences solubility. Literature has shown that carboxylic acids with fatty acid chains of less than 12 carbons were unable to bind to the surface of bacterial cells due to low hydrophobicity (Kjelleberg et al., 1980). Because fraction 1 has been previously shown to contain NAs with $\leq$ 15 carbons (Frank et al., 2008) it is likely that the high solubility of some of these NAs contributed to low toxicity. Additionally, smaller NA molecules may be more easily metabolized, therefore rendering fraction 1 less toxic overall.

TABLE 3.2: Summary of most toxic fractions to Japanese medaka and fathead minnow at respective endpoints tested.

Summary Table

Endpoints	Most Toxic Fractions	
	Japanese Medaka	Fathead Minnow
Mortality[1]	3	5
Time to Hatch	3[a]	4[b]
Percent Normal	5	3,4
Hatch Length	4	4

[a] delayed time to hatch; [b] shorter time to hatch

Fraction 5 appears to be one of the least toxic fractions (except to fathead minnow mortality), yet has the highest molecular range. Compared to fraction 1, fraction 5 contains many heteroatoms (60 oxygen, nitrogen or sulfur-containing ions) and a higher abundance of diaromatics (Table 2.2, Chapter 2). The presence of heteroatoms and aromatic structures has been shown to significantly increase the solubility of hydrocarbons by decreasing hydrophobic surface area (Stanford et al., 2007). This means that with the increase in aromaticity and heteroatoms in fractions 4-5, a decrease in potency would be expected. Moreover, fraction 5 may contain dicarboxylic acids, which have been previously shown to have lower toxicity than more common monocarboxylic acids present in oils sands NAs (Frank et al., 2009). This is possibly

due to the decrease in hydrophobicity that carboxylic acids typically confer, resulting in compounds that are less likely to enter cell membranes. Because fraction 5 contains the greatest number of heteroatomic structures, a high degree of aromaticity (Figure 2.5), and dicarboxy moieties, its low potency is warranted due to its high solubility.

The most toxic fractions based on relative potency to all endpoints, in both fish species, are fractions 2, 3, and 4 (mid to high molecular weight). Increased mortality and reduced growth were associated with fractions 3 and 2, and fraction 4, respectively. Studies have revealed that an increase in NA alkyl chain length serves to increase the toxicity of these acids (Jones et al., 2011). According to narcotic properties and their association with solubility, an increase in chain length increases hydrophobic surface area, and therefore, toxic potential. It is likely that fractions 3 and 4 contain long alkyl chains, as indicated by their molecular weight, which promoted greater entry into fish cell membranes, enacting a greater toxicity. In addition, these larger mid to high molecular weight NAs contain a high degree of ring structures, which potentially served to cause more disruption within cell membranes, resulting in the greater toxicities observed.

Although, solubility and molecular weight can explain some of the observed toxicity in the present study, I do not propose that they are the sole factors involved. Toxicity is likely due to a number of factors including molecular weight, surface area, membrane binding potential, solubility, as well as, organism metabolism, sensitivity, and behaviour.

3.4.4 Species Comparison

The two fish species, Japanese medaka and fathead minnow, varied greatly in their sensitivity and their relative response to different fractions. In general, fathead minnow were more sensitive than Japanese medaka as indicated by lower LC_{50} values and threshold estimates

for growth, and the presence of developmental abnormalities for two of the fractions. The fact that acute toxicity was more variable between species for a given fraction (> 3-fold for fraction 5) than toxicity between fractions for a given species (>2-fold for fathead minnow) (Table 3.1), emphasizing the need for multi-species toxicity evaluations to assess the effectiveness of remediation and reclamation strategies of oil sands tailings.

Observations of varying species sensitivity may be a function of differences in the morphology of the protective membrane, the chorion. The chorion of the fathead minnow embryo is very porous, with pores ~0.2 μm in diameter spread consistently over the chorion surface ~2 μm apart (Lillicrap, 2010). In contrast, the embryonic chorion of Japanese medaka possesses many filamentous protrusions 130-140 μm in diameter, distributed over the surface at ~23 μm intervals and lacks large pores (Iwamatsu, 1992). Thus, it is possible that the large quantity of pores associated with the chorion of fathead minnow embryos could facilitate increased permeability to NAs and subsequently increased NA exposure to the embryo, in comparison to Japanese medaka. The protection nature of the chorion could explain the higher tolerance of Japanese medaka to oil sands NAs, minimizing effects on survival, growth and development, despite the fact that Japanese medaka have an extended incubation period and therefore were exposed to NAs for a longer duration (~12 days) than fathead minnow (~7 days).

Also, Japanese medaka showed general trends of delayed time to hatch with increasing concentration for most fractions yet for fathead minnow there was no effect or early time to hatch (fraction 4 only). Delayed hatch for Japanese medaka, allowing more time for embryonic growth within the protective chorion, may explain the higher threshold values of hatch length for Japanese medaka compared to fathead minnow based on fractions 3-5. Studies of yellow perch

and Japanese medaka exposed to OSPW and NAs have also reported reduced growth, measured as hatch length (Peters et al., 2007).

These two species also experience different developmental regimes. Where Japanese medaka completes organogenesis prior to hatch (Villalobos et al., 2000), fathead minnow larvae undergo the mid-final stages of organogenesis up to 4 days post-hatch (Scudder et al., 1988). Differences in organ development, particularly the liver, could impact the potential for embryonic metabolism of NAs. Although both species have been found to exhibit embryonic mechanisms of detoxification for select xenobiotics (Colavecchia et al., 2007; Jovanovic et al., 2011; Lindstrom-Seppa et al., 1994; Wisk and Cooper, 1992; Wu et al., 2011), the potential and level of NA induction of detoxification enzymes in embryos among these two species remains unknown. Detoxification and excretion of metabolites would alter the level of impact on growth and development.

3.5 Conclusions

The findings presented in this study suggest that the toxicity of this complex mixture of oil sands NAs is not associated simply with the molecular weight ranges of NA fractions generated by distillation. The higher acute toxicity and proportion of intermediate molecular range fractions (2 and 3) suggests constituents within these fractions likely represent higher environmental risk relative to the other fractions and require further study. Biological differences between the fish species tested may explain the varied response and greater sensitivity and variability associated with measurement endpoints for fathead minnow compared Japanese medaka. Of all endpoints tested, mortality and growth (as hatch length) were the more sensitive endpoints for both species. Time to hatch was a sensitive endpoint for Japanese medaka only and

percent normal, derived from the incidence of developmental abnormalities, for fathead minnow only. Greater variability among species than fractions indicates the need to assess environmental risk of current and future remediation and reclamation strategies for OSPW using a suite of sensitive and environmentally relevant endpoints (survival and growth), beyond the common and frequently used bacterial assay (Microtox®).

Chapter 4. General Discussion and Conclusions

4.1 Synthesis and Significance of Results

4.1.1 Fractionation

The NA fractionation procedure outline by Frank et al. (2008) was previously performed on the NAE used in this study. In order for bioassays to be run, the NA fractions had to be de-methylated and reconstituted in NaOH. When analysed under high resolution ESI-MS the mean molecular weights of the fractions observed were very similar to those reported previously on the same fractions (Frank et al., 2008). This indicates the high repeatability of the demethylation procedure and verifies analytical findings by Frank et al. (2008) that this procedure produces fractions of increasing molecular weight. Additionally, the high resolution analysis was able to verify NA structures contributing to increased molecular weight observed in the fractions. Most notably, there was an observed increase in cyclic structures, carboxylic acid content, hydroxyl groups, aromaticity, and heteroatoms, which all contributed to increased molecular weight of NAs.

4.1.2 Structures of Interest

A major finding in this study relates to the aromaticity of oil sands NAs of increasing molecular weight. Generally, it was found that with increasing molecular weight there was a concurrent increase in NA aromaticity. More specifically, fractions 1 -3 display increasing presence of monoaromatics, fraction 3 additionally shows a minor presence of diaromatics, while fractions 4 and 5 display increasing presence of diaromatics and decreasing presence of

monoaromatics. Thus, this study reveals that in oil sands extracts, the degree of aromaticity

contributes to the mass in the higher molecular weight range of acids present.

Of additional interest is the presence of potential estrogenic NA structures, particularly in

fraction 4. The spectrofluorometry performed on the NA fractions revealed that fraction 4

contained a high proportion of monoaromatic compounds, while DBE data indicated a high

proportion of 4-ring structures. Additionally, the potential presence of dihydroxy groups in

fraction 4 suggested by mass spectra and DBE data further substantiate the estrogenic potential

of NAs. Previous studies have suggested the presence of estrogenic NAs through

spectrofluorometric analysis and toxicity assays (Kavanagh et al., 2009; Kavanagh et al., 2011),

and in this current study we have been able to reveal their presence in the mid-high mass range

NAs.

ESI-MS analysis indicated a "hump" in the m/z range of 140-250 for fractions 4 and 5,

which Xcalibur v 2.1 software revealed was due to a double charge, suggesting the presence of

dicarboxy and/or dihydroxy groups. If in fact dihydroxy groups were present, it would further

confirm the existence of estrogenic compounds in fraction 4. The presence of dihydroxy

compounds in oil sands NAs have not been reported in literature to this point. But, the presence

of dicarboxy compounds in oil sands NAs has been identified in literature and linked to a marked

decrease in acute toxicity of NAs (Frank et al., 2009). Therefore, it is quite possible that both

dihydroxy and dicarboxy components exist within oil sands-derived NAs.

When observing class distribution data, there appears to be an abundance of nitrogen-,

sulfur-, and oxygen-containing NA moieties present in the higher molecular weight fractions.

This suggests the presence of heteroatomic branching on oil sands NA compounds, which has

been previously documented in OSPW (Grewer et al., 2010; Headley et al., 2009a; Stanford et

al., 2007). The NSO compounds present appear to contribute greatly to molecular weight as they are most detected in fractions 4 and 5. Heteroatomic branching could have implications in NA monitoring as they have the potential to affect solubility and acute toxicity of oil sands NAs by increasing hydrophobic surface area, and potentially decreasing toxicity.

4.1.3 Fathead Minnow-Japanese Medaka Differences

In comparing Japanese medaka acute toxicity results with those of fathead minnow, the Japanese medaka yielded more significant trends. Firstly, for time to hatch and hatch length endpoints Japanese medaka displayed more defined concentration-response relationships allowing for better comparison between fractions. The fact that Japanese medaka take ~3 more days to hatch, and are therefore exposed to NAs for a longer period of time, could explain this difference. Secondly, Japanese medaka mortality data appeared to contrast with earlier fraction toxicity findings using *Vibrio fischeri* (Frank et al., 2008), which revealed a decrease in acute toxicity to oil sands NAs of higher molecular weight. Although mid molecular weight NAs displayed a greater toxicity, the lowest molecular weight NAs (fraction 1) were least toxic. Mortality data for fathead minnow was highly variable, limiting the calculation of confidance limits needed to compare LC_{50}s between fractions. These findings suggest that different species display different relative toxicities with respect to NA molecular weight. Therefore, one should be careful when extrapolating toxicity data between species or other aquatic organisms (ie. *Vibrio fischeri*).

Although fathead minnow displayed weak concentration-response relationships, they were more sensitive than Japanese medaka to NA exposure. Differences in chorion ultrastructure between the two species may explain the greater sensitivity of fathead minnow.

Where the Japanese medaka possess filamentous chorion protrusions, the fathead minnow chorion is porous. The benefit of greater sensitivity in this species, in addition to their shorter incubation period, is that less test material is required when performing bioassays. This is potentially useful when test material is limited, or many bioassays are required.

Lastly, fathead minnow consistently displayed greater variability at most endpoints tested, possibly due to differences in exposure methods, incubation times, and physiology. This made it more difficult to compare fractions, and to determine significant differences from controls for most endpoints. Specifically, for mortality endpoints, confidence intervals could not be generated for estimated LC_{50} values even at higher α.

4.1.4 Useful Endpoints

Of all the endpoints tested, mortality and hatch length appeared most useful as clear concentration response-relationships were established and allowed for the interpretation of population-level effects in both fish species. Additionally, mortality data enabled the comparison between fractions and their respective acute toxicities. Hatch length data also coincides with current literature reporting a decrease in yellow perch and Japanese medaka hatch length with exposure to OSPW and NAs (Peters et al., 2007). For Japanese medaka, time to hatch also showed defined concentration response relationships allowing for the establishment of thresholds which displayed fraction associated trends.

4.1.5 Overall Fraction Toxicities and Trends

Generally, no strong trends were observed in time-to-hatch thresholds, hatch-length thresholds, and LC_{50}s for Japanese medaka and fathead minnow with respect to NA molecular

weight (alpha 0.2). Although the null hypothesis (fraction toxicities were equal) was rejected, the expected alternate hypothesis, that lower molecular weight fractions would be more toxic than higher fractions, was not confirmed.

The most toxic fractions overall were fractions 2, 3 and 4 which displayed considerable potencies at multiple endpoints. Fraction 3 displayed the lowest concentration threshold for time to hatch and the lowest LC_{50} values for mortality in Japanese medaka. Fraction 2 showed similar results displaying the second highest potency for these endpoints in Japanese medaka. For fathead minnow, fraction 5 displayed the lowest and fraction 2 displayed the second lowest LC_{50} for mortality, while fraction 4 displayed the lowest threshold concentration for hatch length for both species.

The least toxic fractions overall were fractions 1 and 5 given that they displayed the lowest potency in a number of endpoints. For Japanese medaka fraction 5 displayed the highest hatch length and time-to-hatch threshold concentrations. Fraction 1 displayed the second lowest effect on time to hatch for Japanese medaka. Finally, fraction 1 had no effect on hatch length, while displaying the lowest toxicity for mortality in both fish species.

In addition to performing embryo/larval bioassays of NA fractions, an assay was also performed using the whole NAE on Japanese medaka. Being that the LC_{50} for mortality of the whole NAE was significantly similar to fractions 2 and 3 LC_{50}s suggests that the toxicity of the extract is driven by mid molecular weight range NAs. This is inconsistent with previously published results on these same fractions, which, when subject to Microtox assays, display an NAE toxicity not significantly different from any of the fractions (Frank et al., 2008). When each fraction's relative proportion to the whole extract is taken into account fraction 2 and 3 have the greatest contribution (totalling 65.7%) further substantiating the results of the present study.

Thus, from a remediation standpoint this study proposes that removing low-mid molecular weight NAs can greatly reduce the acute toxicity of OSPW to aquatic organisms.

4.1.6 Toxicity is Structure Dependent

The fact that no NA molecular weight-toxicity relationship was observed, deviates from the idea that molecular weight drives toxicity. Therefore, toxicity may be more structure dependent. As NA acute toxicity is proposed to display a narcotic mode of action, drivers of narcotic toxicity must be explored. The toxic potency of narcotics is a function of solubility and molecular size (Schultz, 1989), meaning larger, less water soluble molecules can more easily penetrate and cause more disruption within a cell's lipid bilayer. For example, polar functional groups, alkyl branching, and aromatization increase solubility by decreasing hydrophobic surface area of the compound, while alkyl chains increase hydrophobic surface area thus decreasing solubility. When solubility is considered in conjunction with toxicity of each fraction, some hypotheses can be postulated with regard to the potential NA compounds present in each fraction.

This study revealed that molecular size of NAs is not the only driver of toxicity and that solubility must be taken into account. This relationship is particularly seen in fractions displaying lower toxicities. For example, fraction 1 likely contains short alkyl chains with very little branching, few rings, and low aromaticity. Toxicity data suggests that short alkyl chains were not sufficient to overcome the hydrophilicity of the carboxylic moiety present. In other words, these smaller NA compounds may not be able to easily penetrate the lipid bilayer. Another explanation may be that the smaller NAs present in fraction 1 are possibly more easily metabolised by the fish rendering them ineffective. Contrarily, although fraction 2 and 3 contain a slightly higher

degree of cyclic structures, mid-high monoaromatic content, and carboxyl groups, mass spectral analysis indicated an increase in alkyl chain length. Toxicity data suggests that the increase in chain length was able to overcome the hydrophilicity of the carboxylic acid moieties and cyclic structures. Additionally, the NAs in fraction 2 and 3 are large enough to cause adequate membrane disruption and are potentially harder to metabolise. Fraction 5 contains a relatively low degree of cyclic structures, but possibly a high degree of diaromatics, and dicarboxylic acids, both of which contribute to decreased hydrophobic surface area. The fact that there is a low degree of cyclic structures but high molecular weight with an abundance of NSO compounds, indicates a high degree of molecular branching which also contributes to decreased hydrophobic surface area. Therefore, because a decrease in hydrophobic surface area translates to an increase in solubility, the fact that fraction 5 displays a low toxicity is warranted.

Unfortunately, the solubility-toxicity relationship could not explain toxicities observed in fractions displaying high toxicities, such as fractions 3 and 4. Therefore, other factors such as organism physiological differences appear to drive NA toxicity.

In summation, although LC_{50} values are within a factor of ~2, this study suggests that low- to mid-molecular weight acids are of greatest concern. Thus, from a remediation and monitoring standpoint, it may be beneficial to focus on long-chained, monocarboxylic acids, with low aromaticity as these acids appear to be contributing greatly to acute toxicity in oil sands NAEs.

4.2 Recommendations and Future Research

4.2.1 Modified Fractionation

Although, the fractions analysed herein represent oil sands-extracted NAs of increasing molecular weight and varying structural components, there is a degree of overlap between fractions in both molecular weight and structures present. For example fractions 1 and 2 contain very similar structures, such as degree of cyclic structures, aromaticity, and ions present. This makes it difficult to associate toxicity to individual properties. Therefore, a recommendation is to explore the refinement of existing fractionation techniques in order to lower the degree of overlap in molecular weight and potentially further separate NA structures. Refinement to the procedure should include generating more than 5 fractions based on molecular weight, which would allow for the collection of fewer NA structure overlaps within each fraction. In addition, this new procedure may help to equalize the fractional contribution to the whole NAE, and reduce contributions such as was seen with the more toxic fractions 2 and 3 contributing 65.7% to the whole. Once fractions are collected, toxicity tests should be conducted using Microtox® assays, and Japanese Medaka embryo/larval bioassays allowing for toxicity comparisons between fractions (NA structures). This would help to isolate the most toxic fraction of oil sands NAs which can be used in future chemical identification and toxicity, as well as to generate fractions which will aid in monitoring/regulatory purposes.

4.2.2 Chronic Tests

The results of this study are related to the acute toxicity of NAs based on a narcotic mode of action. Based on new findings, specifically with regard to NA structures, it may be beneficial to explore the chronic toxicity of NAs. Thusfar, research regarding oil sands NAs has largely

focused on acute and sub-acute toxicities (Lai et al., 1996; Nero et al., 2006a; Peters et al., 2007) and information is therefore lacking in longer tests such as partial lifecycle tests which investigate chronic toxicity endpoints such as growth and reproduction. It is possible that an increase in detrimental effects would be observed with longer exposure periods to aquatic organisms.

4.2.3 Estrogenic/Anti-androgenic Properties

Current literature, including results found herein, have uncovered sex steroid-like NA compounds (Kavanagh et al., 2011; Rowland et al., 2011b). The presence of 4-ring structures, monoaromatics, and hydroxyl moieties discovered in this study suggest the presence of estrogen-like NAs which present a mode of toxic action different from narcosis. With this in mind, it would be beneficial to test the estrogenic and anti-androgenic properties of oil sands NAs in order to expand on current literature reporting the reproductive impairment of NAs (Kavanagh et al., 2011). The steroidal properties of NA fractions which contain estrogenic structures could be evaluated by exposing them to MELN and/or H295R human carcinoma cell lines.

4.2.4 Toxicity Testing on Other Organisms

As seen by comparing some results found herein with Microtox assays conducted using oil sands NAs (Clemente et al., 2004; Frank et al., 2008), the acute toxicity of NA fractions can vary between organisms. The toxicity of NAs to the microbe *Vibrio fischeri* and multiple fish species are well documented, but organisms of intermediate complexity have been largely ignored. In order to make assessments on an aquatic ecosystem level it would be beneficial to conduct bioassays on organisms such as *Daphnia magna* (water flea) and *Hyalella azteca* to

determine their sensitivity to NAs. It has been reported in literature that there are differences between *Daphnia magna* and *Vibrio fischeri* (Jones et al., 2011; MacKinnon and Boerger, 1986), as well as between *Vibrio fischeri* and fish (Oris et al., 1990) with regard to toxicity of NAs and OSPW. Therefore, a study comparing the toxicity of *Vibrio fischeri*, *Daphnia magna*, and *Pimephales promelas,* would be beneficial in determining differences in species' sensitivity to NAs. The relationship between these three organisms is such that juvenile fish will feed on *Daphnia*, while bacteria degrade NAs reducing overall toxicity to the system. Thus, the overall health of a system could be better assessed by comparing each organisms' relative sensitivity with their ecosystem role and the NA levels in the system. A hypothesis would be tested to determine whether an organisms level on the food-chain is related to their relative sensitivity to NAs. Research in this regard should therefore be planned in order to better assess the bottom-up and top-down effects NAs have on a system rather than simply an individual organism.

4.2.5 Testing Individual NAs with Varying Structures

Because the fractions in this study contain a degree of overlap in both molecular weight and structures present, it is difficult to attribute observed toxicities to an individual structure. Thus, it may be beneficial to conduct research involving individual NAs, isolating for one structural component. Recently, literature has deduced toxicities using NAE based on single structural components such as degree of aromaticity (Kavanagh et al., 2009; Kavanagh et al., 2011), carboxylic acid content (Frank et al., 2009), and degree of cyclic structures (Lo et al., 2006). Studies in this fashion that evaluate toxicities using NA surrogates or synthesised NAs, help to better understand the contribution of a single structure by negating confounding influences by other structures present in an extract. Additionally, it is becoming increasingly

important to study individual components of oil sands extracted NAs, as more "non-classical" structures are being discovered. Studying the effects of "non-classical" NA heteroatoms and hydroxyl moieties for example, would be beneficial in understanding toxicities associated with oil sands NAs. One such study may involve testing the relative toxicities of carboxylic acids with increasing heteroatomic complexity to determine the influence heteroatoms have on toxicity. Another study may involve testing relative toxicities of carboxylic acids with increasing aromaticity, which could be compared with the previous study. When individual structural toxicities are well categorized, then toxicities involving whole extracts may be better interpreted.

4.2.6 NAs From Different Sources

It is presently well documented that NAEs from different sources afford vastly different compositions and toxicities. There are differences between commercial and oil sands NAEs, between different industry lease sites, and between tailings ponds on the same lease site (Clemente et al., 2003; Holowenko et al., 2002; Kavanagh et al., 2009; Lo et al., 2006). Analytical and toxicological testing should therefore continue to elucidate the differences between NAs from different sources in order to better assist in the monitoring and remediation of oil sands NAs. Studies of this manner may also help determine industrial practices that are detrimental to remediation efforts, benefiting industry in the long term.

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Appendix A: Spectra from ESI-HRMS of Five Oil Sands-Derived NA Fractions

Fraction 1

Fraction 2

Fraction 3

Fraction 4

Mass spectrum with labeled peaks: 140.0844, 153.0922, 167.1078, 188.1312, 243.1388, 257.1544, 271.1699, 285.1856, 299.2012, 307.1721, 315.1420, 334.8954, 367.8810, 400.8667, 440.8590, 474.8471, 506.8309, 546.8237, 580.8091. X-axis: m/z ranging from ~150 to 600. Y-axis: relative intensity 0 to 100.
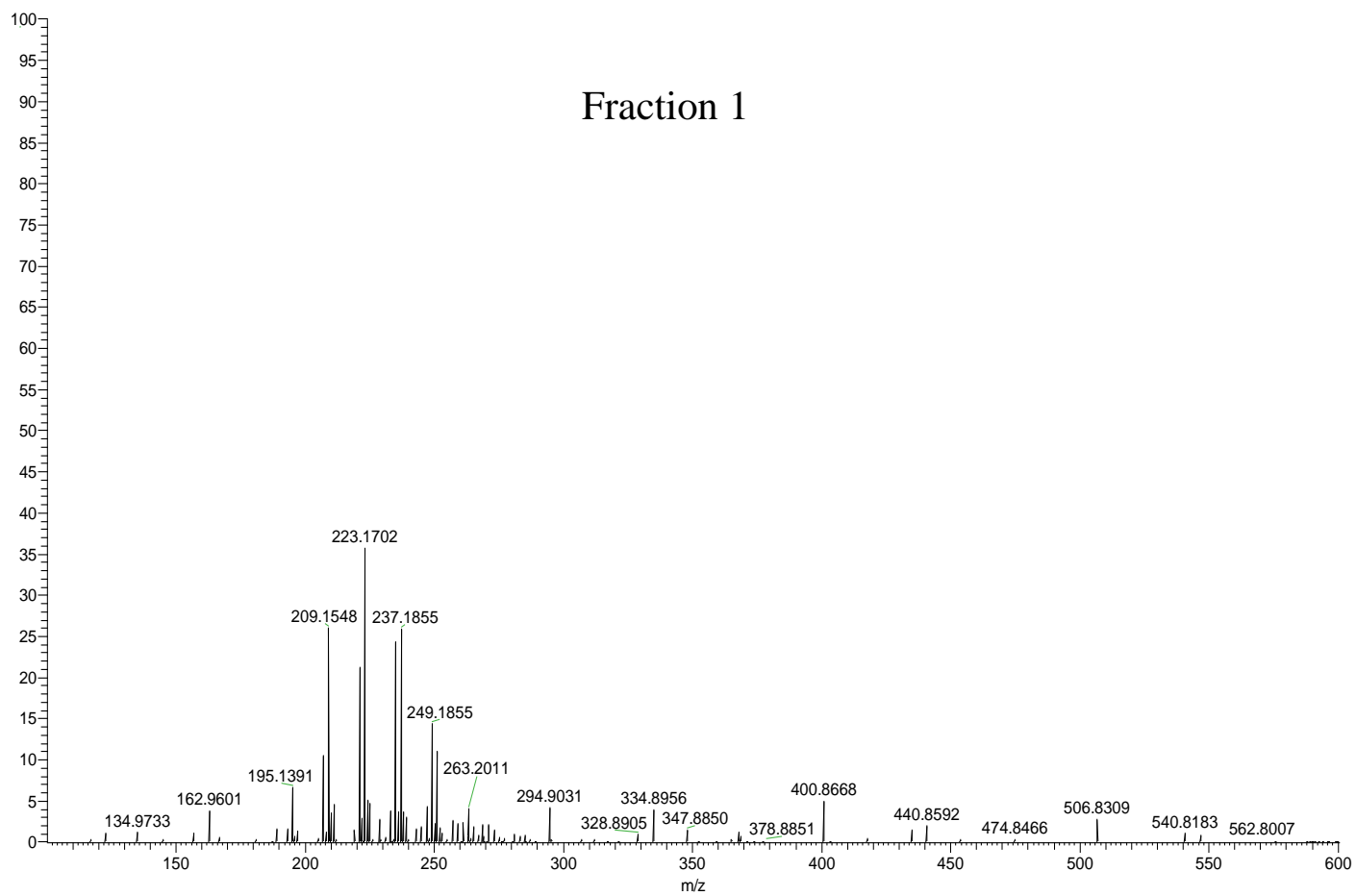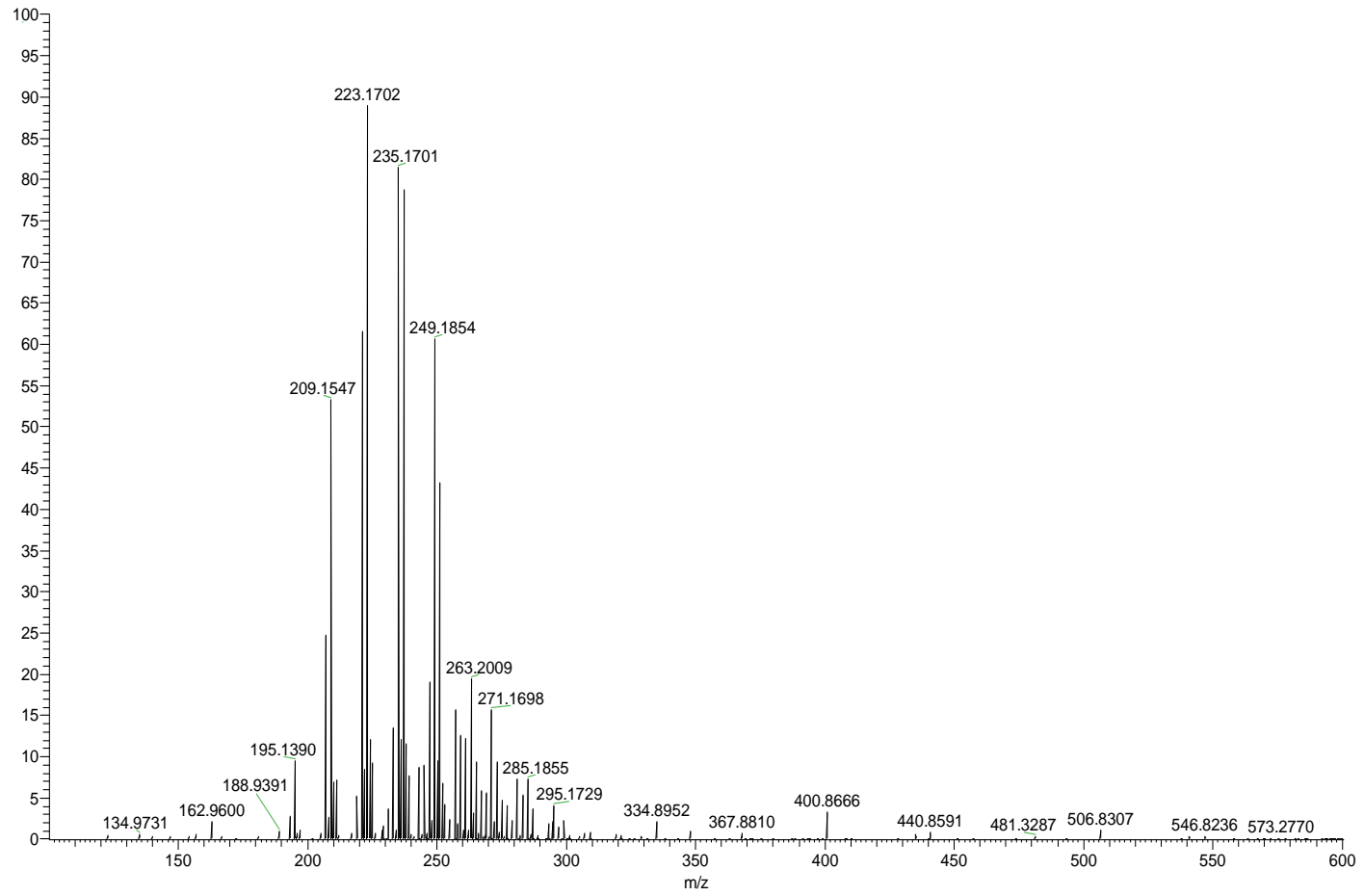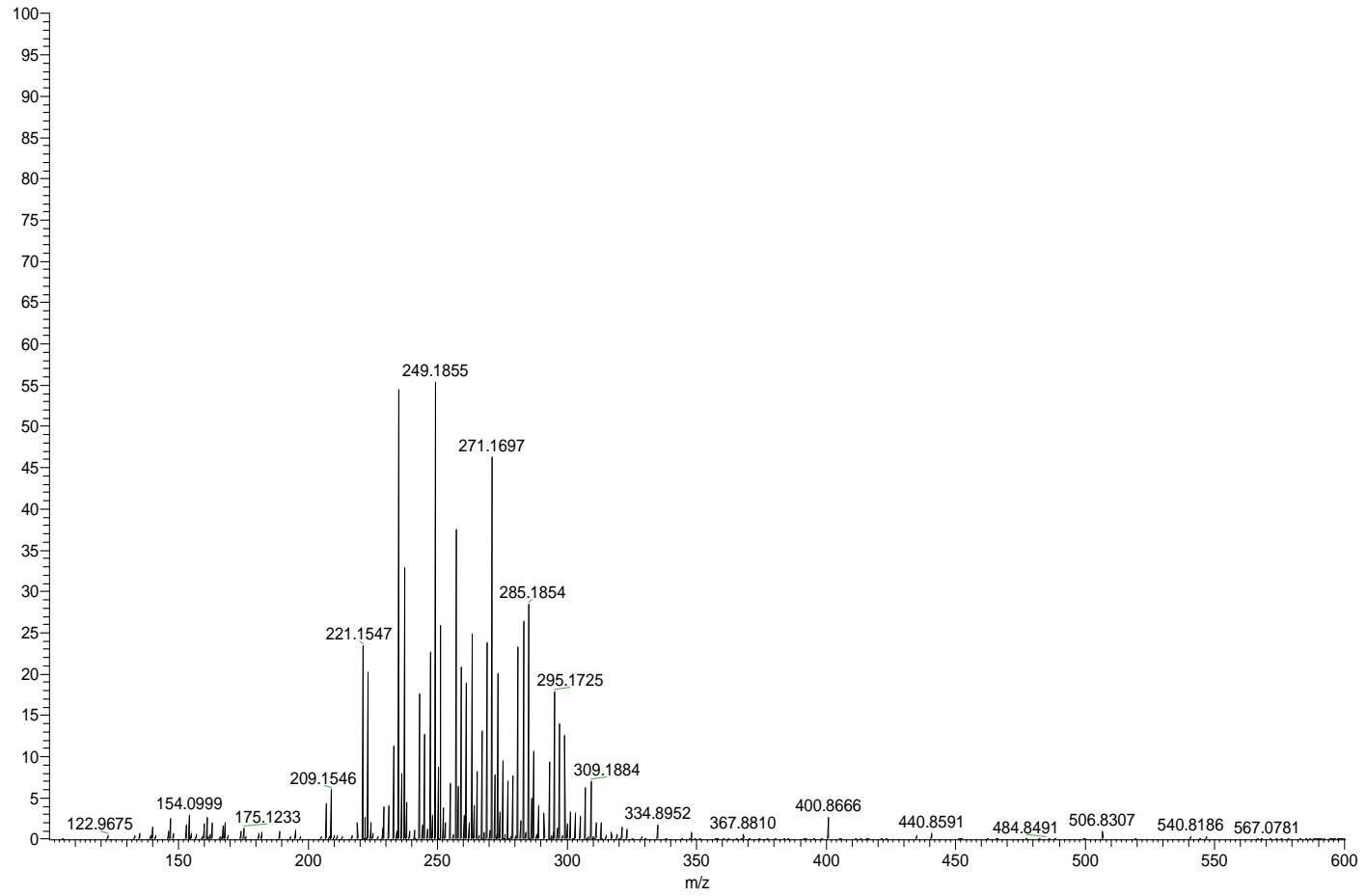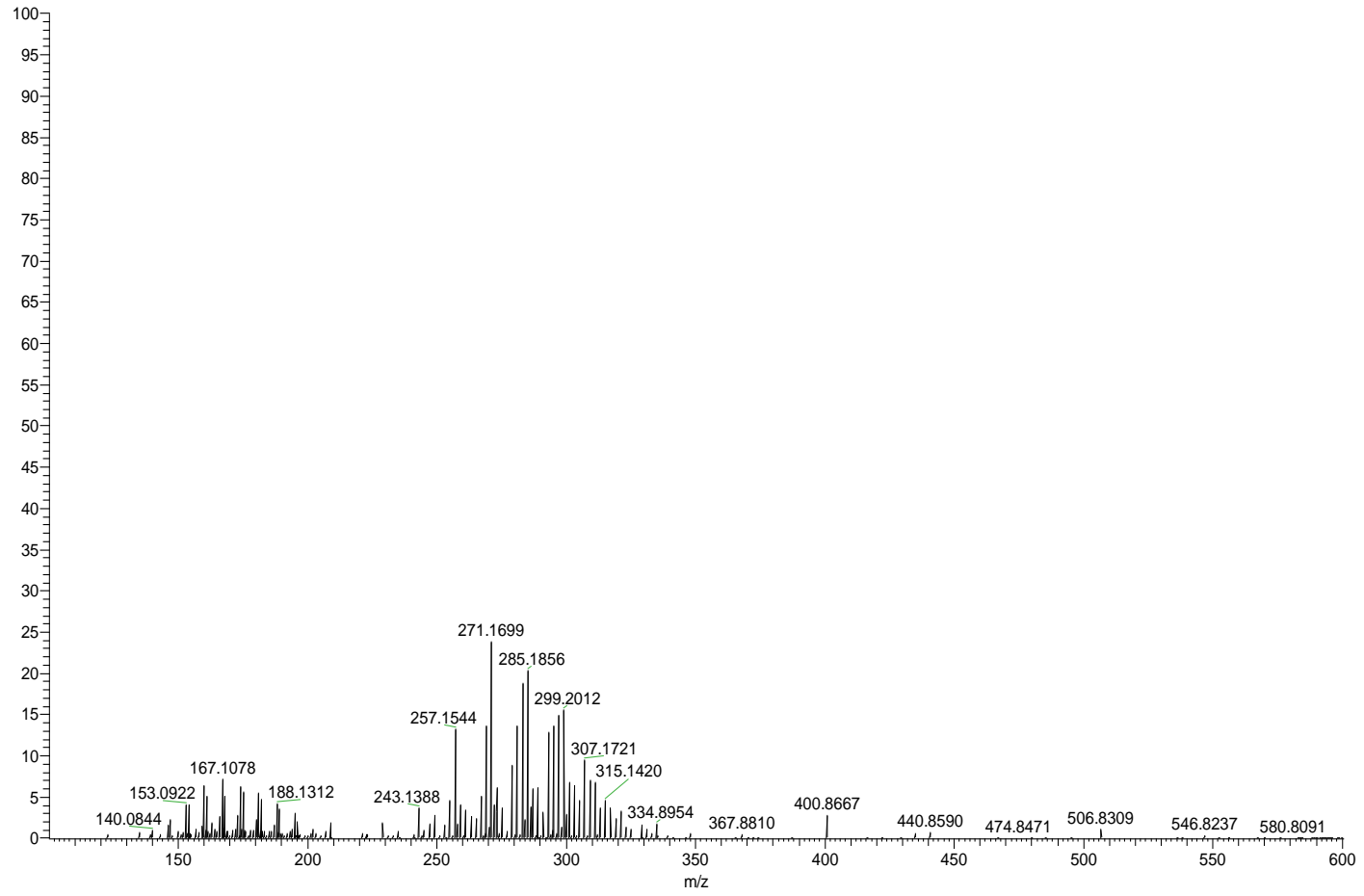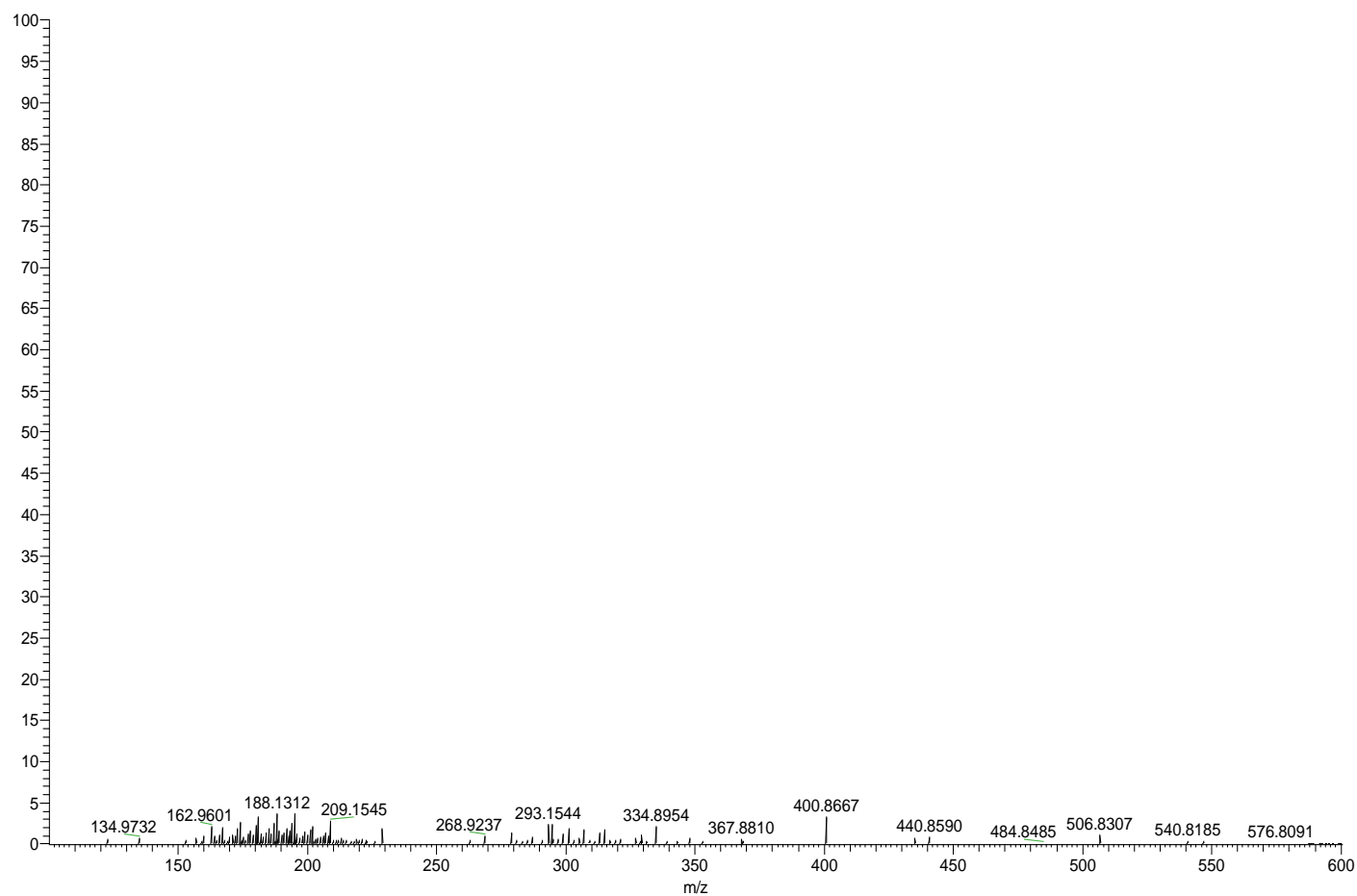
Fraction 5

FIGURE A.1: Mass spectra from ESI-HRMS of five oil sands-derived NA fractions. Bars represent the respective ion's relative abundance. Numbers above bars represent the respective ion's mass-to-charge ratio (m/z) and is indicative of atomic mass.

Appendix B: Time to Hatch and Hatch Length Data for Japanese Medaka and Fathead Minnows Exposed to Five NA Fractions
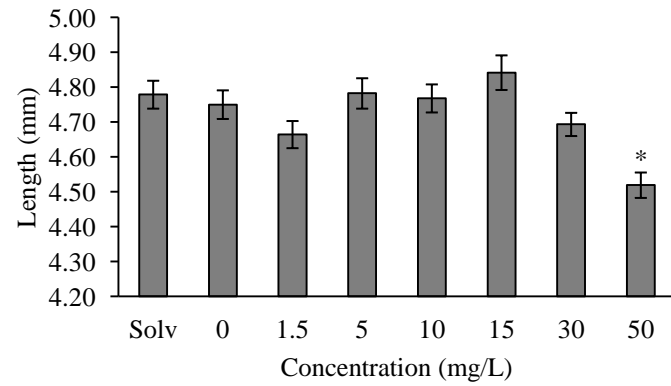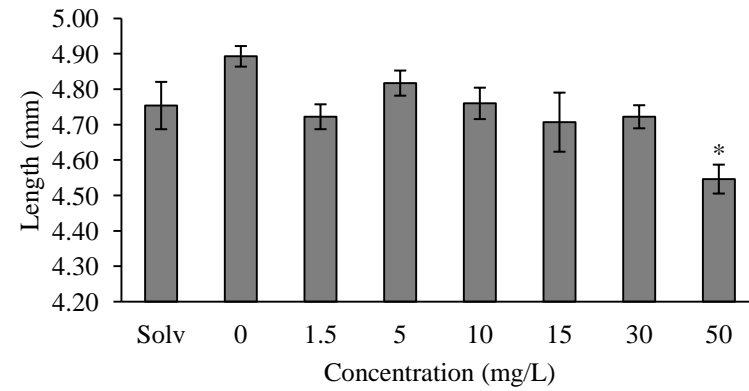
Japanese Medaka Time to Hatch

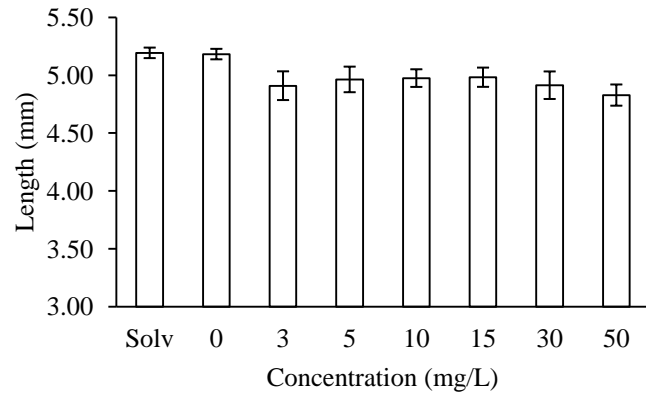
Fraction 1

Fraction 2

Fraction 3
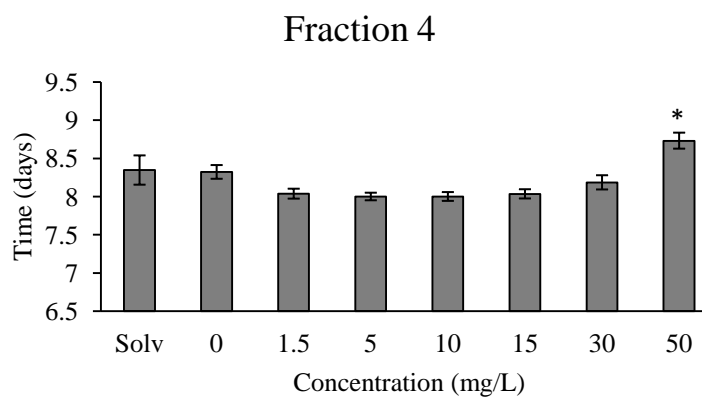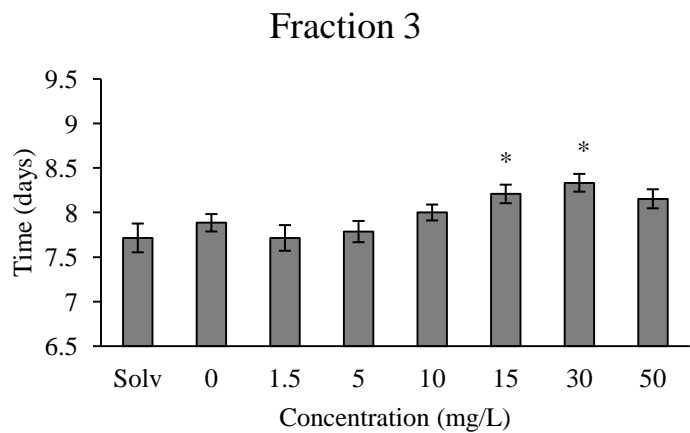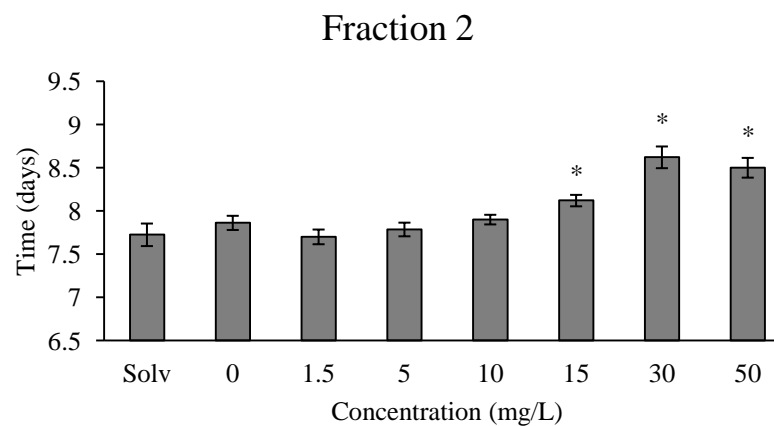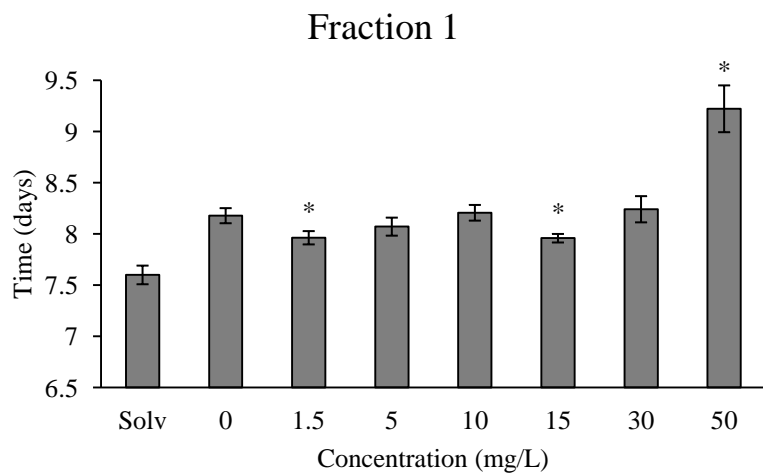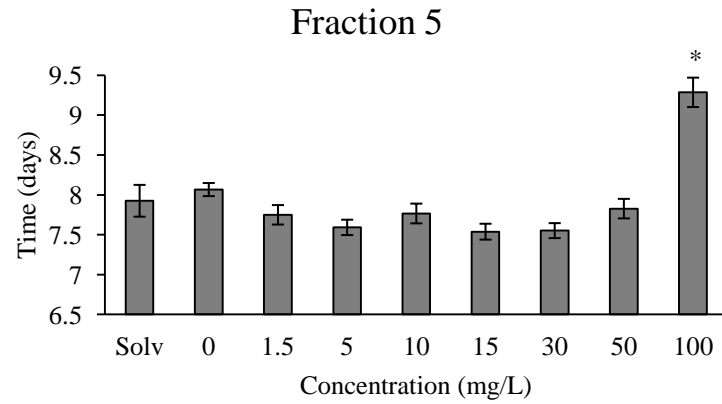
Fraction 4

Figure C.1: Effects of five NA fractions on Japanese medaka time to hatch embryo larval endpoint. Bars represent mean time to hatch (days) $\pm$ SEM. X-axis represents nominal concentrations; "Solv" represents solvent controls, and concentration at 0 represents water controls. Those concentrations which are significantly different from both controls are indicated by an asterisk ($p \le 0.05$).

Fathead Minnow Time to Hatch

Fraction 1

Fraction 2

Fraction 3

Fraction 4
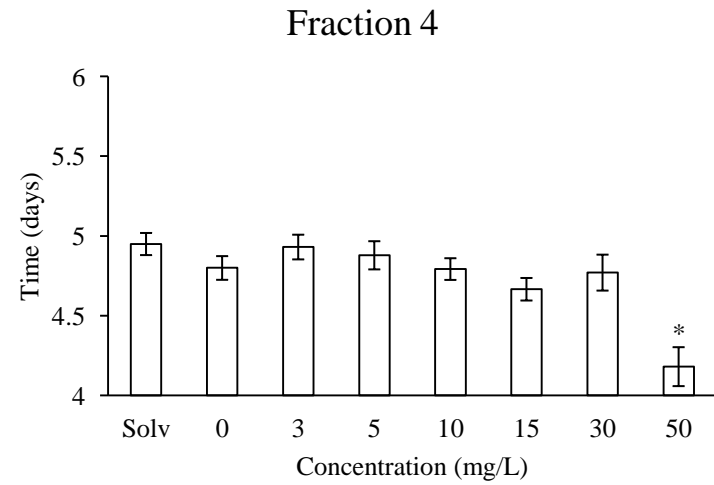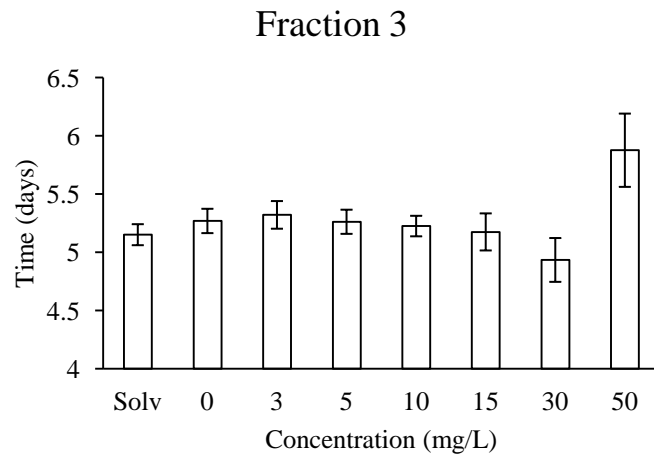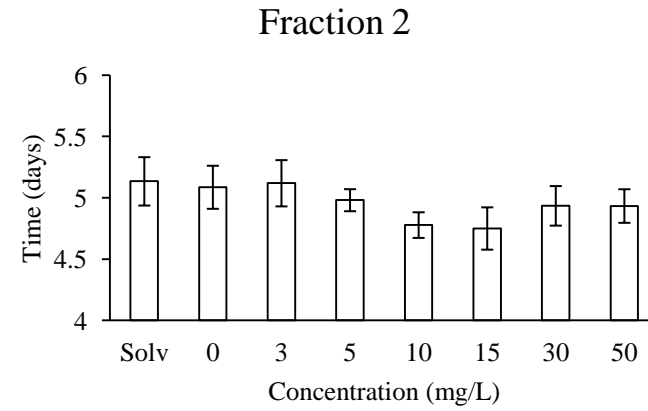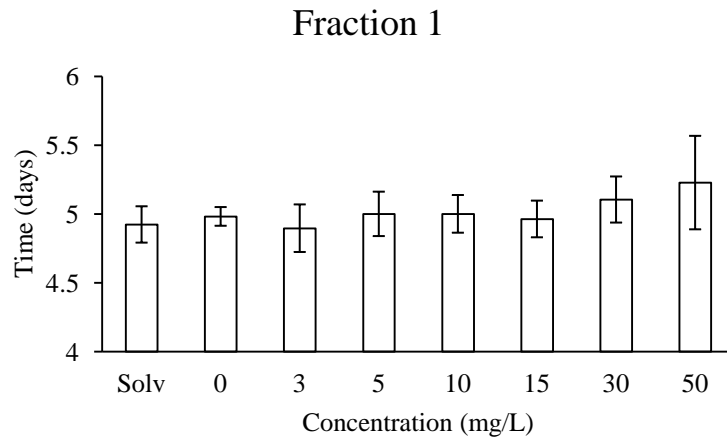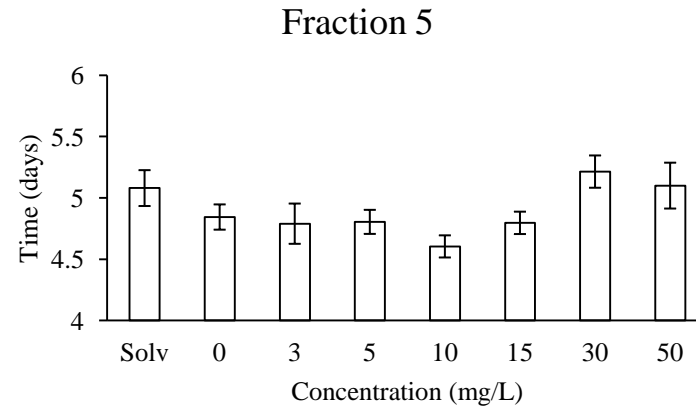
Fraction 5

Figure C.2: Effects of five NA fractions on fathead minnow time to hatch embryo larval endpoint. Bars represent mean time to hatch (days) $\pm$ SEM. X-axis represents nominal concentrations; "Solv" represents solvent controls, and concentration at 0 represents water controls. Those concentrations which are significantly different from both controls are indicated by an asterisk ($p \leq 0.05$).

Japanese Medaka Hatch Length

Fraction 1

Fraction 2
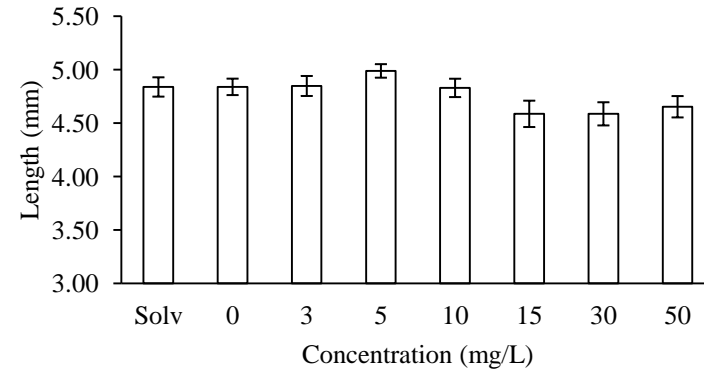

Fraction 3

Fraction 4
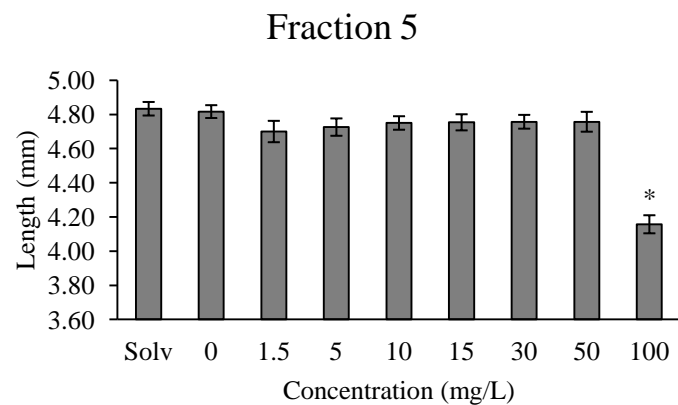
Fraction 5

Figure C.3: Effects of five NA fractions on Japanese medaka hatch length embryo larval endpoint. Bars represent mean hatch length (mm) $\pm$ SEM. X-axis represents nominal concentrations; "Solv" represents solvent controls, and concentration at 0 represents water controls. Those concentrations which are significantly different from both controls are indicated by an asterisk ($p \leq 0.05$).
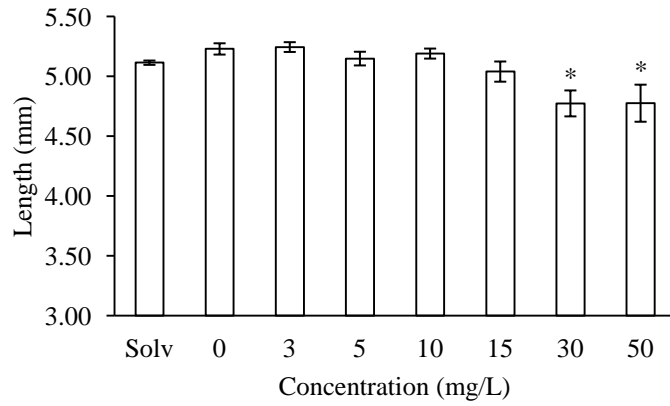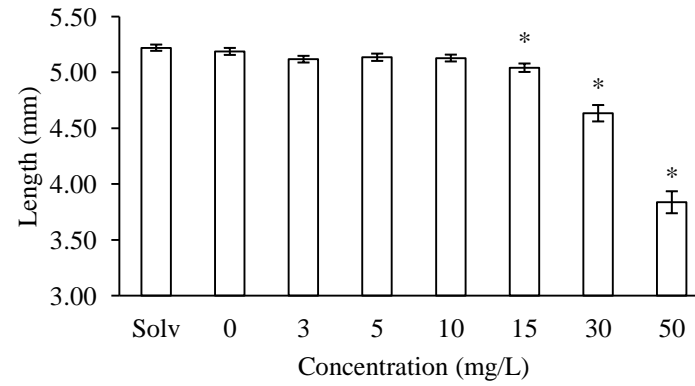
Fathead Minnow Hatch Length
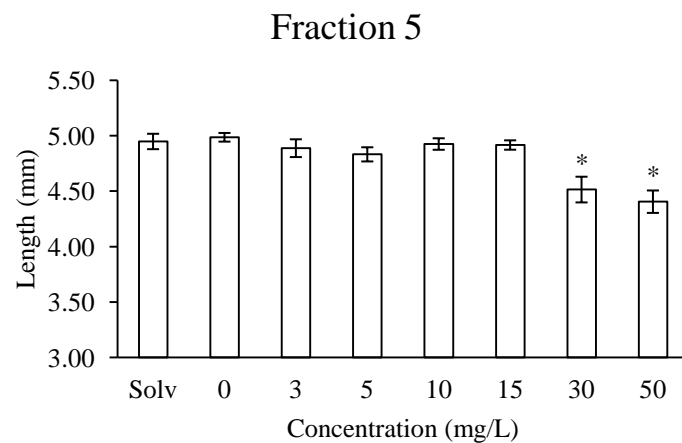

Fraction 1

Fraction 2

Fraction 3

Fraction 4

Fraction 5

Figure C.4: Effects of five NA fractions on fathead minnow hatch length embryo larval endpoint. Bars represent mean hatch length (mm) $\pm$ SEM. X-axis represents nominal concentrations; "Solv" represents solvent controls, and concentration at 0 represents water controls. Those concentrations which are significantly different from both controls are indicated by an asterisk ($p \leq 0.05$).

Appendix C: Japanese Medaka and Fathead Minnow Mortality Expressed in mg/L

Fraction	Japanese Medaka (mg/L)	confidence interval		Fathead Minnow	confidence interval	
		lower	upper		lower	upper
Whole NAE	37.6[a]	36.6	38.7	nc		
1	68.9[b]	66.3	71.5	37.7	-	-
2	37.6[a]	36.3	39.0	19.2	-	-
3	38.4[a]	37.7	39.1	26.6	21.7	32.7
4	64.3[b]	53.4	77.4	28.4	23.7	34.1
5	68.0[b]	64.9	71.3	21.7	-	-

Table C.1: Spearman-Karber mortality LC_{50}s ($\alpha = 0.2$) for five NA fractions and a whole NAE for fathead minnow and Japanese medaka. The "-" indicates values that could not be derived at $\alpha = 0.2$. Tests that were not conducted are indicated by "nc". Significantly different LC_{50}s are denoted by letters (a-d).

Appendix D: Pooled Abnormality Incidences for Japanese Medaka and Fathead Minnow
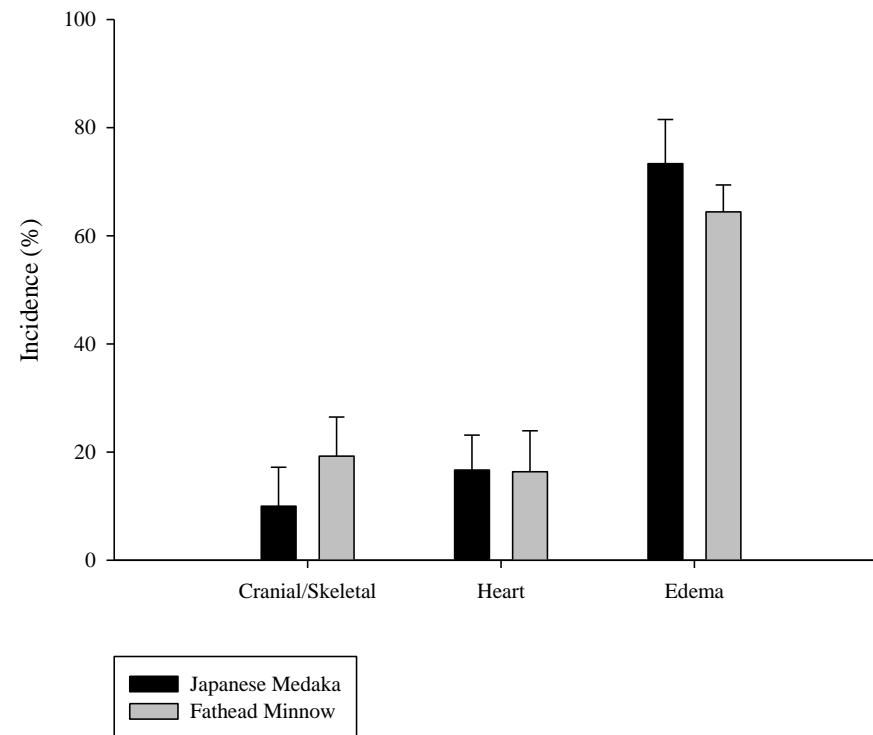

Figure D.1: Pooled percentage incidence of abnormality type for Japanese medaka and fathead minnow. Bars represent mean percentage incidence + standard error (error bars).